Optimizing bacterial expression and purification of Phactr4 protein and its fragments

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Sarah Gaynor

Metropolia

Helsinki Metropolia University of Applied Sciences

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Author/Authors

Sarah Gaynor

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ABSTRACT

This final project was carried out in co-operation with a research group led by Maria Vartiainen Ph.D at the Helsinki Institute of Biotechnology. The aims of this work were: 1. Cloning Phactr4 protein's fragment Ph4 1-528 into bacterial expression vector pET41a producing a GST-tag, 2. Optimizing the expression of Phactr4 fusion protein and its C-terminal and N-terminal fragments consisting of optimizing temperature, time and testing the solubility of the fusion proteins and 3. Optimizing the purification of the two fragments of Phactr4 protein.

The cloning process was successfully performed after some optimization and change of vector. The optimal combination of temperature and time for expressing the fusion proteins was +37^{o}C for duration of three hours. The two fragments were soluble according to the solubility test, but no result was obtained for Phactr4 because the protein was not expressed sufficiently. The protein purification was optimized to a certain extent, but further optimization is still needed. Some N-terminal fragment protein was obtained with acceptable purity.

The cloned protein fragment can be expressed in suitable bacteria and then purified. The efficiency of the purification of the proteins still needs to be improved further. The purified proteins obtained will be utilized for biochemical binding studies to clarify the molecular mechanism by which Phactr proteins function.

Keywords

recombinant DNA technology, gene cloning, Phactr4 protein, recombinant protein production, GST affinity purification, SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

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TIIVISTELMÄ

Tämä opinnäytetyö tehtiin Maria Vartiaisen tutkimusryhmälle Biotekniikan Instituuttiin. Työn tavoitteina olivat: 1. Optimoida Phactr4 proteiinin fragmentti Ph4 1-528 kloonaus pET41a vektoriin, 2. Optimoida Phactr4 proteiinin ja sen C- sekä N-terminaalisten fragmenttien tuoton lämpötila, aika sekä suorittaa liukoisuustesti kyseisille proteiineille sekä 3. Optimoida Phactr4 C- ja N-terminaalisten fragmenttien puhdistus.

Kloonauksen eri osia optimoitiin erilaisin tuloksin, mutta kloonaus saatiin suoritettua, kun käytetty vektori vaihdettiin toiseen erään pET41a vektoria. Paras lämpötila ja aika proteiinien tuottamiseen olivat +37^{o}C ja kolme tuntia. Liukoisuustestin tulos kahden fragmenttien osalta oli, että molemmat proteiinit olivat liukoisia. Valitettavasti Phactr4 liukoisuustesti epäonnistui, koska proteiinia ei tuottunut, mutta sen kuitenkin oletetaan olevan myös liukoinen. Proteiinien puhdistuksen optimointi onnistui suhteellisen hyvin ja proteiineja saatiin puhdistettua vaihtelevalla menestyksellä. N-terminaalisen proteiinin tuotto ja puhdistus onnistui toista proteiinia paremmin ja puhdistuksilla saatiin pieni määrä kohtalaisen puhdasta proteiinia.

Kloonattua Phactr4 fragmenttia voidaan kokeilla tuottaa sopivissa bakteereissa sekä yrittää sen puhdistamista. Proteiinien tuotto onnistui hyvin, mutta sitä voidaan halutessa optimoida vielä lisää käyttäen pidempiä tuottoaikoja. Proteiinien puhdistus onnistui melko hyvin, mutta proteiinien puhdistusta on vielä optimoitava runsaasti lisää, jotta saataisiin suurempi määrä vielä puhtaampaa proteiinia talteen. Nyt saatua proteiinia tutkimusryhmä voi käyttää omissa tutkimuksissaan, joilla he pyrkivät selvittämään miten Phactr4 proteiini toimii.

Avainsanat

yhdistelmä-DNA tekniikka, kloonaus, rekombinanttiproteiinin tuotto ja puhdistus, SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

ABBREVIATIONS

amp^R	ampicillin-resistance gene
APS	ammonium persulfate
ATP-wash	adenosine triphosphate-wash
cDNA	complementary DNA
$CaCl_2$	calcium chloride
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded DNA
E. coli	Escherichia coli
EtBr	ethidium bromide
FPLC	fast performance liquid chromatograph
g	times gravity (same as relative centrifugal force)
G-actin	globular / monomeric actin
GST	glutathione S-transferase
$(His)_6$	6 x histidine residues
IPTG	isopropyl-β-D-1-thiogalactopyranosid
kb	kilo base
kDa	kilodalton
LB	luria bertani
MAL	megacaryocytic acute leukemia protein
mRNA	messenger RNA
N-/C-terminus	amino terminus / carboxy terminus
OD	optical density
o/n	overnight

PBS	phosphate buffered saline
PCR	polymerase chain reaction
Phactr	phosphatase and actin regulating protein
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase 1
RNA	ribonucleic acid
RNAi	RNA interference
RPEL	Arginine - Proline – Glutamic acid – Leucine
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ssDNA	single-stranded DNA
TAE	Tris-acetate (buffer)
TBE	Tris-boric acid-EDTA (buffer)
TEMED	tetramethylethylenediamine
tRNA	transfer ribonucleic acid
U	unit
UV	ultra-violet
V	volts

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1 INTRODUCTION

Despite the discovery of deoxyribonucleic acid (DNA) in the early 1950's and the subsequent discovery of the structure of DNA by the 1960's, it was only during the 1970's, when a new methodology called *recombinant DNA technology* or *genetic engineering* was developed. This new methodology made it possible to determine the structure of individual genes. (Brown 2006: 3-4.)

Recombinant DNA technology, which is a collection of methods, can be used to isolate genetic material from different sources, to modify and recombine genetic material outside of the cell and to transfer genetic material into other organisms. The technology can be used in many different fields of study, such as biochemical studies of cells, protein studies, molecular genetics, medical studies, in the creation of transgenic animals and plants, in gene therapy and in the biotechnical industry. (Suominen – Ollikka 2006: 45-46.)

Recombinant DNA technology is also an important part of this work. Different methods are used for example in cloning Phactr4 fragment into bacterial expression vector and in the creation and production of recombinant proteins. These methods are described in a later chapter.

The topic of this final work is: *Optimizing the bacterial expression and purification of Phactr4 protein and its fragments*. This final work is done under the supervision of Maria Vartiainen, Ph.D. - Academy Research Fellow, who leads a research group at the Helsinki Institute of Biotechnology and Johanna Puusaari, Ph.D. student. The group is studying the role of actin and actin-binding proteins in the nucleus and they have identified Phactr-proteins as novel nuclear actin-regulating proteins. Their cell biological assays have shown that Phactr-proteins bind protein phosphatase 1 (PP1), actin and plasma membrane, but the biochemical details of these interactions remain unclear. The Phactr proteins are also interesting to the group, because they contain a G-actin binding RPEL domain, which is similar to the RPEL domain of transcriptional coactivator MAL, whose function is regulated by G-actin levels. Mammals contain four Phactr proteins, but the Phactr4 protein was chosen for further studies, because it is expressed

more widely compared to Phactr3, for example, whose expression is largely restricted to brain.

There currently exists very little published research information about Phactr4, since it has only very recently been studied in depth. It belongs to a family of phosphatase and actin regulating (Phactr) proteins and these proteins are present for instance in worms (*Caenorhabditis elegans*), insects (*Drosophila melanogaster*), mice and humans. This family of Phactr proteins contains four members, which are Phactr1, Phactr2, Phactr3/Scapinin and Phactr4. These proteins show varied tissue distribution, and many are expressed in the nervous system, in the brain for example. (Allen – Greenfield – Svenningsson – Haspeslagh - Greengard 2004: 7187; Sagara – Arata - Taniguchi 2009:1; Sagara et al. 2003: 45616.)

The research group is focused on elucidating the basic concepts of actin in the nucleus, which is significant in order to understand how actin functions in several vital nuclear processes. The results will have an impact on a wide variety of basic cell biological fields, for example from regulation of gene expression to wiring of cellular signalling pathways. The group uses a wide range of cell biological and biochemical techniques for studying different aspects of nuclear actin. Their current projects include for example identification of novel factors regulating nuclear actin levels, of which Phactr-proteins is one example, studies on nuclear actin dynamics and understanding the molecular mechanism by which actin participates in gene regulation. (Nuclear actin laboratory: Maria Vartiainen.)

My role is to express and purify Phactr4 protein and its fragments as recombinant proteins in *Escherichia coli*. These purified proteins will then be utilized in the laboratory for biochemical binding studies to clarify the molecular mechanism by which Phactr proteins function. My written work can be used as a guideline for expressing and purifying these proteins in the future.

A brief catalogue of abbreviations used in this work is included after the abstract section.

2 AIMS

The main aim of this work is to express and purify Phactr proteins successfully in a laboratory environment for use in associated biochemical binding studies. Another important aim is to write a guideline, which could be used in future production of these proteins. More specific aims of this work include the following:

- Cloning Phactr4 protein's fragment Ph4 1-528 into bacterial expression vector pET41a producing a GST-tag.
- Optimization of the expression of Phactr4 fusion protein and its C-terminal and N-terminal fragments, which consist of optimizing temperature, time and testing the solubility of the fusion proteins.
- Optimizing the purification of the C-terminal and N-terminal fragments of Phactr4.

3 METHODS

The use of optimized methods and protocols is essential in order to assure that consistent, reliable and acceptable results are obtained. However, although pre-existing methods are available to many assays in biological sciences, they might be suboptimal for the studied DNA fragment or protein, which are all unique. Optimization allows one to narrow down the best options to use for a given method or protocol. For example, one can optimize, which reagents and amounts of used reagents work best or what kind of environmental conditions (e.g. temperature), or other experimental factor (e.g. incubation time) produce the best experimental results.

The use of commercial kits and ready protocols are often selected on the basis that they already assure the required optimization for different stages of the experimental work. Some experimental factors are also an important part of the overall optimization process. Examples include the annealing temperatures in PCR, the time and temperature used in protein production and the washing buffers used in protein purifications.

In this work, the research group's own protocols were used throughout, but these were also further optimized to improve the cloning of Phactr4 fragment, the expressing of the Phactr4 protein and its fragments and the purification of the two fragments.

3.1 Introduction to recombinant DNA technology methods

Genetic recombination takes place in nature both in animals and plants, where both parents of an individual are the original sources of the DNA, which recombine during meiosis. This recombinant DNA differs only from the parental DNA in the combination of the alleles it contains, but the sequence of the genes remains the same. Such restrictions do not however exist in the laboratory. Basically any segment of DNA can nowadays be cut out from any genome and be joined back together with any other piece of DNA. (Becker – Kleinsmith – Hardin – Bertoni 2009: 629.)

Recombinant DNA technology was made possible by the discovery of restriction enzymes, which have the ability to slice DNA molecules at precise sequences called restriction sites. They are powerful tools for slicing large DNA molecules into smaller fragments, which can then be recombined in different ways. The restriction enzymes, which create single-stranded sticky ends by making staggering cuts into the DNA, are especially useful because the sticky ends create a simple way for joining DNA fragments obtained from different sources. Basically, this means that any two DNA fragments, which have been generated by the same restriction enzyme, can be joined together by complementary base pairing between their single-stranded sticky ends and then be covalently sealed together by ligation (i.e. by DNA ligase enzyme). (See Figure 1.). (Becker et al. 2009: 629-630.)

The use of both restriction enzymes and DNA ligase enzymes enables two or more strands of DNA to be joined together, irrespective of their origins. For example, a strand of human DNA can be joined to bacterial DNA, thus creating recombinant DNA molecules, which would never occur in nature. The power of recombinant DNA technology lies in the fact that humans can create recombinant DNA molecules without regard for the natural barriers that would otherwise limit recombination to genomes of the same or closely related species. (Becker et al. 2009: 629-630.)

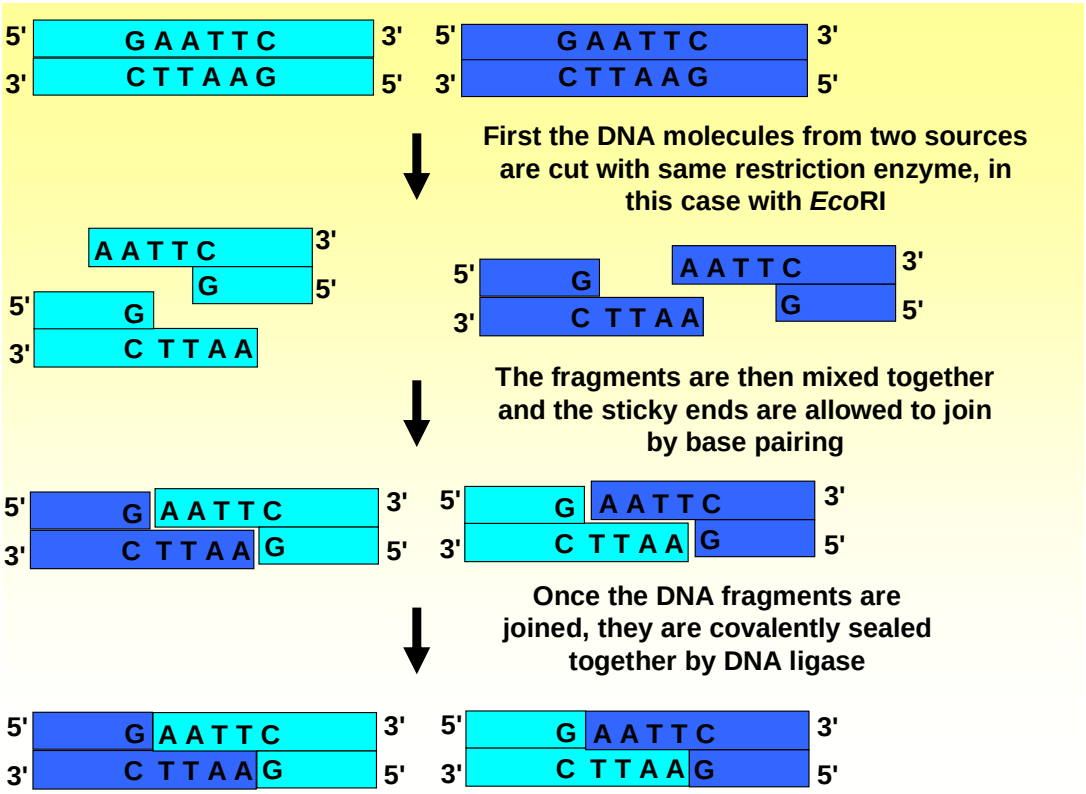

FIGURE 1. Creating recombinant DNA molecules (Modified from Becker et al. 2009: 630.)

