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Characterization of the recombinant proteins

Ser 3 and BARN

Bachelor Thesis

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Annotation

This bachelor thesis deals with the expression of two recombinant proteins in a bacterial expression system. These proteins are derived from adhesive proteins originating from silkworm (*Bombyx mori*) silk and cement from barnacle (*Megabalanus rosa*). This thesis includes optimization of the protocol for protein expression and simple characterization of recombinant proteins involving examination of its biocompatibility in tissue culture and ability to form films on different substrates using electron microscopy.

Declaration

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.

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Abstract

Biopolymer materials with adhesive properties have proven significant purpose in modern medicine and in fields of cosmetics. These “Bioadhesives” that stand out due to their biocompatibility and degradability, often replace mechanic alternatives and are of great interest to the scientific community (Khanlari and Dubé 2013; Ebnesajjad 2008). This Bachelor’s Thesis concerns the characterization of two recombinant proteins with adhesive properties. On the one hand the Ser 3 protein, originating from the silk glands of the domestic silkworm *Bombyx mori*, and on the other hand the BARN protein, derived from the cement of the barnacle *Megabalanus rosa*. The aim of this thesis is to develop an adapted protocol for the efficient expression of both proteins and to test their adhesive properties in a cell culture experiment. After expressing the target sequences in *Escherichia coli* and the subsequent purification procedure, the proteins were utilized to coat a hydrophobic surface to cultivate insect cells. The protein films on the surface further examined by SEM. The expression process was monitored by several analysis techniques and the results were discussed to further improve and adapt the protocol for further research.

Index

List of Abbreviations

AA	Acrylamide
AFM	Atomic force microscopy
APS	Ammonium persulphate
BSA	Bovine Serum Albumin
Da	Dalton
diH_2O	Water (deionized)
DTT	Dithiothreitol
CM	Cl8+ medium
EB	Elution buffer
EDTA	Ethylenediamine tetra-acetic acid
ESI	Electrospray Ionisation
ETB	Electro-transfer buffer
IBB	Inclusion body buffer
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranosid
LB	Luria Bertani
LDS	Lithium dodecyl sulphate
NBB	Natural binding buffer
NC	negative control
NCBI	National Center for Biotechnology Information
NLB	Natural lysis buffer
OD	Optical density
PBS	Phosphate buffered saline
Q-TOF	Quadrupole-Time of Flight
RNAP	Ribonucleic acid polymerase
SB	Super broth
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
TB	Terrific broth

TCA	Trichloroacetic acid
TRIS	tris(hydroxymethyl)aminomethane.
WB	Washing buffer

1 Introduction

1.1 Adhesive biopolymers

The applications of adhesive biopolymers are various and range from medical applications like wound closure, bleeding control, drug delivery, dental and bone applications and implantation of medical devices to cosmetic application (Khanlari and Dubé 2013; Mehdizadeh and Yang 2013). There are many advantages of utilizing adhesive materials, as they provide uniform distribution of stress and a favorable strength/ weight ratio compared to mechanical fastening. They are also quickly applied and cheap compared to their mechanical alternatives. They further successfully bind materials of different size and shape and may shield the materials from adverse effects that they may have on each other (e.g. electrochemical corrosion). On the contrary, the usage of adhesives also has certain disadvantages, as the application of an adhesive requires care and cleanliness. The cure time of the adhesive may be long, and the lifetime of the adhesive bond varies depending on the temperature and general environment. Naturally derived adhesives may experience damage due to fungal or bacterial attacks. Also, health hazards are a potential disadvantage of adhesives (Ebnesajjad 2008).

1.2 Applications of adhesives

Over the years, utilizing tissue adhesives instead of staples and sutures, has proven to be a reasonable alternative for wound closure. The adhesives in use are required to be non-toxic, sterilizable and should rapidly solidify to prevent bleeding. Moreover, it is important for the operator to be able to easily and rapidly prepare the adhesive and precisely administer the substance. The adhesive should maintain strong bonding for the time needed but also be degradable in reasonable time. Tissue adhesives and sealants can be divided into two major groups: naturally derived glues and synthetic adhesives/ sealants. While the synthetic glues are artificially produced polymerizing substances, the naturally derived adhesives are either directly extracted from living tissue or based on isolated proteins. They can either work independent of active chemicals or in combination with them (Mehdizadeh and Yang 2013).

Due to their fast and pain-free application and their ability to prevent wound infection, the use of most tissue adhesives shows clear advantages over wound closure by suturing (Khanlari and Dubé 2013). The premise is of course fulfilling the requirements listed above. In case of naturally derived glues, lack of biocompatibility can be a problem and allergic reactions can be triggered upon application of the glue (Mehdizadeh and Yang 2013).

In case of complicated bone fractures, in which regular bone healing is not applicable, adhesives are used as substituents, to align the bone pieces and fill in the space between. According to the research of *Xie* et. al *(2015)*, substances using proteins derived from deep-sea mussels show promising characteristics for this task, as they have strong adhesive properties and adhere well to soft tissue (Xie et al. 2015).

For the administration of drugs, biopolymers can be used to attach the active compound to the designated site. As *Lehr* et. al (2000) proposes, the advantages of such an application are the prolonged residence time of the drug on the site and the potential to modify mucosal sites to enable faster transport to epithelial transport (Lehr 2000). According to *Khanlari* et al. (2013), bioadhesives can also prevent fast degradation of unstable drugs and therefore limit the administration frequency, as they keep more of the active compound functional (Khanlari and Dubé 2013). The major disadvantage, however, is the lack in binding specificity, as the bioadhesives may adhere to surfaces that are similar to the target one and are therefore difficult to control (Lehr 2000). A solution to this could be provided by the technology of triggered adhesion that allows the adhesive agent to attach to a surface after it has experienced a distinct stimulus. This is important not only for controlled drug administration but also for implantation of medical devices or dental surgery. To address certain environments where a lot of fluid is present. For example in the oral cavity or blood vessels, adhesive substances are a preferred choice as they prevent the therapeutic agent from getting flushed away and support the implantation (Helmus et al. 2008).

1.3 Surface treatment

Various surfaces can be coated using adhesive materials. To facilitate proper bonding, it is important to directly administer the adhesive to the surface without intervening layers of impurities (Ebnesajjad 2008). It further is important to evenly spread the adhesive onto the surface to achieve efficient “wetting” that guarantees strong bonding (Duncan et al. 2005): Depending on the type of surface, different treatment is suggested to prepare it for adhesive bonding. According to *Ebnesajjad (2008)*, metal surfaces should be cleaned by means of organic solvents without changing the physical and chemical properties of the target material. Methods include vapor degreasing and ultrasound treatment. After a subsequent chemical treatment to improve the adhesion qualities, application (priming) of the adhesive can be performed (Ebnesajjad 2008). In case of wood composite materials, a combination of mechanical abrasion and corona discharge treatment has proven as successful surface treatment (Moghadamzadeh et

al. 2011). For non-metallic surfaces, treatment with aqueous detergent solutions is recommended. In case of solvent resistance, proper surface preparation can be accomplished with plasma treatment (Ebnesajjad 2008). Application of adhesives on living tissue is greatly dependent on enhanced binding efficiency. As *Matsuda et al. (2012)* discovered, the bonding strength highly depends on the penetration strength that can be enhanced by customizing the side-chains of the polymer to adapt them to the target tissue (Matsuda et al. 2012).

1.4 Biocompatibility

Compatibility of adhesive polymers with living tissue is especially important for application in the fields of medicine. Even though glues may facilitate strong bonding, degradation products may disqualify the compounds as they may have adverse effect on the tissue (Matsuda et al. 2012). As *Mehdizadeh et al.* (2013) describes, certain adhesives may trigger allergic reaction when used on human tissue and may even cause the antibodies that are produced in response, to interfere with bodily processes to cause severe health problems. Also, utilizing adhesives compounds that were gained from animal systems, bears the risk of facilitating the transmission of infectious agents (Mehdizadeh and Yang 2013).

1.5 Adhesive producing organisms

There are various organisms that produce compounds with adhesive properties. We further focus on the organisms relevant for this research.

1.5.1 Barnacles

Barnacles are cirriped arthropods that belong to the Superorder *Thoracica*. They exist as a sessile type (Order *Sessilia*) or pedunculate type (Order *Pedunculata)* that both have segmented calcareous shells. The discrimination of the two different types is based upon the different way the arthropod attaches itself to the substrate. The pedunculate type that is typified by the “goose” barnacles (Suborder *Lepadomorpha*), is attached through a stalk-like peduncle. The sessile order is typified by the “acorn” barnacles (Suborder *Balanomorpha*) and is directly cemented to the substrate (DOYLE et al. 1996). Most significant for this research is the *Megabalanus rosa* that is a representative of the *Megabalaninae*, a subfamily of the *Balanomorpha* (Pitombo 2004). The life cycle of the barnacle is divided into two distinct phases. A free-swimming phase, where the barnacle exists in a larval state, and a sessile phase, were the barnacle settles down and glues itself to surface and grows adult. This process is referred to as cementation and is facilitated by the release of a certain adhesive substance called the barnacle cement (Okano et al. 1996). There

are two types of cement that are produced by this organism. On the one hand, the primary cement that is produced while the barnacle is sessile and on the other hand, the secondary cement that is secreted by the barnacle when it is free from the substrate (Kamino et al. 1996). Both types of cement show a very similar amino acid composition (Naldrett 1993). The barnacle cement is stored in two kidney-shaped glands that are positioned ventrolateral. Inside of the glands, the cement is stored in secretory granules that show, in case of *Megabalanus rosa*, a diameter of 1-4 μm. The secretion from the granules is however, not yet fully understood. (Pitombo 2004). The cement is difficult to dissolve in low concentrations of SDS and is prone to repolymerize quickly. The reason for its strong resistance is not yet fully understood and has yet hindered scientists from fully understanding the exact binding mechanism and all biochemical properties of the cement. As *Naldrett (1993)* suggests, cross-linkage by di-sulfide bonds of the generally cysteine-rich cement could be the major factor for adhesion, together with hydrophobic interactions (Naldrett 1993; Kamino et al. 2000).

