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MASTER'S THESIS

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Writer: Pralav Karki	……………………………………… (Writer's signature)
Faculty supervisor: Prof. Dr. Lutz Eichacker External supervisor(s):	
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I. Abstract:

Transglutaminases (TGs) are a family of enzymes that catalyzes cross linking of proteins by forming covalent bonds between lysine residues and glutamine residues in different polypeptides. Cross-linking reactions are involved in blood clots, skin formation, embryogenesis and apoptosis. Clinically, they are interesting since they seem to be implicated in neurodegenerative diseases, tumours and celiac diseases. Transglutaminases also have a great potential in the food industry because of its ability to cross bind proteins that are not normally linked.

A gene coding for transglutaminase from Atlantic cod was cloned into a bacterial expression vector pET151-D-TOPO. The recombinant plasmid was used to transform the bacterial expression strain BL21 and expression products were analyzed with SDS-PAGE, Immunoblotting and mass spectrometry analysis. The protein was expressed in the host in the soluble fraction, which was confirmed by SDS PAGE, imunoblotting and mass spectrometry analysis. A polyhistidine tag introduced at the amino-acid terminus of the transglutaminase protein allowed for the purification of the protein by using His-Trap columns. Large scale purification of the protein was successful after optimizing the washing and eluting conditions of the buffer. Based on these results, the bacterial expression system successfully expressed transglutaminase and possible use of the enzyme as a meat-glue in food industry is investigated.

II. Acknowledgements:

First of all, I would like to thank Prof. Dr. Lutz Eichacker for supporting and helping this project. Without him, this project would not have happened. Clemens Furnes was behind the whole idea of this project. Thanks to him, that this idea was able to take off and get to the next step. I would like to thank Clemens for all the innovative ideas and patience throughout the project. I learned so much about research and science from his perception and will use it in the near future. I would also like to thank Ann Kristine for all the tips about safety and cleanliness which should be followed in any lab.

Thank you all, who were helpful throughout this project.

I made a lot of mistakes throughout the project, but in return I gained a lot of knowledge and learned so much through my own errors and mistakes.

III. Abbreviations:

APS: Ammonium persulfate

Asn: Asparagine

Asp: Aspartic acid

Cys: Cysteine

ddH$_2$O: Double distilled water

His: Histidine

IPTG: Isopropyl β-D-1-thiogalactopyranoside

kDa: kilo Dalton

PAGE: Polyacrylamide gel electrophoresis

SDS: Sodium dodecyl sulfate

SOC: Super Optimal broth with catabolite repression

TGs: Transglutaminase

1. Introduction:

1.1 Cold Adapted Organisms:

Organisms inhabit different parts of the world and have to adapt different conditions like temperature, salt concentration, pH, pressure, etc. The largest proportion of the Earth's biosphere is comprised of organisms that live in cold environments (psychrophiles) (Siddiqui and Cavicchioli 2006). Since nearly three quarters of the planet is covered by ocean which has a mean temperature of 4-5°C, many organisms are psychrophiles. In other terms, psychrophiles are the organisms that live permanently at temperature close to freezing zero and these organism include yeast which has natural propensity to grow well at low temperature, algae which are responsible for the red to green color of the ice layers, some invertebrates and polar fish which are the biggest psychrophiles (Feller 2007).

1.2 Cold Adapted Enzymes:

The ability of these organisms to live in cold conditions may be attributed to their ability to produce cold adapted enzymes. Such enzymes are characterized by an increased thermo sensitivity and, most of them, by a higher catalytic efficiency at low and moderate temperatures, when compared to their mesophilic counterparts (Georlette, Bentahir et al. 2002). In most cases of the cold adapted enzymes, the high catalytic efficiency and reduced thermal stability is connected. The adaptation to cold is achieved through a reduction in the activation energy that possibly originates from an increased flexibility of either a selected area or of the overall protein structure (D'Amico, Claverie et al. 2002). Due to these unique characteristics of these enzymes, it has been an attraction in the field of enzyme technology and industrial biotechnology. Apart from that, this enzyme could be a model for the study of protein folding, protein flexibility, stability and catalysis.

Due to their reduced stability and high catalytic efficiency, these enzymes are a potential alternative to mesophilic enzymes in the field of industrial biotechnology. The fact that these enzymes function at low temperature and it could be easily deactivated (due to its reduced stability) make them good candidates to replace other widely used mesophilic enzymes. Proteases, lipases, amylases and cellulases are few of the enzymes that are being used as ingredients in some of the industrial products like detergents, clothing, food etc. In food industry, cold adapted enzymes like β-galactosidase has been used to remove lactose at low temperature. Proteases from cold adapted organisms are used in the meat industry to tenderize the meat at low temperature. Cold adapted enzymes could be used in brewing, cheese manufacturing. The only drawback for the use of cold adapted enzymes could be its instability. But this problem could be overcome with the use of side directed mutagenesis and recombinant enzyme which show improvement in the stability of the enzyme (Narinx, Baise et al. 1997).