3.1.1 Gene cloning

An essential feature of recombinant DNA technology is its ability to produce specific pieces of DNA in large enough quantities for research purposes. This method of generating numerous copies of particular DNA fragments is called DNA cloning. DNA cloning typically involves the following five steps: (1) DNA fragments are inserted into a cloning vector, (2) The recombinant vector is then introduced into the bacteria, (3) The recombinant vector is amplified in the bacteria, (4) The clones that are carrying recombinant vector DNA are selected and (5) The clones containing the gene of interest are identified. (See Figure 2.) (Becker et al. 2009: 630-631.)

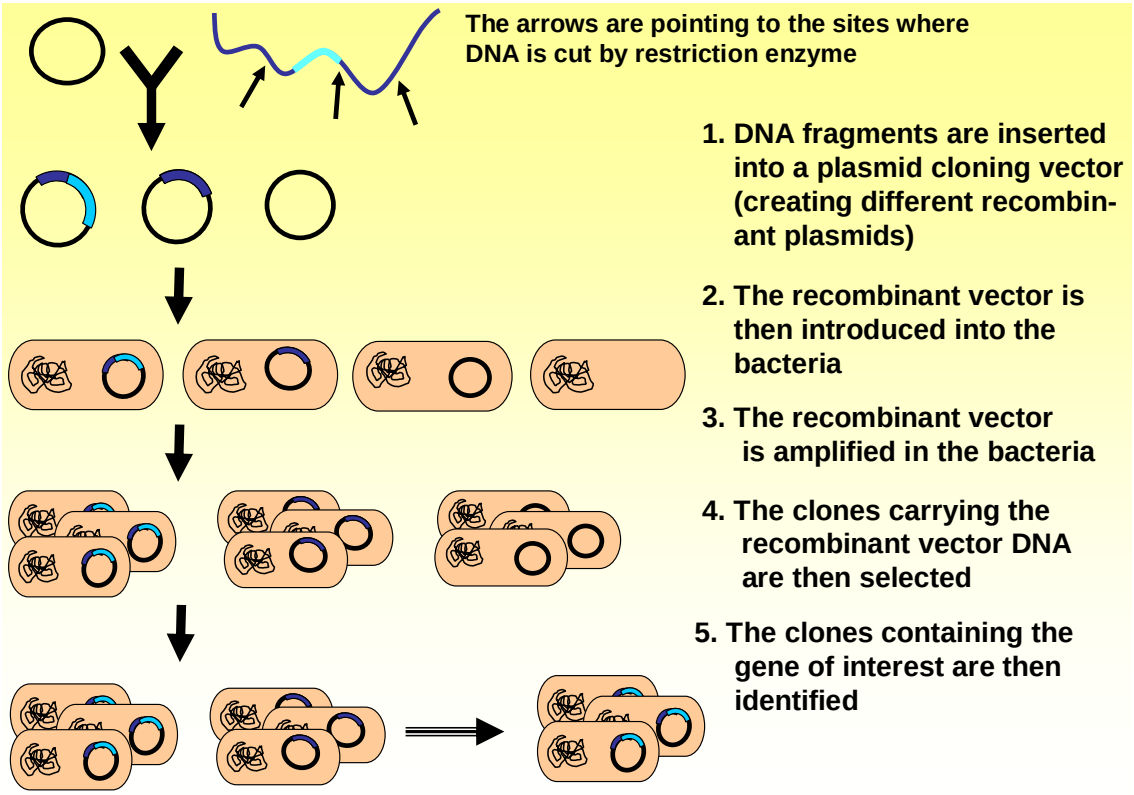

FIGURE 2. Gene cloning (Modified from Becker et al. 2009: 631.)

The first step of cloning is to insert the desired section of DNA into a suitable vector, for example into a plasmid or into a bacteriophage. A plasmid is a circular and double-stranded DNA (dsDNA) molecule that leads an independent existence in a bacterial cell. A bacteriophage is a virus, which infects bacteria. The plasmids, which are used for cloning, often have a variety of restriction sites and they often carry antibiotic-resistance genes giving antibiotic resistance in the associated host cells. The antibiotic-resistance genes make the selection stage possible and the presence of numerous restriction sites allow the plasmid to incorporate DNA fragments prepared with a variety of restriction enzymes. (Becker et al. 2009: 630; Brown 2006: 14.)

The second step is to introduce the recombinant vector into the bacterial cell, for example into the cells of *E. coli*, where the vector is replicated. Plasmids are introduced into the medium that surrounds the target cells, where in certain conditions a small amount of the cells will take up the plasmid DNA. The efficiency of this process is often enhanced by special treatments for example by a chemical treatment or by electroporation. In one chemical treatment, the bacteria cells are treated with calcium chloride, $CaCl_2$,

and then heat-shocked by raising the temperature momentarily to +42^{o}C. This enhances the uptake of plasmid-DNA into the bacteria. In electroporation, the bacteria cells are subjected to a short electrical pulse, which cause transient pores in the cell membrane, through which the plasmid-DNA enters into the cells. (Becker et al. 2009: 631; Brown 2006: 90-92, 105.)

The third step is to amplify the vector in bacteria. After the host bacteria have taken up the recombinant vector, they are then plated out on nutrient medium. As the bacteria replicates, the recombinant plasmids are also replicated generating a vast number of vector molecules, which contain the foreign DNA fragments. (Becker et al. 2009: 631-632.)

The fourth step is to select the cells that have successfully incorporated the recombinant vector. This selection can be based on the antibiotic-resistance genes. For example, if the plasmids carry an ampicillin-resistance gene, amp^R, the bacteria will be resistant to the antibiotic ampicillin. When bacteria cells are grown in a culture medium, which contains ampicillin, then only the bacteria that have the plasmids carrying the ampicillin-resistance gene will survive in the medium. (Becker et al. 2009: 632-633.)

The fifth step is to identify the clones that have the desired DNA. Usually the previous steps have created a huge number of bacteria producing many different kinds of recombinant DNA, but with only a few that are relevant to the desired application. The bacterial colonies have to be screened to identify those colonies containing the specific DNA fragment of interest. There are several techniques that can be used to screen the colonies of bacteria, for example by restriction digestion and DNA sequencing. (Becker et al. 2009: 633; Brown 2006: 166-180.)

3.1.2 Polymerase chain reaction

Nowadays, scientists have determined the genome sequences of hundreds of bacteria and several dozen eukaryotic organisms, which also include humans. This means that there is a simple and fast method available to clone genes, called polymerase chain reaction (PCR). This method can be used to clone genes from complementary DNA (cDNA) or from genomic DNA libraries. The method requires that one knows part of the base

sequence of the target gene to be amplified, and requires use of short single-stranded DNA (ssDNA) primers, which are complementary to sequences located at opposite ends of the gene under synthesis. The primers are then used to target the intervening DNA for amplification. PCR also allows genes to be modified by adding a desired base sequence to the primers being used. (Becker et al. 2009: 636.)

Polymerase chain reaction can be used to amplify a selected DNA region, whose border sequences are known. The PCR reactions are done in microcentrifuge tubes by mixing the DNA with needed reagents for the reactions and then placing the tubes into a thermal cycler, which controls the incubation temperatures. The amplification of the desired DNA is carried out by a heat durable DNA polymerase enzyme, for example *Taq*, *Pfu, Vent, Tth* or *Phusion* polymerase. (Brown 2006: 6-7, 181-183; Suominen – Ollikka 2006: 107.)

The template DNA, which is a dsDNA, is denatured when the mixture is heated to +94^{o}C. The mixture is then cooled down to between +50^{o}C and +60^{o}C, whereby annealing of the oligonucleotide primers then occurs. The temperature is then raised again to +72^{o}C, whereby the DNA polymerase attaches to the end of each primer and it synthesizes from dNTPs new strands of DNA, which are complementary to the template. The temperature is then raised back to +94^{o}C, thereby causing a second reaction cycle to start. The cycle is then repeated 25-30 times, to create enough copies of the template DNAs and at the end of the last cycle a final elongation at +72^{o}C can be done to ensure that all of the single stranded DNA are fully extended. (See Figure 3.). Please note that used temperatures and the number of repeated cycles can vary in different PCR programs. (Brown 2006: 6-7; Suominen – Ollikka 2006: 107-109.)

When all of the PCR cycles are completed, the produced PCR product is checked by subjecting a portion of it to agarose gel electrophoresis. The result of the electrophoresis confirms if the produced PCR is the correct size and if the PCR has worked correctly. (Brown 2006: 189.)

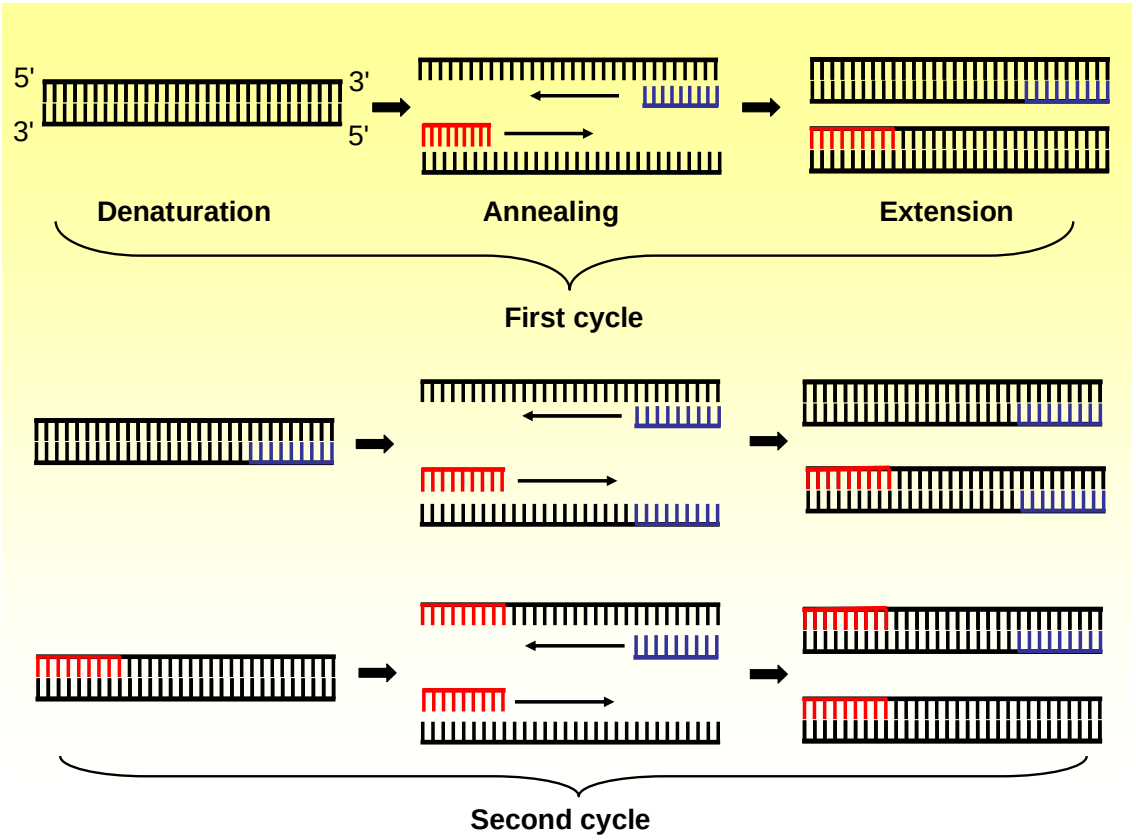

FIGURE 3. PCR reaction (Modified from Suominen – Ollikka 2006: 109.)

3.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis can be used to separate DNA or RNA according to their size, or more specifically to analyse nucleic acids that are between 0,1-50 kb. Nucleic acids are negatively charged so they move through a gel, which is subjected to an electric field, towards the anode. Because of the web structure of the gel, smaller molecules move faster through the gel than larger molecules and the molecules are separated into different bands according to their size. These bands are visualised with ethidium bromide (EtBr) staining, which penetrates between nucleic acids and emits an orange - red fluorescence, when the gel is exposed to ultraviolet light. (Brown 2006: 189; Miesfeld 1999: 18-19; Suominen – Ollikka 2006: 72-75.)