1.5.2 Bombyx mori

The *Bombyx mori,* also referred to as the domestic silkworm, is a commonly used organism in biological and biochemical research. It is further used excessively for the research of its physiology and metamorphosis. After hatching, the *B. mori* stays in a larval state for 36 days, before spinning a cocoon and emerging as a mature moth after another 15-21 days. The exact time depends on the predominant conditions (Hsueh and Tang 1944). Especially the silk of the *B. mori*, is a subject of investigation. It has been used for centuries in textile industry due to its strength and appearance. Also, due to its biocompatibility and slow degradation, it has gained interest as a biomaterial. Of special interest to my research are sericins, a group of soluble glycoproteins that are expressed in the middle silk gland of the organism (Rockwood et al. 2011; Hardy and Scheibel 2010). “Sericins” have a high content of the amino acid serine (S) in its sequence, for which sericin owes its name. When the silk is used for textile manufacturing, the sericins are mostly removed. However, studies have shown that sericin acts as an antioxidant and inhibits the activity of tyrosinase. Importantly, the tyrosinase is known to trigger the browning reaction of various foods and the biosynthesis of melanin in the skin. Sericin has revealed its potential for use in the cosmetic industry and food industry. Moreover, sericins have been shown to work against wrinkles and have moisturizing effects. The downside in utilizing sericins is their potential to cause allergic reaction in humans. As research has shown, silk containing a high

number of sericin triggers immune response and therefore, the sericin had to be removed to guarantee biocompatibility (Kato et al. 1998).

1.6 Recombinant protein expression

A recombinant protein results from cloning a gene of interest into an expression vector, that is further transformed into a host of choice. The initiation of the protein expression is called induction and is triggered by the addition of a certain agent.

1.6.1 Host expression systems

Several expression systems can be utilized to express the desired protein. Utilizing yeast (Saccharomyces cerevisiae) as an expression host brings the advantage of working with a eukaryotic microorganism, that grows on cheap media and guarantees better protein folding pathways than other expression systems like *Escherichia coli* (*E.coli*) The dependence of the yeast expression system on specific yeast promotors, however, is a disadvantage. Also, the growth kinetics are inferior to bacterial expression *(Verma et al. 1998).* Baculovirus-mediated systems can handle large proteins and facilitates most post-translational modifications. However, utilizing this expression system is pricy and labor-intensive (Geisse et al. 1996; Verma et al. 1998). Mammalian cells in culture can stably express recombinant constructs and facilitate proper folding and glycosylation of the products. Disadvantageous are the relative high expenses (Geisse et al. 1996; Verma et al. 1998). The most commonly used cell lines utilized for protein expression are the Chinese Hamster Ovary (CHO) and NS0 (mouse myeloma) cells. *E. coli* as a representative of the bacterial expression systems excels due to its rapid growth (20 minutes doubling time at optimal conditions and media) on cheap media and its capacity to be cultivated in high density (1 x 10^{13} viable cells per mL) (Rosano and Ceccarelli 2014). The *E. coli* expression system, however, cannot perform post- translational modifications and is prone express misfolded proteins (Baneyx 1999; Rosano and Ceccarelli 2014).

1.6.2 Expression in *E. coli*

The recombinant proteins that are part of this research, are produced by utilizing *E. coli* as a host. *E. coli* is the most common gram-negative bacteria in a human intestinal system. While it is predominantly harmless and lives in symbiosis with its host, there are various pathogenic strains that can cause several diseases (Nataro and Kaper 1998). Laboratory strains contain multiple auxotrophic mutations. This gram-negative bacterium shows rapid growth at high density on cheap substrate and possesses many cloning vectors. The bacterial strains used for the

recombinant expression are also deficient for some endogenous proteases. All those features make it one of the most frequently used systems for heterologous protein production (Baneyx 1999).

In this research the protein expression in *E. coli* (One Shot™ BL21 (DE3)) is performed by using the pET-15b vector system (Novagen™) containing strong bacteriophage T7 transcription signals. This type of vector system is specifically designed for the λDE3 lysogen strains of *E. coli* (Gräslund et al. 2008) expressing the highly active T7 RNA polymerase (T7 RNAP). The transcriptional activity of the T7 RNAP is under control of a lac promotor that is activated by the addition of the non-hydrolysable lactose analog Isopropyl-β-D-thiogalactopyranosid (IPTG). Unintended premature expression of the protein is prevented by the presence of glucose that inhibits the lac promotor by binding to it and prevents T7 RNAP production. After induction of the promoter by IPTG, expression of the protein takes place (Rosano and Ceccarelli 2014). Overexpression of the recombinant protein can result in overloading the folding machinery of the prokaryotes (Prasad et al. 2011). When the bacteria fail to successfully fold the polypeptide chain, insoluble aggregates form – so called "inclusion bodies" (Yamaguchi and Miyazaki 2014).

1.7 Principles of Methods used for the expression process

1.7.1 Bacterial Cell cultivation

The *E. coli* cells containing the plasmid vector are cultivated on streak-plates from sterile Luria-Bertani (LB) Broth. After one day of incubation, single colonies from the plates are selected and spread on new streak-plates according to Figure 1. To ensure the selective cultivation of the cell containing the plasmids that guarantee antibiotic resistance, the LB media can be spiked with different antibiotics. The cells from the streak-plate are further moved to liquid media, that is incubated until the culture enters the log phase (Optical Density (OD) ~0.6). Then induction is triggered by the addition of IPTG.

Figure 1: Streak-Plate from Luria-Bertani Broth with E. coli cells spread in distinct manner.

1.7.2 Bacterial Cell Lysis

The broth containing induced *E. coli* cells, is centrifuged and the resulting pellet is treated with a binding buffer (NBB, Table 5). Cell lysis is commonly facilitated by the addition of lysozyme that disrupts the bacterial cell wall. Additionally, sonication can be performed. In case of protein expression, it is important to add RNAse and DNAse to digest the contaminating genetic material of the cell.

1.7.3 Nickel-Agarose affinity chromatography.

To purify a recombinant protein, Nickel-Agarose affinity chromatography is performed, that belongs to the immobilized metal affinity chromatography (IMAC) techniques. The advantages of this type of purification method are strong specific binding of the protein and the mild elution conditions. Furthermore, the resins used as stationary phase show adequate tolerance to harsh cleaning conditions (Gräslund et al. 2008).

The immobilized metal ions of the resin bind by co-ordinate bonding to the exposed electron donor groups of the amino acid histidine on the surface of the protein structure. (Mooney et al. 2013). For selective binding, the proteins used in this research are thus tagged with a hexa-histidine tail at the N-terminus. The Nickel-Agarose that is used as a stationary phase will bind to the protein sequence, while potential impurities are washed away by addition of washing buffer. By keeping track of the absorbance of the flow through fractions, by an Eppendorf Biophotometer (OD_{600} nm), the quantity of the sample can be estimated. For elution, an Elution buffer (Table 7) is applied to the chromatography tube that contains a high amount of imidazole to compete for the binding sites of the stationary phase and therefore, causes the elution of the desired protein.

1.7.4 Inclusion Body purification

Recombinant proteins produced in E. coli are frequently deposited in the form of inclusion bodies, insoluble aggregates of misfolded protein lacking biological activity. Inclusion Body purification protocol allows to retrieve aggregated proteins in pure form. After the lysate is centrifuged the inclusion bodies sediment and the resulting pellet is suspended in a buffer (IBB 1, Table 8). To the suspension, lysozyme is added, and the container is incubated on ice until the mixture turns viscous. Lysis is supported by sonication. Protease inhibitors are added to shield the desired proteins from degradation by some protease contamination. Next, RNAse and hygroscopic agents like $CaCl_2$ or Mg_2Cl_2 are added. The mixture is then centrifuged, and the

pellet is suspended in a different buffer (IBB 2, Table 9,) (2x) before repeating the procedure with the final buffer solution (IBB 3, Table 10) (4x). The pellet is finally dissolved in urea to denature the protein. Alternatively, dissolving can be also performed in 9M LiBr or concentrated formic acid.

1.7.5 Dialysis

To facilitate protein refolding and to get rid of contaminants and denaturants (including imidazole), dialysis is performed (Yamaguchi and Miyazaki 2014). The dissolved proteins are transferred to a dialysis tubing and placed in a pre-cooled container with refolding buffer on a stirring plate. Dialysis is performed for 24 hrs., while the refolding buffer is replaced every 8 hrs.

1.8 Principles of Methods used for analysis

1.8.1 Sodium Dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a technique to separate proteins according to their molecular mass. The separation of the different proteins of a sample is facilitated by applying voltage. Figure 2 shows the corresponding set-up. The set-up consists of a separation polyacrylamide gel (Table 12) and a stacking gel (Table 13) that are polymerized between two glass plates. A comb that is inserted into the upper stacking gel is used to create wells for sample loading. The glass plates are fixed in a container filled with Running Buffer (Table 15) that provides ions allowing to perform electrophoresis. After the comb is removed, the samples can be loaded. The samples are diluted with sample buffer (Table 14) to denature the proteins and apply charge, so the proteins start moving towards the positive electrode once the voltage is applied. To assist the denaturation, the samples are heated shortly before loading them onto the gel. A protein marker containing coloured components of known sizes is added to at least one of the wells, to provide a scale. After loading, voltage is applied, and the proteins start to separate. When fully separated, the proteins on the gel can be visualized by different staining techniques.

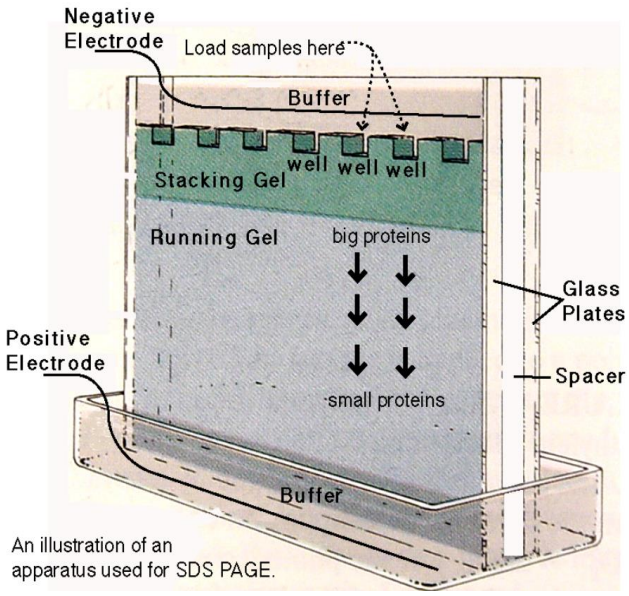

Figure 2: SDS-PAGE set-up (Peek and Williams 2001).

1.8.2 Western Blot

Using the Western Blot method, makes it possible to visualize specific protein bands by electroblotting the content of an SDS-PAGE gel onto a nylon membrane. Therefore, the polyacrylamide gel is arranged in a "sandwich" according to Figure 3. The membrane is previously treated with methanol (to lower its hydrophobicity) and deionized water (diH_2O). The transfer stack is placed in a buffer solution (Table 17). When voltage is applied, the proteins from the gel move onto the membrane as they travel towards the positive electrode. The proteins can further be visualized on the membrane by Ponceau S stain or antibody staining.