1.3 Transglutaminase:

Transglutaminase (TG) (protein-glutamine γ-glutamyl-transferase, E.C. 2.3.2.13) is an enzyme that catalyses the acyl transfer between the γ-carboxyamide groups of glutamine (Q) residues within peptides and the primary amino groups of various amines, where a glutamine residue serves as a acyl donar and the most common acyl acceptors are the ε-amino groups of peptide-bound lysine (K) residues or primary amino groups of naturally occuring polyamines (Noguchi, Ishikawa et al. 2001). When the ε-amino group of lysine or lysyl residue acts as a acyl acceptor, ε-(γ-glutamyl)lysine crosslinks are formed. Apart from the crosslink between glutamine and lysine residues in proteins, TG is invovled in two other reactions. In the presence of primary amine, TG can cross link glutamine residue of the protein to the primary amine which is known as acyl transfer reaction. In the absence of lysine or primary amine, TG catalyses the reaction between water and glutamine, resulting in the deamidation of the glutamine residue. . In all the reactions, ammonia (NH_3) is liberated. All the three reaction catalyzed by TG is shown in the Figure 1.
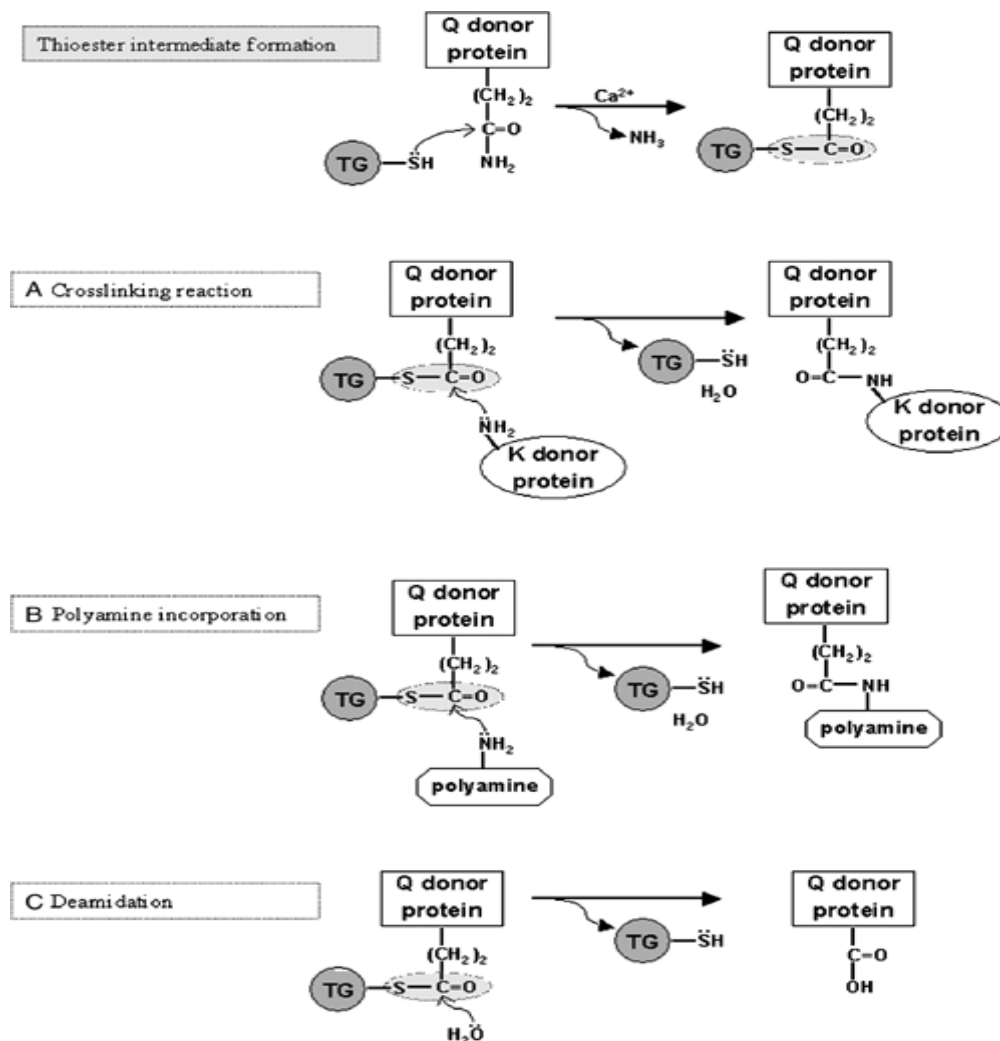

Figure 1. TG catalysed reactions; A: reaction between glutamine (Q) and lysine (K). B: reaction between a glutamine (Q) and a polyamine. C: Reaction between Glutamine (Q) and water. The figure is taken from (Esposito and Caputo 2005)

Transglutaminase are found in various organisms such as mammals, fish, bacteria and plants. But the most diverse and studied TGs are from mammals.

Mammalian TGs:

Mammalian TGs are distributed in various parts of the body. They are found in blood plasma, platelets, erythrocytes, prostrate etc. Nine TGs genes have been identified in humans, eight of which encode active enzymes and six have been isolated and characterized at protein level (Esposito and Caputo 2005). The list of mammalian TGs are shown in the table below.

Table 1: Lists of mammalian TGs found:

Name	Molecular weight (kDa)	Tissue	Location
TG1	90	Epithelia	Cytosolic, membrane
TG2	80	Ubiquitous	Cytosolic, nuclear, extracellular
TG3	77	Epithelia	Cytosolic
TG4	77	Prostate	Extracellular
TG5	81	Epithelia	Cytosolic
TG6	Unknown	Unknown	Unknown
TG7	80	Ubiquitous	Unknown
Factor XIIIA	83	Blood plasma, platelet	Extracellular
Band 4.2	77	Erythrocytes	Membrane

Source (Esposito and Caputo 2005)

All the mammalian TGs, except a catalytically inactive homolog (band 4.2), require calcium for their catalytic activity and all the TGs have the same amino acid sequence at their active site (Mehta 2005). By the structure and the sequence of the active site of the enzyme, TGs belong to the superfamily of papain-like cysteine proteases, all of which have the catalytic triad—Cysteine (Cys), Histidine (His), Aspartic acid (Asp) or Cysteine (Cys), Histidine (His), Asparagine acid (Asn) (Shleikin and Danilov 2011).