Several factors affect the migration of molecules in the gel: the conformation and size of the DNA, the concentration of the agarose in the gel and the applied voltage. In particular, the concentration of the agarose determines the pore size of the gel and therefore affects the migration of different sized molecules. The denser the gel, the slower the

molecules migrate through it. The applied voltage determines the electrical current, which affects the velocity of electrophoresis. The higher the voltage, the faster the molecules move, but if the voltage is increased too much, the heat it creates will melt the gel. (Miesfeld 1999: 18-19; Suominen – Ollikka 2006: 75.)

3.1.4 The principles of DNA extraction from agarose gel

The basic principle of the used commercial kit NucleoSpin® Extract II (Macherey-Nagel), which is designed for DNA purification from TAE or TBE buffer agarose gels is the following: the desired DNA band is visualized by EtBr-staining under UV light and cut from the agarose gel with a clean scalpel, then the agarose is melted and the DNA is bound to the silica membrane found in the NucleoSpin® Extract II columns in the presence of chaotropic salt. Contaminants such as salts and soluble macromolecular components are removed by washing the membrane with ethanol. The purified DNA is then eluted from the membrane under low ionic strength conditions. See Appendix 4 for more details on the extraction process. (PCR clean-up, gel extraction: NucleoSpin® Extract II Users manual 2009:6)

3.1.5 The principle of plasmid-DNA purification with DNA minipreps

The principle of the DNA miniprep (Fermentas) method is based on SDS/alkaline lysis of bacterial cells, which is then followed by adsorption of DNA onto silica in the presence of high salt concentration. The method consists of four essential steps whereby the bacteria are first lysed under alkaline conditions after which the lysate is neutralized and adjusted to high-salt binding conditions. In the second step, the plasmid DNA is adsorbed onto the silica membrane and in the third step contaminants are washed away. In the fourth step the plasmid-DNA is eluted with elution buffer or sterile water. The purified DNA is then ready for further use. See Appendix 5 for more details on the purification process. (GeneJET^{TM} Plasmid Miniprep kit 2008: 2.)

3.1.6 Description of cloning process used in this work

The PCR of the cloning process was carried out with the following reagents and PCR program, which were used to amplify the desired sequence. See Charts 1 and 2. The used PCR machine was PTC-100 MJ^{TM} research. After PCR, the PCR product was sub-

jected to agarose gel electrophoresis. A 1% agarose gel containing a few drops of ethidium bromide was used to perform all of the agarose gel electrophoresis. Loading buffer with a final concentration of 1x was added to the samples to give colour and to make them heavier. Samples and 15 µl of 1 kb ladder (New England biolabs) were then subjected to electrophoresis for approximately 45 minutes at 80V. After electrophoresis was completed, the gel was transferred onto a UV-light table and photographed.

CHART 1. The reagents needed in PCR reactions.

Amount	Reagents	Final concentration
1 µl	Ph4-GFP maxiprep	100 ng/µl
1,5 µl	dNTP mix (Finnzyme)	300 µM
5 µl	5' primer V215 (BamHI)	1mM
5 µl	3' primer V175 (HindIII)	1mM
10 µl	HF buffer (Finnzyme)	1x
0,5 µl	Phusion hot start-polymerase (Finnzyme)	1 U
27 µl	Sterile H_2O	---

CHART 2. The utilised PCR program

Phase	Temperature	Time	Repetitions
1. Pre-denaturation	98°C	3 minutes	Once
2. Denaturation	98°C	30 seconds	x 30
3. Annealing	60°C	45 seconds	
4. Elongation	72°C	2 minutes	
5. Final elongation	72°C	10 minutes	Once
6. End	4°C	End-less	Once

The desired insert, obtained by PCR, was inserted into a suitable vector by restriction digestion. The needed reagents (see Chart 3) were mixed together in a microcentrifuge tube, which was then incubated for one hour at +37°C. After the incubation, the digestion was pipeted onto an agarose gel with a 1 kb ladder and subjected to electrophoresis. Upon completion of the electrophoresis, the correct size PCR product and vector were cut from the gel and the DNA was extracted as described in section 3.1.4.

CHART 3. The samples and reagents needed in the digestion. (* The concentration was not stated on the manufacturer's product information).

Amount	Reagents	Final concentration
25 µl / 5 µl	PCR product / Vector	*unknown* / 0,65 µg/µl
3 µl	Fast digest buffer green (Fermentas)	1x
1 µl	Fast digest enzyme BamHI (Fermentas)	1 µl*
1 µl	Fast digest enzyme HindIII (Fermentas)	1 µl*
0 µl / 20 µl	Sterile water (making a total volume of 30 µl)	---

The ligation was then performed by mixing the digested and extracted insert and vector DNAs and the reagents (see Chart 4) into a microcentrifuge tube, and then subjected to overnight (o/n) incubation at +17^{o}C.

CHART 4. Ligation reagents.

Amount	Reagents	Final concentration
16,5 µl	Digested insert Ph4 1-258	*unknown*
5 µl	Digested vector pET41a	*unknown*
2,5 µl	T4 DNA ligase buffer (New England Biolabs)	1x
1 µl	T4 DNA ligase (New England Biolabs)	200 U

If the used vectors tend to seal back together without the desired insert, the digested vectors can be treated in order to avoid this. The needed reagents used are shown in Chart 5. The mixture is left to incubate for 20 minutes at +37^{o}C, and then subjected to agarose gel eletrophoresis and gel extraction before it is used in ligation.

CHART 5. Vector dephosphorylation reagents.

Amount	Reagents	Final concentration
20 µl	Digested vector pET41a	*unknown*
2,5 µl	FastAP^{TM} buffer (Fermentas)	1x
1 µl	FastAP^{TM} Thermosensitive alkaline phosphatase (Fermentas)	1 U
1,5 µl	Sterile water	---

The ligated recombinant vector was transformed into a chemically competent *E. coli* bacteria strain. The transformation was performed as follows: 200 µl of DH5α-cells were thawed on ice and transferred into a bigger tube with a round bottom. The ligation was then added, mixed by gently tapping the tube and incubated on ice for 30 minutes. Cells were heat-shocked by inserting the tube into +42^{o}C water bath for 30 seconds. After the heat-shock, the tube was placed back on ice for 1-2 minutes and 900

µl of 1x LB-medium was then added. The tube was then placed on a shaker for one hour at +37°C.

After the incubation, the transformed bacteria were transferred into a microcentrifuge tube and centrifuged at 11 000 x g for 1 minute at room temperature (RT) (Heraeus Pico 17 centrifuge Thermo Electron Corporation). Most of the media was then pipeted away until there was only 100 µl remaining. The pellet was re-suspended into the remaining media and transferred onto a plate containing LB-agar and 50 µg/ml of kanamycin. The re-suspension was spread across the surface of the agar with the help of small glass beads to ensure the appearance of single colonies. The plate was then placed for o/n growth at +37°C. The selection of correct bacteria was done by using antibiotic kanamycin. This antibiotic ensured that only bacterium containing plasmids with a kanamycin resistant gene encoding the GST-fusion protein, which was being cloned, could grow.

The identification of the correct clones was done by restriction digestion. First, some small liquid-cultures were grown from the colonies on the plate. These cultures contain 6ml of 1x LB-medium, kanamycin with a final concentration of 25 µg/ml and a single colony of bacteria. These cultures were left to grow o/n on a shaker at +37°C. On the following day plasmid-DNA purification was performed with a commercial kit as described in section 3.1.5. After the purification, each miniprep, which are purified plasmids eluted into sterile water, were tested with restriction digestion (see Chart 6). The concentration of the minipreps that contained the correct sized insert, were measured with Nanodrop ND-1000 spectrophotometer and they were then stored at -18°C for further use.

CHART 6. Testdigestion reagents. (* The concentration was not stated on the manufacture's product information).

Amount	Reagents	Final concentration
5 µl	Plasmid-DNA	*unknown*
2 µl	Fast digest buffer green (Fermentas)	1x
0,5 µl	Fast digest enzyme BamHI (Fermentas)	0,5 µl*
0,5 µl	Fast digest enzyme HindIII (Fermentas)	0,5 µl*
12 µl	Sterile water (making a total volume of 20 µl)	---

3.2 Recombinant protein production

One of the key methods used in this experimental work is recombinant protein production, which is a specific application of recombinant DNA technology. See also Figure 4. First, the desired recombinant DNA, which contains DNA sequences derived from two different sources, is inserted into an appropriate vector, for example into a plasmid or into a bacteriophage with the help of DNA ligases. The vector is governed by the host, which can either be phage, bacteria, yeast, plants, filamentous fungi, insect or mammalian cells grown in culture or transgenic animals. It is also influenced by the behaviour of the target protein. The amplification of a fusion protein, which contains a tag of known size and biological function, can greatly simplify subsequent isolation, purification and detection. This factor has led to the increased use of fusion protein vectors. The two most commonly used tags are glutathione S-transferase (GST tag) and 6 x histidine residues $(His)_6$ tag and they are chosen according to the needs of the specific application. (Becker et al. 2009: 630; Lodish et al. 2008: 176-178; The recombinant protein handbook: protein amplification and simple purification 2000: 6-8.)

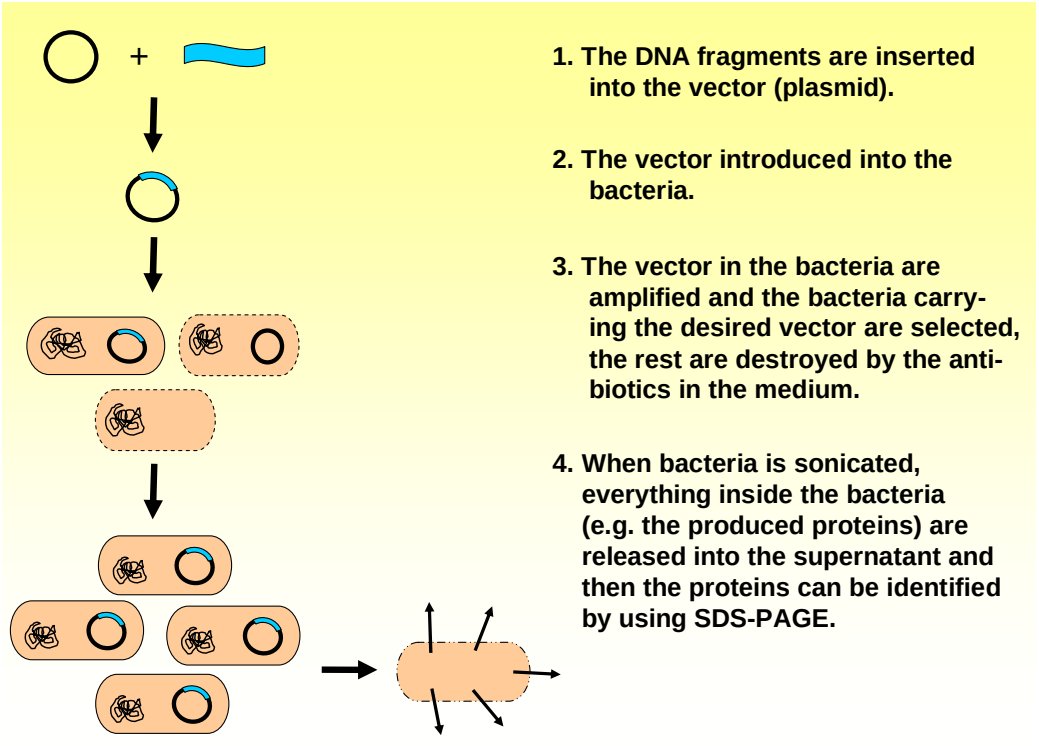

FIGURE 4. Protein production using the method of DNA cloning. (Modified from Becker et al. 2009: 631.)

The transformation of the vector into the bacteria, the amplification of the bacteria and the bacteria selection are done as previously described in section 3.1.1. Bacteria containing the plasmid of interest can then produce the recombinant protein. In most cases, the desired recombinant protein accumulates inside the bacteria, but it can in some cases be secreted out of the cells into the medium. (Becker et al. 2009: 631-633; Lodish et al. 2008: 178-179.)

3.2.1 Expression system

There are many types of expression systems, which are designed to produce several copies of the desired protein in a host cell. The T7 expression system is one of them. It is one of the most commonly used, because it can produce high levels of most proteins in bacteria. (The pET Expression System 2009; pET system manual 2006:3.)

In this system, the expression of the protein coding mRNA is driven by the T7 promoter, which is dependent on the T7 RNA polymerase. It originates from a phage, and is thus not normally expressed in bacteria. Therefore a host strain is used, which is genetically engineered to carry the T7 RNA polymerase, lac promoter and lac operator in its genome. The lac operator ensures that the T7 RNA polymerase, and thus the protein expression, only occurs in the presence of lactose, or a similar molecule IPTG, which binds to the lac repressor, and displaces it from the lac operator. The lac repressor is in turn encoded by the expression plasmid, thus ensuring efficient repression of the expression in the absence of lactose. (The pET Expression System 2009; pET system manual 2006:3.)

3.2.2 GST affinity purification

The use of GST affinity tag with recombinant proteins has become popular, after its introduction in 1988. It is based on GST's affinity to glutathione ligand coupled to a matrix creating an affinity column that allows GST-tagged proteins to be easily purified from bacterial lysates. The non-bound proteins are then easily washed away. Binding of the GST-tag to the ligand is reversible and the proteins can also be eluted under mild and non-denaturing conditions by the addition of reduced glutathione to the elution

buffer. (Recombinant Protein Purification Handbook: Principles and Methods 2009: 113-120.)

This method therefore provides a mild purification process, which does not affect the native structure and function of proteins. If the proteins need to be separated from the GST-tag, an appropriate protease cleavage site can be introduced between the GST-tag and the protein. The most commonly used protease for this application is thrombin but other proteases can also be used, for example 3C protease. (Recombinant Protein Purification Handbook: Principles and Methods 2009: 113-120.)

