Optimization of recombinant antibody production in the form of a single chain variable fragment (scFv) antibody

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MEDICINSKI FAKULTET

DIPLOMSKI SVEUČILIŠNI STUDIJ

SANITARNOG INŽENJERSTVA

Merima Ćulah

OPTIMIZACIJA PROIZVODNJE REKOMBINANTNIH PROTUTIJELA U OBLIKU JEDNOLANČANOG VARIJABILNOG FRAGMENTA (scFv) PROTUTIJELA

Diplomski rad

Rijeka, 2022

Mentor rada: Prof. dr. sc. Tihana Lenac Roviš

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M. Ć.

Contents

SUMMARY

The development of recombinant insulin protein as a drug was a major step in the history of medicine. Today, the use of recombinant biological drugs is growing, which includes recombinant antibodies, a third of which is produced in *Escherichia coli*. Although these prokaryotic cells do not possess all the mechanisms for posttranslational modifications of proteins, *E. coli* has an oxidative environment for proteins in its periplasmic space where proteins can be directed for proper structuring. An important form of recombinant antibody, characterized by a very low molecular weight with preserved target molecule recognition properties, is the single-chain variable antibody fragment (scFv). The aim of this work is to investigate which factors result in higher yield and purity during scFv production in *E. coli*. Three methods of protein isolation from periplasm were used. Plasmid pOPE101 was used to transform *E. coli* pREP-DE3 strain with the scFv protein gene. The identity of the pOPE101-scFv expression vector was verified by restriction analysis, and high protein expression was achieved by overnight induction at 18°C. The scFv expression was confirmed by SDS-PAGE technique and Western blot analysis. We have shown that protein isolation from the periplasmic space is improved by vigorous stirring after cell resuspension and that double osmotic shock is the best technique for releasing soluble scFVs. It was observed that a large amount of scFv still remains in insoluble form. The obtained scFvs were also successfully isolated using the immobilized metal affinity chromatography (IMAC) and binding of the His tag, without loss during chromatography. To remove residual contaminants, a subsequent purification step based on size exclusion is recommended.

Keywords: recombinant protein production, antibody, antibody fragments, *Escherichia coli*, protein isolation.

SAŽETAK

Razvoj rekombinantnog inzulinskog proteina kao lijeka bio je veliki korak u povijesti medicine. Danas je sve veća upotreba rekombinantnih bioloških lijekova, što uključuje rekombinantna protutijela, od kojih se trećina proizvodi u *Escherichia coli*. Iako ove prokariotske stanice ne posjeduju sve mehanizme za posttranslacijske modifikacije proteina, *E. coli* ima oksidativno okruženje za proteine u svom periplazmatskom prostoru gdje se proteini mogu usmjeriti na pravilno strukturiranje. Važan oblik rekombinantnog protutijela, kojeg karakterizira vrlo niska molekularna težina sa očuvanim svojstvima prepoznavanja ciljne molekule, je jednolančani varijabilni fragment protutijela (scFv). Cilj ovog rada je istražiti koji čimbenici rezultiraju većim prinosom i čistoćom tijekom proizvodnje scFv u *E. coli*. Korištene su tri metode izolacije proteina iz periplazme. Plazmid pOPE101 korišten je za transformaciju *E. coli* pREP-DE3 soja s scFv proteinskim genom. Identitet ekspresijskog vektora pOPE101-scFv verificiran je restrikcijskom analizom, a visoka ekspresija proteina postignuta je indukcijom preko noći na 18°C. Ekspresija scFv potvrđena je SDS-PAGE tehnikom i Western blot analizom. Pokazali smo da se izolacija proteina iz periplazmatskog prostora poboljšava snažnim miješanjem nakon resuspenzije stanica i da je dvostruki osmotski šok najbolja tehnika za otpuštanje topljivih scFV-a. Uočeno je da velika količina scFv još uvijek ostaje u netopivom obliku. Dobiveni scFvs također su uspješno izolirani korištenjem imobilizirane metalne afinitetne kromatografije (IMAC) i vezanja His oznake, bez gubitka tijekom kromatografije. Za uklanjanje zaostalih kontaminanata preporučuje se naknadni korak pročišćavanja koji se temelji na isključenju veličine.

Ključne riječi: proizvodnja rekombinantnih proteina, protutijela, fragmenti protutijela, *Escherichia coli*, izolacija proteina.

1.INTRODUCTION

1.1. RECOMBINANT DNA TECHNOLOGY

The history of recombinant technology goes back to 1972 when a scientific conference on plasmids took place in Honolulu. These early discussions were later published as a scientific paper titled "Construction of biologically functional bacterial plasmids in vitro" in 1973 by Stanley N. Cohen, Herbert Boyer, and others (1). They discussed the basis of recombinant DNA technology, or the merging of genetic information from different organisms to create new genetic structures.

Just three years later, in 1976, the biotechnical company Genentech isolated the gene for human insulin, which was transferred using a plasmid vector to *E. coli* to produce the two chains of human insulin. Insulin chains were then individually purified and by chemical reactions disulfide bonds were made to create active form, which resulted in a recombinant insulin used to treat diabetes in humans. Recombinant insulin also addressed the issue of allergic events to pork-derived insulin. The production of an insulin that is no different from the one produced naturally in the human body, was a massive step in medical history. In the 1980s, the FDA approved recombinant human insulin for diabetes treatment (2). Insulin thus became the first recombinant protein to be used as a drug, and today the use of recombinant biological drugs is increasing, predominantly antibody preparations. Today, more than 150 recombinant therapeutic drugs have been approved by the FDA (Food and Drug Administration), and one third of these are produced in *E.coli,* still a important host for recombinant therapeutics production (2).

Recombinant DNA technology is a set of techniques for manipulating genes or other DNA sequences of interest. It usually involves methods that take intact or modified genes from one organism and transfer them into another organism (3). Genes are generally fused to a vector (i.e. virus or plasmid), which allows them to enter the host organism, where protein synthesis can take place (4). Resulting that gene encoding a particular protein in the original host is now able to be expressed in another organism. Moreover, in many cases, the original genes have been modified to give the protein some additional desirable properties, such as improved pharmacokinetics or higher protein expression. One of the most used methods to generate plasmids is by insertion of the gene using restriction endonucleases. Restriction endonucleases are enzymes that recognize and cleave specific nucleotide sequences (restriction sites) in the double-stranded DNA plasmid. Restriction enzymes were originally found in bacteria, where

they play the role of destroying foreign invading DNA. Today, there are more than 400 different isolated restriction endonucleases, and the first isolated from *Escherichia coli* RY13 was EcoRI (5).

Gene of interest is then cloned into the cut destination vector by ligation, a process that uses a ligase, which fuses the gene fragment with the receiving vector. Finally, the resulting plasmid, carrying the gene of interest, is inserted into the host organism where it replicates together with the bacterial cell (3). In the case of bacterial strains, there are two main types based on their function, those used to store and multiply such plasmids and strains used for protein expression, which will be explained in more detail in the following sections.

1.2. PLASMIDS

Plasmids are small (two to few hundred kilobase pairs) circular DNA molecules that are found in bacteria and in some other microscopic organisms (6). They are separated from chromosomal DNA and they replicate independently of it. Typically, plasmids have a small number of genes associated with antibiotic resistance, virulence factors, metabolic pathways, or nitrogen fixation and can be passed from one cell to another (7). Plasmid DNA is used in several applications, such as transformation (entry of the vector into a prokaryotic cell), transfection (entry of the vector into a eukaryotic cell), sequencing, restriction digestion, cloning, and PCR (8).

All natural plasmids contain an origin of replication (which controls the host range and copy number of the plasmid) and typically include a gene that gives selective advantage for bacterial surviving in some conditions (9). Plasmids created in the laboratory are designed to carry foreign DNA into another cell and they have at least an origin of replication, selection marker, and cloning site (9). Plasmids are used as vectors to clone fragments of up to 10 kb (5).

The brief description of plasmid properties is given bellow:

- **Origin of Replication (*ori*)** is a DNA sequence that allows replication initiation by recruiting replication machinery proteins, necessary for self-replication.
- **Antibiotic resistance gene or selectable marker** important for selection of plasmid-containing bacteria.
- **Promoter region** drives transcription of the target gene, it determines in which types of cells the gene can be expressed, and the amount of recombinant protein obtained.

- **Multiple Cloning Site (MCS)** is a short segment of DNA which contains several restriction sites allowing for the easy insertion of the gene. In expression plasmids, the MCS is often downstream from a promoter.
- **Insert** is a gene, promoter or other DNA fragment cloned into the MCS for further study.

The pOPE101 expression vector used in this work, is designed for protein production in the periplasm of *E.coli*. It contains an IPTG-inducible synthetic promoter for lac operon, a pe1B leader sequence for the secretion of functional recombinant proteins into the periplasm, and a c-myc/His tags to facilitate recombinant protein detection and purification (10).

A brief explanation of the terms mentioned above:

- **lac operon** is a genetic regulatory mechanism in prokaryotes, has the function in nature to control the metabolism of lactose in absence of glucose. This system is regulated by a repressor (lac repressor) positioned in proximity to the gene promoter, which blocks the binding of the RNA polymerase, turning the gene off. The lac repressor, however, is an allosteric protein, and upon binding of allolactose to the allosteric site, changes the repressor conformation, releasing itself from the DNA. This allows the free binding of the RNA polymerase to the promoter, resulting in gene expression and production of the respective proteins. This gene regulation system has been used for the controlled production of recombinant proteins in bacteria (11).
- **IPTG (Isopropyl β-D-1-thiogalactopyranoside)** is an inducer of the lac operon. Gene transcription is achieved by IPTG mimicking allolactose, an isomer of lactose, which binds and releases the repressor from the lac operon. To sum up, when IPTG enters the cell containing the expression vector, it is a signal to start the target protein production. IPTG is typically used as the main lac inducer owing to its stability and effectiveness (12).
- **pe1B leader** is a signal peptide that, when attached to a protein, directs the protein to the bacterial periplasm, where this sequence is removed by a signal peptidase (13). The pelB gene that encodes pectate lyase B is one of three pectate lyases identified in *Erwinia carotovora* EC (14). Pectate lyase B was purified from *E. coli* containing the pelB gene on a recombinant plasmid. In *E. coli*, pe1B leader is linked to the N-terminus of the recombinant protein and enables passing through the cytoplasmic membrane (15).

- **c-myc tag** is a peptide protein tag derived from the c-myc gene product that can be fused to either the C- or N-terminus of a protein. It can ease isolation and detection of the tagged protein (16).
- **His tag** is a peptide sequence specifying a sequence of six to nine histidine amino acids. When used in the production of recombinant proteins, results in the expression of a recombinant-His-tagged protein, with the His tag typically fused to its N- or C-terminus. His-tagging of recombinant protein makes production easier since the sequence of histidine residues binds to immobilized metal ions such as nickel, which is used in Immobilized metal affinity chromatography (IMAC) purification (explained in detail in the methods section).

Anti-His-tag antibodies are commercially available for use in a variety of methods for detecting recombinant protein, without the need for protein-specific antibodies (17). In this work, we applied the detection of the target scFv using anti-His antibodies.

1.3. *Escherichia Coli*

The most common bacteria used in recombinant technology is *E. coli*. In addition, *E. coli* is the most used for large-scale production of recombinant proteins. There are many known tools for *E.coli* systems such as expression vectors, production strains, protein folding and fermentation technologies that are well constructed for industrial needs (2).