Mammalian Factor XIIIa is nowadays used to treat severe pathology as fatal bleeding, since its role in disease has been well assessed and the role of TGs in Alzheimer's, Huntington's and Parkinson's disease has also been suggested (Mariniello and Porta 2005).

Bacterial TGs:

Since TGs have the property of polymerization and forming isopeptide, its use in food industry was thought be immense. The ability of the enzyme to produce food of cheap food with nutritional value is one of the main cause for the researchers to isolate the enzyme since mammalian TGs are expensive and calcium dependent.

In 1989, Ando and others screened for 5000 microorganisms isolated from the soil for the detection of transglutaminase activity and a strong transglutaminase activity was found in one of the strain of *Streptoverticillium sp* S-8112. The transglutaminase purified was found to have a molecular weight of 40 kDa (SDS-PAGE), isoelectric point 8.9 and optimal pH for reaction was found to be 6-7, and moreover it was calcium independent unlike its mammalian counterpart (H Ando 1989).

Bacterial cells have the ability to survive in extreme physiological conditions and stress by developing a protective spore around them. Spores are complex structures composed of carbohydrates and cross linked proteins. ε-(γ-glutamyl)lysine cross links and transglutaminase activity have been detected in the spore coat proteins of *Bacillus subtilis* (Kobayashi 1996). TGs from *Streptoverticillium sp* strain S-8112 has been isolated and its primary structure analyzed. The enzyme contains a sole Cysteine residue, which is essential for its catalytic activity and hydropathy analysis indicated that the secondary structure of the region around the active site Cysteine residue is similar to those of mammalian TGs (Kanaji, Ozaki et al. 1993).

Fish TGs:

Fishes live in a different environment that their terrestrial counterparts. They have to cope with extreme conditions such as low temperature, low availability of oxygen, etc. So, the enzymes and proteins produced in fish could be of great interest as it high catalytic efficiency at lower temperature, which could play an immense role in food industry. The extraction and purification of the fish enzyme is difficult because of commercial reasons but with the help of recombinant DNA technology, it can be produced in microorganisms. So, TGs from fish is of great interest as it catalytic efficiency in lower temperature and thermostabilty.

A cDNA for TG from the liver of red sea bream (*Pagrus major*) was cloned in a vector and expressed in *E.coli* and the amino acid sequence was compared with tissue type TGs from guinea pig liver. The overall sequence similarity was not high but the 25 amino acid sequence of the active site of the enzyme was identical and the recombinant enzyme was calcium dependent (Yasueda, Nakanishi et al. 1995).

In 1996, TG from the liver extract of walleye pollack (*Theragra Chalcogramma)* was purified, and the molecular weight was estimated to be 77 kDa by SDS-PAGE, the optimal pH and temperature was found to 9.0 and 50° C respectively and calcium was required for the maximum activity (Yoshiyuki Kumazawa 1996).

Comparison of Bacterial TGs and Fish TGs:

A commercial tranglutaminase is sold in the market under the brand name of Activa^R TG-BP by Ajinomoto Co. Inc, Japan. As already noted, since this commercially available TG is from bacterial origin, it is calcium independent. The various TGs purified and characterized from fish shows that the transglutaminase require calcium for its activity. The presence of metal ions such as Cu^{2+} inhibits the activity of both types of TGs. The commercial TG showed optimum activity at pH 6.0- 6.5 and the optimum temperature for the activity was found to be 40° C (Alves Macedo, Durães Sette et al. 2010).

The transglutaminase purified from squid gills showed pH optimum at 8.0 and the optimum temperature for the activity was found to be 20° C (Nozawa 2001).

Plant TGs:

In plants, it is ubiquitous. It has been detected in algae and in angiosperms in different organs and sub-cellular compartments, chloroplasts being the best-studied organelles (Serafini-Fracassini and Del Duca 2008).

Both bacterial and plant TGs are found to be calcium independent.

Application of TGs in food industry:

As already noted, TGs crosslinks proteins to form polymers which are chemically and mechanically stronger. So TGs have a high potential in food industry to covalently link protein and covalently link essential amino acid to food products. This could generate food products which are nutrional and could be supplied to person suffering from malnutrition.

The major area for the use of TGs could be found in milk production. Since milk contains casein which is a good substrate for TGs. TGs could be used to cross link the caseins in the milk products. This property can be used for the production of cheese, yogurt etc in food industry.

The use of TGs could also be found in the sea food products such as surimi. A surimi is a fish based product pulverizied to a thick paste and has the property of a dense and rubbery food item, and it is well known that "the setting" is a very important process to produce surimi based products with greater water holding capacity and elasticity. There are no doubts TGs are able to improve the texture of the raw materials by catalyzing the formation of ε-(γ-glutamyl)lysine cross-links between fish proteins which is certainly responsible for the elasticity and firmness of the surimi gel (Mariniello and Porta 2005).

Meat proteins such as myosin and actin act as good substrate for TGs. Cross linking of the myosin molecules lead to the gelation of the myosin which is very helpful in the preparation of ham and sausages.

Advantages of cold adapted TGs are outlined below:

The benefits of using a cold adapted enzyme over warm adapted are outlined below.