3.2.3 Gel filtration chromatography

In gel filtration, also called size exclusion chromatography, proteins flow through the column that separates the proteins according to their size. The gel filtration column contains gel filtration matrix, which is made of porous matrix in the form of spherical beads, and it is equilibrated with a suitable buffer that fills the pores of the matrix. The matrix is chosen according to its chemical and physical stability and also by its lack of reactivity and adsorptive properties. This material can be for example polyacrylamide, dextran, which is a bacterial polysaccharide, or agarose. (Gel filtration: principles and methods 2007: 6, 9-10; Lodish et al. 2008: 96-97.)

When buffer is added to the column to wash the proteins through the column, smaller proteins travel through the column more slowly compared to bigger proteins. The smaller proteins penetrate the depressions in the porous beads of the column more eagerly than bigger proteins and consequently take more time to travel through the column. (Lodish et al. 2008: 96-97.)

Gel filtration can be performed according to the requirements of the experiment for instance in the presence of essential ions or cofactors, detergents, at high or low ionic strength and at +37°C or in a cold room. Also any chosen buffer can be used to collect the purified proteins. This is why this technique is suitable for biomolecules that can be very sensitive to changes in pH, concentration of metal ions or to harsh environmental conditions. (Gel filtration: principles and methods 2007: 9-10.)

3.2.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins according to their molecular weight. The technique is popular because it is easy to set up and perform, inexpensive and the resolution is high with a fast separation. The separation of the molecules on the gel takes place according to their size. Small proteins migrate faster through the gel, because they can manoeuvre more swiftly through the pores of the gel. (Janson, Jan-Christer – Rydén, Lars 1998: 481-483; Lodish et al. 2008: 94-95.)

SDS-PAGE consists of two gels, the stacking gel (the upper gel) and the resolving gel (the lower gel). The stacking gel stacks the proteins into tight bands, or in other words concentrates the proteins and the resolving gel separates proteins on the gel. (Demystifying SDS-PAGE 2006.)

In SDS-PAGE, the sample, which is often a mixture of proteins, is first denatured with sodium dodecyl sulphate, which also gives the sample a negative charge, dissociates multimeric proteins and denatures all the polypeptide chains. The sample is then placed into the wells of the stacking gel and an electric field is applied. During the electrophoresis the proteins migrate and separate into bands according to their sizes and they are visualized by staining with a protein-binding dye. The molecular weight of a protein can be estimated by comparing the distance it travels to the distance of a reference protein with a known molecular weight. The proteins that have been separated with SDS-PAGE can be extracted from the gel and used for additional analysis. (Lodish et al. 2008: 94-95.)

3.2.5 Description of protein expression procedure used in this work

The recombinant proteins containing a GST-tag were produced as follows: The plasmids were transformed into suitable *E. coli* with electroporation. 1 µl of the bacterial expression plasmids were pipeted into a microcentrifuge tube containing the Rosetta strain bacteria and they were mixed together. After that, 50 µl of the mixture was pipeted into an electroporation cuvette, which was placed into the electroporation machine, with *bacteria mode* selected, and a single shock was administered. 1 ml of LB

medium was then added to the cuvette and the mixture was transferred to a 2 ml micro-centrifuge tube that was incubated in a shaker for one hour at + 37^{o}C.

During the incubation, four LB plates were prepared with the appropriate antibiotics for the bacterial cultures. The plates contained 1x LB agar, 50μg/ml of kanamycin and 34 μg/ml of chloramphenicol. The same concentration of the antibiotic chloramphenicol was used throughout different parts of the task. In liquid-growths kanamycin was used at 25 μg/ml. 100 ml of solid LB agar was melted in a microwave oven and then left to cool down at room temperature, until it was safe to touch. The antibiotics were then mixed into the agar, which was poured into four plates, avoiding bubbles. They were then left to set, with the lids a little open to allow moisture to escape.

After the incubation, the bacteria were treated as described in section 3.1.6, and the plates were grown o/n at +37^{o}C. Master-cultures were prepared by mixing 1x LB-medium, the appropriate antibiotics, and 1% glucose into suitable bottles and by inoculating a single colony into the mix. The cultures were grown o/n at +37^{o}C in a shaker. The bacteria plates were saved for possible later use and they were stored at +4^{o}C with parafilm around the edges, to prevent the plates from drying. Next day sub-cultures were diluted, for example 1:100, which were then grown on a shaker for a few hours at +37^{o}C, until the OD_{600} (optical density) was in the range of 0.5-0.6.

The optical density measurement was done as follows: first a reference sample was measured at 600 nm, which in this case was 1 ml of 1x LB medium in a transparent cuvette. Then, a 1 ml sample was taken from one of the bacterial cultures and placed into a similar cuvette and the OD was measured with the spectrophotometer (Ultrospec 2000 UV/visible spectrophotometer Pharmacia biotech).

The protein expression was induced with 0.2 mM IPTG and the protein production was allowed to proceed at suitable temperature and for the required time. After both uninduced and induced samples were taken, the rest of the cultures were centrifuged depending on the size of the cultures. For example 1L cultures were centrifuged at 4550 x g for 25 minutes at +4^{o}C (Beckman Coulter J6-MI centrifuge). The pellets were re-suspended into suitable amount (2-10 ml) of 1x PBS containing 15 μg/ml benzamidine and 1 mM PMSF. Benzamidine and PMSF are protease inhibitors, and will thus prevent degrada-

tion of the proteins during the subsequent steps. The bacteria were then stored in Falcon tubes at -80°C for preservation and purification at a later stage.

3.2.6 Description of protein purification procedure used in this work

The GST affinity purification was performed as follows to the expressed proteins: First, the bacteria cells stored at -80°C were melted and sonicated for 6x 20 seconds (Branson sonifier cell disruptor B15) and cooled in-between on ice. Sonication is a method that uses ultra high-frequency sound to break open cells. With this method it is possible to break *E. coli's* tough cell wall and release the proteins. (Lodish et al. 2008: 391.)

They were then centrifuged depending on the volume of the bacterial pellet. Small volumes, such as 1-2 ml, were centrifuged at 16 000 x g for 15 minutes at +4°C (Eppendorf Centrifuge 5415 R). Bigger volumes were centrifuged at 9680 x g for 30 minutes at +4°C (SS-34 rotor, Sorvall Instruments RC5C centrifuge) using special tubes capable of withstanding the high pressure. During the centrifugation, a suitable amount of glutathione sepharose™ 4B beads (GE healthcare) were washed in a Falcon tube with 1x PBS, by centrifuging at 650 x g for 2 minutes at +4°C (Eppendorf centrifuge 5810 R). The supernatant was removed by suction and the tube with the beads was placed on ice. This washing was done to remove the ethanol in which the beads were preserved.

The cleared supernatants were added onto the beads and the beads were left to incubate on rotation for two hours at +4°C. After the incubation, the beads were centrifuged as above. The supernatant was removed by suction and the beads were then washed by adding suitable washing buffer, mixing the beads and centrifuging as before. The supernatant was again removed by suction. This washing step was repeated as many times as needed (typically 4 – 7 times). The beads were then transferred into a microcentrifuge tube and treated with 3C protease, which was added onto the beads and left to incubate o/n on rotation at +4°C. The 3C protease cleaves the proteins off the GST and releases the proteins into the supernatant.

The following day, the beads were centrifuged at 1800 x g for 1 minute at +4°C and the supernatant was collected into a separate tube placed on ice. The beads were then washed with a suitable washing buffer for example 3 times collecting all the

supernatants in the separate tube. The supernatants were concentrated with Amicon® Ultra-4 centrifugal filter device (Millipore) by centrifuging the filter until only the desired volume of the supernatant was remaining. The concentraded supernatants were then divided into microcentrifuge tubes (50 µl/ tube) and quick-frozen with liquid nitrogen. They were then stored in a freezer at –80^{o}C.

When the pooled supernatants obtained by GST affinity purification contained mostly only the desired protein, it was subjected to gel filtration chromatography. With gel filtration chromatography, which separates the proteins according to their size, the desired protein is separated from rest of the unwanted proteins and pure protein is obtained. The equipment used for the gel filtration chromatography was FPLC (ÄKTAFPLC^{TM} GE Healthcare). The used gel filtration buffer was 10 mM Tris (pH 7,0) – 100 mM NaCl, and 3 ml fractions were collected after the proteins passed through a UV-spectrophotometer. When molecules passed the spectrophotometer, they were shown as different size peaks on the curve, following the purification. Some samples were taken from the peak fractions and some were taken from the fractions, which did not show any signs of proteins. These samples, taken in the same way as in the previous purification rounds, were analyzed with SDS-PAGE. The concentration of the purified protein after gel filtration was obtained as follows: The absorbance was measured at 280 nm and 320 nm and counting the result with Lambert-Beer's law $A= \varepsilon cl$, with the ε_{280} = 11000 M^{-1} cm^{-1} (ProtParam), $A= A_{280} – A_{320}$ and $l = 0,1$ cm.

All the samples taken during expression and purifications were melted and sonicated (Bandelin Sonorex RK52) until the samples could be pipeted. The samples where then boiled for 5 minutes at +100^{o}C and then pipeted onto the wells of SDS-PAGE gels together with PageRuler^{TM} pre-stained protein ladder (Fermentas). See Appendix 6 for further details and instructions on how to make the SDS-PAGE gels. The electrophoresis was started at 100V but was later increased to 150V, in order to finish it faster. In total, the samples were subject to electrophoresis for about 1,5 hours.

After the electrophoresis was finished, the glass moulds were detached and one of the glasses was carefully removed off the gel. The upper stacking gel was scraped away and the lower resolving gel was detached onto a staining dish with great care. Coomassie brilliant blue dye, which stains the proteins allowing their visualization, was then used

to dye the gels. The gels were left to stain for about 15 minutes and after that the dye was rinsed with water and destain (5 % methanol, 7 % acetic acid) was added to the dishes. When the destaining solution became blue, it was changed and the gels were left to destain overnight.

4 EXPERIMENTAL WORK AND OBTAINED RESULTS

There are three aims in this experimental work: the optimization of the cloning of a protein fragment of Phactr4 into a bacterial expression vector, the optimization of the expression of the full-length Phactr4 protein (Ph4-fl) and its two fragments: C- (Ph4-C) and N-terminus (Ph4-N) and the optimization of the purifications of the two fragments. An outline of all stages of the experimental work is described through three related Appendices. See Appendix 1, Appendix 2 and Appendix 3.

4.1 Cloning a protein fragment into bacterial expression vector

The first step in cloning Ph4 1-528 into pET41a vector involves use of PCR to amplify the nucleotide sequence corresponding to amino acids 1-528 of Ph4 for cloning. The PCR was performed as described in section 3.1.6, and as a template the Ph4-GFP vector was used, which contains the coding sequence for Phactr4 protein in another vector. After the PCR program was finished the sample was subjected to agarose gel electrophoresis. Unfortunately the PCR product at 1,5 kb was very weak, but it was cut from the gel and the DNA was extracted with an extraction kit called NucleoSpin® Extract II (Macherey-Nagel). See also Appendix 4 for details of the extraction protocol.

Next the extracted PCR product and the pET vector were digested with BamHI and HindIII enzymes as described in section 3.1.6, and subjected to agarose gel electrophoresis. The correct sized bands were cut from the gel and PCR and vector DNAs were extracted and ligated. On the following day the ligation was chemically transformed into DH5α-cells and after o/n incubation the plate was checked for colonies - the sign of successful transformation. There were 3 colonies on the plate, which were used to grow small liquid-cultures for performing plasmid-DNA purification using a commercial kit

GeneJET™ Plasmid Miniprep Kit (Fermentas). See also Appendix 5 for details of the purification protocol.

After the purification, a testdigestion was performed for all minipreps. The result of the testdigestions indicated that all minipreps had only a band corresponding to an empty vector, and none contained the desired insert. In other words, the cloning had failed during some step, most likely because there was not enough insert after PCR.

4.1.1 Optimizing the PCR of the cloning

Because it was obvious that the PCR had not worked as well as it should have, it was further tested if increasing the annealing temperature from +60°C to +65°C would boost the amplification, by making the primers work more specifically. This made no difference to the result however, since no band was visualized after electrophoresis. After this result, it was suspected that the PCR machine itself was not working correctly and all the remaining PCR reactions were performed on a different PCR machine (DNA Engine Tetrad 2 Cycler, Biorad), with better success.

Next, the PCR was performed at two different annealing temperatures and with two different buffers. PCR was performed on the first two samples with an annealing temperature of +50°C. The same HF-buffer was used on the first sample and a GC-buffer (Finnzyme) was used on the second sample. PCR was then performed on the second set of samples with an annealing temperature of +55°C. Again a HF-buffer was used on the first sample and a GC-buffer on the second sample. The number of cycles of steps 2-4 of the PCR program was increased from 30 to 40. Different annealing temperatures were tested to find the annealing temperature, where both used primers work best. Both HF- and GC-buffers came with the Phusion hot start-polymerase and were tested to see which of them works better in this cloning.

All of the PCR reactions worked this time and there was a correct band of 1,5 kb on the gel for each of the samples. The other PCR machine was obviously somehow broken and this was probably the reason why the PCR did not succeed the first two times. Out of the four samples taken, the two best were sample 1 with an annealing temperature of

+55°C and HF-buffer and sample 4 with an annealing temperature of +50°C and GC-buffer. These two PCR products were then used for the rest of the cloning procedure.