Cellular organization

On average, *E.coli* cells are usually 1-1.5 μm wide and 2 t-6 μm long (18), and contain an inner and outer membrane that separates the cell into two main subcellular compartments− the cytoplasm and the periplasm (**Figure 1**). Therefore, *E. coli* has four different compartments where proteins can be localized: outer membrane, inner membrane, cytoplasm, or periplasm (19).

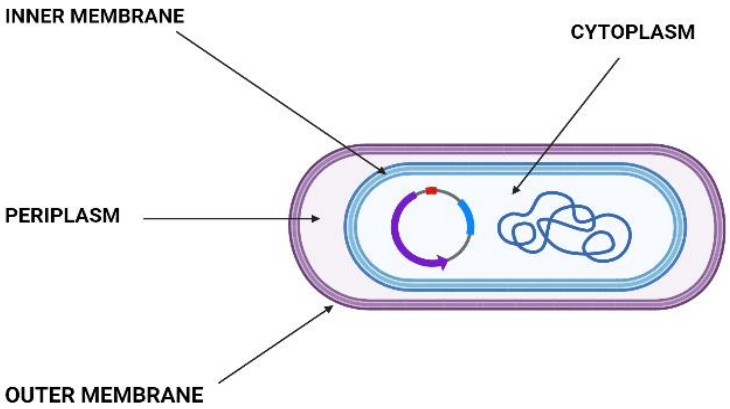


Figure 1. Cellular organization of *E. coli.* Created with BioRender.com

Prokaryotic expression system

Besides *E. coli*, other hosts, such as yeasts, filamentous fungi, insect cells and mammalian cells are used for producing different or more complex recombinant therapeutics (such as monoclonal antibodies (mAbs)) (2). Accordingly, the expression system should be selected based on the target protein. The most common protein expression systems are bacterial and mammalian systems, and both have their advantages and disadvantages, which are shown in **Tables 1** and **2**.

Table 1. Advantages and disadvantages of prokaryotic, *E. coli*. expression system

PROKARYOTIC EXPRESSION SYSTEM (*E. coli*)	
Advantages	**Disadvantages**
Very high yield (often 500mg / l L culture)	No/limited posttranslational modifications (disulfide bonds, glycosylation, acetylation, phosphorylation, proteolytic cleavage of precursors, etc.)
Low-cost	Limited DNA insert size (15 kb)
Known and well-described structure; well-characterized genetics	The difference in synonymus codons in relation to eukaryotes
Technologically simple/Simple fermentation process	Protein product instability
Rapid growth (doubling time is 20 min)	Possible formation of inactive, dysfunctional protein
Versatile cloning tools	Formation of inclusion bodies

Table 2. Advantages and disadvantages of eukaryotic, mammalian, expression system.

EUKARYOTIC EXPRESSION SYSTEM	
Advantages	**Disadvantages**
Sophisticated posttranslational modifications	Low yield
Cell biology similar to humans, (localization, cell cycle, etc.)	Expensive
Improved expression vectors and transfection methods	

In summary, with *E. coli*, there are two main problems: difficult/insufficient expression of an heterologous gene and poor solubility of recombinant proteins upon over expression (20).

The lack of some posttranslational modifications in bacterial cells can, to some extent, be overcome by protein engineering (21). Nowadays, many biotechnology companies offer different types of genetically modified strains of *E. coli* (**Table 3**).

Table 3. Characteristics of E.coli strains used in recombinant protein expression (20).

E. coli strain	***Characteristics***
BL21(DE3)	Expression under T7 promoter
BL21(DE3) pLys S/E	Controlled expression
Lemo21(DE3)	Membrane, globular and toxic protein expression
Shuffle	Proper disulfide bond formation in cytoplasm

E.coli BL21 (DE3) strains are the most widely used hosts for protein expression in the periplasmic space. This *E. coli* strain has the advantage of being deficient in both *lon* and *ompT* proteases, which aids the production of high yields of protein. This protease-deficient strain is unable to degrade heterologous protein (20). Subsequent improvement of *E.coli* BL21 (DE3) was achieved by addition of the T7 RNA polymerase gene, under the control of the IPTG-inducible lac UV5 promoter (15)(22). The resulting strain, known as *E.coli* BL21 (DE3) allows a tighter control of protein expression as this promoter is controlled by the addition of lactose/IPTG. *E.coli* BL21 (DE3) is commonly used in combination with the pET vector system, which contains a leader peptide sequence. This pET vector is specially developed for expression of recombinant proteins in *E.coli,* usually it comes in commercially available kits with other companion products (23).

Other relevant strains include BL21(DE3) pLysS and BL21(DE3) pLysE**,** which have an additional lysozyme coding plasmid. Lysozyme is inhibitor of T7 polymerases which inhibits residual T7 polymerase and therefore prevents leaky expression (20).

Periplasmic space

Bacterial expression systems, due to their evolutionary differences in relation to humans, are often not able to produce a recombinant human protein. Bacteria did not e.g. develop mechanisms for performing posttranslational modifications which are present in eukaryotic organisms (21). In bacteria, cytoplasm is a reductive environment in which disulfide bridges cannot be formed (21). *E. coli* do have an oxidative environment for proteins in their periplasmic space where the proteins can be directed (19), and it was shown that in the periplasm *E.coli* can produce human proteins which require disulfide bridges (21). Periplasm is more suitable for protein folding because of the oxidizing environment when disulfide bonds are required, it provides conditions for formation of the disulfide bonds, due to the presence of specific chaperons and foldases, which enable the production of soluble and active recombinant proteins (10).

The periplasm has a lower concentration of host cell proteins and lower proteolytic activity, therefore, purification of the target protein can be more easily achieved (2). In the case of *E.coli*, passage to periplasm is secured by protein-conducting Sec-translocon, the main protein-conducting channel that helps the biogenesis of both bacterial membrane and secretory proteins (15). Recombinant proteins are therefore often produced in the periplasm because it is easier to isolate them from this cellular compartment than from whole cell lysate.

Gentle periplasmic extraction is important inner membrane disruption results in sample contamination with cytoplasmic proteins. Standard methods use EDTA-lysozyme, cold osmotic shock, or both. EDTA is used to destabilize the outer membrane, allowing the lysozyme to enter the periplasm and hydrolyze the peptidoglycan cell wall. This step releases the periplasm and leaves a remnant of a bacterial cell, called spheroplast. The method using osmotic shock is performed by adding a hypertonic solution to bacterial cells that results in weakening outer membrane, followed by hypotonic solution to partially disrupt the outer membrane without compromising the inner membrane (19). In this work, we used three variations of the osmotic shock method for isolating periplasmic scFv from bacterial expression system.

1.4. ANTIBODIES

Antibodies, also known as immunoglobulins, are serum glycoproteins composed of a monomer shaped as letter "Y". Antibodies enable antigen recognition in the serum and are produced by B-cell-derived plasma cells. They are manly found in blood, bone, spleen, marrowbone, interstitial fluids and in exocrine secretions. They play a major role in the body's defenses against any foreign compounds such as bacteria, viruses, parasites, toxins etc. (24). Antibodies' defensive roles are mediated through the recognition of an epitope on an antigen's surface. The next step is the binding of effector proteins or cells to the antibody's Fc domain, which trigger diverse biological functions, such as natural killer cell activation, activation of the classical complement pathway, and phagocytosis (25).

There are 5 isotypes of mammalian immunoglobulins (Ig): A, D, E, G and M. IgGs are formed by two light and two heavy chains connected by disulfide bonds, with a size of about 150 kDa. All 4 chains contain variable (N-terminal) and constant regions (C-terminal). Each light chain contains one variable domain (VL) and one constant (CL), whereas each heavy chain has one variable domain (VH) and three constant domains (CH 1-3) (**Figure 2**) (24). Therefore, immunoglobulins have twofold-symmetrical structure (26). Specific binding to the antigens goes through variable regions (24).

Antibodies function by recognizing a part of an antigen called epitope, that is a group of amino acids or other chemical groups on the surface of the antigen. They can recognize epitopes of different sizes, and this binding is reversible. Antibody can bind to epitopes using one or all of its six complementary-determining regions (CDRs) and even minor changes in antigen structure can modify the strength of the binding between antibody and the epitope, resulting in decreased affinity of the antibody towards the antigen (25). Binding specificity means the recognition of a specific epitope in presence of other epitopes, and this is the main difference between monoclonal and polyclonal antibodies. Polyclonal antibodies recognize multiple epitopes whereas monoclonal antibodies recognize only one specific epitope (25).

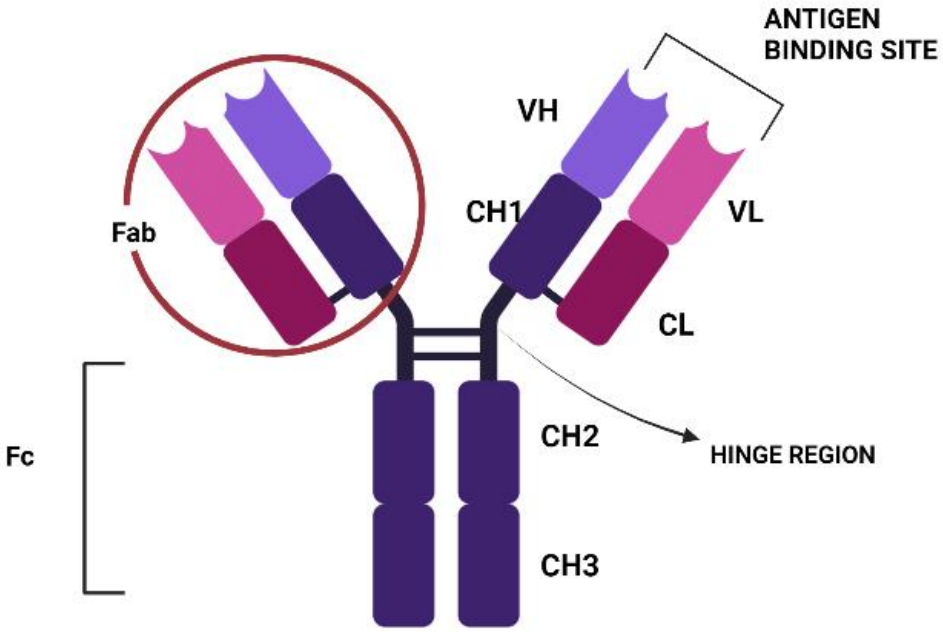


Figure 2. General antibody structure of human /mouse IgG. VH = variable region of antibody heavy chain; VL = variable region of antibody light chain; CL = constant region of antibody light chain; CH1,2,3 = constant domain one, two and three of antibody heavy chain; hinge region = short sequence of the heavy chains (H) of antibodies linking the Fab (Fragment antigen binding) region to the Fc (Fragment crystallizable) region. Created with BioRender.com

Antibodies in diagnosis and therapy

In the pre-antibiotic era, passive antibody administration or so called serum therapy, was the only effective treatment for many infectious diseases (27). The administration of immune serum (usually animal) for the treatment was introduced in the 1890s for the treatment of diphtheria. By the 1930s, antibody-based therapy was among others widely used for the treatment of pneumonia, meningitis, scarlet fever ,whooping cough, anthrax, botulism, gas gangrene, tetanus brucellosis, dysentery, tularemia, diphtheria and chickenpox. (27). As there were no antibiotics, passive antibody therapy was useful, but nowadays, with the development of technology, its disadvantages are visible. Some negative effects of transferring passive antibody serum from an immune-protected animal/human to a patient are allergic reactions associated with injecting foreign proteins and lack of standardized sera (24). Furthermore, traditional intravenous immunotherapy product contains many more antibodies against many more antigens, while only a fraction binds the antigen of interest, explaining why a larger dose is required to have an effective treatment (28).