1. Enzyme reaction is performed at low temperature so that products are not destroyed.

2. The enzyme is easily inactivated at moderate temperatures after the reaction is carried out without destroying the products.

3. The process can be done in low temperature meaning the quality and preservation of the food is more easily maintained.

4. The operation at low temperature means that growth of bacteria is kept at a minimum.

1.3   Expression of recombinant protein in *E. coli*:

Bacterial expression system has been widely used in the expression of recombinant proteins. Prokaryotes such *E.coli* has been widely because of several reasons such as low cost, high productivity, easy manipulation, a strong knowledge about the genetics and physiology of the bacterial cells etc. Purification of recombinant protein is also easy when bacterial expression system is used. Use of other expression systems such as insect expression system, mammalian expression system has their own drawbacks. The insect cell line uses baculovirus to transfect the cell line and working with viruses is not as easy as bacteria. Mammalian cells are time consuming, expensive and difficult to perform at large scale.

One major disadvantage of bacterial expression system is that the recombinant protein does not undergo post translational modification. The correct folding on the protein may not occur since the cytoplasm of the bacteria is always in a reduced condition which prevents the formation of disulfide bonds (Terpe 2006).

1.4   Host for the expression of the recombinant protein:

BL21 Star ^{TM} (DE3) *E. coli*:

The host for the expression of the recombinant protein chosen in the experiment was BL21 Star ^{TM} (DE3) *E. coli*. This strain was from the Champion^{TM} pET Expression System (Invitrogen). This expression system utilizes the elements from bacteriophage T7 and the strain has DE3 bacteriophage lambda lysogen inserted in its genome. This lambda DE3 lysogen contains a part of *lac* operon. It includes
   a) The gene for the production of the lac repressor; *lac* I
   b) The T7 RNA polymerase gene under the control of l*acUV5* promoter.
   c) A small portion of *lacZ* gene.

The vector used in the expression utilizes the promoter of T7 bacteriophage that has been modified to contain a *lac* operator sequence. T7 RNA polymerase has a strong recognition for this promoter. So in order to transcribe the gene from the vector, the RNA polymerase has to

be supplied by the host strain. T7 RNA polymerase transcribes mRNA more quickly than the polymerase from the host E. *coli.*

The transcription of the T7 RNA polymerase from the host cell is controlled by the l*acUV5* promoter. So, the production of the T7 RNA polymerase has to be induced by isopropyl β-D-thiogalactoside (IPTG). The reason for the use of *lacUV5* promoter, the altered form of the regular l*ac* promoter is that it eliminates the requirement of CAP/cAMP in the transcription of the RNA polymerase. So the expression of the RNA polymerase in the host is fully controlled by the presence of IPTG.
(The text is taken from the Invitrogen Champion pET directional TOPO Expression Kits, Manual part no 25-0400).

1.5 Vector used in the expression:

Vector used for the insertion of the gene of interest in this experiment was chosen as pET151/D-TOPO.

The map of pET151/D-TOPO:
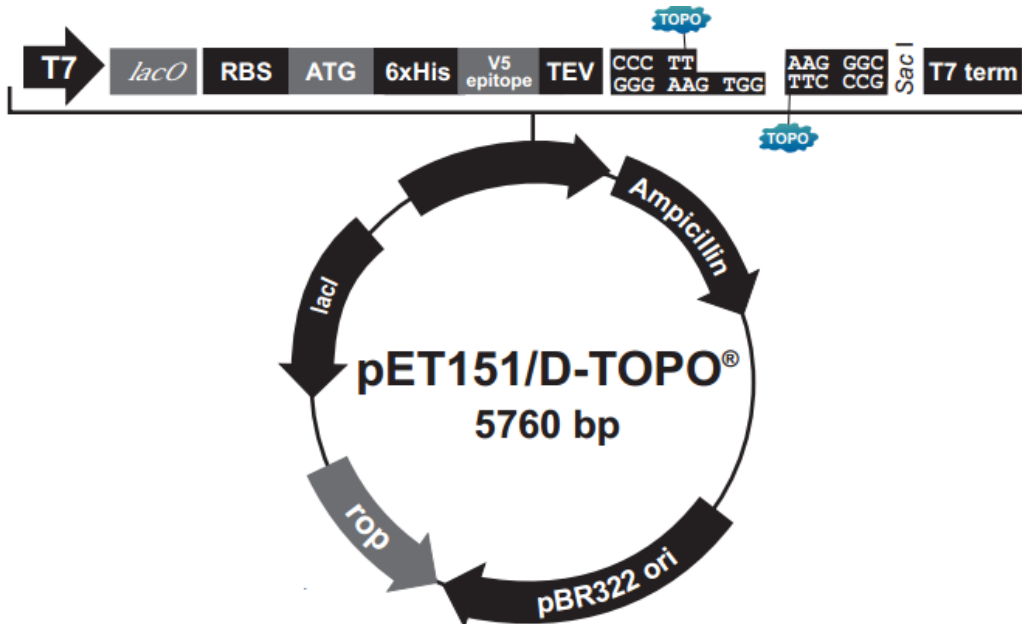
Comments for pET151/D-TOPO®
5760 nucleotides

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-289
Initiation ATG: bases 297-299
Polyhistidine (6xHis) region: bases 300-317
V5 epitope: bases 318-359
TEV recognition site: bases 360-380
TOPO® cloning site (directional): bases 387-400
T7 reverse priming site: bases 455-474
T7 transcription termination region: bases 416-544
bla promoter: bases 849-947
Ampicillin (*bla*) resistance gene: bases 948-1808
pBR322 origin: bases 1953-2626
ROP ORF: bases 2997-3188 (complementary strand)
lacI ORF: bases 4500-5612 (complementary strand)