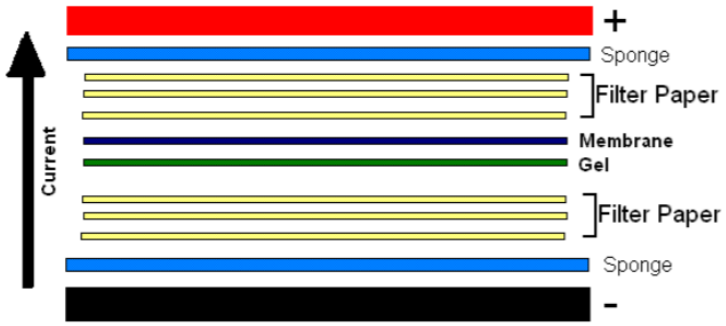

Figure 3: Western Blot transfer stack (Zeeck 2003).

1.8.3 Densitometry

To quantify the yield of expressed proteins, densitometry from SDS-PAGE can be performed. Therefore, besides the protein to analyze, standard solution bands (BSA) of different concentration are visualized on a gel. The integrated density of each band on the gel is gained

using an adequate scanner and analytical software. The density measured for the standards is used to create a trendline (Concentration vs Density). The resulting formula of the linear fit is then used to calculate the concentration of the protein band.

1.8.4 Mass spectrometry

Mass spectrometry is a technique that can be used to determine the protein content of a sample by analysing the mass and amino acid sequence or even the site of attachment and the type of posttranslational modification of a polypeptide. The sample is digested by trypsin and resulting peptides are ionized and accelerated by electric or magnetic fields. An analyser machine selects peptides –based on their mass to charge ratio (m/z) – that are recognised by a detector. The resulting sequences are compared to databases to determine the identity of the proteins (Domon and Aebersold 2006).

1.8.5 Animal Cell Culture

Cell culture is a method which allows growing animal cells in a controlled artificial environment. Depending on their type, animal cells grow either in suspension as an adherent monolayer on the surface of small plastic or glass vessels. Adherent cells require a charged or otherwise specially prepared vessel surface for their attachment. Our cell culture experiment involved the coating of a non-adherent surface with the pure protein to selectively cultivate cells. The cells plates are then incubated and for several days and pictures of the distinct, marked spots are taken at fixed times. The success of the cell growth is evaluated by counting the vivid cells. A substance with no adhesive properties can be used as negative control.

1.8.6 Scanning Electron Microscope (SEM)

The scanning electron microscope uses a focused beam of electrons to build an image of the object to investigate by scanning the probe spot by spot. The electric current that leaves the object is detected an modulates the brightness of the image (Smith and Oatley 1955). In our work we used electron microscopy to examine the quality of glass or wood coating with recombinant protein.

2 Aims of the thesis

- Optimization of the protocol for adhesive protein expression in bacterial system
- Preliminary characterization of biocompatibility of recombinant proteins in tissue culture
- Examination of recombinant protein films deposited on glass or plastic surfaces using electron microscopy

3 Material and Methods

3.1 Materials

- Expression system: *Escherichia coli strains:* One Shot™ BL21 (DE3), pET-15b vector system (Novagen™), T7 RNA polymerase (T7 RNAP)
- SDS-PAGE and Western blot analysis: Coomassie Brilliant Blue R250™, Ponceau S., Immobilon™-P Polyvinylidene membrane
- Mass spectrometry: ESI-Q-TOF premier (Waters ®), nanoACQUITY-UPLC® (Waters®)
- Dialysis tubing (Servapor 29mm, 12000-14000 MWCO)
- Scanning Electron Microscope (SEM, Jeol 6300)
- Freeze dryer ALPHA 1-2 / LD p l u s (Christ)
- Ultrasonic homogenizer 4710 (Cole parmer)
- Orion 3 STAR pH Benchtop (Thermo Scientific)
- Bio Photometer / AG 22331(Eppendorf)
- Centrifuge 5804R (Eppendorf)
- Analysis Software: GS-900™ Calibrated Densitometer (BioRad), ImageQuant™ TL (GE Healthcare Life Sciences), ImageJ™ (National Institutes of Health (NIH)).
- Growth Media: Luria-Bertani Broth (LB-medium, Table 3), Cl8+ Medium (CM, Table 11)

Buffer solutions:

- Affinity chromatography: Natural Lysis Buffer (NLB, Table 4), Natural Binding Buffer (NBB, Table 5), Washing Buffer (WB, Table 6), Elution Buffer (EB, Table 7)

  Inclusion Body purification: Inclusion Body Buffer (IBB 1-3, Table 8-10),

  SDS-PAGE: Separation Gel (Table 12), Stacking Gel (Table 13) Sample Buffer (Table 14), Running Buffer (RB, Table 15), Destaining solution (Table 16)

  Western Blot: Electro-transfer Buffer (ETB, Table 17-18)

(All medium & solution compositions are listed in the appendix)

3.2 Recombinant DNA encoding adhesives

Sequences were derived from natural proteins, their coding region was optimized for the expression in *E. coli* and were synthesized *de novo* by Gene Universal Inc. (Newark, DE, USA).

- Ser 3 amino acid sequence, *Bombyx mori* (16.8 kDa)
  MGSSQSSSSKNSSGSKGSGSSESGDKKSSSRGSSGDNSDDDQTDSARSNSKRSTSSDA
  STKKSSSRKSSNHRSSRSQQAHSSSSKQAQSSSSQQAQNSRSQQAHSSRSQQAHSSSS
  KQAQSSSSKQAQSSSSKQAQSSSSKQAQSSSSQQAQSSRSHHHHHH

- BARN amino acid sequence, *Megabalanus rosa* (17.8 kDa)
  MGDLEIASLERRSTGKAKTQTAIKGRSLAKAKLRQAATDQGLNQDLQTDVLGQCQT
  NTVTRQSGSFVQLEKKGKKCDHCTSTLKKLTKGTTFGSSRERLQEQTRQKKRDTLRG
  RAGQGATLVSTGKETNGFRRAKQSGSGSDVQEKYKGANSHLSTHHHHH

  Ser 3 was derived from the *Bombyx mori* Sericin 3 protein (NP_001108116.1) (Geer et al. 2010). and BARN was derived from the mrcp-20k protein (Okano et al. 1996).

3.3 Bacterial Cell Cultures

- 10 mL of LB medium with ampicillin (final concentration: 0.6 µL/ mL) and Chloramphenicol (final concentration: 0.3 µL/ mL) were inoculated with bacterial culture and grown overnight (o/n) at 37°C.
- In the morning of the following day, 1.5 mL of the culture was used to inoculate 250 mL and grown for approx. 2-3 hrs (37°C).

- When the culture entered the log phase (OD600 ~0.6) of their growth; the protein expression was triggered by the addition of IPTG (final concentration: 1mM).
- This was followed by incubation for 3.5 hrs on a shaker (220 rpm, 37 °C).
- The samples were centrifuged for 10 min (3000 rpm, 4 °C). The supernatant was discarded, and the pellet was suspended in 20 mL of NLB.

3.4 Cell lysis

- The suspended pellet was treated with 0.8 mL lysozyme (100 mg/ mL) and RNAse (final concentration: 10 mg/ mL) and incubated on ice on a shaker (30 min).
- The viscous mixture was sonicated (4710 series ultrasonic homogenizer) 10 times (60% power) for 10 s with 10 s of break in between (on ice).
- Next, the suspension was centrifuged for 15 min (3000 rpm, 4 °C).

3.5 Inclusion Body purification (BARN)

- The pellet of the cell lysate was suspended in 40 mL of IBB 1and 4 mL of lysozyme (100 mg/ mL).
- The container was incubated on a shaker for 45 min (on ice).
- Then, the viscous mixture was treated with protease inhibitor (1:1000) and sonicated 5 times (60% power) for 30 s with 30 s break in between (on ice).
- 40 µL RNAse, 20 µL $MgCl_2$ (2M) and 10 µL $CaCl_2$ (50 mM) were added and the mixture was incubated on ice for 60 min.
- The mixture was centrifuged for 15 min (3000 rpm, 4 °C). The supernatant was discarded, and the pellet is suspended in 2 mL IBB 2 (2x).
- Afterwards, the mixture was centrifuged for 15 min (3000 rpm, 4 °C). The supernatant was discarded, and the pellet was suspended in 50 mL IBB 3 (4x).
- Finally, the pellet was dissolved in 10 mL urea (8M)

3.6 Affinity Chromatography

- 2 mL of Nickel-Agarose resin were added to falcon tube 1 (15 mL) and suspended in ~ 4 mL diH_2O. The homogenized mixture was centrifuged for 2 min (3000 rpm, 4 °C) (2x).
- The resin was then suspended in 2 mL NLB (Table 4). The homogenized mixture was centrifuged for 2 min (3000 rpm, 4 °C) (2x).
- The pellet from the cell lysate was suspended in 2 mL NLB in falcon tube 2 (15 mL).

- Next, the resin was suspended in 2 mL NLB and added to the container with the suspended pellet. The mixture was left on a rotor for 60 min (4 °C).
- Then, the mixture was centrifuged for 10 min (3000 rpm, 4 °C) and the pellet was suspended in 2 mL of the supernatant (the rest is discarded).
- The suspension was transferred to a falcon tube 3 (15 mL) and centrifuged for 10 min (3000 rpm, 4 °C). The supernatant was discarded.
- The mixture was centrifuged for 1 min (3000 rpm, 4 °C). The supernatant was discarded, and the pellet was suspended in 400 µL WB (Table 6).
- Then the mixture was centrifuged for 5 min (3000 rpm, 4 °C). An aliquot of the supernatant was used to measure the absorbance OD_{250}. The pellet was suspended in WB and centrifuged until the absorbance reached 0.
- The protein was then eluted using 300 µL EB (Table 7).

3.7 Dialysis

Single-step dialysis was performed for the elution fractions after purification that were dialysed in a dialysis tubing (Servapor 29mm, 12000-14000 MWCO) in 1 l diH_2O on a stirring plate (4°C). Dialysis was performed for 24 hrs. The water was exchanged every 8 hrs. Dialysis of BARN expressed in inclusion bodies was performed against 10 mM NH_4HCO_3 (pH: 7.0).

3.8 Cell Culture

- The protein was lyophilized and dissolved in formic acid (10 %).
- Six circles (5 mm of diameter) were drawn on a polystyrene petri dish (55 mm of diameter (Figure 4). Each circle was visibly pinched three times to mark the control spots for the pictures.
- The proteins (0.5 mg/ mL, 1 mg/ mL) were applied to each two of the circles. The remaining two circles were treated with Bovine Serum Albumin (BSA, 0.5 mg/ mL). The substances were left to dry in sterile environment.
- 5 mL of Shield and Sang medium (Table 11) and 10 µL of Fungin™ were added to the petri dish.
- 3 mL of Cl8+ cells ($5*10^5$) were added to the petri dish. The cells were cultivated for 3 days at 37 °C.