Polyclonal antibodies

Polyclonal antibodies (PAbs) derive from several B-lymphocyte clones unlike monoclonal that are produced by a single B-lymphocyte clone. PAbs recognize multiple epitopes of the antigen. In case they need to be produced it requires *in vivo* settings and involves the repeated immunization of an animal with a desired antigen followed by purification from blood serum (29). Although PAbs development is rapid, inexpensive and easy to perform, PAbs lack the homogeneity and consistency of monoclonal antibodies as well as require a living host (25).

Monoclonal antibodies

Monoclonal antibodies (mAbs), unlike polyclonal antibodies, are homogeneous, highly specific for a particular epitope and can be produced in higher quantities. MAbs have numerous applications in diagnostics, therapeutics, targeted drug delivery systems, infectious diseases, cancer, metabolic and hormonal disorders, allergy, asthma and autoimmune diseases (30) (31). MAbs can detect a single antigenic determinant (epitope), and this restricted reactivity allows for precise pathogen identification (30). The screening test most used in diagnostics to detect antibodies or - by which antibodies are put into the function of identifying a particular product - is the enzyme-linked immunosorbent assay (ELISA). ELISA is a fast, sensitive test easily performed with many samples (30). Depending on the choice of technique, mAbs can be produced without a live donor, through existing libraries, or by involving only one laboratory animal, after which continuous further production of the recombinant preparation no longer involves the need for live donors.

Recombinant antibodies

Blood-derived products not only have limited availability, but also carry the risk of blood-borne disease transmission and batch-to-batch variability, highlighting the importance of producing pure recombinant antibodies or antibody fragments (28). Since antibodies are molecules of complex quaternary structure many years have been invested to test expression systems that are optimal for their production while maintaining full functionality. For example, it is possible to produce different antibody fragments which retain functional. Recombinant antibodies do not exist in nature as such, they are made in laboratory by expressing antibody heavy-chain and light-chain gene sequences together. The most used fragments for recombinant antibody

production are single-chain fragment (scFv) and antigen-binding region (Fab), these fragments are preferred due to their short generation time, structural stability and retained antigen affinity (24).

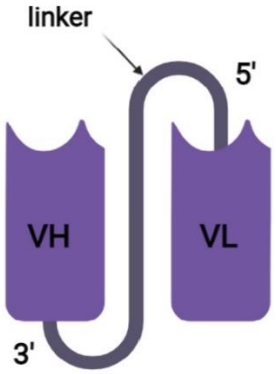


Figure 3. Single chain variable fragment (scFv), molecular weight of ~ 25 kDa, connected with a short linker peptide, normally ten to 25 amino acids.

Recombinant DNA technology through genetic engineering has successfully led to the possibility of reconstruction of monoclonal antibodies, including the humanization of antibodies originally derived from animals so that they can be used to treat humans (30). In the case of antibodies currently approved for treatment, they are almost always IgG-type antibodies. On the other hand, in the laboratory and in experimental procedures, the most commonly used fragments are scFv and Fab. The scFv and Fab became the most popular forms of antibodies as these fragments are easily produced in e.g. *E. coli* (32) and the smaller size of these antibody fragments offers some advantages over full IgG antibody. Antibody fragments can penetrate tissue more quickly than the whole antibody molecule, they also exhibits reduced serum half-life and are less immunogenic compared to whole antibodies (32). Some of the antibody fragments that currently have attracted a high interest for their production are:

- **Fab**

  Fab is antigen-binding antibody fragment, heterodimeric and monovalent (Figure 3). The molecular weight is about 50 kDa and is approximately double the size of the scFv fragment and includes variable and constant domain of heavy and light chains(32).

- **Single-chain variable fragment (scFv)**

  Single-chain variable fragment (scFv) is composed of the VH and the VL domains which are connected by a flexible peptide linker (Figure 3)**.** It has a molecular weight of approximately 25 kDa. The scFv fragment and its derivates are the smallest parts of the antibody that still carry the complete antigen binding site (27).

- **Fab dimers F(ab'2) and Fab trimers F(ab')3**

  Fab dimers F(ab'2) and Fab trimers F(ab')3 are multivalent fragments and can be engineered to bind to two or three different antigens at once. Like other forms, these fragments are also created using genetic engineering tools (32).

The ability of antibodies to bind an antigen with a high degree of affinity and specificity has led to their ubiquitous use in a variety of scientific and medical disciplines (25). Because recombinant antibodies can be produced, tested, and modified relatively rapidly, desired characteristics can be selected based on affinity, epitope specificity, and protective mechanism (e.g., by selecting an appropriate Fc type or antibody fragment) to ensure that each therapeutic achieves optimal pharmacological effect.

2. AIM OF THE RESEARCH

The aim of this work is to compare different isolation methods for the production and purification of antibody single-chain variable fragments (scFv) expressed in the periplasm of *E. coli* BL21 (DE3) transformed with pOPE101. Here, we compare three periplasmic protein extraction methods to determine the factors resulting in the highest yield and purity for recombinant scFV antibody fragment.

3. MATERIALS AND METHODS

3.1.MATERIALS

3.1.1. CHEMICALS

- Acetic acid glacial, BDH PROLABO, Art.-Nr. 20104.298
- Ampicillin:stock 100 mg/mL (H_2O); working solution 50 µg/mL
- EDTA (Ethylendiamine tetraacetic acid), Carl Roth GmbH, Art.-Nr. 8040.2
- Gel loading purple dye (6x), VIAL, New England Biolabs
- HCl, BDH PROLABO, Art.-Nr. 20252.290
- Imidazol, Carl Roth GmbH, Art.-Nr. X998.4
- IPTG (Isopropyl β-D-1-thiogalactopyranoside), Sigma-Aldrich
- Kanamycin: stock 50mg/mL (H_2O); working solution 50 µg/mL
- Milkpowder, Carl Roth GmbH Art.-Nr. T145.3
- NaCl, Kemig d.o.o., Control Number 1500723
- PageRuler™ Prestained Protein Ladder, Thermo Fisher Scientific Inc., Product# 26616
- Quick load®, Purple 1kb plus DNA ladder, New England Biolabs
- Rothiphorese® Gel 30 (37, 5:1), Carl Roth GmbH Art.-Nr. 3029.2
- Sucrose, Kemig d.o.o. Control Number 1101397
- TRIS, Trizma® base, Sigma-Aldrich Pcode: 1001681492

3.1.2. EQUIPMENT

- Laboratory equipment (pipets, pipette extensions, petri dish, flasks, measurement jugs, beakers…)
- Spectrometer, Biomate 3, Thermo Fisher Scientific Inc., UK
- pH/mV/°C meter, pH 510, Thermo Fisher Scientific Inc., UK
- Vortex mixer, RS-VA 10, Phoenix Instrument GmbH, Germany
- Mini orbital shaker, SSM1, Cole-Parmer, UK
- Centrifuge, Rotina 380 R, Andreas Hettich GmbH & Co. KG, Germany
- Centrifuge 5415 R, Eppendorf®, Germany
- Heating magnetic stirrer, HPS 630, WIGGENHAUSER, Germany
- Mini vertical electrophoresis system, Amersham Biosciences, UK
- Mini electrophoresis system for proteins, Model TE22, Hoefer Scientific Instruments, California

- Electrophoresis Power Supply- EPS 301, Amersham Biosciences, UK
- Electrophoresis hardware: a tank, lid with power cables, electrode assembly, cell buffer dam, casting stands, casting frames, combs (10-well or 15-well), glass plates (thickness 0.75mm or 1.0mm or 1.5mm)
- Nitrocelulose Blotting Membrane, Catalogue No 10600002, GE Healthcare, Life Sciences, Germany
- NucleoSpin®Plasmid/-Plasmid (NoLid) by Marcherey- Nagel kit
- Pur-A-Lyzer ™ Mega Dialysis Kit by Sigma-Aldrich
- HisTrap™ HP nickel column
- 0.45 µm Whatman® Syringe Filter
- GE AKTA Prime Plus Liquid Chromatography System, GMI, USA
- ImageQuant LAS 4000 camera system, ImageQuant LAS 4000 Control Software, GMI, USA

3.1.3. BUFFERS AND SOLUTIONS

- 1.5 M Tris-HCl, pH 8.8: Dissolve 18.15 g of Tris base in 80 mL distilled water. Adjust pH to 8.8 using 6N HCl. Make up the final volume to 100 mL with distilled water.
- 10% SDS: Dissolve 10 g of SDS in distilled water up to 100 mL volume.
- 1x Running (Laemmli) Buffer (For SDS-PAGE and Western blot): 25 mM Tris-HCl, 200 mM Glycine, 0.1% (w/v) SDS, pH 8.3
- 5X Sample buffer (reducing buffer) 16 mL: 5.2 mL dH_2O, 2.0 mL 0.5M TRIS pH 6.8, 3.2 mL glycerol, 3.2 mL 10% SDS, 0.8 mL β-mercaptoethanol, 1.6 mL 1% bromophenol blue
- APS: Dissolve 1g ammonium persulphate in 10 ml of distilled water.
- Destaining reagent: 5% ethanol, 7.5% acetic acid (For 1L: 50 mL ethanol, 75 mL acetic acid, 875 mL H_2O)
- Dialysis buffer (30 mM TRIS, 400 mM NaCl, pH 7.5)
- Hypertonic solution (30 mM TRIS-HCl, 200 g/L sucrose, 5 mM EDTA, pH 8.0)
- Hypotonic solution (5 mM $MgSO_4$; optional:protease inhibitors 1 x complete EDTA free)
- IMAC Binding buffer (200 mL), pH 7.5 (30 mM TRIS, 400 mM NaCl, 10 mM Imidazol (1M solution))
- IMAC Elution buffer (100 mL), ph 7.5 (30 mM TRIS, 400 mM NaCl, 300 mM Imidazol (1M solution))

- rCutSmart™ Buffer, New England Biolabs, Ipswich, MA, USA
- Spheroblast solution buffer: 1 mM EDTA, 50 mM Tris, 20% sucrose, pH 8.0
- Staining reagent for Coomassie Brilliant Blue technique: 0.25 g of Coomassie Brilliant Blue R-250 in 90 ml of ethanol:H_2O (1:1, v/v) and 10 ml of glacial acetic acid. Filtered through a Whatman No. 1 filter.
- TBS buffer 10x: 0.2 M Tris base, 1.5 M NaCl, H_2O up to volume, adjust pH to 7.2-7.4
- TBST, 1x: For 1L use 100 mL of TBS and add 0.5 mL of Tween 20 (Polyoxymethylene sorbitane monolaureate)
- TEMED : add one TEMED polymerization tablet to 1.0 mL of distilled water.
  -Transfer buffer 1x: 100 mL of 10x Transfer buffer, 200 mL methanol, 700 mL H_2O, 0.5 mL 10% SDS
  -Blocking buffer: 5% Milk powder in TPBS (For 1 gel = 1.5 g in 15 mL)
  -ECL™ Prime Western Blotting Detection Reagents, Amercham™, GE Healthcare, Life Sciences, Germany
- Western Blot:
  - Transfer buffer 10x: 30.3 g Tris base, 144 g glycine, dilute in 1 L of H_2O, adjust pH to 8.3

3.1.4. CULTURE MEDIUM

- LB Broth with agar (Lennox), by Sigma-Aldrich (Agar, 15 g/L, NaCl, 5 g/L, Tryptone, 10 g/L, Yeast Extract, 5 g/L)

3.1.5. ENZYMES

- MluI-HF®, New England Biolabs, Ipswich, MA, USA
- NotI-HF®, New England Biolabs, Ipswich, MA, USA
- HindIII-HF®, New England Biolabs, Ipswich, MA, USA
- NcoI®, New England Biolabs, Ipswich, MA, USA

3.1.6. BACTERIAL STRAIN

In this experimental work was used *E.coli* BL21(DE3) bacterial strain subsequently transformed with pREP4 plasmid. Plasmid pREP4 contains genes encoding the enzyme for antibiotic resistance to kanamycin and the lac I gene encoding the lac operon repressor (lac repressor). Because of the pREP4 plasmid this bacterial strain has an additional lac repressor, which is used to prevent early (premature) protein induction, i.e. the so-called leakage before adding inductor.