Both samples were treated as described before and transformation into DH5α-cells with the ligations was attempted. Unfortunately, all attempts failed. The remaining steps of the cloning procedure also needed to be further optimized.

4.1.2 Optimizing the other steps of the cloning

Several unsuccessful attempts were made to transform the ligation into the desired cells, the main reason for failure was suspected to be a lack of insert after digestion. One further attempt produced a vector, which contained an insert, however the size of the insert proved to be too large and thus could not be used. At this point in time, the main reason for failure was suspected to be contamination during the ligation process.

Further attempts were made, using increased amounts of the PCR product, to try to result in greater amounts of the desired insert after the digestion, but this also failed to produce a successful result. The transformation was then tried again, with some recently made chemically competent DH5α-cells, but this did not make any difference to the result of the transformation.

In the next transformation attempt, the bacteria was left to grow for two hours instead of one hour, to see if this would have any effect on the transformation. This time there were more colonies on the plates, but unfortunately DNA minipreps isolated from these colonies contained only the used vectors.

To prevent the cloning vectors from sealing back together without the desired insert during ligation, the digested vectors were dephosphorylated with a phosphatase enzyme. Next, a new ligation was made using the treated vectors and this time the ligation was performed using two different temperatures and reaction times to see for which conditions the ligase would work best. First, the ligation was left at room temperature for one hour and then half of the ligation was transformed, as before. The rest of the ligation was transferred into an incubator for o/n incubation at +17°C and transformed the next day. The first transformation did not produce any colonies, but the second transforma-

tion produced a single colony, which was treated as described earlier. The result was that it indeed contained both vector and insert, but this time the insert's size was a little bit too small to be the desired insert. Again, the sample seemed to have been contaminated during some step of the cloning, but the source of the contamination remained unclear.

After this result, it was contemplated if the used vector was really working correctly during the cloning. A different batch of the vector was used to test this hypothesis. As a source of the vector, a pET41-Ph4 plasmid, which contains the full-length Phactr4 sequence cloned with the same enzymes used for this cloning, was used for digestion and overnight ligation with the digested PCR product was performed, as before. The ligation was transformed and the transformation produced several colonies, of which 12 were treated as before and then testdigested. Out of the 12 colonies, 7 contained both the vector and the desired insert. Finally, the cloning had worked. Clearly, the reason for all of the previous troubles was the used vector, which was not working correctly during the cloning. Unfortunately, when the concentrations were measured it turned out that the concentrations were really low, even though they were pure enough. It is recommended that new plasmid-DNA miniprep should be performed, to ascertain if better concentrations of the plasmids could be obtained.

4.2 Optimizing the expression of Phactr4 and its fragments

First the bacterial expression plasmids for GST-tagged Ph4-C, Ph4-N and Ph4-fl were transformed into a Rosetta expression strain by electroporation and bacteria were plated on plates containing kanamycin and chloramphenicol. The use of these antibiotics ensured that only bacterium containing plasmids encoding the GST-fusion proteins with a kanamycin resistant gene could grow. The Rosetta strain contains plasmids that encode for secreted lysozyme and tRNAs, which are specific to human codons that help the bacteria protein production. These plasmids contain a chloramphenicol resistant gene.

The following day, the plates were checked for single colonies to tell if the transformation had worked correctly, which it had. Liquid-cultures of single colonies were prepared, and after o/n growth, they were diluted into 300 ml so that OD was 0.1, and grown until OD was 0.5-0.6, when an uninduced sample of 1 ml was taken from the

cultures and placed into microcentrifuge tubes, which were centrifuged at 11 000 x g for 1 minute at RT. The supernatant was removed and the bacterial pellet was re-suspended in 100 µl 1x SDS-PAGE loading buffer and subsequently stored at –18°C.

The protein expression was induced with IPTG and cultures were divided into three 100 ml cultures. The protein production was allowed to proceed at different temperatures: +37°C (3 hours), +24°C (3 hours) and +15°C (overnight). After these incubations, 1 ml samples were taken from and treated as described above. Rests of the cultures were handled as described in section 3.2.5. The samples where then subjected to SDS-PAGE gel electrophoresis and the gels were stained with Coomassie, and subsequently destained. See Chart 7 for sample order in SDS-PAGE.

On the next day, the gels were checked and it was noticed that they contained the correct bands for the desired proteins. The Ph4-N band was slightly larger than expected from its calculated molecular weight and this trend seemed to continue throughout all of the performed expressions. The Ph4-fl bands were very small, so the protein had not been expressed very well and it was barely discernible from the gel. This meant that it was not possible to express the protein sufficiently well to permit proper test purification. According to these results, the best temperature for expressing the proteins Ph4-N and Ph4-C was +37°C. The correct size bands for the three proteins are as shown in Chart 8. (See also to Figure 5.)

CHART 7. Order of the samples from the expression test on the gels.

Sample	Amount	Sample	Amount
1. Prestained protein ladder	5 µl	1. Prestained protein ladder	5 µl
2. Ph4-N: uninduced sample	10 µl	2. Ph4-fl: uninduced sample	10 µl
3. Ph4-N: +37°C	10 µl	3. Ph4-fl: +37°C	10 µl
4. Ph4-N: +24°C	10 µl	4. Ph4-fl: +24°C	10 µl
5. Ph4-N: +24°C	10 µl	5. Ph4-fl: +15°C	10 µl
6. Ph4-N: +15°C	10 µl		
7. Ph4-C: uninduced sample	10 µl		
8. Ph4-C: +37°C	10 µl		
9. Ph4-C: +24°C	10 µl		
10. Ph4-C: +15°C	10 µl		

CHART 8. Protein Band Sizes.

Protein	Combined Band Size (kDa)	Protein Size (kDa)	GST-tag Size (kDa)
Ph4-N	42	12	30
Ph4-C	48	18	30
Ph4-fl	108	78	30

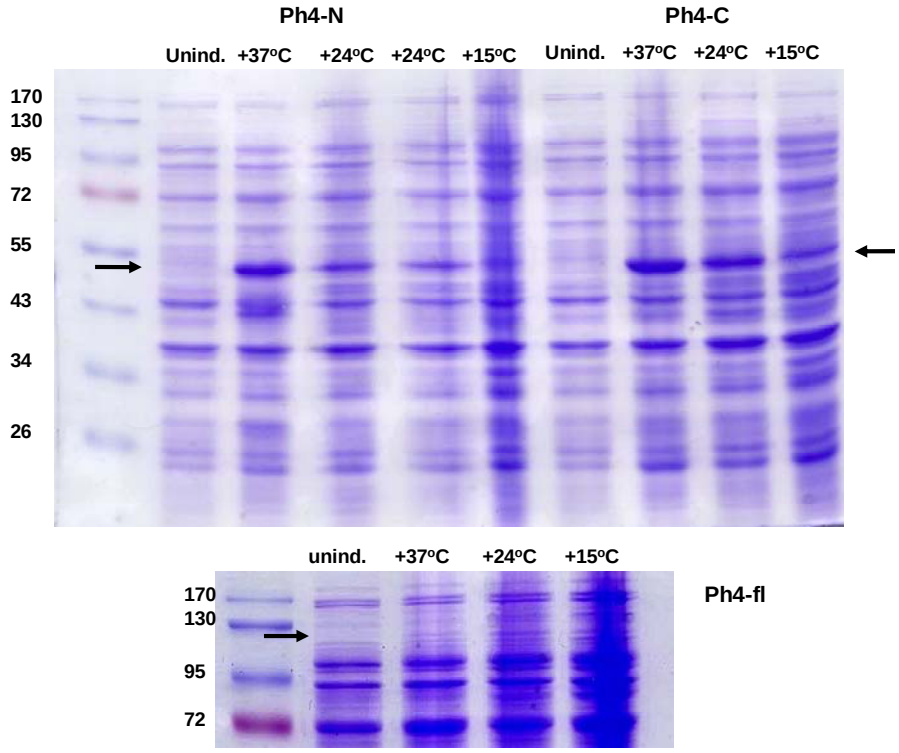

FIGURE 5. The results of the expression of the proteins with arrows pointing to the correct sized bands. (Unind. stands for uninduced sample.)

4.2.1 Solubility tests

The solubility screen was performed to ascertain that the proteins were soluble, and remained in the supernatant after lysing the bacteria. If the bacterium has problems in protein production, the proteins may cluster to inclusion bodies, and later when the bacteria is lyzed by sonication, proteins will not be released into the supernatant, as needed.

Ph4-C and Ph4-N were expressed for 3 hours at +37°C as described above. After the induced samples were taken, the cultures were then divided into five tubes, each containing 10 ml of the culture and they were centrifuged at 3200 x g for 10 minutes at +4°C.

The supernatants were discarded and the pellets were re-suspended in 1 ml of different lysis buffers as shown in Chart 9 and then placed on ice. The bacteria were then sonicated 4x 10 seconds and cooled in-between the sonications on ice to avoid the disintegration of the proteins. After the sonications, 25 µl lysis samples (L) were taken and mixed together with 25 µl of the lysis buffer and 15 µl of 4x SDS-PAGE loading buffer.

CHART 9. The used lysis buffers

	Lysis Buffers	Abbreviated names
1.	1x PBS	PBS
2.	1x PBS + 1 % Triton	1 % Triton
3.	1x PBS + 5 % Glycerol	5 % Glycerol
4.	1x PBS + 500 mM NaCl	500 mM NaCl
5.	1x PBS + 1 % Triton + 5 % Glycerol	1 % Triton + 5 % Glycerol

The remaining solutions were centrifuged, as before, and 50 µl supernatant samples (S) were taken from the supernatants and mixed together with 15 µl of 4x SDS-PAGE loading buffer. The remainders of the supernatants were discarded and the pellets were dissolved in 1 ml of 1% SDS. 50 µl pellet samples (P) were taken from the dissolved pellets and 15 µl of 4x SDS-PAGE loading buffer was again added. The obtained samples were subjected to SDS-PAGE, as described before.

The result of the solubility tests can be seen in Figures 6-7. The proteins had been expressed, but it seemed that the bacteria had not been sonicated enough and this caused most of the proteins to remain in the pellets. The proteins were, however, soluble and the best lysis buffer for both Ph4-C and Ph4-N was 1x PBS containing 1% Triton and 5% Glycerol, as seen from supernatant samples on the gels. The worst lysis buffer was 500 mM NaCl.

The solubility test was also done to Ph4-fl, but unfortunately the protein was not expressed following induction of the culture, thus there was no result for the test. Since the proteins were soluble, test purification of the two shorter fragments was however possible.

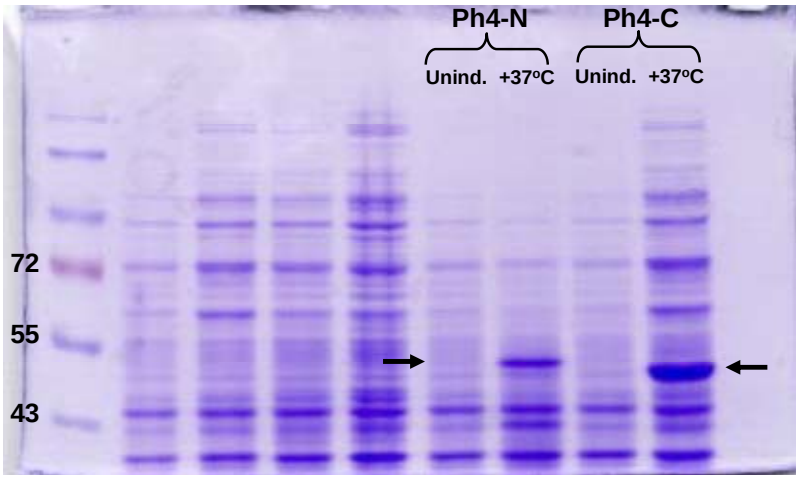

FIGURE 6. The expression of Ph4-N and Ph4-C in the solubility test with arrows pointing to the correct sized bands. (Unind. stands for uninduced sample and +37°C for induced sample grown at +37°C.)

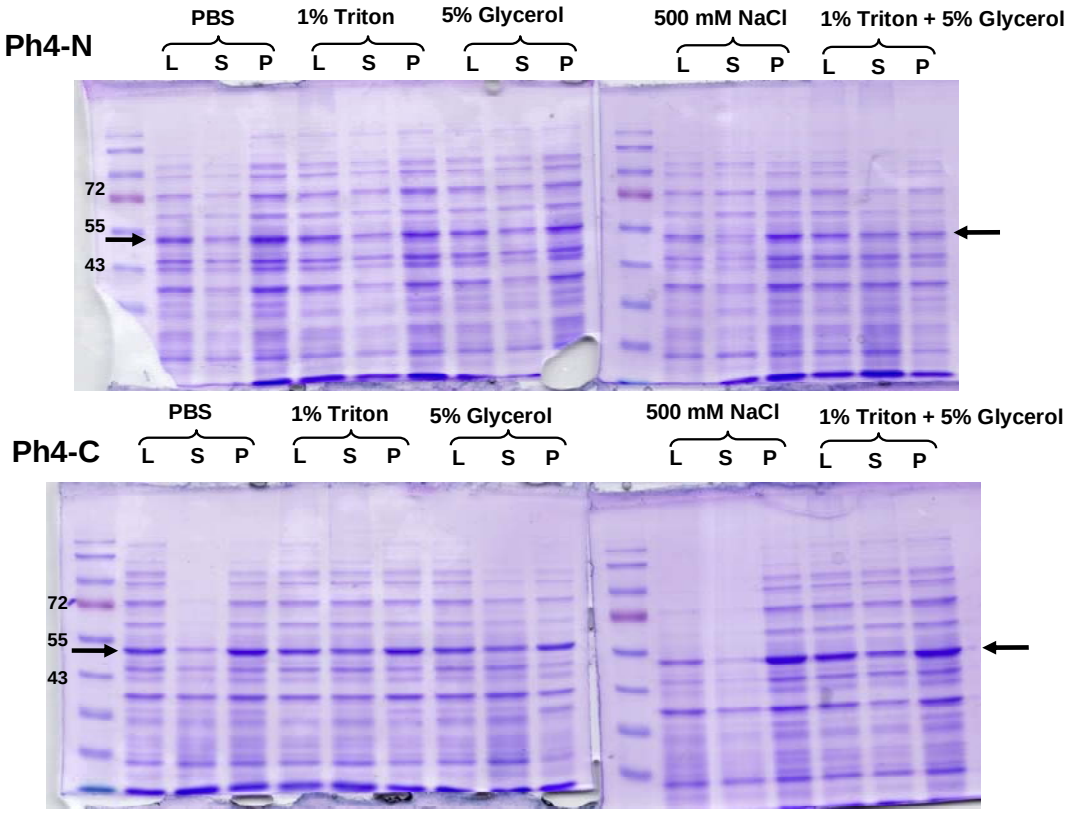

FIGURE 7. The results of the solubility tests with arrows pointing to the correct sized bands (From Sample L to Sample P of each buffer). (L stands for lysis sample, S for supernatant sample and P for pellet sample).