Pictures of the control spots were taking every 24 h. The success of the cell growth was evaluated by counting the viable cells in all marked spots for both the proteins and the negative control (BSA). The cells counted as vital were determined by their elongated and fibroblast-like shape. On the other hand, unhealthy cells appeared in a round cell shape (Broz 2017). Also, cell aggregates were not counted.

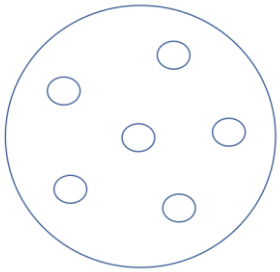

Figure 4: Cell culture positioning scheme.

3.9 Analysis techniques

3.9.1 SDS-PAGE

Self-made and pre-casted gels were used. The samples were treated with 10x concentrated sampled buffer (1:10) and were heated for 10 min (95 °C) shortly before loading the gel.

The gel was stained with Coomassie Brilliant Blue R-250™ for 24 hrs before de-staining the background (Table 16). Alternatively, the gels were used for western blot analysis instead of Coomassie staining.

3.9.2 Western Blot

- An Immobilon™-P Polyvinylidene membrane was treated with 100% methanol (20 s) followed by diH_2O (2 min).
- The membrane, together with 4 pieces of Whatman paper, two sponges and a gel holder cassette with electrodes, were transferred to a container with 1x Electro-Transfer Buffer.
- The transfer stack was arranged, and 50 Volts were applied for 200 min. The set-up was surrounded with ice and water to keep the temperature moderate.
- The membrane was stained with TCA (3%) and Ponceau powder (0.5 mg/ L).

3.9.3 Densitometry

For densitometric analysis, gels from SDS-PAGE stained with Coomassie Brilliant blue were used for scanning using GS-900™ Calibrated Densitometer (BioRad). The integrated density of the protein bands was determined by the ImageQuant™ and ImageJ™ software.

3.9.4 Mass spectrometry

For this mass spectrometric analysis, a protein band was carefully cut out using a razor and analyzed according to the description on "Identification of protein fragments by mass spectrometry" according to *Zurovec et al.* (Zurovec et al. 2016) was performed by Peter Konik (Facility "Makrokomplex" of the Faculty of Sciences, JCU). The recombinant protein was eluted from the gel, digested by trypsin and analyzed using an ESI Q-TOF premier (Waters ®) mass spectrometer and a nanoACQUITY UPLC ® (Waters ®).

3.9.5 Electron Microscopy

The protein was coated onto a polystyrene surface and analyzed by SEM according to the description on "Histology and Electron Microscopy" according to *Zurovec et al.* (Zurovec et al. 2016), to determine the spreading efficiency of the adhesive. The solvent formic acid was removed from the protein and it was partially dissolved in PBS and a minor fraction of urea (8M). A negative (PBS) control was used to put the obtained images into perspective. A Jeol 6300 scanning electron microscope was used.

4 Results

4.1 Protein expression in *E. coli*

A pilot expression of the recombinant protein was performed under the following conditions: Inoculation of 20 ml of LB media, induction with 0.2 mM IPTG, expression temperature of 37° C and expression duration of 1 to 4 hours, 1 ml aliquots of bacterial culture were collected for analysis after 1 to 4 hours, centrifuged, and pellets stored at -20 °C until bacterial cell lysis and (Figure 5).

We further tested the amount of proteins in bacterial lysates in order to optimize the cultivation and purification conditions. For Ser 3, affinity chromatography was performed followed by subsequent dialysis against diH_2O. For BARN, the inclusion bodies were dissolved in 8M urea and samples were compared on SDS-PAGE (see the gels). The amount of isolated Ser 3 was quite low (Figure 6) compared to the BARN (Figure 7). For Ser 3 the amount of protein was quantified by densitometry (Figure 9). We repeated the protocol several times with slight modification of

incubation times, lowered incubation temperature to 20 °C or increase of IPTG for induction, however the yield still was much lower than expected.

To confirm that the protein bands we observed in PAGE are the expected recombinant proteins, we performed western blotting and stained the membrane with a different method, using Ponceau S red (Figure 8) In addition, we also cut the appropriate band for sericin 3 for mass spectroscopy analysis and confirmed by 3 peptides that the band indeed was our desired protein (Table 1). Other proteins detected on a single peptide were most probably artifacts and contaminations (Table 2).

In next experiments we also tested the solubilization of recombinant proteins in inclusion bodies by lithium bromide and formic acid. Both solvents seemed to solubilize the inclusion bodies completely. Finally, formic acid was used for protein solubilization, because it can be removed from the sample by evaporation. The formic acid treated, and dried protein samples were then possible to dissolve in 8 M urea and analyze on PAGE (Figure 10).

4.1.1 SDS-PAGE

In Figure 5, the first SDS-PAGE results of the final candidates are shown. The impure proteins reveal several bands for all samples. For the Ser 3 protein, very similar results can be observed from the samples after IPTG induction (lane 1 & 3, ~24 and 49 kDA). The same bands are not seen in equal intensity for the uninduced sample. In case of the BARN protein, the most significant band appears for the pellet after induction (lane 4, ~19 kDa). It shows more than double the size of the bands seen for Ser 3. This band, however, is not found in the supernatant.

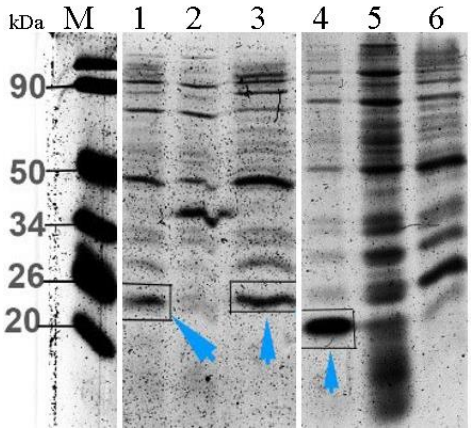

Figure 5: SDS-PAGE gel (10%) of impure recombinant proteins BARN (Megabalanus rosa, lanes 4-6) and Ser 3 (Bombyx mori, lanes 1-3). Staining of the gel was performed with Coomassie

Brilliant Blue R-250. For each protein (Name and molecular weight given), three samples were analyzed. M: protein marker (Fermentas #SM0671); 1, 4: induced expression in the pellet; 2, 5: uninduced;3, 6: induced expression in the cell cytosis. The significant bands for the proteins are highlighted by boxes and arrows.

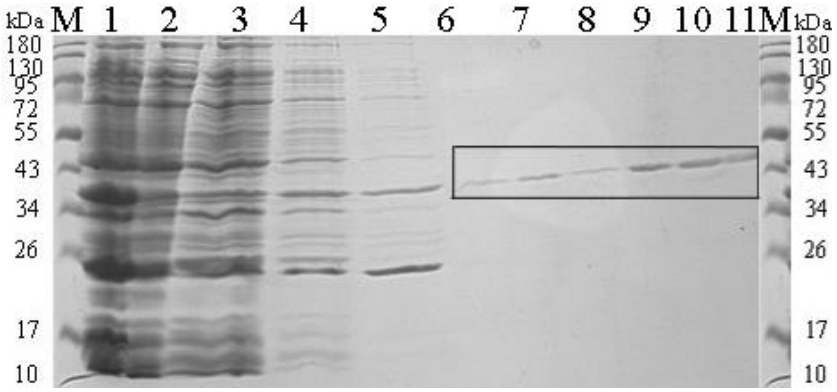

Figure 6: SDS-PAGE gel (10%) of the Ser 3 protein after affinity chromatographic purification. M: protein marker (Fermentas #SM0671); 1: induced expression in the pellet; 2: induced expression in the cell cytosis; 3: Flow-through fraction of the purification process; 4-5: Washing fraction. 6-11: Elution fractions. Loading volume: 10 µL protein marker, 15 µL samples. The marker column on the right side was added as a copy of the original marker column loaded onto the gel (left side). The significant bands for the elution fractions are highlighted by a box.

Figure 6 shows an SDS-PAGE gel of different fractions drawn during the purification procedure of Ser 3. The progress in purification can be seen from left to right, as the number of bands decreases. For "WF", the gel shows two similarly strong bands (lane 4-5, ~24 kDA and ~37 kDa). The lower band, however, disappears for the elution fractions (lane 6-11) and only the higher one remains. Especially the bands from lane 6-8 show very low intensity and suggest a small amount of protein. The bands from lane 9-11 are stronger in comparison. Throughout the elution fractions, the single band shifts upwards (~37 kDa → ~49 kDa).

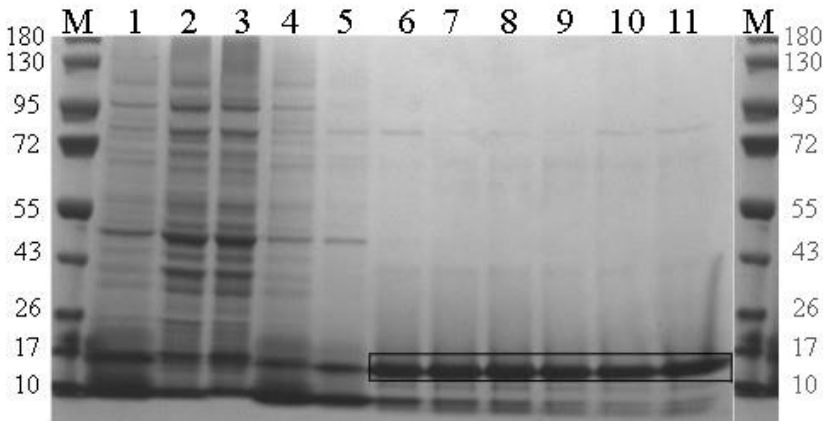

Figure 7: Pre-casted gradient SDS-PAGE gel (BioRad 4-15%) of different fractions from the purification process of BARN. M: protein marker (Fermentas #SM0671); 1: induced expression in the pellet; 2: Flow-through; 3-5: Washing fraction; 6-11: Elution fractions. Loading volume: 10 µL protein marker, 15 µL samples. The marker column on the right side was added as a copy of the original marker loaded onto the gel (left side). The significant bands for the elution fractions are highlighted by a box.

Figure 7 shows the SDS-PAGE analysis of the purified Barnacle protein, gained after affinity chromatography purification. It reveals a strong response at ~15 kDa for all elution fractions (lane 6-11), suggesting a high amount of protein. Another band at ~9 kDa, that is most significant in the washing fractions (lane 3-5), gets weaker throughout the elution fractions (6-11).