3.1.7. PLASMID

Bacterial strains used in this experiment were transformed with expression vector pOPE101 (Genebank accession no. Y14585) (Figure 4) by PROGEN Biotechnik GmbH laboratory, Germany. This plasmid is designed for the cloning of antibody heavy and light chain variable regions (VH and VL) for the production of recombinant single-chain Fv antibody fragments in *E.coli.* Nevertheless, this plasmid already contains the VH/VL genes for the expression of a functional scFv that recognizes epitope PIII-215 (Yol) (33). This plasmid was selected for this work because it is a plasmid that has been widely and successfully used for production of scFv antibody fragments. Cloning sites: MluI and NotI for light chain VL genes and NcoI and HindIII for heavy chain VH genes.

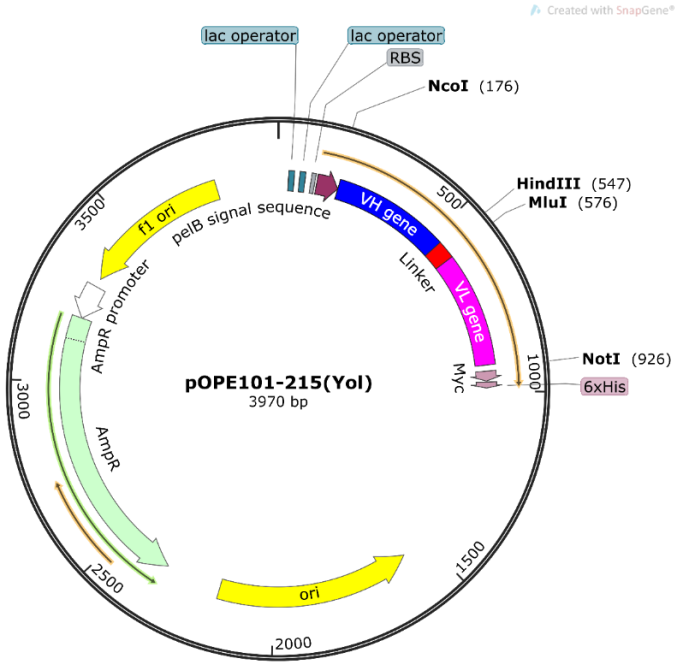


Figure 4: Map of pOPE101-215 (Yol) made with SnapGene Viewer software.

Table 4. Explanation of abbreviations showed on plasmid map.

ABBREVATION	DESCRIPTION
***LAC* OPERATOR**	The *lac* repressor binds to the *lac* operator to inhibit transcription in *E. coli*. This inhibition can be relieved by adding IPTG.
f1 *ori*	f1 bacteriophage origin of replication
AmpR	Resistance to ampicillin
ORI	Origin of replication
RBS	Strong bacterial ribosome binding site
Myc	Human c-myc proto-oncogene epitope tag
6xHis	6xHis affinity tag

3.2. METHODS

SDS-PAGE

SDS-PAGE is an analytical electrophoresis technique that separates proteins based on molecular weight. SDS, sodium dodecyl sulfate, is known as a detergent that denatures proteins by disrupting non-covalent bonds and destabilizing native conformations. By acting as an anionic surfactant, it imparts a negative charge on proteins causing repulsions between amino acids which leads to protein unfolding. By denaturing the proteins and giving them a uniform negative charge, it is possible to separate them on the gel, therefore PAGE (Polyacrylamide gel electrophoresis), based on the size as they migrate towards the positive electrode (34).

COOMASSIE BRILIANT BLUE

Coomassie Brilliant Blue is a dye used for staining a polyacrylamide gel for visualizing the proteins. It works on the principle of Van der Waals force and electrostatic interactions. The negatively charged anionic form of the dye stabilizes the formation of the dye-protein complex and produces a blue dye which can then be seen on the gel. The number of bounds-color molecules is approximately proportional to the amount of protein in each signal, which means that the color intensity of each signal correlates with the amount of protein in the same. This technique was used for visualizing the proteins obtained in *E. coli.*

SDS-PAGE Protocol

Assemble the cassette and prepare 12% polyacrylamide gel (Table 5)

Table 5. Preparation of 12% polyacrylamide gel.

SEPARATING GEL	***AMOUNT***
H_2O	3.9 mL
1.5M TRIS pH 8.8	3 mL
30% polyacrylamide	4.8 mL
10% SDS	120 µL
TEMED	15 µL
APS	120 µL
STACKING GEL	
H_2O	2 mL

0.5M TRIS pH 6.8	0.375 µL
30% polyacrylamide	500 µL
10% SDS	30 µL
TEMED	10 µL
APS	30 µL

- Between separating and stacking gel put 300 µL 100% isopropanol to get rid of the air bubbles
- Stacking gel is poured on top of the separating gel, they are different in density
- Insert gel comb after pouring stacking gel
- Put the cassette in the tank and fill with Running buffer until the mark for maximum
- Prepare the samples by mixing them with 5 x sample buffer (loading buffer), heat them in the thermo block at 95°C for 5-10 min
- Load protein marker (ladder)
- Load prepared samples according to the selected well volume
- Start the running of the gel (100V-150V, 30mA per gel) until the samples reach the distance of 1 cm to the bottom of the gel
- Put the gel in the plate with the staining reagent and leave it on shaker for about 10 to 20 minutes
- After staining, discard the staining reagent and pour the distaining reagent in the plate with the gel (make sure the plate is covered), heat in the microwave for 30 seconds and put back to the shaker until cooled down (do not move the cover of the plate until cooled), discard the distaining reagent put new amount and repeat the steps 2-4 times in one hour.

WESTERN BLOT ANALYSIS

Western blot is used for protein analysis and is a method of specific protein detection i.e., a specific target protein is detected using an appropriate antibody. First, samples are put on polyacrylamide gel and separated by electrophoresis (SDS-PAGE). Next step is to transfer the proteins from gel to the membrane using the electro-transfer. After that, membrane is incubated with antibody specific for the protein and then with enzyme-labelled secondary antibody. After the addition of an enzyme substrate, a specific, mainly chemiluminescent signal is detected on the membrane, using a special visualization instrument.

Gel transfer:

- Cut an 8x8 cm membrane per gel
- Cut 2 pieces of 8x8 cm filter paper per gel
- Prepare 3 chambers: Wash the membrane in methanol (10 seconds), water (1-2 minutes), and Transfer buffer (5 minutes)
- Place a sponge in the transfer cassette, wet a filter paper in Transfer buffer and place it on top of the sponge, place the membrane on top of the filter paper, then gel, another filter paper and second sponge
- Close the cassette
- Insert the cassette in transfer box and run at 150V, 30 mA per gel, 120 minutes
- Prepare the Blocking buffer
- Incubate membrane in the Blocking buffer overnight
- On Day 2:
- Wash the membrane with TBST buffer
- Add primary antibody (A-His-POD), 1: 3000 dilutions in TPBS
- Wash the membrane with TBST (10 minutes)
- Development of the membrane
- Gel reading- Chemiluminescence

PROTEIN PURIFICATION USING IMMOBILIZED METAL CHELATE AFFINITY CHROMATOGRAPHY (IMAC)

Recombinant antibodies can be purified based on their specific and reversible interaction with immobilized target antigens or tags. IMAC is based on the principle which is separation of protein containing specific groups (e.g., histidine that is usually located on the C-terminal end to avoid purification of non-functional proteins) by coordinate covalent binding with metal ions (e.g. nickel) immobilized on the resin (35). IMAC purification consists of four steps which are: equilibration, binding, washing and elution. Washing enables non-specific proteins and impurities to be washed away (35).

The dialysis fraction was used for histidine-tagged protein purification in volume of 19.5 mL. In this case, HisTrap™ HP nickel column was used. Binding and elution buffer are described in materials section.

TRANSFORMATION

Host strains: *E. coli*, BL21 (DE3) pREP4

Vector name: pOPE 101 (Yol) Expression Vector
Host strains and vector are stored at -80°C and they were put on ice for 30 minutes to achieve the temperature around 4°C.
The antibiotics ampicillin and kanamycin were used for selection since *E. coli* -pREP4 DE3 are kanamycin resistant and vector pOPE 101 is ampicillin resistant.

PREPARATION OF LB BROTH

LB-broth was heated using the microwave until melted (approximate time is 5 minutes in intervals of 30 to 15 seconds). After melting it was cooled down at the room temperature. Cooled LB-broth was put in the glass bottle in volume of 15 mL and antibiotics were added each in volume of 15 μL (1:1000 dilution). Mixture was then homogenized with pipette.

PETRI PLATE PREPARATION FOR *E. COLI* CULTURING

Petri plate was used for growing bacteria cultures. One petri plate was used in which was added 15 mL of mixture LB-agar- antibiotics. While the mixture is getting solid petri dishes were not completely closed to prevent condensation.

For preparing bacterial cultures in the Eppendorf tube was mixed 50 μL of bacteria strains + 0.1 μL of plasmid. Mixture was homogenized using the pipette then incubated on ice for 30 min. After incubation, mixture was subjected to heat shock at 42°C for 45 seconds, then was placed again on ice for 2 minutes. Then, 800 μL of fresh LB-broth was added to the mixture (this step is called „rescuing bacteria" because it prevents bacteria from dying of the heat shock) and it was placed on incubation for 1 hour at 37°C and shaking at 750 rpm. Mixture was centrifuged at 5000 G/5 min, supernatant was discarded. Pellet was resuspended in 50 μL of

fresh LB-broth which was then transferred to the Petri dish using glass beads, it was places on room temperature until dried and then set on incubation at 37°C overnight.

GROWING OF STARTER CULTURES

Three colonies (colony 1, colony 2 and colony 3) (Figure 6, results) were picked from plate, pOPE 101 in pREP DE3 0.1." and were transferred one in each test tube that contained 8 mL of LB-broth (in each was added 8 µL of ampicillin and kanamycin for selection). Test tubes were placed in incubator at 37°C overnight.

PLASMID DNA ISOLATION

The following kit and protocol NucleoSpin®Plasmid/-Plasmid (NoLid) by Marcherey- Nagel was used for isolation of high-copy plasmid DNA from E. coli.