4.2.2 Test purification

The test purifications of the three proteins were done only to the bacteria grown at +37°C, since the proteins were expressed best at this temperature. First, the bacteria cells were melted and sonicated. They were then centrifuged and before the cleared supernatants were added onto the beads, a 15 µl sample was taken from the supernatant and 5 µl of 4x SDS-PAGE loading was added to the sample, which was then stored at -18°C. The same steps were repeated for the samples taken later. The beads were left to incubate on rotation and after the incubation, the beads were centrifuged. A second sample, containing the unbound fraction, was taken from the supernatant. The remainder of the supernatant was removed and the beads were then washed by adding 3 ml of 1x PBS, mixing the beads and centrifuging as before. A third sample was taken after the first wash from the supernatant and the remainder of the supernatant was again removed by suction. This washing step was repeated three times and after the last wash 25 µl of 1x SDS-PAGE loading buffer was added onto the beads for elution, thereby forming the fourth sample.

The four samples taken (i.e. 1-4) with un-induced and induced samples were subjected to SDS-PAGE gel electrophoresis. The gels of Ph4-N and Ph4-C are shown in Figure 8. The quantities of samples pipeted in the wells were 10 µl for the first three samples (1-3) and 20 µl for the fourth sample (4). The Ph4-fl gel is not shown since the bands are not visible in the taken picture and the gel staining had not functioned correctly. As can be seen from sample 4 on the gels, the beads contained too much of other proteins and there is a need to improve the efficiency of the washing steps. From the smearing of the samples 1 and 2 it can be noted that the bacteria also needs further sonication, since evidently a lot of the proteins have remained in the pellet during sonication of the bacteria.

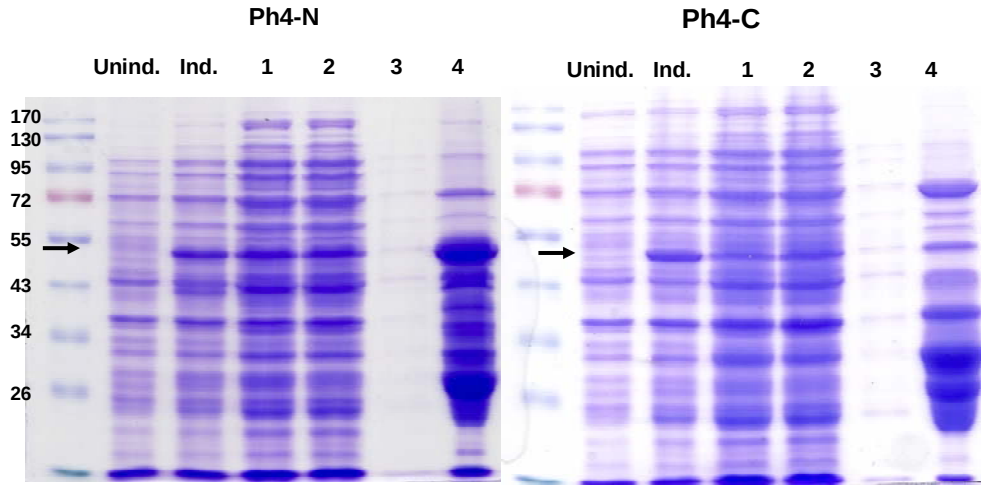

FIGURE 8. Test purification of Ph4-N and Ph4-C with arrows pointing to the correct size bands (From Uninduced sample to Sample 4). (Unind. stands for uninduced sample and ind. for induced sample grown at +37°C. 1-4 represent Sample1 to Sample 4 taken during purification).

4.3 Optimizing the purification of the proteins

The proteins were expressed in the manner previously described section 3.2.5, after which the purifications were then performed.

4.3.1 The first purification

From the test purification, it was noticed that the beads sample contained many other unwanted proteins and to get rid of them new washing buffers with different salt concentrations were used. The different concentration of salt should elute most of the unwanted proteins off the beads. Also different detergents could be used to help the removal of unwanted proteins.

To start the purification, bacteria from the 3L culture were melted and sonicated until the bacteria were no longer slimy. Otherwise, the first parts of the purifications were done as before, but the amount of used glutathione sepharose beads was 4x 750 µl.

After incubation, the beads were washed with 5 ml of 50 mM Tris (pH 7,5) - 50 mM NaCl buffer. The beads were then mixed and placed for further 5 minute incubation on rotation at +4°C. After this incubation, the beads were centrifuged, as before. The beads

were then washed a second time with the same buffer and after that 2x 50 mM Tris (pH 7,5) - 150 mM NaCl buffer, 2x 50 mM Tris (pH 7,5) - 300 mM NaCl buffer and 1x 50 mM Tris (pH 7,5) - 150 mM NaCl buffer. After the last wash, 500 µl of the last wash buffer was added onto the beads, which were then transferred into four microcentrifuge tubes. 75 µg of 3C protease was added into each tube, which were then left to incubate with the caps sealed with parafilm on rotation at +4^{o}C for o/n.

Next morning, the beads were centrifuged and the supernatants were collected into two separate tubes, which were placed on ice. The beads were then washed three times with 125 µl of 50 mM Tris (pH 7,5) - 150 mM NaCl buffer and all the supernatants were pooled. The fourth samples were taken from the pooled supernatants and the fifth samples were taken from the beads. The supernatants were centrifuged at 3200 x g for 4 minutes at +4^{o}C to get rid of any beads remaining in the supernatant. The supernatants were concentrated into 500 µl, which were then divided into microcentrifuge tubes and quick-frozen with liquid nitrogen. They were then stored in a freezer at –80^{o}C.

The samples collected during expression and purification of the proteins were treated as before and subjected to SDS-PAGE gels with the 14% resolving gels. The gels are shown in Figure 9. From the results of the purifications of Ph4-N and Ph4-C, it was apparent that additional purification is needed, as there were too many proteins still remaining in the supernatant, which can be seen from sample 4. Also from sample 5, it can be seen that the beads contained too many unwanted proteins. Additional changes noted necessary for the next purification included increased sonication of the bacteria, which can be seen from samples 1-3, and more effective washing of the beads.

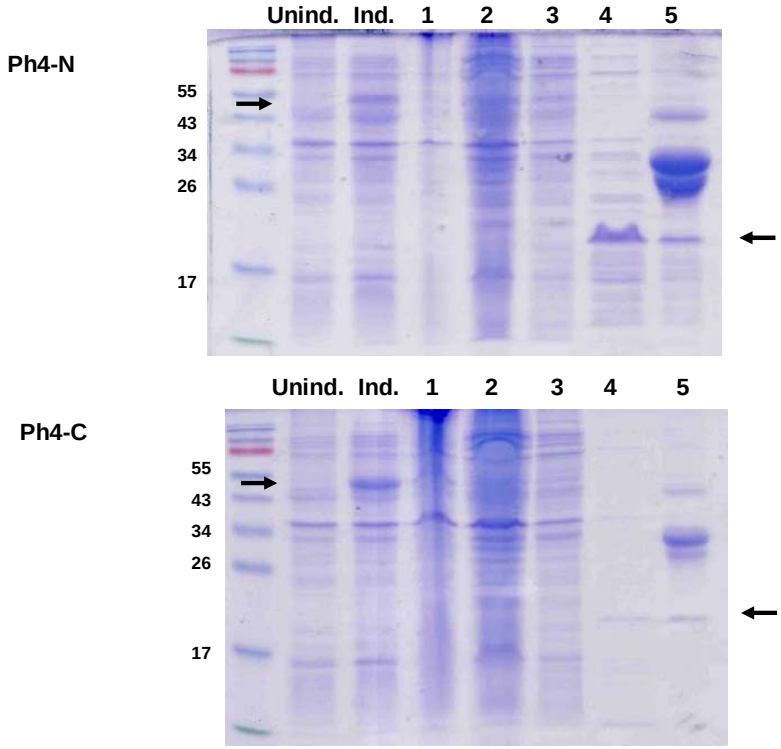

FIGURE 9. The results of the first purifications. The upper arrows on the gels are pointing to the correct sized bands of the proteins with the GST-tag (From Uninduced sample to Sample 3). The lower arrows are pointing to the correct size bands of the proteins after the GST-tag has been removed (Sample 4 and Sample 5). (Unind. stands for uninduced sample and ind. for induced sample grown at +37^{o}C. 1-5 represent Sample1 to Sample 5 taken during purification).

4.3.2 The second purification

In the second purification, 1L of bacteria containing Ph4-N and 3L of bacteria containing Ph4-C were purified as described in section 4.3.1 with some minor changes. 0,1 % of Triton X-100, a non-ionic detergent, was added to the first washing buffer to increase the strength of the wash. Also new beads, Protino® Glutathione Agarose 4B (Macherey-Nagel), were tested and used from this point forward in all purifications. A new marker was used in SDS-PAGE called SeeBlue® Plus2 prestained standard (Invitrogen) to produce a better scale.

Unfortunately, one mistake occurred during the purification and some of Ph4-C got erroneously mixed into the Ph4-N pooled supernatants. Because of this, the Ph4-N supernatants were not stored, but instead two samples were taken both from the contaminated

supernatant and from the non-contaminated supernatant, and these were subjected to SDS-PAGE.

The results of these purifications were clearly improved, because less other proteins were visible in the pooled supernatants, but more efficient purification is still clearly required. This time there was also much more of the Ph4-N, and it was decided that after the next purification, the pooled supernatant would be purified even more with the help of FPLC equipment, which utilises gel filtration chromatography. With the help of gel filtration chromatography, the desired protein would thus be separated from rest of the proteins. See Figure 10.

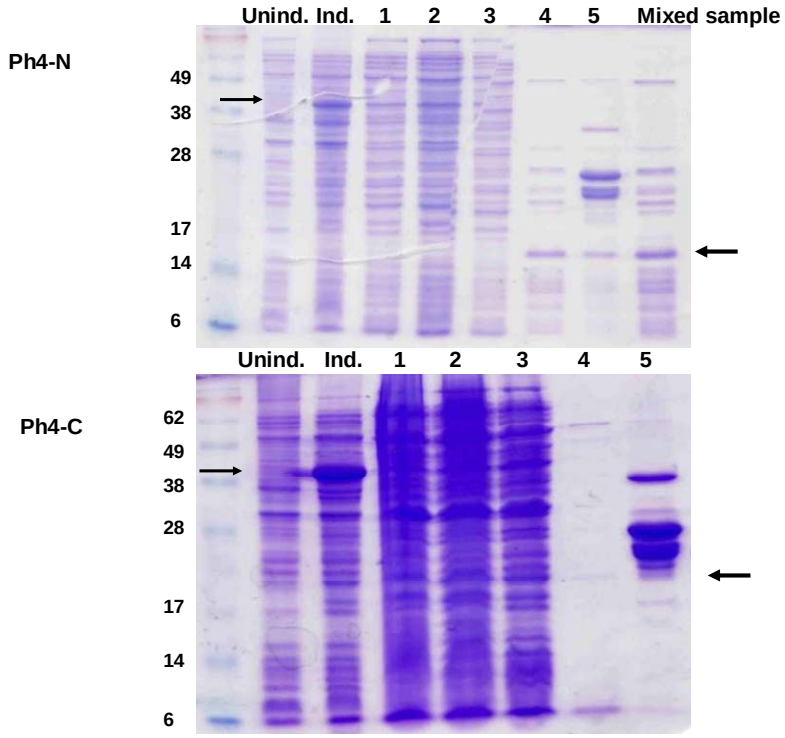

FIGURE 10. The results of the second purifications. The upper arrows on the gels are pointing to the correct sized bands of the proteins with the GST-tag (From Uninduced sample to Sample 3). The lower arrows are pointing to the correct size bands of the proteins after the GST-tag has been removed (Sample 4, Sample 5 and Mixed sample). (Unind. stands for uninduced sample and ind. for induced sample grown at +37^{o}C. 1-5 represent Sample1 to Sample 5 taken during purification).