4.1.2 Western Blot

Figure 8 shows the response for a sample of induced expression of Ser 3 in the pellet on a stained membrane (bottom) and an SDS-PAGE gel (top). When comparing both pictures, a particular band (~36-42 kDA) catches the eye, that shows high intensity on both the gel (lane 1) and the membrane (lane 2).

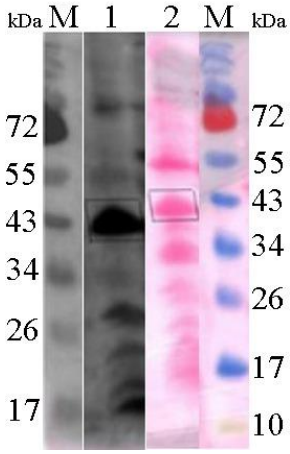

Figure 8: Composition of a stained membrane (Right M+2) and a stained gel (Left M+1) after Western blot electroblotting. Staining: Ponceau S (Membrane), Coomassie Brilliant Blue R-250 (Gel). M: protein marker (Fermentas #SM0671); 1: induced expression in the pellet (gel); 2: induced expression in the pellet (membrane). The significant bands for Ser 3 are highlighted by boxes.

4.1.3 Densitometry

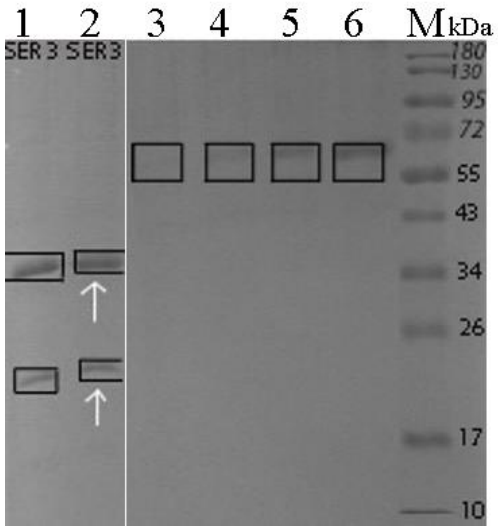

Figure 9: Gel (10%) after purification of Ser 3 (18 µL per column). Staining: Coomassie Brilliant Blue R-250. M: protein marker (Fermentas #SM0671); 1-2: Elution fractions; 3-6: BSA, concentration: 0.1875, 0.375, 0.75, 1.5 mg/ mL. Loading volume: 10 µL protein marker, 10µL BSA standards, 15 µL samples. The bands for the protein and the standards are highlighted by boxes. The white arrows mark the bands used for densitometric analysis.

The gel shown in Figure 9, shows two different bands for both samples containing the purified Ser 3 protein (lane 1-2, ~22-23 kDa, ~35 kDa). The intensity for the upper bands appears to be higher in both lane 1 & 2. The differently concentrated BSA standards (lane 3-6), were used to quantify the protein amount by densitometric analysis (ImageQuant ™). The outcome of the calculation revealed 1.895 mg/ mL for the upper band and 0.496 mg/ mL (lane 2, R^2: 0.998) (Ito et al. 2018).

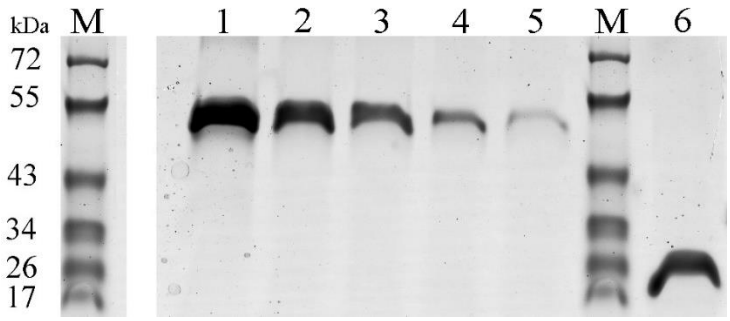

Figure 10: Gel (Gradient 4-15%, BioRad-Mini-Protean Precast Gel) after dissolution of the inclusion bodies in formic acid. M: protein marker (Fermentas #SM0671); 1-5: BSA, concentration: 1, 0.4, 0.2, 0.1, 0.05 mg/ mL; 6: BARN after inclusion body purification and single-step dialysis. Loading volume: 10 µL protein marker, 10 µL BSA standards; 15 µL BARN.

Figure 10 shows BARN at ~28 kDa (lane 6). The protein was further quantified by densitometric analysis, utilizing the ImageJ™ software. The result was calculated, utilizing the BSA standards (lane 1-5) to form a trendline (R^2: 0.9497) and deriving the concentration from the formula of the applied linear fit. The concentration of BARN on this gel is 0.655 mg/ mL (Schneider et al. 2012).

4.1.4 Mass spectrometry

Table 1: List of sequences detected from a protein band that was analyzed by mass spectrometry. The 3 sequences shown here, were matched in two different databases with the Bombyx mori sericin 3 sequence and have proved to be peptides included in the sequence. The table shows the which databases were used, along with the corresponding accession number for Bombyx mori sericin 3. Besides the amino acid sequence, also the retention time and the peak molecular weight of the peptides is given.

Database	Accession number	Sequence	Retention Time/ min	Molecular Weight/ Da
NCBI, UniProt	BAF81029, A8CEQ1	(K)QAQSSSSQQAQSSR(S)	8,9794	1479,676
		(R)GSSGDNSDDDQTDSAR(S)	9,5563	1626,611
		(R)LSVINNTEGHATSSSFDEQSSSARQSSSSYQSQSYNK(D)	24,0294	3998,774

Table 2: List of contaminants found by mass spectrometry analysis of Ser 3. The list shows the name of each database that was used to determine the identity of each contaminant as well as the corresponding accession number. The list further shows the description and the molecular weight of each contaminant.

Database	Accession number	Description	Molecular Weight/ Da
NCBI	XP_004923293.1	uncharacterized protein LOC101743063 [Bombyx mori]	79512
	XP_004928370.1	homeobox protein MOX-2 [Bombyx mori]	20143
	XP_004931616.1	ER membrane protein complex subunit 8/9 homolog [Bombyx mori]	24149
	XP_012552293.1	uncharacterized protein LOC105842664, partial [Bombyx mori]	27214
	XP_021205902.1	uncharacterized protein LOC101743893 isoform X2 [Bombyx mori]	68454
	BAK82124.1	glycosyltransferase [Bombyx mori]	47691
Uniprot	H9IW46	Uncharacterized protein OS=Bombyx mori OX=7091 GN=LOC101743893 PE=4 SV=1	69517
	H9JFW2	Uncharacterized protein OS=Bombyx mori OX=7091 PE=4 SV=1	17834
	H9JKH0	Uncharacterized protein OS=Bombyx mori OX=7091 GN=LOC101743063 PE=4 SV=1	79512
	H9JQS8	Uncharacterized protein OS=Bombyx mori OX=7091 GN=LOC101745108 PE=4 SV=1	24149

	H9JD88	Uncharacterized protein OS=Bombyx mori OX=7091 GN=_Bre4 PE=4 SV=1	42615
Swissprot	P17169	Glucosamine--fructose-6-phosphate aminotransferase	66721
	P77398	Hypothetical protein yfbG.	74242
	P04264	Keratin_ type II cytoskeletal 1 (Cytokeratin 1)	65846
	P13645	Keratin_ type I cytoskeletal 10 (Cytokeratin 10)	59482
	P35908	Keratin_ type II cytoskeletal 2 epidermal	65825
	P35527	Keratin_ type I cytoskeletal 9 (Cytokeratin 9)	61949
	P13645	Keratin_ type I cytoskeletal 10 (Cytokeratin 10)	59877
	P35908	Keratin_ type II cytoskeletal 2 epidermal	16228
	P35527	Keratin_ type I cytoskeletal 9 (Cytokeratin 9)	53854
	P02538	Keratin_ type II cytoskeletal 6A (Cytokeratin 6A)	24393
	P00698	Lysozyme C precursor (EC 3.2.1.17)	62423
	Q10758	Keratin_ type II cytoskeletal 8 (Cytokeratin 8)	11276
	P00761	Trypsin precursor (EC 3.4.21.4).	29887
	P13647	Keratin_ type II cytoskeletal 5 (Cytokeratin 5)	61120
	P81605	Dermcidin precursor (Preproteolysin)	73966
	P05782	Keratin_ type I cytoskeletal 47 kDa (Fragment).	51458
	P02349	30S ribosomal protein S1.	65405
	P00955	Threonyl-tRNA synthetase (EC 6.1.1.3)	64381
	P02533	Keratin_ type I cytoskeletal 14 (Cytokeratin 14)	27240
	P32132	GTP-binding protein typA/BipA (Tyrosine phosphoryl	71378

Table 2 shows all contaminants found after mass spectrometric analysis of a band from the expressed recombinant Ser 3 protein. Several matches from the databases NCBI™ and uniprot™, could not be exactly determined but were linked to *Bombyx mori*. Many matches found by the swissprot™ database, were determined as keratin (*Homo Sapiens*). Further findings, include remains of the LB media (Trypsin precursor, *Sus scofra*), remains of the lysozyme (Lysozyme C precursor, *Gallus gallus*) and proteins that stem from the expression host (e.g. Glucosamine-fructose-6-phosphate aminotransferase, *E. coli*) (UniProt: a worldwide hub of protein knowledge 2019; Geer et al. 2010).

As the gel in Figure 6 suggests, the expression and purification of Ser 3 (37-49 kDa) was successful as only a single band remains in the elution fractions. Figures 5 (~49 kDa), 8 (36-42 kDa) and 9 (~35 kDa) suggest bands of different size for the same protein. Mass spectrometric analysis of a sample that was expressed analogously, revealed peptides overlapping with the sericin 3 sequence (Table 1). Therefore, the expression protocol used yielded the desired protein. The purification procedure was not flawless in all cases as a list of contaminants shows that were found by mass spectrometry (Table 2). Also, Figure 9 revealed a contaminant/degradation product that was still present after affinity chromatography. Densitometric analysis of Figure 9 quantified the expressed protein (~35 kDa) to yield 1.895 mg/ mL.

4.2 Characterization of biocompatibility

To test the biocompatibility of the recombinant proteins, polystyrene surfaces were covered with the lyophilized proteins (0.5 mg/ mL, 1 mg/ mL) and cells were cultivated in adequate media. The surfaces were sectioned into different areas containing different proteins and a negative control (BSA). Pictures were taken from these areas (Figure 12) every 24 hrs. for three days and the cells were counted. The surfaces coated with BARN (Figure 11, 13) showed higher cell counts than those covered with Ser 3 (Figure 13). No significant difference was observed for the different concentrations, the proteins were used in (Figure 15).