- **Cultivate and harvest bacterial cells**

  Use 1-5 mL of a saturated E. coli LB culture, pellet cells for 30 seconds at 11, 000 x g. Discard the supernatant.

- **Cell lysis**

  Add 250 µL Buffer A1. Resuspend the pellet completely by pipetting up and down.

  Add 250 µL Buffer A2 and mix gently by inverting the tube 6-8 times. Incubate at room temperature for up to 5 min.

  Add 300 µL Buffer A3 and mix by inverting the tube 6-8 times until blue samples turn colorless.

- **Clarification of lysate**

  Centrifuge for 5-10 min at 11,000 x g at room temperature until the supernatant is clear.

- **Bind DNA**

  Place NucleoSpin®Plasmid/-Plasmid (NoLid) column in a Collection Tube and pipette a maximum of the supernatant onto the column. Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the NucleoSpin®Plasmid/-Plasmid (NoLid) Column back into the collection tube.

- **Wash silica membrane**

Add 600 µL Buffer A4 and centrifuge for 1 min at 11,000 x g. Discard flowtrough and place the NucleoSpin®Plasmid/-Plasmid (NoLid) Column back into the empty collection tube.

- **Dry silica membrane**

  Centrifuge for 2 min at 11,000 x g and discard the collection tube.

- **Elute DNA**

  Place the NucleoSpin®Plasmid/-Plasmid (NoLid) Column in a 1.5 mL eppendorf tube and add 50 µL Buffer AE. Incubate for 1 min at room temperature. Centrifuge for 1 min at 11,000 x g.

RESTRICTION ANALYSIS

Restriction analysis of pOPE101 in *E.coli* BL21 (DE3) was performed with aid of computer software.

SNAP GENE VIEWER

Using "SnapGene Viewer" restriction enzymes were chosen for restriction analysis of the plasmid. Restriction enzymes used:

- MluI-HF
- NotI-HF
- HindIII-HF
- NcoI

NcoI and HindIII cut the variable heavy chain and MluI and NotI cut the variable light chain.

IMAGEJ

Using the "ImageJ" image processing program, the amount of plasmid DNA in induced bacteria was calculated after DNA isolation to see how much DNA was obtained. By knowing the enzymes that are needed for cutting the plasmid and the quantity of plasmid DNA in the samples, it was possible to carry out the double digestion (Table 6).

Table 6. Reaction mixture for double digestion.

COMPONENT	50 µL REACTION
DNA	1 µg
10 X RCUTSMARTBUFFER	5 µL(1x)
MLUI-HF	1.0 µL (20 units)
NOTI-HF	1.0 µL (20 units)
NUCLEASE-FREE WATER	To 50 µL

Incubate at 37°C for 45 min.

LARGE SCALE PRODUCTION

Starter culture "pOPE 101 in pREP DE3 0.1. " from colony 3 was used for large scale production. For this purpose, LB-broth in volume of 1L was poured into an Erlenmeyer glass flask with 4 mL starter culture (1:250 dilution), 1 mL ampicillin and 1 mL kanamycin. Glass flask was then covered with aluminum foil and placed in the incubator at 37°C /250 rpm until the culture reached optical density (OD) at 600 nm wavelength within values 0.6-0.8. This optical density is ideal because at mentioned values bacteria are in the logarithmic phase of growth that is ideal for protein production. When the culture reached desired OD value, which amounted to 0.671, sample in volume of 2 ml was took and labeled as "pOPE 101 in pREP DE3 0.1.-pre induced". The culture was supplemented with 1 mL (1:1000 dilution) inducer IPTG. Culture was incubated at 16°C overnight. This temperature is optimal for *E. coli* when producing proteins, since the production is slower and prevents the formation of inclusion bodies (aggregates of insoluble proteins) that are not desired.

ISOLATION OF PROTEINS FROM PERIPLASMIC SPACE

For isolation of periplasmic scFv from *E.coli* BL21 (DE3) were used three methods. These methods used in this paper were created by searching the literature and similar isolation protocols, to finally compile and select three methods that represent different isolation conditions for protein isolation from periplasm.

METHOD 1

- Transfer induced bacteria in centrifugation bottles and centrifuge at 4000 x g, 10 min, 4°C to completely remove the rest of the medium
- Resuspend the pellet in 1/16 volume of cold Spheroblast Solution Buffer
- Stir lightly for 3h at 4°C
- Aliquot into smaller centrifugation bottles and centrifuge at 30 000 x g, 1 h, 4°C
- Supernatant contains the periplasmic fraction
- Filter through 0.45 μm Whatman® Syringe Filter
- Dialyze overnight at 4°C against buffer (30 mM TRIS, 400 mM NaCl, pH 7.5.)
- Repeat against new cold buffer (30 mM TRIS, 400 mM NaCl, pH 7.5.) overnight at 4°C (in ice water)
- Supernatant is periplasmic fraction containing the protein

METHOD 2

- Transfer induced bacteria in centrifugation bottles and centrifuge at 5000 x g,10 min, 4°C
- Discard the supernatant and resuspend the pellet in 1/10 volume (50 mL) of Spheroblast Buffer (50 mM Tris-HCl, 20 % (w/v) sucrose, 1 mM EDTA, pH 8.0)
- Incubate on ice for 20 min
- Centrifuge at 6000 x g, 15 min, 4°C
- Discard the supernatant, resuspend the pellet in 1/10 volume (50 mL) of $MgSO_4$ (5 mM)
- Incubate on ice for 20 min
- Centrifuge max speed, 1h, 4°C
- Pellet is containing the periplasmic fraction, resuspend it in 25 mL of water
- Dialyze overnight at 4°C against buffer (30 mM TRIS, 400 mM NaCl, pH 7.5.)
- Perform purification with IMAC

METHOD 3

- Transfer induced bacteria in centrifugation bottles and centrifuge at 4000 g x 20 min at 4°C
- Discard the supernatant and resuspend the pellet in 200 mL of hypertonic solution (30 mM TRIS-HCl, 200 g/L sucrose, 5 mM EDTA, pH 8.0)
- Mix for 30 min on magnetic stirrer at 4°C

- Centrifuge at 8000 g x 10 min at 4°C
- Discard the supernatant and resuspend the pellet in 50 mL of cooled hypotonic solution (5 mM $MgSO_4$; optional add protease inhibitors 1 x complete EDTA free)
- Mix on magnetic stirrer for 30 min at 4°C
- Centrifuge at 10 000 x g, 10 min, 4°C
- Supernatant contains periplasmic fraction
- Dialyze overnight at 4°C against buffer (30 mM TRIS, 400 mM NaCl, pH 7.5.)
- Perform purification with IMAC

For dialysis of the supernatant was used tube from „Pur-A-Lyzer ™ Mega Dialysis Kit" by Sigma-Aldrich. Dialysis tube has molecular cut of 3.5 kDa and capacity range of 15 to 20 mL. Sample in volume of 20 mL was loaded in the tube which was placed in the supplied floating rack and then placed in the glass flask that contained buffer (30 mM TRIS, 400 mM NaCl, pH 7.5.). Buffer was changed three times (2 x 2h, 1 x overnight). This together was put on a magnetic stirrer at 4°C for 2 days. After the dialysis 2 mL of dialysis buffer was taken as a sample for analysis to verify that we did not have any proteins in the buffer. Further, from the dialysis fracture was taken 500 µL for SB and WB analysis.

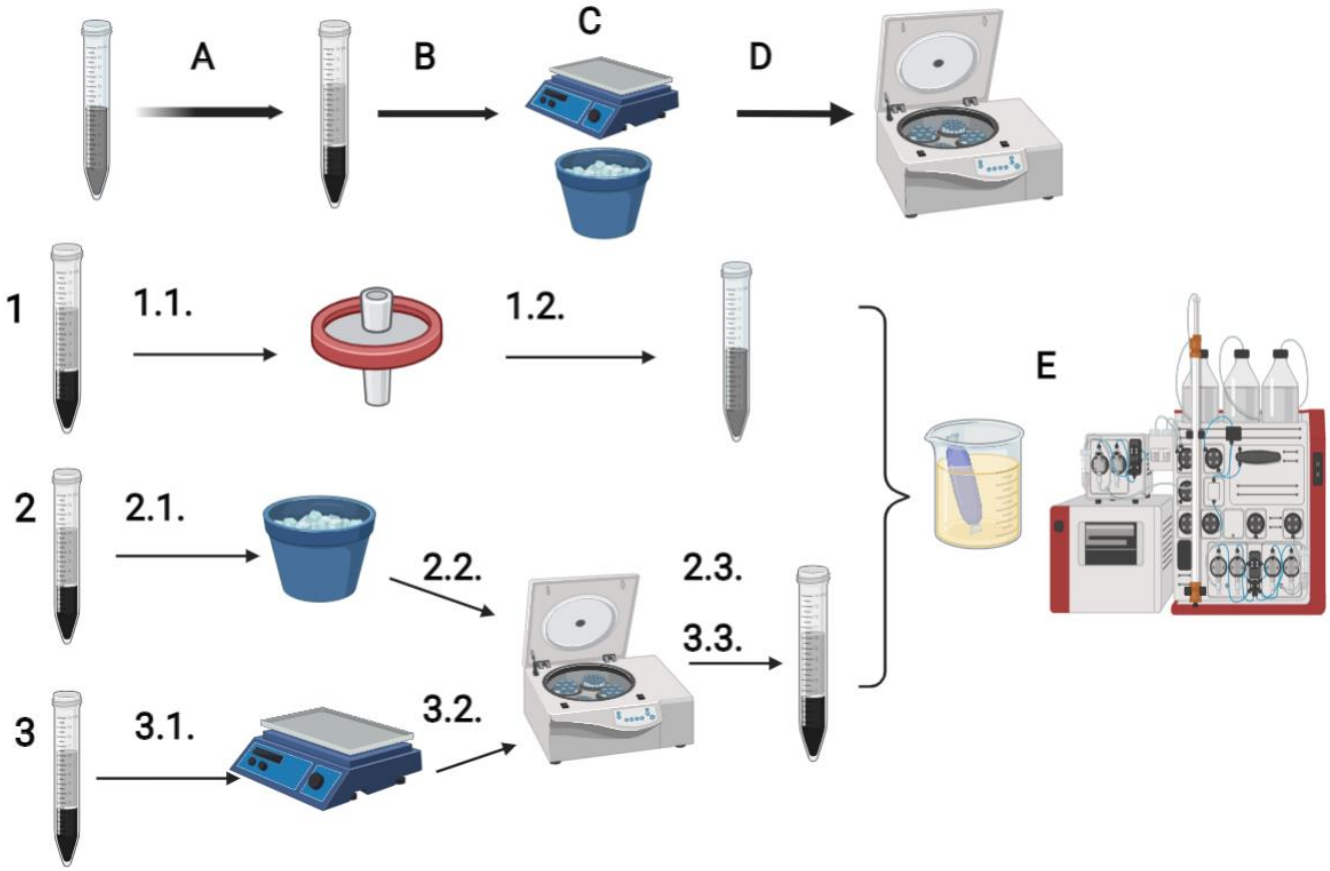


Figure 5. **Simplified presentation of all three methods of periplasmic fraction isolation.** Common steps for all methods are marked with letters (A, B, C, D and E). Steps that differ by method are marked with numbers (1,2,3). Explanation of each step: **A=centrifugation** (method 1- 4000 x g, 10 min, 4°C; method 2-5000 x g, 10 min, 4°C; method 3- 4000 x g, 20 min, 4°C), **B= resuspend the pellet** (method 1-in 1/16 of original volume spheroblast solution buffer; method 2-in 1/10 of original volume spheroblast solution buffer; method 3- in 150 mL hypertonic solution) , **C=mix on the stirrer** (method 1-3h, 4°C; method 3- 30 min, 4°C) **or on ice incubation** (method 2- 20 min on ice), **D= centrifugation** (method 1- 30000 x g, 30 min, 4°C; method 2-6000 x g, 15 min, 4°C; method 3- 8000 x g, 10 min, 4°C), **E= dialysis overnight and IMAC purification. Method 1**: **Step 1.1.** = Supernatant contains periplasmic fraction, filter it through 0.45µm syringe filter; **step 1.2**. = filtered periplasmic fraction goes on dialysis. **Method 2: Step 2.1.** = resuspend the pellet in 1/10 of original volume in $MgSO_4$ (5 mM) solution and put on ice incubation for 20 min; **step 2.2.** = centrifuge 23 000 x g, 1 hour, 4°C; **step 2.3**. = supernatant contains periplasmic fraction. **Method 3: Step 3.1.** = resuspend the pellet in 50 mL of hypotonic solution and mix on magnetic stirrer 30 min, 4°C: **step 3.2.** = centrifuge 10 000 x g, 10 min, 4°C; **step 3.3**. = supernatant contains periplasmic fraction. Created with BioRender.com