Figure 1: pET151/D-TOPO vector carries a promoter called T7/*lac*. This promoter contains a promoter for the T7 bacteriophage RNA polymerase and a *lac* operator. This *lac* operator is the site where the repressor molecules bind. This operator helps to repress the expression of the gene until the inducer is added. Apart from repressor and operator, the vector contains pBR322 origin of replication. The vector contains sequences for hexa-histidine peptide and

V5. This epitope helps to detect the protein of interest using antibodies against them. The vector also contains gene for the production of a small peptide of amino acids which serves as a recognition site for the Tobacco Etch Virus (TEV) protease. After the production of the protein, this helps in the cleavage of the protein of interest from the rest of the tags or epitope. The vector codes for the enzyme β-lactamase which helps in the resistance to ampicillin. In addition to the vector, it contains Topoisomerase I from *Vaccinia* virus. This enzyme helps to clone the gene of interest in the vector.

(This figure and text is taken from the Invitrogen Champion pET directional TOPO Expression Kits, Manual part no 25-0400).

2. Materials:

2.1 List of solutions according to the methods:

Concentrations are given in Molarity (M). For simplicity, for some of the reagents, exact weights and volumes are specified.

2.1.1 Competent Cells:

- $MgCl_2$-$CaCl_2$ Solution: (300 ml) (80 mM $MgCl_2$ and 20 mM $CaCl_2$)
  - 12 ml 2M $MgCl_2$
  - 6 ml 1M $CaCl_2$
  - Add 282 ml ddH_2O to make final volume of 300 ml.

- $CaCl_2$ Solution:
  - 1ml 1M $CaCl_2$
  - 2.5 ml glycerol
  - Add 6.5 ml ddH_2O to make final volume of 10 ml.

2.1.2 Expression:

- Luria-Bertania (LB) media: (1000 ml)
  - 1% Peptone
  - 0.5% Yeast Extract
  - 1% NaCl
  - Solubilize in ddH_2O, autoclave and store at 4^oC.

- 1 M KH_2PO_4 (100 ml)
  - 13.6 g KH_2PO_4 in 100 ml ddH_2O.
  - Autoclave and store at 4^oC

- 1M K_2HPO_4 (100 ml)
  - 17.4 g K_2HPO_4 in 100 ml ddH_2O.
  - Autoclave and store at 4^oC

- Lysis Buffer (100 ml)
  - 0.3 ml of 1M KH_2PO_4
  - 4.7 ml of 1M K_2HPO_4
  - 2.3 g NaCl

- 0.75 g KCl
- 10 ml glycerol
- 0.5 ml Triton X-100
- Mixed thoroughly in 90 ml ddH$_2$O. Adjust the pH to 7.8 and bring the volume to 100 ml.

- IPTG (aqueous):
  - 100 mM (aqueous); premade aliquot of 1ml and stored at -20 ^{o} C.

- Lysozyme (aqueous): (7.5 µg/µl)
  - 0.1 gram lysozyme
  - 0.1 ml 1M Tris (pH 8.0)
  - 9.9 ml ddH$_2$O
  - 5 ml glycerol
  - Total volume = 15 ml, aliquot 1 ml and store at -20° C.

- SOC media (15 ml)
  - 2% Peptone
  - 0.5% Yeast Extract
  - 0.05% NaCl
  - 10 mM MgCl$_2$
  - 10mM MgSO$_4$
  - 20mM Glucose
  - Solubilize in 15 ml ddH$_2$O, autoclave and aliquot and store at -20° C

- Ampicillin (100 mg/ml) (1000 X stock)
  - Dissolve 1 gram ampicillin in 10 ml of ddH$_2$O
  - Filter through 0.22µm filter
  - Aliquot and store at -20°C.

2.1.3 SDS-PAGE:

- 10% APS (aqueous)
  - 1 gram of APS in 10 ml of ddH$_2$O
  - Aliquot 1 ml at -20° C.

- 10% SDS (100 ml)
  - 10 gram of SDS in 100 ml ddH$_2$O. Store at room temperature.

- 12% SDS Resolving gel (30 ml for 5 gels)
  - 9.9 ml ddH$_2$O.
  - 12 ml 30% acrylamide mix
  - 7.5ml of 1.5 M Tris (pH 8.8)
  - 0.3 ml of 10% SDS
  - 0.3 ml of 10% APS
  - 0.012 ml of TEMED
  - Mix all the ingredients (APS and TEMED is added at the end). Mix it and cast the gel. Immediately add 0.5 ml of water saturated butanol at the top of each gel.

- 8% SDS Resolving gel (30 ml for 5 gels)
  - 13.9 ml ddH$_2$O.
  - 8.0 ml 30% acrylamide mix
  - 7.5 ml of 1.5 M Tris (pH 8.8)
  - 0.3 ml of 10% SDS
  - 0.3 ml of 10% APS
  - 0.018 ml of TEMED
  - Mix all the ingredients (APS and TEMED is added at the end). Mix it and cast the gel. Immediately add 0.5 ml of water saturated butanol at the top of each gel.

- 5% Stacking gel (6 ml for 5 gels)
  - 4.1 ml of ddH$_2$O.
  - 1.0 ml of 30% acrylamide mix.
  - 0.75 ml of 1.0 M Tris (pH 6.8)
  - 0.06 ml of 10% SDS
  - 0.06 ml of 10% APS
  - 0.006 ml of TEMED
  - Mix it by pipetting and apply 1 ml for 1 gel. Put the comb in for the wells.