4.2.1 Cell culture

4.2.1.1 Results for BARN protein

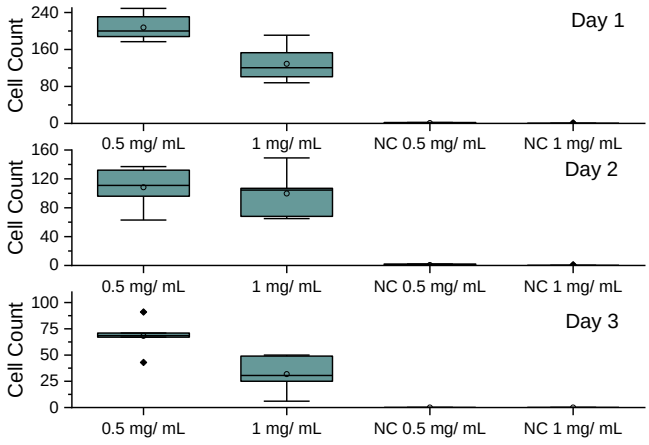

Figure 11: Cell Count for the first Culture Experiments using the BARN protein. NC: negative control (BSA). Y-axis: Median of the number of vital cells counted for each time point. X-axis: Concentration of protein utilized for surface coating in mg/ mL. Day 1: Median: 34 (0.5 mg/ mL), 27.5 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL); Day 2: Median: 188.5 (0.5 mg/ mL), 86.5 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL); Day 3: Median: 20.5 (0.5 mg/ mL), 43.5 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL).

Figure 11 shows the results of the cell count after performing a Cell Culture experiment, using the BARN after inclusion body purification. Figure 11 reveals a higher average cell count throughout all 3 days for the spots coated with 0.5 mg/ mL BARN (Figure 11), thus suggests a preference over the higher concentrated protein. For all surfaces coated with BARN, a general decrease in cell count can be observed as time passes.

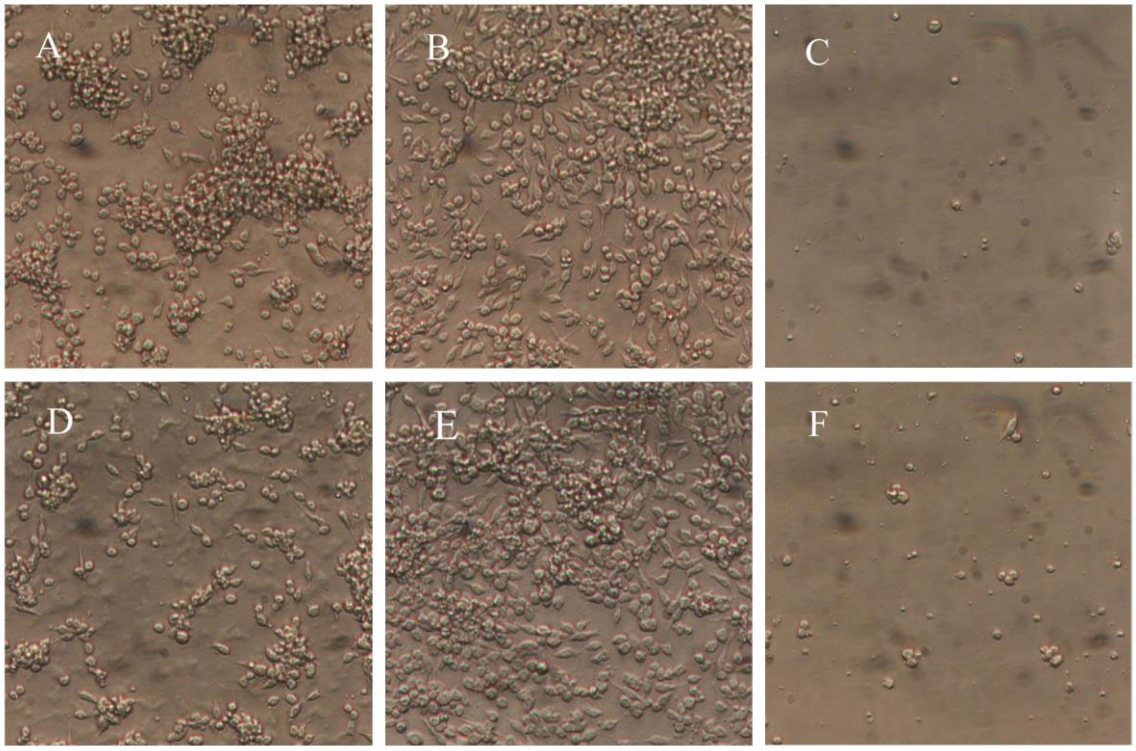

Figure 12: Tissue culture using Cl8+ cells on polystyrene surface, grown at Ser 3, BARN and BSA in different concentrations after 48 hrs. of incubation (37°C). The pictures were taken by light microscope. A: Ser 3 0.5 mg/ mL; B: BARN 0.5 mg/ mL; C: BSA: 0.5 mg/ mL; D: Ser 3 1 mg/ mL; E: BARN 1 mg/ mL; F: BSA 1 mg/ mL.

Figure 12 depicts the difference in biocompatibility dependent on what substance the cells were grown. The images were taken after 48 hrs. of incubation at 37°C. After 48 hrs. cultivation without changing the medium, the amount of cell clusters was already high in most cases (A-B, E). For Ser 3(A, D), the number of viable cells was lower than for BARN (B, E). “A” (0.5 mg/ mL, Ser 3) revealed a higher number of cell aggregates compared to “D” (1 mg/ mL, Ser 3). For “B” & “E”, there is no clear difference in response. “C” & “F” show no viable cells, however, for “F” (1 mg/ mL, BSA) there is a higher number of deformed cells.

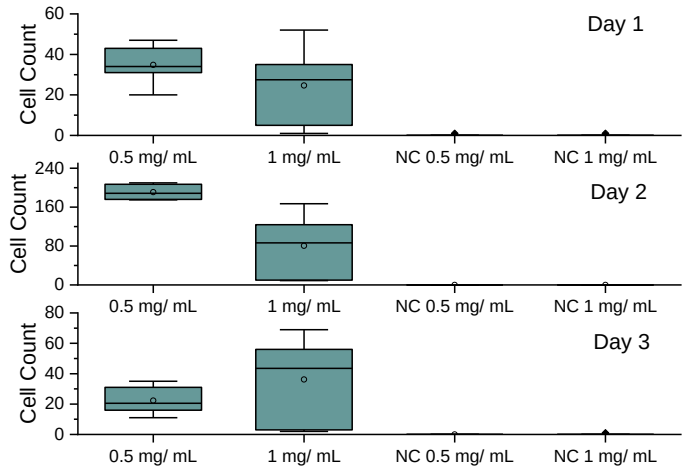

Figure 13: Cell Count for the second Culture Experiments using the BARN protein. NC: negative control (BSA). Y-axis: Median of the number of vital cells counted for each time point. X-axis: Concentration of protein utilized for surface coating in mg/ mL. Day 1: Median: 189 (0.5 mg/ mL), 105 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL); Day 2: Median: 109 (0.5 mg/ mL), 103 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL); Day 3: Median: 67 (0.5 mg/ mL), 34 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL).

Throughout all 3 days of the experiment, vivid cells were counted for the spots coated with BARN whereas the surfaces coated with BSA showed no healthy cells. For the first 24 hrs. of the experiment, the number of healthy cells is higher for the surfaces with lower concentration of BARN. However, after the third day, the cell count for the higher concentrated protein is greater in average. This contradicts the trend seen in Figure 11. The average response for the BSA coated surfaces in Figure 13 is identical with the response in Figure 11, as no vivid cells were counted. In contrast to Figure 11, the cell count in Figure 13 increases within the first 24 hrs. and decreases throughout the following day. Especially for 0.5 mg/ mL, the decrease appears drastically.

4.2.1.2 Results for Ser 3

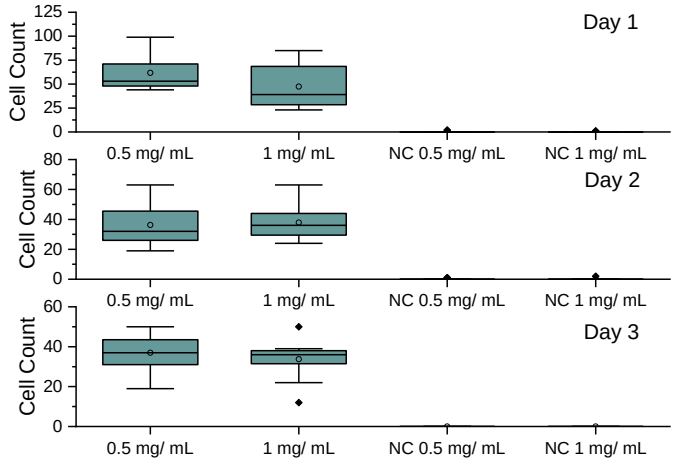

Figure 14: Cell Count of Ser 3 Cell Culture Experiments. NC: negative control (BSA). Y-axis: Median of the number of vital cells counted for each time point. X-axis: Concentration of protein utilized for surface coating in mg/ mL. Day 1: Median: 53 (0.5 mg/ mL), 34 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL); Day 2: Median: 33 (0.5 mg/ mL), 36 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL); Day 3: Median: 39 (0.5 mg/ mL), 35 (1 mg/ mL), 0 (NC 0.5 mg/ mL), 0 (NC 1 mg/ mL).

Figure 14 shows the successful cultivation of Cl8+ cells on Ser 3 coated surface areas. The response for the average cell growth on the protein in different concentrations, appears similar and does not suggest any clear preference. The response for BSA coated surfaces was negative. Interestingly, the number of healthy cells does not change significantly throughout the experiment.

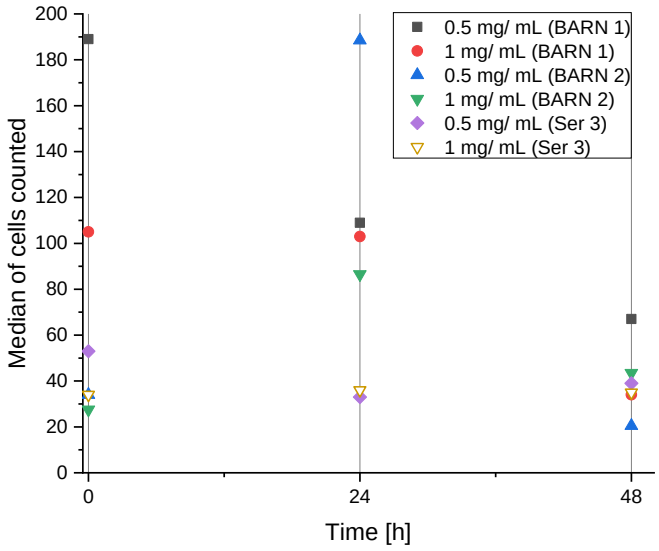

Figure 15: Graphical display of the trend in cell count throughout the cell culture experiments described above. BARN 1: results displayed in Figure 11; BARN 2: results displayed in Figure 13; Ser 3: results displayed in Figure 14. The proteins were applied in two different concentrations (0.5, 1 mg/ mL). The cell count for the negative control is not shown in this graph as the median for all counts resulted in 0 (Figures 11, 13-14). Y-axis: median of the number of vital cells counted for each time point. X-axis: time passed since performing the initial count.