4. RESULTS

Starter cultures

For growing of starter cultures of *E.coli* BL21 (DE3) transformed with pOPE101 three colonies (colony 1, colony 2 and colony 3) (Figure 6) were picked from plate, pOPE 101 in pREP DE3 0.1." . Figure 6. shows these cultures on petri plate which indicates that the selection and growing of induced bacteria was successful.

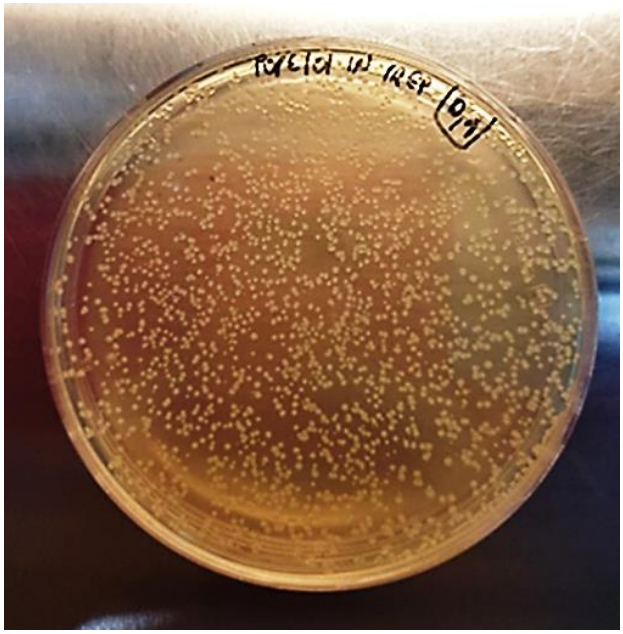


Figure 6. Petri dish with pOPE101 transformed *E. coli* DE3-pREP colonies, 3 colonies were picked for plasmid DNA isolation/analysis.

2. Restriction analysis

Plasmid pOPE101 was used for transformation of *E. coli* pREP-DE3. Transformation was verified by isolation of plasmids from selected transformed colonies (so called 'mini prep' plasmid isolation format). The mini preps were analyzed by gel electrophoresis to confirm the plasmid presence and size (Figure 7A and 8B). Three bands can be observed at around 3 kbp, 10 kbp and >10 kbp corresponding to different forms of circular plasmid DNA (Figure 7A).

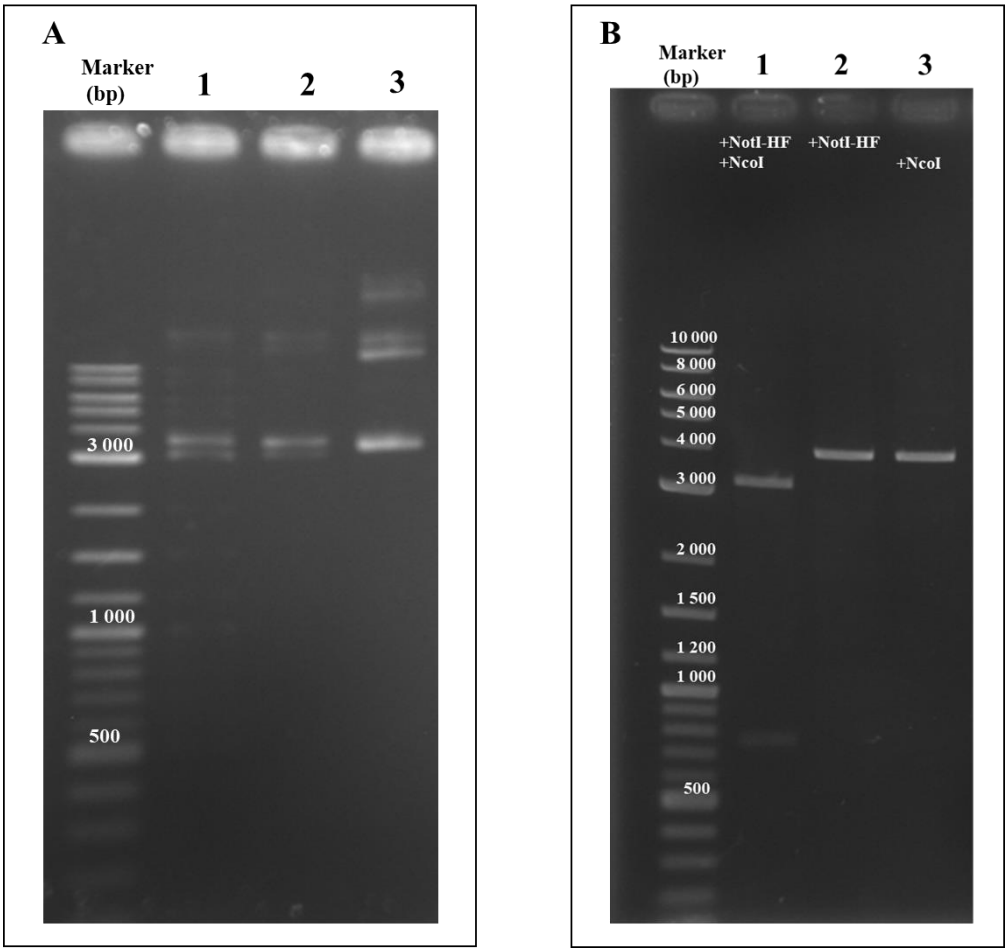


Figure 7. pOPE101 A. Plasmid colony screening and B. Restriction analysis

Restriction analysis was performed on pOPE101 to verify both the size of the insert and the size of the plasmid in linearized form (Figure 7B). After treatment with the restriction enzymes NotI-HF and NcoI, two bands were observed, one band at ~750 bp and another at ~ 3000 bp (Figure 7, lane 1). The size of this insert confirms the identity of the plasmids, as taking the two fragments together, they account for a size of ~4000 bp. When the plasmid was only treated individually with either NotI-HF and NcoI (lane 2 and 3) only a single band was observed at ~4000 bp. The latter is another confirmation for the correct plasmid size.

3. Protein expression

After confirmation of the plasmid pOPE101, the next step was to verify that the plasmid was able to produce the scFv protein in the selected expression system. The induced culture (Figure 8A, lane 2) clearly shows the appearance of a band at ~35 kDa, which is not seen in the non-induced culture (Figure 8A, lane 1). The specificity of the obtained scFv was confirmed by

Western blot analysis (Figure 8B), which shows a strong His signal at a position of approximately 35 kDa. Weaker His-positive signals observed at lower and higher molecular weights in induced culture may indicate degraded protein products or some aggregated forms that travel irregularly, but they are negligible in quantity. Taken together, these analyzes confirm scFv is expressed as a monomeric protein, which contains a histidine tag.

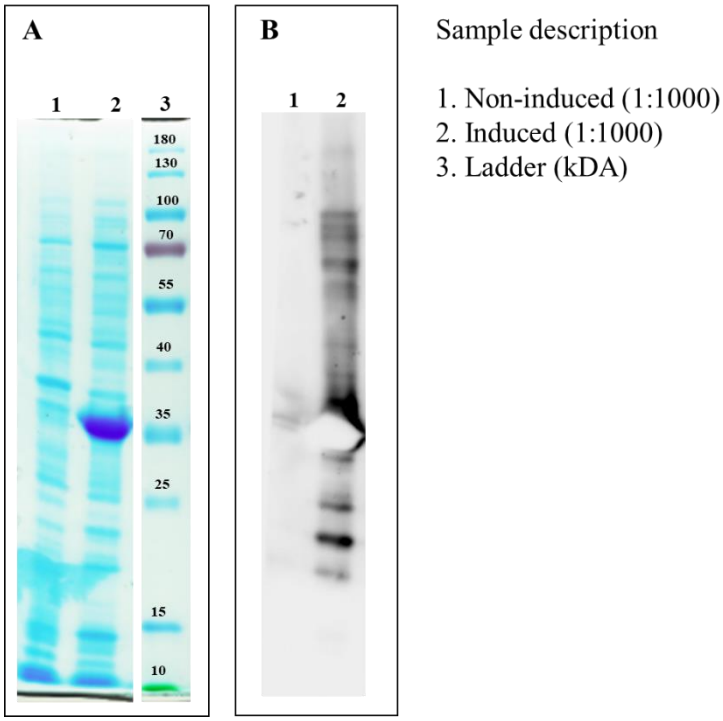
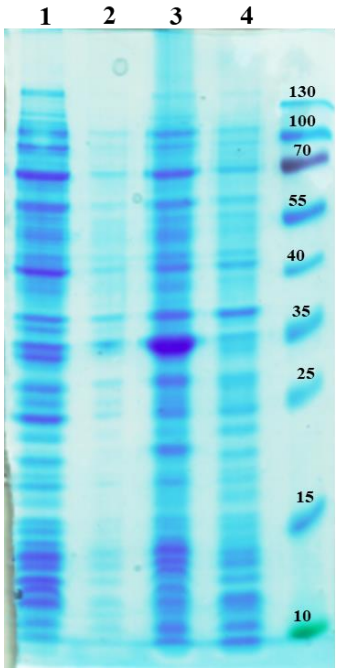


Figure 8. Small scale pOPE101 induction. A. Coomassie brilliant blue and B. Western blot analysis.1. Non-induced 2. Induced 3. Ladder (kDa)

4. Protein isolation and purification

Once the expression was confirmed in small-scale, large-scale productions (0.5 to 1.0 L) were performed to evaluate different methods. Isolation method 1 (Figure 9) resulted in poor isolation of the protein band of interest (lane 1), as almost all the proteins from the bacteria were also present in the periplasmic fraction. Moreover, the analysis of the pellet (lane 2) indicates that the scFv remained in this fraction, as is present in the band shows higher quantity in this sample. HPLC purification of this fractions was not performed since the initial samples were not pure enough and the protein of interest was not present as a dominant protein.