4.3.3 The third purification

In the third purification, 5L of Ph4-N and 3L of Ph4-C were purified with one change whereby 0,1 % Triton was added into the buffers of the first six washes. Otherwise, the

purification was done as described earlier; except Ph4-N was concentrated into 2 ml. Ph4-N was also further purified with the help of gel filtration chromatography as described in section 3.2.6. In this case, there was one nice sharp peak and one low wide peak on the curve. The taken samples were analyzed with SDS-PAGE. See the results of the purifications in Figure 11.

The gel filtration purified some of the Ph4-N and the fractions containing the correct protein were joined together and concentrated into 500 µl. The fractions from the sharp peak had too many other proteins in them along with the desired protein, so they had to be discarded, because they could not be used in any further studies. The concentration of the saved proteins from the lower peak was 54 µM, which should have been a lot better.

As seen from sample 4, the purification of Ph4-C had gone well and the obtained protein was fairly pure. The beads should probably have been washed better to remove all of the desired proteins off the beads. Nevertheless, both proteins still need to be purer for some of the studies planned by the research group. Further purification steps will still need to be introduced before the use of gel filtration in the future.

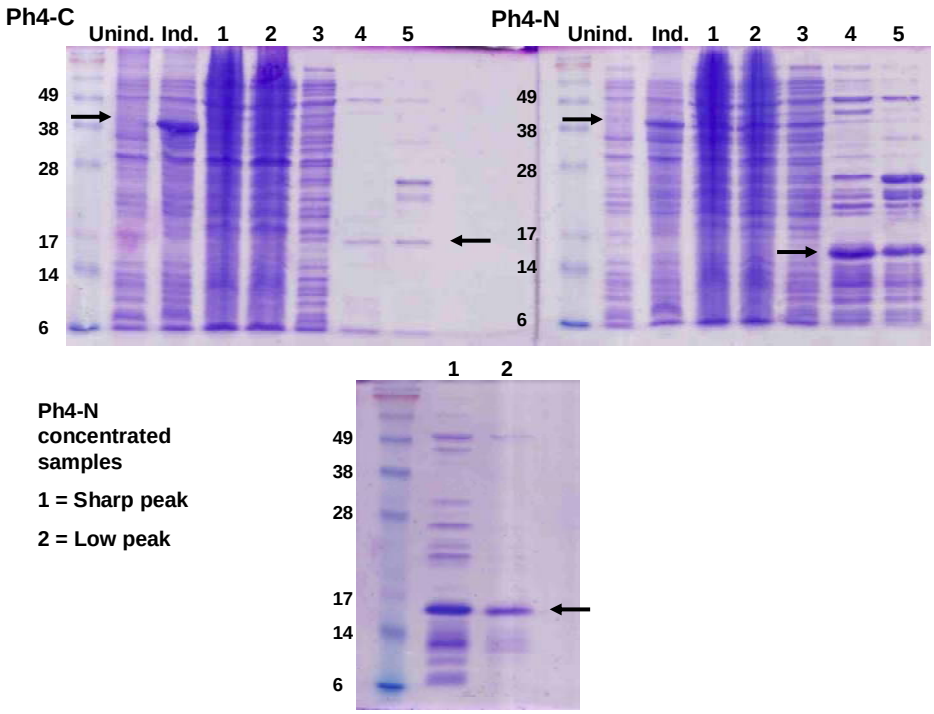

FIGURE 11. The results of the third purifications and gel filtration. The upper arrows on the gels are pointing to the correct sized bands of the proteins with the GST-tag (From Uninduced sample to Sample 3). The lower arrows are pointing to the correct

size bands of the proteins after the GST-tag has been removed (Sample 4 and Sample 5). (Unind. stands for uninduced sample and ind. for induced sample grown at +37°C. 1-5 represent Sample1 to Sample 5 taken during purification).

4.4 Summary of key results

Cloning of the protein fragment - result

The result of cloning Ph4 1-528 into bacterial expression vector pET41a producing a GST-tag was that after optimization, the cloning was successful. The first used pET41a-vector did not work as desired causing most of the problems during the cloning, but after a different batch of the pET41a-vector was used, there were no problems in getting the desired insert into the vector.

Optimization of expression - result

The optimization of the expression of Ph4-C, Ph4-N and Ph4-fl fusion proteins mostly worked well. The optimal combination of temperature and time for expressing the proteins was +37°C for three hours. However, Ph4-fl was expressed only very little and the expression of this protein still needs to be optimized further. The solubility test was performed successfully for Ph4-C and Ph4-N and the result of both of the tests was that both proteins were soluble as desired. Solubility test was also performed on Ph4-fl, but unfortunately the protein was not expressed during the test and thus no result was obtained. It is however assumed from the other two solubility test's results that Ph4-fl is also soluble.

Optimization of the purification - result

The purification of Ph4-C and Ph4-N were optimized a lot, but further optimization of the purifications is still needed. The third purification of Ph4-C removed most of the unwanted proteins from the supernatant and the gel filtration helped to obtain fairly pure Ph4-N. In both cases, the proteins still need to be purer before successful large scale purification with gel filtration can be performed. After the gel filtration and following analysis of the samples taken from the fractions, the protein was stored at +4°C. It was at first feared that the protein might breakdown and be lost before it could be stored at -80°C, but it transpired that the protein was still fine, even after storage for several days at +4°C. It was also noted that during the concentration, the protein did not get stuck to the filter device, contrary to an earlier suspicion by the research group. Throughout all

of the performed purifications, it was noted that the proteins seem to disappear for an unknown reason and although the filter element of the concentration device was suspected to be involved, this was not adequately proven and thus this theory was discounted.

5 DISCUSSION

The cloning process in the experimental work proved much more difficult than expected and was very susceptible to the quality of the utilised vector. Often, several experimental attempts were required in order to obtain the desired result. This indicates a need for special care and vigilance during the preparation of the vector.

During this experimental work the desired result was obtained only after optimization of several parts of the cloning process. The two most significant encountered problems were: malfunction of the PCR machine and failure to obtain sufficient quantity of the correct PCR product. It was suspected, but not confirmed, that temperature played a role in the machine malfunction and the malfunction was in turn a factor for the poor yield of the required PCR product. After the PCR machine was changed, the PCR part of this work then worked as expected. In addition to the poor yield of PCR product, the overall cloning process also failed to achieve the expected results, due to poor performance of the utilised vector. In particular, the vector did not work as expected during the digestion or ligation phases of the work, which resulted in either missing insert in the vector or a wrong insert in the vector. It was concluded that a new batch of working vectors will need to be expressed, before further continuation of any cloning is attempted. It was shown through this work that it is possible to clone Ph4 1-528 into bacterial expression vector pET41a producing a GST-tag. However, due to the poor concentrations of obtained minipreps, it was concluded that new minipreps should be prepared before the expression of the protein in a suitable bacteria is further continued.

The expression part of this work succeeded well for the fragments of the protein, but the expression of full length protein did not succeed very well. This was however the first occasion where any expression of the full length protein was actually achieved, which was at least encouraging for the research group. It is suspected that a longer expression

(e.g. six hours) at a lower temperature (e.g. +24^{o}C) may result in an improved expression of these proteins, but this hypothesis was not specifically tested. As a conclusion, the expression of the proteins for three hours at +37^{o}C seems to work satisfactorily.

It was concluded that further improvements in the optimization of the purification are still needed in future experimental work. The purification of both proteins worked reasonably well when 0,1 % Triton was added to the first three washing buffers. These washes did not remove all of the other proteins from the beads, so additional washes or purifications are clearly required. It was suggested by my instructors that the use of an ATP-wash might help remove some of the proteins from the beads, but due to time constraints this suggested improvement could not yet be verified.

Gel filtration can be used to obtain very pure protein, when most of the other proteins have been removed from the beads. The purified Ph4-N protein obtained in this work represents the best result -currently obtained- by the research group, but it is noted that the obtained purity is still just within the bounds of what is experimentally acceptable. The research group plan to use the obtained protein for some of their future studies. These include creating an antibody against the Phactr4 protein, which could be used in Western Blotting to confer the result obtained with RNA interference (RNAi) and possibly also be used in immunofluorescence. When there are enough of the purified proteins, they will be used in GST pull-downs to confer their interaction with actin and PP1.

I believe in the reliability of the results obtained in this work, since the expression, solubility test and all of the purifications were monitored with samples taken during different parts of each process. None of the obtained results were contradictory to one another. All known mistakes in the work were reported promptly to the research group and were thus taken into consideration, when the results were concluded. The cloning was monitored constantly by subjecting the different reactions to agarose gel electrophoresis. The final quality control was made by performing the test digestions to ascertain if the vectors had the correct inserts. In the event of any suspicious results appearing, the minipreps could have been sequenced to ascertain that they really had the correct sequence, but this was not found to be necessary. The desired insert had already been sequenced earlier and it contained the correct sequence.

Since one aim of the work was to produce a suitable guideline for the future performing of cloning, expression, purification and the optimisation of the different procedures, the overall process is described in Appendix 1, Appendix 2 and Appendix 3. Some of the procedures described in these appendices require more detailed information, which has already been described in chapters 3 and 4. References to the relevant sections of chapter 3 and 4 are also included in the three appendices.

Throughout this work I learned how to perform all of the technical tasks, which are part of the experimental work. This was made possible through the help and instruction given by the research group instructors. In particular, the instructors explained in detail the experimental instructions, and gave expert advice on the needed optimizations and showed me how to interpret the obtained results correctly. After this initial help, I was able to conduct the experimental tasks quite independently. Overall, I have learned that one needs to be patient, methodical, persistent and above all well prepared when working in this kind of environment. My work has helped the research group to gain more information about how these proteins express and the required purification processes. I have learned valuable knowledge and useful practical skills through this work, complimenting skills already learned in earlier previous work in this area.

I wish to sincerely thank my instructors Maria Vartiainen and Johanna Puusaari for their extensive guidance and help throughout this work. I would also like to sincerely thank Riitta Lumme for valuable feedback and guidance throughout this work. Finally, I would also like to thank Colum Gaynor for help with the language proof reading of this work.