Figure 15 suggests a general preference of the cells for the BARN, as the average cell count for all experiments utilizing BARN, is higher than for Ser 3 after 24 hrs. of incubation. Also, the change in cell count is far more significant for BARN as 0.5 mg/ mL (BARN 2) shows (Figure 15).

Despite of the ability of recombinant proteins to support cell growth, the adhesivity and the relatively short period of time for which the cells were able to grow on the recombinant protein films, suggest that protein purity and quality of coating would need improvement in future experiments.

4.3 SEM Microscopy

To examine the quality of surface coating, a polystyrene surface coated with BARN was analyzed by SEM to determine its spreading efficiency compared to PBS (negative control).

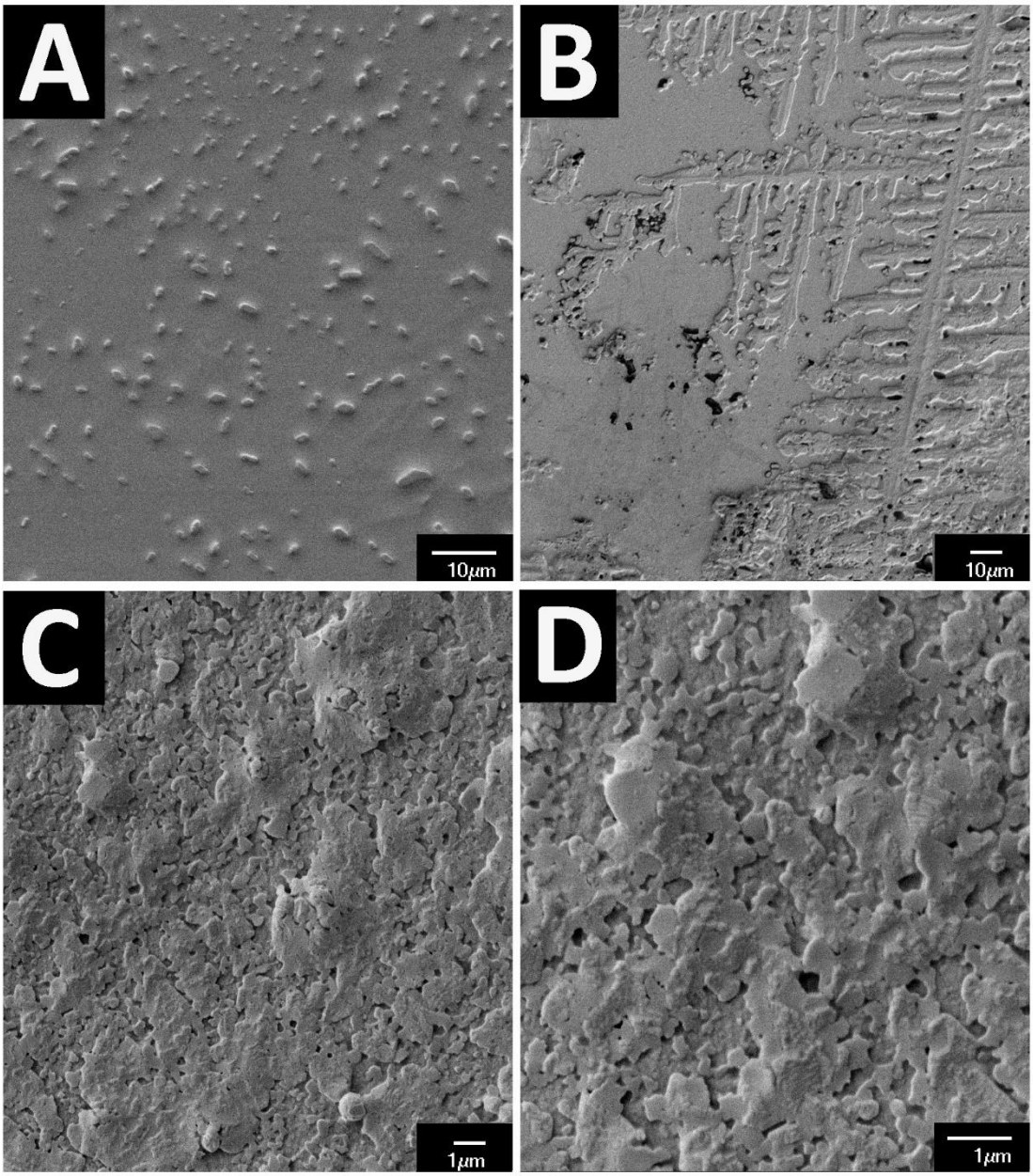

Figure 16: Images showing a BARN coated polystyrene surface in different magnification, as well as a PBS coating of the same type of surface. The images were taken by SEM.
A: Negative Control PBS 1 000x, B: BARN layer 500x, C: BARN layer 5,000x, D: BARN layer 10,000x.

Figure 16 shows a selection of representative images of the recombinant barnacle protein as coating material. The images of BARN coating a polystyrene surface are presented in different

magnification (B-D). “A”, shows the negative control using PBS as coating material. Images B-C reveal porous structures and show uneven spreading. From image A, also an uneven surface can be observed, showing various aggregates of the components throughout the figure.
The results from electron microscopy suggest that the protein coating contained contaminated salts and higher protein purity would be needed for future applications.

5 Discussion

5.1 Protein Expression

5.1.1 Cultivation

Ser 3 was successfully expressed by *E. coli* in cytosol and verified by mass spectrometry (Table 1). However, low yield and impurities that were detected by mass spectrometry (Table 2), suggest poor efficiency for the purification procedure. As the insoluble fraction of Ser 3 showed equal amount of protein as the soluble (Figure 5), either measures should be taken to avoid the formation of inclusion bodies, or the protein has to be extracted from the aggregates to increase the yield. According to *Collins et al. (2013)*, the use of medium richer in nutrients as terrific broth (TB) and super broth (SB) has proven to increase the yield (Collins et al. 2013). For moving to larger scale production of the proteins, utilizing a fermenter is recommended (Hardy and Scheibel 2009).

Affinity chromatography of his-tag modified recombinant proteins, expressed by bacterial expression systems, using imidazole containing buffers, as performed in this study, is also reported in literature (Hoffmann and Roeder 1991; Scheich et al. 2003). A more cost-intensive variation of this techniques involves the use of magnetic affinity beads (Scheich et al. 2003).

Cultivation of BARN led to increased expression of the protein in inclusion bodies. According to *Rosano et al. (2014)* the formation of inclusion bodies results from either incorrect folding of the proteins or incorrect disulfide bond formation that results from high-level expression that exhausts the host. Therefore, the protein not only has to be extracted from these aggregates but also the yield decreases due to the higher misfolding rate. Cultivating the *E. coli* at lower temperature would cause the culture to grow slower and decrease the rate of misfolding. Literature therefore suggests, to utilize strains with cold-adapted chaperones. As this would cause the production rate to decrease, *Rosano et al. (2014)* also suggests other alternatives, like co-expressing molecular chaperones or supplementing them via the media (Rosano and Ceccarelli 2014; Singh et al. 2015). *Baneyx et al. (1999)* suggests, to utilize *E. coli* the cold shock promotor

CspA, as it is a well characterized promotor that is stable at low temperatures (Baneyx 1999). *Yang et al. (2011)*, was successful in expressing insoluble proteins in soluble form after cold shock expression (pCOLD DNA vector system) and proving them by Western Blot. However, the yield was too low to visualize the protein on SDS-PAGE (Yang et al. 2011).

Palmer et al. (2004), suggest the use of guanidine HCl instead of urea, to extract the protein from the inclusion bodies. *Batas et al. (1999),* approves the approach to purify means of centrifugation as performed in this research, as it separates the protein from the cell debris. They further suggest gel filtration and membrane filtration as adequate alternatives (BATAS et al. 1999). Also, Nickel-chelate affinity chromatography is recommended, as the protein is his-tagged (Palmer and Wingfield 2004)

After extracting BARN by dissolution in urea, single-step dialysis was performed to facilitate refolding of the protein. As *Yamaguchi et al. (2014)* suggests, multi-step dialysis is preferred over one-step dialysis, however, also more time intensive (Yamaguchi and Miyazaki 2014). Successful stepwise dialysis from urea has been reported in literature (Jung et al. 2003). This might represent the most difficult step in the entire procedure of recombinant protein preparation.

5.1.2Analysis

SDS-PAGE and Western Blot analysis of the Ser 3 revealed a different band size (~ 36-49 kDa Figures 6,7&9) than was to expect from the originally suggested molecular weight of the protein (16.8). However, literature reports that due to its unique amino acid composition, the mass of the Ser 3 protein may appear higher when visualized on SDS-PAGE (Takasu et al. 2007). Huang et al., also observed this phenomenon when analysing “sericins” using LDS-PAGE (Lithium dodecyl sulphate-PAGE). Literature further suggests potential intrinsic net charge or protein oligomerisation to cause the band to appear with double of the size (Huang et al. 2003).

In case of Figure 9, a second band appeared (lane 1-2, ~22-23 kDa) that may resemble a degradation product of *E. coli* origin (Hengen 1995). As *Hengen (1995)* suggests, expression using an empty plasmid could be conducted analogously to verify whether the band is of *E. coli* origin or resembles external contamination (Hengen 1995).

The amount of Ser 3 (1.895 mg/ mL) calculated by densitometry (lane 2, Figure 9) appears high, as *Collins et al. (2013)* expects 5-300 mg/ mL from expressing biopolymers in shake flasks

(Collins et al. 2013). However, as mass spectrometry shows, the protein was not fully purified and thus the value is incomparable to literature.

BARN (Mw: 17.8 kDa) also showed different results on SDS-PAGE. The protein expressed in cytosol showed a band around 19 kDa (Figure 5) and 15 kDa (Figure 8). After inclusion body purification, a band at 28 kDa (Figure 11) was visualized. Deviation of the size estimated by SDS-PAGE from the molecular size, has also been reported for a different protein contained in the barnacle cement of *M. rosa* (Urushida et al. 2007). The difference in size, however, was not as significant as seen for BARN. The protein mentioned by *Urushida et al.* (2007), was also expressed in *E. coli* , however, it was expressed in cytosol (Urushida et al. 2007). Literature further shows the testing of rMrcp-20k that BARN was derived from. SDS-PAGE analysis of rMrcp-20k also showed a deviation of mass from the originally evaluated molecular mass (Mori et al. 2007). Hence, it is suggested to support the verification by SDS-PAGE with additional methods as mass spectrometry.