- 2X SDS Sample buffer: (10 ml)
  - 2.5 ml of 0.5 M Tris-HCl (pH 6.8)
  - 2.0 ml of glycerol
  - 0.2 ml DTT

- 0.02 gram of Bromophenol Blue
- 0.4 g SDS
- Solubilize in 10 ml ddH$_2$O, aliquot and store at -20° C.

- 1X SDS Sample buffer: (10 ml)
  - 1.25 ml of 0.5 M Tris-HCl (pH 6.8)
  - 1.0 ml of glycerol
  - 0.1 ml DTT
  - 0.01 gram of Bromophenol Blue
  - 0.2 g SDS
  - Solubilize in 10 ml ddH$_2$O, aliquot and store at -20° C.

- SDS Running Buffer for SDS-PAGE (1 litre)
  - 3 % Tris
  - 14.4% Glycine
  - 10% (v/v) of SDS (stock solution of 10%)
  - Solubilize in 1000 ml of ddH$_2$O.

- 1 M Tris (pH 6.8) 1 liter.
  - 121.14 gm of Tris in 900 ml ddH$_2$O, adjust pH by HCl and adjust the volume upto 1000ml.
  - Store at room temperature.

- 1.5 M Tris (pH 8.8) 1 liter
  - 181.71 14 gm of Tris in 900 ml ddH$_2$O, adjust pH by HCl and adjust the volume upto 1000ml.
  - Store at room temperature.

2.1.4 Immunoblotting:

- TBS 1X (1 liter)
  - 10 ml of 1 M Tris-HCl (pH 7.5)
  - 30 ml of 5 M NaCl
  - Bring the volume upto 1 litre and store at room temperature.

- TBS-Tween 1 X (1 liter)
    - 10 ml of 1 M Tris-HCl (pH 7.5)
    - 30 ml of 5 M NaCl
    - 500 µl Tween-20
    - Bring the volume upto 1 litre and store at room temperature.

- Blocking solution 40 ml for 2 gels
    - 5 % milk powder
    - 40 ml TBS 1 X
    - Mix before use

- Primary antibody (1°) solution for 2 gels, 40 ml (anti-his or anti-myc)
    - 40 ml of blocking solution + 13.33 µl of primary antibody solution (to make a dilution of 1:3000)
    - This could be reused later and was stored at -20°C.

- Secondary antibody (2°) solution for 2 gels, 30 ml
    - 30 ml of blocking solution + 6 µl of secondary antibody (goat anti mouse IgG-HRP) to make a dilution of 1:5000.

- ECL1 (5 ml for 1 membrane) (tubes are covered with aluminum foil as it is light sensitive)
    - 250 µl of 2M Tris-HCl (pH 8.3)
    - 50 µl of 250mM luminol
    - 50 µl of 40 mM p-coumaracid
    - Add ddH$_2$O to make 5 ml

- ECL2 (5ml for 1 membrane)
    - 250 µl of 2M Tris-HCl (pH 8.3)
    - 3.05 µl of 30% hydrogen peroxide
    - Add ddH$_2$O to make 5 ml.
    - ECL1 and ECL2 are prepared before use only.

- Luminol (250mM):
    - Premade aliquot of 1 ml stored at -20° C.

- p-coumaracid (40 mM):
    - Premade aliquot of 1 ml stored at -20° C.

- Transfer Buffer/Towbin buffer for western blot (900 ml)
    - 96mM Glycine
    - 10 mM Tris
    - ddH$_2$O
    - Store at room temperature.

- 2M Tris HCl pH 8.3 (500 ml)
    - Solubilize in ddH$_2$O and adjust pH to 8.3
    - Autoclave and store at room temperature.

- Methanol Transfer Buffer 100 ml
    - 10 ml methanol
    - 90 ml Transfer buffer

2.1.5 Coomassie Brilliant Blue (500ml)

- 1 gram Coomassie Brilliant Blue R-250
- 37.5 % (v/v) acetic acid
- 50% (v/v) methanol
- Add ddH$_2$O to a volume of 500 ml
- Store at room temperature.

2.1.6 Destaining Coomassie Brilliant Blue solution (500 ml)

- 0.75 % (v/v) acetic acid
- 10% (v/v) methanol

- Add ddH$_2$O to a volume of 500 ml

- Store at room temperature.

2.1.7 Agarose Gel Electrophoresis:

- 1 % agarose gel:
    - 1 gram of agarose in 100 ml of TBE buffer.
    - The mixture was heated in a microwave oven to solubilize the agar.
    - 10 µl of Gel Red DNA stain was added for the purpose of visualization.

- TBE buffer 5X (1 liter)
  - 53 gram of Tris base
  - 27.5 gram of boric acid
  - 20 ml of 0.5 M EDTA (pH 8.0)
  - Dissolve in 1000 ml of ddH$_2$O.

- 6X gel loading dye:
  - 0.25% bromophenol blue
  - 0.25% xylene cyanol FF
  - 30% glycerol in ddH$_2$O.

- 1 kb DNA ladder:
  - 10 µl 1 kb pluss ladder
  - 20 µl loading dye
  - 70 µl TE buffer (pH 8.0)

2.1.8 Protein purification using the His tag:

- Buffer A pH 7.5 (1 liter)
  - 25 mM Hepes
  - 300 mM NaCl
  - 10 mM imidazole
  - Add ddH$_2$O, adjust pH to 7.5
  - Degased by magnetic stirring with vacuum for 15 minutes.
  - Store at 4° C.