Sample description

1. Periplasmic Fraction 1 (PF1)
2. Pellet after PF1 (1:2500)
3. Induced
4. Non induced

Figure 9. Protein solation method 1. 1. Periplasmic fraction 1 of induced bacteria, 2. Remaining pellet after PF1 isolation of induced bacteria, 3. Bacterial lysate induced and 4. Bacterial lysate Non induced. All samples are normalized according to protein quantity contained in 1 ml of bacterial non induced culture

Isolation method 2 resulted in a very clean extraction of the scFv in the periplasmic fraction 1 (Figure 10A&B, lane 3). Very little scFv was additionally extracted in PF2 (lane 4), indicating that most soluble protein was already in PF1. The latter is supported by the analysis of the pellet, which shows that most of the protein is present in the pellet after periplasmic fraction 2 (lane 5). IMAC purification was performed using the PF1, the complete run is shown in Figure 11A, the elution peak (Figure 11B, red arrow) did not show a single resolved peak, instead it looked like two peaks that were not resolved. Representative fractions of the purification were tested by SDS-PAGE (Figure 11C). The results show that a significant portion of the scFv in the initial sample (lane 1) was lost after additional centrifugation when preparing the sample for application to HPLC (lane 2), but this quantity was maintained after further filtration (lane 3). On the other hand, the PAGE also shows that the purification was very effective at separating the protein of interest, since the flow-through fraction (lane 4) shows the presence of most of the bands from the initial sample except for the scFv. Moreover, the elution fraction 5 (lane 5) shows a very pure band at the 35 kDa MW, with little to no presence of other proteins. Elution fractions 6-10, 14 (lanes 6-9) did not show the presence of any proteins, indicating that the absorbance produced for second peak might be coming from a source other than protein.

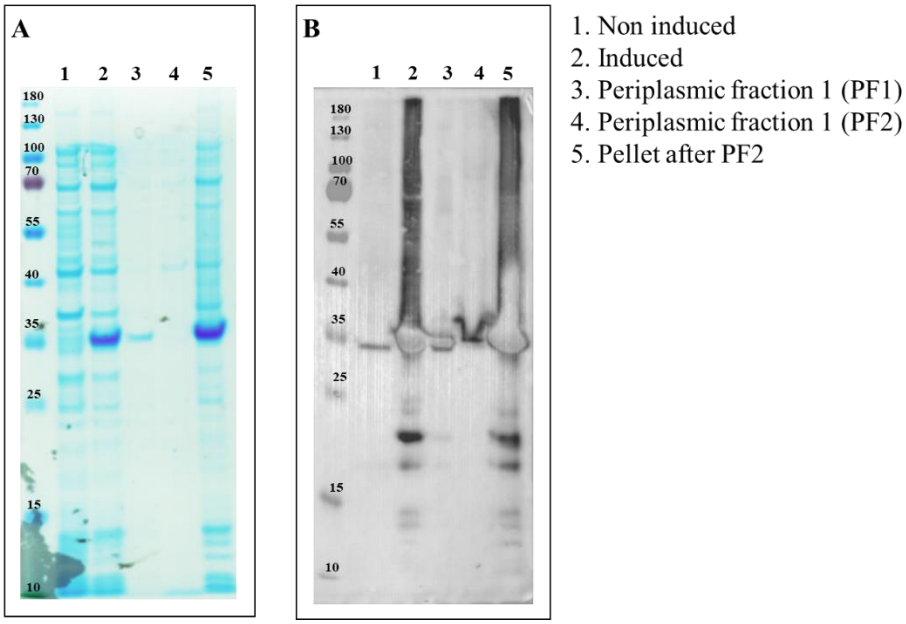


Figure 10. Protein isolation method 2. Cell lysate 1. Non-induced and 2. Induced, 3. Periplasmic fraction 1, 4. Periplasmic fraction 2, 5. Remaining pellet after PF2 isolation. All samples are normalized according to protein quantity contained in 1 ml of bacterial non induced culture.

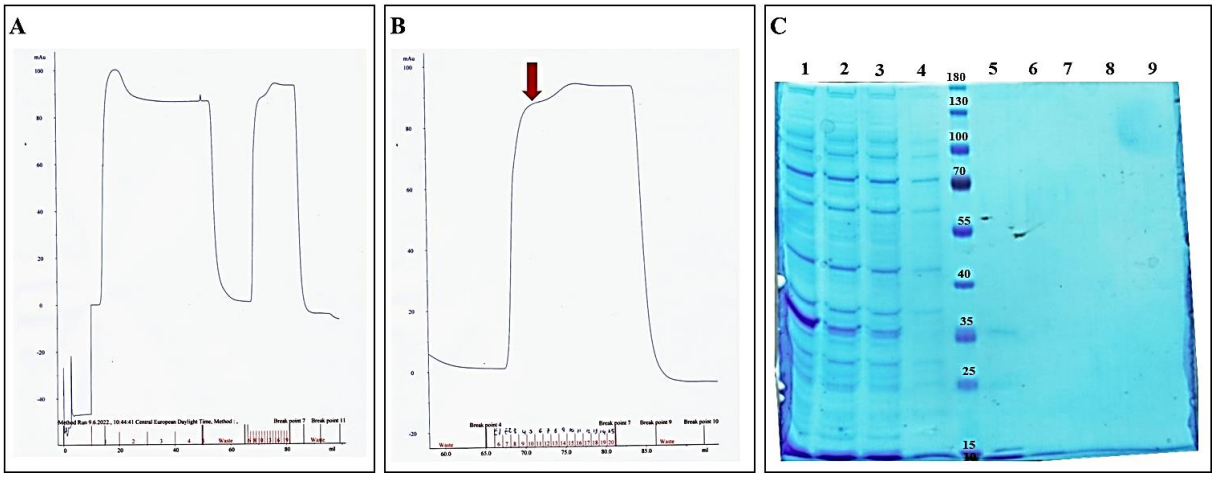


Figure 11: Protein purification method 2. 1. Initial sample, 2. Sample 1 centrifuged 20 min at 10 000 x g, 3. Sample 2 filtrated, 4. Flowthrough 3, 5-6. Elution fractions 5 and 6, 7-8. Elution fractions 9 and 10. Elution fraction 14.

Finally, isolation method 3 (Figure 12A and 12B) resulted in the purification of the scFv in the PF2 (lane 4). However, also with this method, a large portion of the protein was observed in the cell pellet (lane 5). The PF2 was purified by IMAC (Figure 13), a peak eluted during the elution step (Figure 13A), however, similarly to the purification performed with the method 2, the peak did not reach the base line of the chromatogram (Figure 13B red arrow), instead, it seemed like this peak was composed by two or more components. The analysis of the flow-through fraction (Figure 13C, lane 4) showed that IMAC removed some of the contaminating proteins present in the PF2. Moreover, the dominant scFv band occured in elution fractions E4

and 5 (Figure 13C, lane 7 and 8), which indicates the binding of the scFv. Nevertheless, some remaining of the contaminant proteins were eluted together with the antibody fragment.

So far, we have successfully isolated scFv, but the results of both methods implied an unexpectedly small amount of protein obtained with the apparently dominant presence of scFv in the pellet, which indicates the problem of protein insolubility. To confirm that most of the protein was not in soluble form, the PF1 from Protein isolation method 3 was also purified by IMAC (Figure 14A and 14B). SDS-PAGE analysis of the initial simple showed a large quantity of the scFv (Fig 14C and 14D lane 1), however, after strong centrifugation, the amount of this protein was greatly reduced (lane 2). The elution peak (Figure 13. A, B) showed a single-well resolved peak in the elution step. Analysis of the elution fractions (lane 3 to 10) show that this peak indeed corresponded to the expected protein (band at 35 kDA). It must be noted that also in this case the elution fractions contained some contaminant proteins. Flowthrough (lane 11) shows that there was no loss of antibody, and that all of it bound to the column.

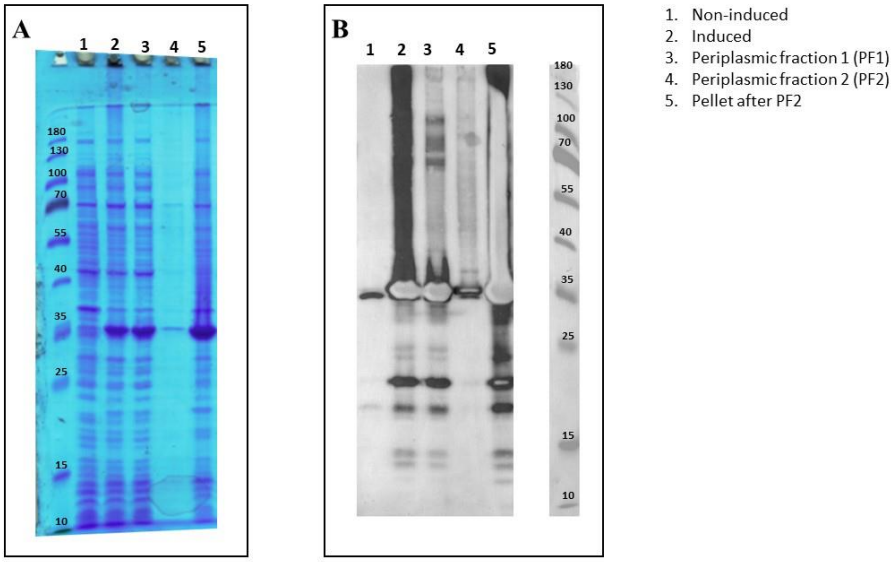


Figure 12. Protein isolation method 3. Cell lysate 1. Non induced and 2. Induced, 3. Periplasmic fraction 1, 4. Periplasmic fraction 2, 5. Remaining pellet after PF2.

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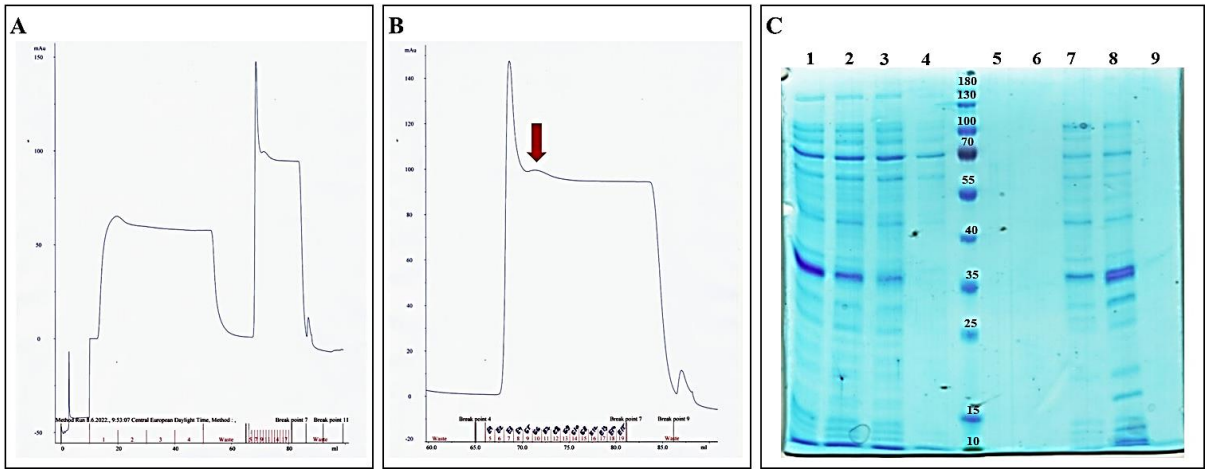

Figure 13. Protein purification method 3, PF2 purification. 1 Initial periplasmic fraction 2, 2. Sample 1 centrifuged 20 min at 10 000 g. 3 Sample 2 filtered. 4. Flowthrough, 5-8. Elution fractions 2-5, 9. Elution fraction 9.