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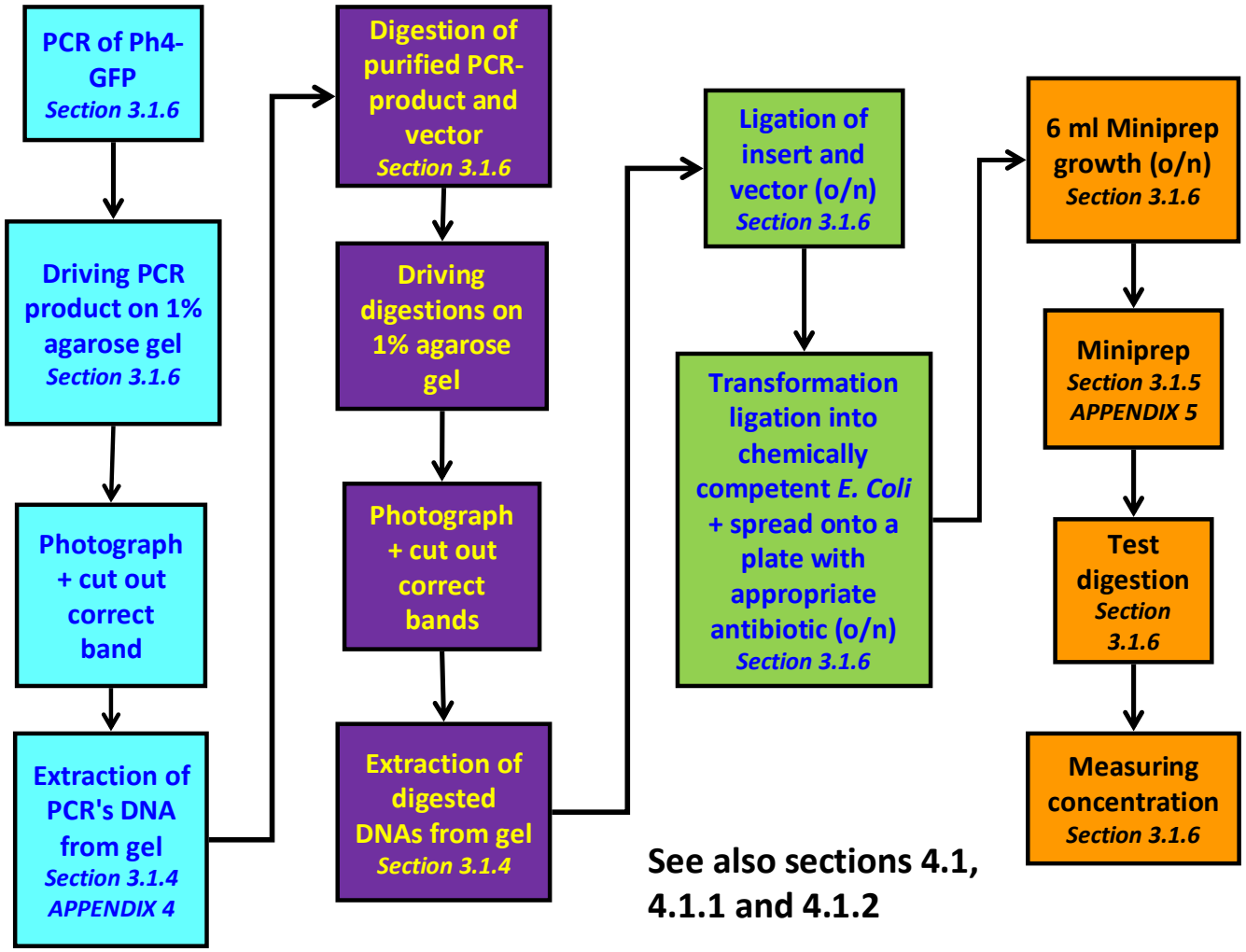
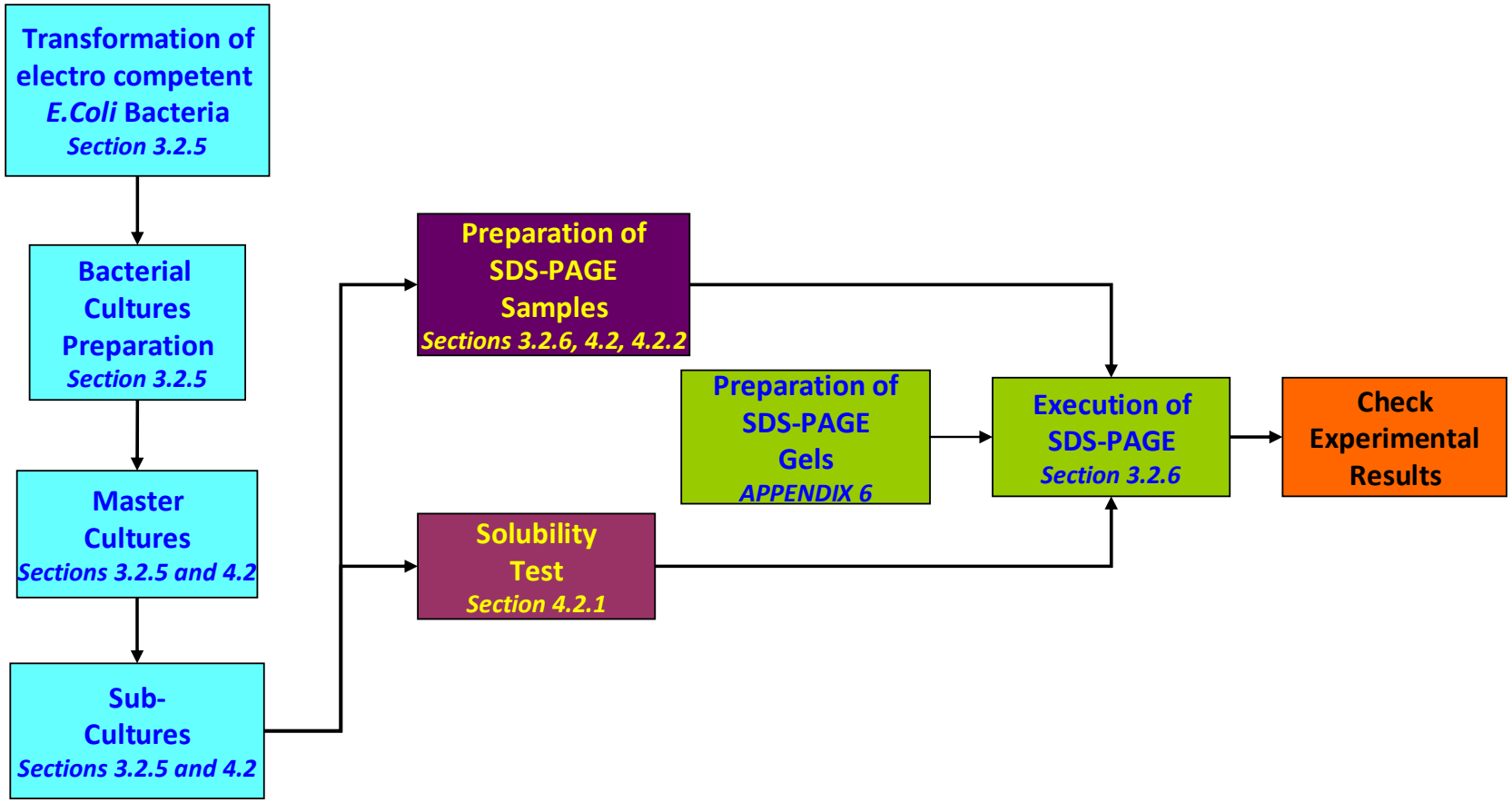
Cloning Ph4 1-528 to pET41a

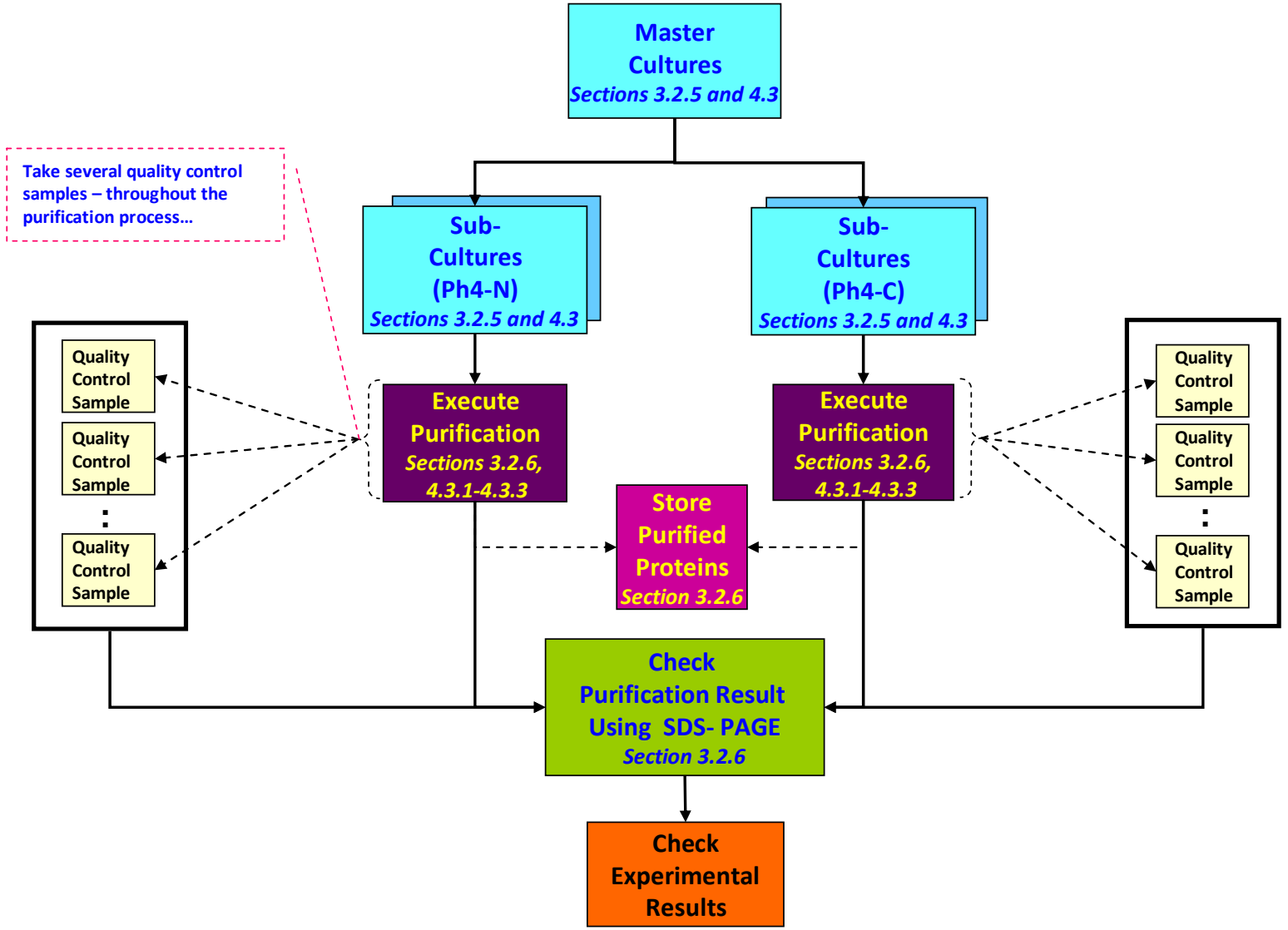


See also sections 4.1, 4.1.1 and 4.1.2

Expression of the proteins



Purification of the proteins



NucleoSpin® Extract II (Macherey-Nagel):
Protocol for DNA extraction from agarose gels

1. Cut the DNA fragment from the agarose gel with a clean scalpel and add 200 µl Buffer NT per 100 mg of agarose gel. Incubate the sample for 5-10 minutes at 50°C and vortex briefly every 2-3 minutes until the gel slice is completely dissolved.

2. To bind the DNA, place a NucleoSpin® Extract II column into a collection tube (2 ml), load the sample and centrifuge for 1 minute at 11 000 x g at RT. After centrifugation discard flow-through and place the NucleoSpin® Extract II column back to the collection tube. (Repeat the step, if there is more of the sample left.)

3. To wash the silica membrane, add 700 µl Buffer NT3 into the NucleoSpin® Extract II column and centrifuge for 1 minute at 11 000 x g at RT. After centrifugation discard flow-through and place the NucleoSpin® Extract II column back to the collection tube.

4. To dry the silica membrane, centrifuge for 2 minutes at 11 000 x g at RT to remove the Buffer NT3 quantitatively. Take care that the NucleoSpin® Extract II column doesn't come in contact with the flow-through while removing it from the centrifuge and from the tube.

5. To elute the DNA, place the NucleoSpin® Extract II column into a clean 1,5 ml microcentrifuge tube. Add 15-50 µl of Buffer NE / H_2O to the column and incubate at RT for 1 minute to increase the yield of eluted DNA. Centrifuge for 1 minute at 11 000 x g at RT.

GeneJET™ Plasmid Miniprep Kit (Fermentas):
Purification protocol

1. To cultivate and harvest bacterial cells, pick a single colony from a fresh streaked selective plate to inoculate 1-10 ml of LB medium containing appropriate selection antibiotic. Incubate for 12-16 hours at +37°C on a shaker. Harvest the bacterial culture by centrifugation at 6800 x g in a microcentrifuge for 2 minutes at room temperature. Discard the supernatant.

2. Add 250 µl of Re-suspension solution and re-suspend completely the pelleted cells until no cell clumps remain. Then transfer the cell suspension into a microcentrifuge tube.

3. Add 250 µl of Lysis solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. Do not vortex to avoid shearing of chromosomal DNA and do not incubate more than 5 minutes to avoid denaturation of supercoiled plasmid DNA.

4. Add 350 µl of Neutralization solution and mix immediately and thoroughly by inverting the tube 4-6 times Centrifuge for 5 minutes at 12 000 x g at RT to pellet cell debris and chromosomal DNA.

5. Transfer the supernatant to the GeneJET™ spin column, but avoid disturbing or transferring the white precipitate. Centrifuge for 1 minute at 12 000 x g at RT, discard the flow-through and place the column back to the collection tube.

6. Add 500 µl of Wash solution (diluted with ethanol) to the GeneJET™ spin column and centrifuge for 30-60 seconds at 12 000 x g at RT. After centrifugation discard the flow-through and place the column back to the collection tube.

7. Repeat the wash procedure using 500 µl of the Wash solution.

8. Discard the flow-through and centrifuge for additional 1 minute to remove residual Wash solution.

9. Transfer the GeneJET™ spin column into a fresh microcentrifuge tube (1,5 ml) and add 50 µl of Elution buffer / H_2O. Incubate for 2 minutes at RT and centrifuge for 2 minutes at 12 000 x g at RT.

10. Discard the column and store the purified plasmid DNA at -20°C.

How to make SDS-PAGE gels:

First, two sets of glass plates are taken, making a mould when put against each other. There is a 1,5 mm gap between the glass plates where the gels are poured. The glass plates, cleaned with ethanol, are set into a stand, which has a small rubber mats at the bottom of the glass plates. After that, the gel mixtures are made in separate Falcon tubes, but the APS and the TEMED were not yet added into the upper gel's mixture. 10% APS and TEMED catalyze the polymerization of acrylamide, to harden the gel. See Charts 10 and 11. The lower gel's mixture is pipeted to the mould all the way up to the mark on the stand and a small amount of water was put on top of the mixture to make the gels harden faster, which occurs when the gel is not in contact with air. The lower gel is left to harden.

CHART 10. The different lower gels with the needed reagents and measures.

Reagents	10% Lower gel 15ml	12% Lower gel 15ml	14% Lower gel 15ml
30 % Acrylamide	4,95 ml	6 ml	7 ml
3M Tris HCl pH 8.8	1860 µl	1860 µl	1860 µl
H_2O	7,95 ml	6,9 ml	5,8 ml
10 % SDS	150 µl	150 µl	150 µl
10 % APS	150 µl	150 µl	150 µl
TEMED	15 µl	15 µl	15 µl

CHART 11. The upper gel's reagents and measures.

Reagents	3,75% Upper gel 7,5ml
30 % Acrylamide	938 µl
3M Tris HCl pH 6.8	312 µl
H_2O	6,12 ml
10 % SDS	75 µl
10 % APS	75 µl
TEMED	7,5 µl

When the lower gel has hardened, the water is removed with blotting paper and the upper gel mixture is mixed ready and pipeted on top of the lower gels all the way to the top. The comb is then added in to the mould and the upper gels were then left to harden.

When the upper gel has hardened, the comb is gently removed and the glass moulds are then removed from the stand and the wells are washed with water. After that, the glass moulds are put into a new stand -the driving stand- and the stand is placed into a electrophoresis chamber (Bio-Rad). A 1x running buffer is added to the middle section, which forms when the glass moulds are in place, all the way to the top. The wells are washed with a 1x running buffer and it is also checked if the stand is leaking any of the buffer. A 1x running buffer is then poured into the bottom of the chamber, until it reached the mark on side of the chamber.