- Buffer B pH 7.5 (1 liter)
  - 25 mM Hepes
  - 300 mM NaCl
  - 50 mM imidazole
  - Add ddH$_2$O, adjust pH to 7.5.
  - Degased by magnetic stirring with vacuum for 15 minutes.
  - Store at 4° C.

- Buffer C pH 7.5 (1 liter)
  - 25 mM Hepes
  - 300 mM NaCl
  - 500 mM imidazole
  - Add ddH$_2$O, adjust pH to 7.5.
  - Degased by magnetic stirring with vacuum for 15 minutes.
  - Store at 4° C.

3.0 Methods:

3.1 Making competent cells:

a) BL21 cells from -80° C were streaked on the LB plate.

b) The plates were grown overnight at 37° C.

c) An isolated colony was picked and transferred to 4 ml of LB media in a Falcon tube and grown overnight at 37° C at 250 rpm.

d) 400 µl of overnight culture was transferred to 400 ml LB media and the cells were grown at 37° C until the OD_{600} reached 0.4.

e) The culture was centrifuged in 2 centrifuge tubes (200 ml each) at 4° C for 10 minutes at 2800 g.

f) The pellets were recovered and supernatant discarded.

g) Each pellet was dissolved in 120 ml of $MgCl_2$-$CaCl_2$ solution.

h) The mixture was centrifuged at 4° C for 10 minutes at 2800 g.

i) The pellet was recovered and dissolved in 4 ml of $CaCl_2$ solution.

j) Aliquot of 500 µl were made and stored at -80° C.

3.2 Expression of the recombinant protein:

Two different temperature (37^oC and 16° C) were used for recombinant protein production.

3.2.1 Transformation and induction:

a) 100 µl of chemically competent cells (BL21) were added to separate eppendorf tube and 1 µl of plasmid were added to their respective tubes.

b) All the tubes were kept in ice for 30 minutes.

c) The cells were heat shocked for 30 seconds at 42° C without shaking and immediately transferred back to ice.

d) 250 µl of SOC (Super Optimal Broth with Catabolite Repression) medium was added to all the tubes.

e) The tubes were capped tightly and incubated at 37° C for 30 minutes with shaking at 250 rpm.

f) All the transformed cells were added to different labeled falcon tubes with 10 ml LB medium with ampicillin (100 µg/ml). Then all tubes were then incubated at 37° C with shaking at 250 rpm and grown overnight.

g) 500 µl of each culture from the overnight incubation were transferred to an appropriately labeled flasks containing 10 ml LB medium. The flasks were incubated for 2 hours at 37° C with shaking at 250 rpm until OD_{600} reached 0.5.

h) Each tube containing 10ml of the culture was split to 5 ml in two tubes. One tube was induced with 100 µl of isopropyl β-D-thiogalactopyranoside (100 mM) (IPTG) and one was not induced. All the tubes were incubated at 37° C with shaking at 250 rpm overnight.

i) All the overnight incubated tubes were taken and collected in different tubes with correct labeling.

j) The culture was centrifuged at 6200 g for 15 minutes. The temperature was kept at 4 to 8° C.

k) The medium were all discarded and the pellets were kept.

3.2.2 Harvesting and Lysis:

a) All the pellets were resuspended in 250 µl of lysis buffer.

b) The resuspended cells were frozen (-80° C) and thawed (42° C) three times.

c) 26 µl of lysozyme (7.5 µg/µl) was added to each tube and left on ice for 1 hour.

d) Each of the tubes was subjected to sonication with 30 amplitude for 10 seconds (3 times).

e) All the cells were transferred to correctly labeled eppendrof tubes and centrifuges for 5 minutes at 4° C (maximum speed).

f) The supernatant were collected in new tubes and the pellets were also kept.

All the samples were now ready for the Coomassie staining and Immonudetection.

3.2.3 Preparation of samples before running SDS gels:

Samples containing the pellet were mixed with 500 µl of 1X SDS-PAGE sample buffer and boiled for 5 minutes at 95° C before running the SDS-PAGE.

Samples containing the supernatant were mixed with equal amount of 2 X SDS-PAGE sample buffers and boiled for 5 minutes at 95° C before running the SDS-PAGE.

10 µl of samples were run along with a prestained molecular marker. The gels were coomassie stained and visualized.

Two different sets of SDS-PAGE were run for pellets and supernatant and were subjected to immunodetection. Immunodetection were based on the use of the antibody against "his" and "myc" tag.

The concentration of stacking gel was chosen to be 5% and the resolving gels were 12%. .

3.3 Amplification of the plasmid:

a) 100 µl of TOP10 chemically competent cells were mixed with 1 µl of plasmid (44).

b) It was incubated in ice for 30 minutes.

c) The cells were then heat shocked in a water bath for 30 seconds at 42° C (without shaking).

d) The cells were immediately placed on ice.

e) 250 µl of SOC media was added and incubated for 1 hour at 37° C at 250 rpm.

f) 2 LB plates with ampicillin were taken. One plate was plated with 50 µl of cells and another was plated with 200 µl of cells.

g) The plates were grown overnight at 37° C.

h) 2 colonies from each plate were chosen and transferred to 5 ml of LB media with ampicillin (100 µg/ml). The cells were grown overnight at 37° C at 250 rpm.