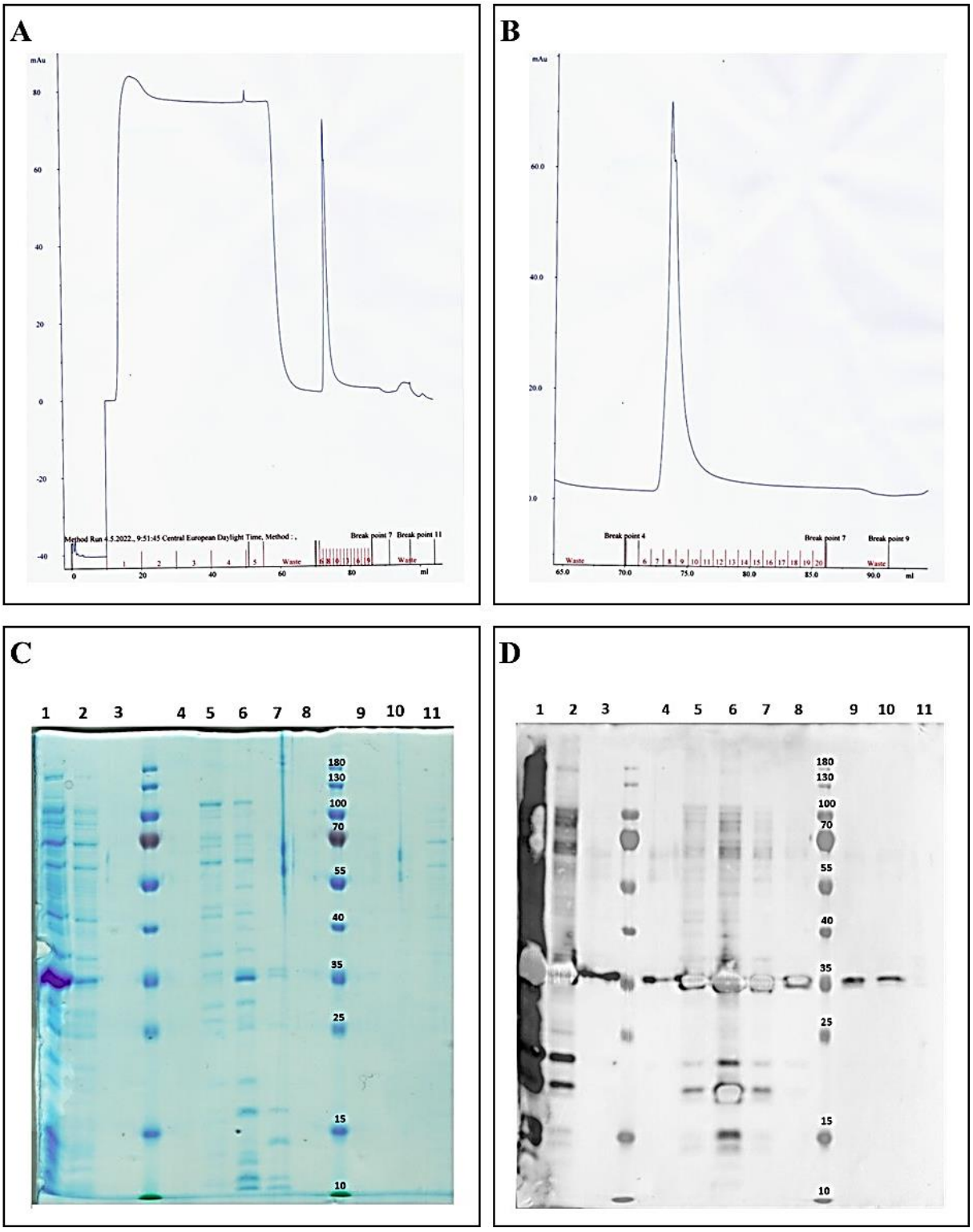

Figure 14. Protein purification method 3. PF1 purification. 1. Initial periplasmic fraction 1, 2. Sample 1 after centrifugation and filtration, 3-10. Elution fractions 1-8, 11. Flowthrough.

In conclusion, a large amount of scFv protein remains in insoluble form. IMAC binds all available soluble scFv without loss during chromatography. The purified scFv contains a certain amount of contaminants and it is recommended to perform a downstream purification step.

5.DISCUSSION

pOPE101 plasmid analysis shows an insert of the expected size

The three pOPE101 plasmid DNA bands observed (Figure 7A, lane 3) correspond to the classical plasmid DNA topologies; nicked, linear, and supercoiled which can be formed from circular intact DNA (36), such as the obtained using miniprep (37). The latter is further supported by the single restriction analysis (Figure 7B, lane 2, 3) where a single band at ~4000 bp can be observed upon complete digestion of the DNA, this corresponds with the theoretical size of 3970 bp of pOPE101 (38). The presence of the 750 bp band after double digestion with NotI and NcoI further corroborates the identity of the plasmid. This restriction sites are located at the VH upstream region and at the VL downstream region, and all this region accounts for the gene coding the scFv, in the form of VH-Linker-VL (39). Overall, these analyses confirm the correct size of the plasmid and insert, although for more detailed data, more powerful techniques such as plasmid sequencing could be performed to corroborate exact bp sequence (40).

Type of stirring and osmotic shock affect the release of soluble protein from the periplasmic space

Initial attempts to release the scFv from the periplasmic space were unsuccessful (see results, isolation method 1, above). This method was not good for isolation of the protein, a likely explanation is that this method is too mild compared to the other two methods, which use an osmotic shock strategy for lysis of the cells. Nevertheless, a similar isolation method applied for large scale production (41), described the successful isolation of recombinant periplasmic protein, however, agitation at 600 rpm with a propeller was employed instead. The later might indicate that strong stirring/shaking might be needed to achieve the lysis of the cell outer membrane in hypertonic buffer-only protein isolation methods.

Next, method 3, which has an additional step of resuspension in hypotonic solution, resulted in extraction of the protein in the hypertonic solution (periplasmic fraction 1), which seems to be contrary to the results obtained with method 1. This is probably because method 3 employs a different method of stirring, magnetic stirrer, which produces more shear force than the tube tumbling stirring. The differences in the shear forces are likely having an impact on the lysis of the outer membrane (19).

Lastly, method 2, which does not have a stirring step of the hypertonic cell suspension, resulted in similar results to method 3. However, the final protein quantity after purification indicates that the stirring step is important for achieving maximal protein extraction, as described in other protocols for antibody fragments isolation (42).

To sum up, it seems that protein isolation in osmotic shock methodologies, such as the ones presented here, benefit from the use of a hypertonic/hypotonic in combination with a high shear stirring method, as it is described by a majority of authors (43). Although it might be the case that only high shear stirring could be enough to release the contents of the periplasmic space, as methods like method 1 have been used previously by other authors (41). Moreover, it must be noted that a large amount of protein is remaining in the bacterial pellet (i.e., Figure 10A, B, lane 5), this could be an indication of another problem not related to the protein isolation, such as protein insolubility. Protein insolubility is a common problem associated with the bacterial expression systems. The latter starts by protein overexpression triggering in a higher synthesis rate in cytoplasm in comparison to the periplasmic transport rate, this disbalance causes the protein in cytoplasm to form insoluble protein aggregates, which are also known as inclusion bodies. This inclusion bodies initially could be denatured and chemically refolded to regain the protein; however this approach adds an extra layer of complexity which is out of the scope of this work.

IMAC was suitable for purification of the scFv protein, but further improvements are required

IMAC purification, intended for purification of HIS-tagged proteins, resulted in very good purifications of the expressed scFv (Figure 11, 13 and 14). The purifications captured all the soluble antibody fragment, as no protein was visible in the flowthrough fraction, resulting from the washing step. This step is intended to remove all the non-his-tagged proteins and further contaminants with low affinity to the HisTrap column. After washing of the column, the next step is to perform the elution of the proteins bound to the column. This step is performed by using of high concentrations of imidazole in the elution buffer, imidazole competes with the histidine tag for the binding to the Ni^{2+} ions. As it can be seen in the elution fractions in the SDS gel, the scFv eluted together with other proteins from the initial sample, this is not an uncommon phenomenon, as it has been stablished that Ni^{2+} IMAC, although providing higher yields, it also results in lower purity if compared with the use of other ions, such as Co^{2+} (44).

Additionally, it is also possible that the low purity was caused by the initial amount of imidazole in the binding buffer. A low concentration of imidazole is used in the binding buffer to prevent the weak binding of irrelevant proteins, which is a common (45), in our case we used 5-10 mM imidazole in the binding buffer, although normal concentrations can be in the range of 40-50 mM (17). Moreover, although this method is not ideal for a single-step purification process, these purifications provide the sample conditions for further purification, it is common to use size exclusion chromatography (SEC) after IMAC, SEC separates the proteins by their molecular weight (46).

6. CONCLUSIONS

- pOPE101 expression vector identity was verified by restriction analysis
- High expression of the protein was achieved by overnight induction at 18°C
- The isolation of protein from the periplasmic space is improved using vigorous stirring after cell resuspension
- Double osmotic shock is the best technique for releasing the majority of the soluble scFv
- IMAC purification is ideal for the purification of scFv proteins, although additional optimization is required for removal of contaminant proteins.
- Overall, the production is not as good as reported in literature by other authors, so therefore alternative expression systems can be used such as mammalian, yeast or insect expression system.

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7. LIST OF ABBREVIATIONS

- CDR- Complementary Determining Region
- CH- Constant Domain Of Antibody Heavy Chain
- CL- Constant Domain Of Antibody Light Chain
- DNA-Deoxyribonucleic Acid
- *E.Coli- Escherichia Coli*
- EDTA- Ethylene Diamine Tetraacetic Acid
- ELISA- Enzyme-Linked Immunosorbent Assay
- Fab- Antigen Binding Region
- FDA- Food And Drug Administration
- IMAC- Immobilized Metal Affinity Chromatography
- IPTG- Isopropyl B-D-1-Thiogalactopyranoside
- Mab-Monoclonal Antibody
- MCS-Multiple Cloning Site
- ORI- Origin Of Replication
- Pab- Polyclonal Antibody
- PCR- Polymerase Chain Reaction
- RNA- Ribonucleic Acid
- Scfv- Single Chain Variable Fragment
- VH- Variable Domain Of Antibody Heavy Chain
- VL- Variable Domain Of Antibody Light Chain

8. BIOGRAPHY

Merima Ćulah was born on April 6, 1997, in Pula. She attended the elementary school "Ivo Lola Ribar" and general high school "Mate Blažine" in Labin. She obtained a bachelor's degree during her undergraduate studies at the Medical Faculty of the University of Rijeka. While she was in her undergraduate studies, she was an active member of the commission at the "Student Congress of Health Care – Sanitas", carrying out the role of fundraising manager. She is currently completing her graduate studies in Environmental and Public Health at the Medical Faculty of the University of Rijeka, where she will earn the title of University Master of Environmental and Public Health.