5.2 Cell Culture

The cell culture experiment was successful for both proteins, as vivid cells were counted throughout all three days of the experiment. No cells were counted for the BSA coated surface that was used as negative control (Figures 11-15). The low number of cells counted for the BSA coated area suggests good spreading of the protein (Humphries 2009). The results suggest a preference for BARN, as the average cell count was predominantly higher than for Ser 3 (Figure 15) The general activity of Ser 3 was arguably low, as the average cell count was similar throughout the 48 hrs. of the experiment (Figure 14). The difference in concentration that the proteins were applied in, appeared to not correlate to the number of cells counted (Figures 11-15). Literature suggests, to use a positive control besides the negative control, to put the quantitative result of a cell adhesion assay into better relation (Wohlrab et al. 2012). The performance of staining assays to distinguish the living cells from the dead is further recommended in case a non-toxic stain is available (Wohlrab et al. 2012). Further research should include the testing of different cell strains on different surfaces to further test the adhesion capacity of the proteins as well as the specific biocompatibility.

5.3 SEM Microscopy

The determination of spreading efficiency of BARN by SEM revealed an uneven, porous surface for the coated polystyrene dish, showing that the protein is clearly not in its native state

(Figure 16, B-C). The poor spreading may have resulted from remaining formic acid that has not been fully removed from the sample before coating and evaporating during the process of visualization. Also, improper folding of the protein may be the cause of the formation of these structures. The negative control (PBS, Figure 16, A), also revealed an uneven surface but shows aggregates of poorly spread components rather than porosity. Literature shows an approach using SEM and phase contrast microscopy to visualize adherent cells. The effectiveness in spreading is quantified by calculating the surface density and surface concentration of the adhesive (Massia and Hubbell 1991). Fluorescence staining and Immunostaining microscopy has also been reported to be promising techniques for assessing the spreading effectiveness (Massia and Hubbell 1991; Singer et al. 1988). To gain further knowledge on bonding efficiency of the proteins, adhesion test as the shear, peel or tensile test should be conducted (Ebnesajjad 2008). Additionally, adhesive force measurements could be performed by atomic force microscopy (AFM) (Bhushan and Dandavate 2000; Hwang et al. 2004).

6 Conclusion

Adhesive biopolymer materials are a promising alternative for chemical substances in many applications in medicine and cosmetic industry. In this bachelor's thesis, the promising adhesive, recombinant proteins Ser 3 and BARN were expressed in a bacterial expression system and after affinity chromatographic purification, their biocompatibility was tested successfully tested in a tissue culture experiment. SDS-PAGE and Western Blot were used to verify the presence of the protein after different steps of the expression and purification process. In case of Ser 3, mass spectrometry was used for analysis. SEM was used to assess the spreading efficiency of BARN. The results revealed the proper folding of the proteins as the major complication of this thesis.

Optimization of protocol expression

SDS-PAGE and Western blot analysis showed bands of a size that do not correspond the originally estimated molecular size. For Ser 3(16.8 kDa), bands appeared in a range of 36-49 kDa (Figures 5-6, 8-9). Deviations of this kind are, however, reported in literature (Huang et al. 2003; Takasu et al. 2007). Ser 3 was further verified by mass spectrometry, that revealed 3 peptides originating from the recombinant sericin 3 sequence (Table 1). However, mass spectrometry also revealed several contaminants (Table 2). For BARN (16.8 kDA), that had to be purified from inclusion bodies, the results showed very different results in a range of 15-28 kDA (Figures 5, 7, 10) Deviations of this kind are also reported for a similar protein (Mori et al. 2007).

Further studies should include adaptations concerning the expression and purification methods, as mentioned in the discussion.

Characterization of biocompatibility

The tissue culture experiments showed success for both proteins, as vivid cells were counted throughout all 3 days of the experiment (Figures 11-15). The results suggest no clear preference for any of the concentrations (0.5, 1 mg/ mL) the proteins were applied but suggest a slightly better biocompatibility for BARN over Ser 3. To be able to distinguish the vivid cells from the dead, fluorescence staining should be performed in further research.

Examination of recombinant protein films

The SEM images from polystyrene surface coated with BARN, show uneven, porous spreading that may result from evaporation of formic acid during the performance of microscopy or improper folding of the protein (Figure 16, B-D). The negative control PBS also showed uneven spreading although the results are not similar to the ones seen for the protein, as PBS contains of non-adherent salts that rather show aggregates than porous structures (Figure 16, A). To assess the binding capacity of the proteins more efficiently, further studies should include adhesion tests and atomic force microscopy.

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8 Appendix:

8.1 Chemicals and Media

The Luria Broth is the liquid medium used to cultivate the *E. coli* bacteria. It consists of tryptone, yeast extract, NaCl and diH_2O, as stated in table 2. The agar-LB medium is used for the cultivation of the *E. coli* on the streak plates and includes agarose alongside the LB ingredients. For both media, the solid compounds are poured together and filled up with H_2O. Subsequently, the mixture is mixed via a magnetic stirrer and afterwards autoclaved.

Table 3: Contents of the LB medium and the Agarose-LB medium*.

Substance	Percentage / %
Tryptone	1.0
NaCl	1.0
Yeast extract	0.5
Agar*	1.5*
diH2O	97.5 /96*

Natural Lysis Buffer (NLB)

Table 4: Composition of NLB. The Buffer is adjusted to pH 8, by addition of HCl (conc.). The lysozyme content is 0.1%.

Substance	Concentration / mM
Na_2HPO_4	25
NaCl	250
Lysozyme	
diH_2O	

Natural Binding Buffer (NBB)

Table 5: Composition of NBB. The Buffer is adjusted to pH 8, by addition of HCl (conc.).

Substance	Concentration / mM
Na_2HPO_4	25
NaCl	250
diH_2O	

Washing Buffer (WB)

Table 6: Composition of WB. The Buffer is adjusted to pH 8, by addition of HCl (conc.).

Substance	Concentration / mM
Na_2HPO_4	25
NaCl	250
Imidazole	20
diH_2O	

Elution Buffer (EB)

Table 7: Composition of EB. The Buffer is adjusted to pH 8, by addition of HCl (conc.).

Substance	Concentration / mM
Na_2HPO_4	25
NaCl	250
Imidazole	250
diH_2O	

Inclusion Body Buffer 1 (IBB 1)

Table 8: Composition of IBB 1. The Buffer is adjusted to pH 7 by NaOH (1M). TRIS is an abbreviation for tris(hydroxymethyl)aminomethane.

Substance	Concentration / mM
NaCl	100
TRIS/ HCl	100
EDTA	1
diH_2O	

Inclusion Body Buffer 2 (IBB 2)

Table 9: Composition of IBB 2. The Buffer is adjusted to pH 7 by HCl conc.

Substance	Content
NaCl	1.5 M
Triton™ X-100	2 %
EDTA	60 mM
diH_2O	

Inclusion Body Buffer 3 (IBB 3)

Table 10: Composition of IBB 3. The Buffer is adjusted to pH 7 by NaOH (1M). TRIS is an abbreviation for tris(hydroxymethyl)aminomethane.

Substance	Concentration / mM
NaCl	100
TRIS/ HCl	100
EDTA	20
diH_2O	

Cl8+ Medium- CM

Table 11: Composition of 250 mL of the Cl8+-CM medium.

The pH of the mixture is adjusted to 6.5 by HCl (conc).

Substance	Content
Shield and Sang M3 (Sigma-Aldrich)	9.85 g
$KHCO_3$	0.125 g
Drosophila extract	2.5 %
BSA (10%)	2 %
Penicillin/ Streptomycin	1 %
Insulin	125 IU^{-1}
diH_2O	250 mL

Separation Gel (10%)

Table 12: Composition of a 10% Separation Gel for a single gel for SDS-PAGE. After addition of Acrylamide, AA and diH_2O, the mixture is shortly boiled in a microwave and subsequently placed on ice to avoid bubble formation when applying the gel to the set-up. The polymerizing agent APS and its catalyst TEMED® are added shortly before adding the mixture between the glass plates to avoid premature polymerization. TRIS is an abbreviation for tris(hydroxymethyl)aminomethane.

Substance	Volume/ µL
Acrylamide-Bis-acrylamide (AA, 29:1, 33%)	2290
4x TRIS-HCl (pH 8.8)	1875
TEMED	5.6
Ammonium persulfate (APS, 10%)	56
diH_2O	3125

Stacking Gel 5% (2 mL)

Table 13: Composition of a 5% stacking gel for a single gel for SDS-PAGE. As for the separation gel, TEMED and APS are added last.
TRIS is an abbreviation for tris(hydroxymethyl)aminomethane.

Substance	Volume/ µL
AA (33%)	525
4x TRIS-HCl (pH 6.8)	940
TEMED	5.6
APS (10%)	56
diH_2O	2250

Sample Buffer 6x

Table 14: Composition of a 6 times concentrated Sample Buffer for samples loaded onto an SDS-PAGE gel. TRIS is an abbreviation for tris(hydroxymethyl)aminomethane.

Substance	Content
Glycerol	3.6 mL
4x TRIS-HCl (pH 6.8)	7 mL
Sodium dodecyl-sulphate (SDS)	1 g
Dithiothreitol (DTT)	0.93 g
Bromophenol Blue	1.2 mg
diH_2O	10 mL

Running Buffer (RB)

Table 15: Composition of the Running Buffer. The percentage of SDS in the mixture is 1%. TRIS is an abbreviation for tris(hydroxymethyl)aminomethane.

Substance	Concentration / M
TRIS Base	0.25
Glycine	1.92
Sodium dodecyl-sulphate (SDS)	
diH_2O	

Destaining-solution

Table 16: Composition of one liter of destaining solution to reduce the background of the polyacrylamide gel after Coomassie-blue staining.

Substance	Volume/ µL
Acetic Acid	400
Methanol	100
diH_2O	500

Electro-transfer Buffer (ETB) 10x (1L)

Table 17: Composition of one liter of the 10x concentrated Electro-transfer Buffer. Salts are dissolved and filled up to one liter with diH_2O. TRIS is an abbreviation for tris(hydroxymethyl)aminomethane.

Substance	Mass / g
Tris (hydroxymethyl)-aminomethane (TRIS)	30.3
Glycine	144
diH_2O	

ETB 1x (1L)

Table 18: Composition of one liter of the 1x concentrated ETB.

Substance	Volume/ mL
ETB 10x	100
Methanol	200
diH_2O	700