3.4 Purification of the plasmid:

a) The overnight grown cells were harvested by centrifuging at 6800 x g for 3 minutes at room temperature in a table top centrifuge.

b) The pellet was recovered.

c) The pellet were resuspended in 250 µl of buffer

d) 250 µl of buffer P2 was added and mixed thoroughly by inverting the tubes 4-6 times.

e) Again 350 µl of buffer N3 is added and mixed thoroughly by inverting 4-6 times.

f) The cells were centrifuged for 10 minutes at 17,900 x g in a table top centrifuge.

g) The supernatant was applied to QIAprep spin column by pipetting.

h) It was centrifuged for 60 seconds at 17,900 x g and the flow through was discarded.

i) 0.5 ml of buffer PB was added and centrifuged for 60 seconds at 17,900 x g.

j) QIAprep spin column was washed by adding 0.75 ml of buffer PE and centrifuged for 1 minute at 17,900 x g.

k) Flow through was discarded and the column was centrifuged for 1 minute at 17,900 x g.

l) The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube and the plasmid was eluted with 50 µl buffer EB (10 mM Tris, pH 8.5) by centrifuging for 1 minute at 17,900 x g.

The procedure for the purification of plasmid was taken from QIAprep Miniprep Handbook. (Source:http://www.qiagen.com/literature/default.aspx?Term=QIAprep%20miniprep%20handbook%2012/2006&LiteratureType=1)

3.5 Agarose gel electrophoresis:

Agarose gel electrophoresis was carried out to determine the length of the purified plasmid.

a) 1% agarose gel was used for the electrophesis. 1gram of agarose was mixed in 100 ml of 0.5 X TBE buffer. 10 µl of GelRed nucleic acid Gel Stain was added.

b) The samples for agarose gel electrophoresis were prepared by adding 8 µl of plasmid with 2 µl of 6X gel loading dye.

c) 6 µl of 1 kb DNA ladder was used as a DNA size marker.

d) 0.5 X TBE buffer was used as the anode and cathode buffer.

e) The gel was run for 45 minutes at 100 V.

f) The bands were visualized in UV lights.

3.6 Determination of DNA concentration:

The concentration of the DNA was measured by NANODROP 2000 spectrophotometer. 2µl of elution buffer (EB) was used as blank. 2µl of the plasmid sample was used for the measurement.

3.7 Sequencing of the purified plasmids:

The sequence of the purified plasmid was determined by sequencing. The samples preparation for the sequencing is given in the table below. Two tubes were used; one with the forward primer and another with the reverse primer.

Reagent for the sequencing:

Reagent	Volume	Final concentration
Plasmid	3 µl	23.4 ng/µl
BIG Dye v 3.1	2 µl	
Primer (forward or reverse) T7 promoter primer (forward primer) T7 reverse sequencing primer (reverse)	1 µl	0.32 µM
Autoclaved water	2 µl	
5X Sequencing buffer	2 µl	1X
Total	10 µl	

After the reagents were mixed, PCR was run.

The set up for PCR is shown below:

Steps	Temperature	Duration	
1	96° C	3 minutes	
2	96 ^{o} C	10 seconds	Repeat steps 2-4 for 25 times
3	50 ^{o} C	5 seconds	
4	60 ^{o} C	4 minutes	
5	4 ^{o} C	∞	

 After the PCR was done 10 µl of autoclaved water was added. The samples were sent to Medisinsk Genetisk avdeling at universitetssykehuset, Nord, Norge, HF (Tromso, Norge)

3.8 Large scale production at 16° C:

3.8.1 Transformation:

a) 1 µl of the plasmid 44 (TG1) was added to tube with 100 µl of BL21 chemically competent cells.

b) The whole mixture was heat shocked at 42° C for 30 seconds.

c) It was put immediately back on ice and 250 µl of SOC media was added.

d) The cells were grown for an hour at 37° C with shaking at 250 rpm.

e) Each mixture was transferred to 10 ml LB media containing ampicillin (100µg/ml) and grown over night at 37° C with shaking at 250 rpm.

3.8.2 Induction:

a) Each tube was added to a flask containing 400 ml of LB media with ampicillin (100 µg/ml). The four flasks were grown at 37° C with shaking at 250 rpm for two hours. The optical density was checked for each flask was checked in between. When the optical density reached around 0.4, the flasks were taken out and induced with 100mM IPTG. For 400 ml media, 4 ml of IPTG was used. After the induction, the cells were incubated over night at 16° C with shaking at 250 rpm.

3.8.3 Harvesting and lysis:

a) The overnight culture was transferred in a 250 ml tube (200 ml culture in one tube) and centrifuged at 6000 x g for 15 minutes at +4° C to +8° C.

b) The supernatant were discarded and the pellets were resuspended in 20 ml lysis buffer. (The lysis buffer contains protease inhibitor).

c) The mixture was transferred to a falcon tube and then frozen (-80° C) and thawed (42° C) for three times.

d) 400 µl of lysozyme (7.5 µg/µl) was added to each falcon tube and kept in ice for 1 hour.

e) Each tube was then sonicated for 6 times for 30 seconds with amplitude of 30.

f) The mixture was transferred to eppendorff tubes and spun at maximum speed (13000 rpm) for 10 minutes at 4 ^{o} C. Table centrifuge was used.

g) The pellets were discarded and the supernatant was collected in a new falcon tube.

h) The supernatant was further filtered with a syringe (20 ml) and a filter unit with a pore size of 0.45 µM.

i) The supernatant (extract) was kept in a fridge at 4° C

3.9 Protein purification:

For the purpose of the purification of the protein from the extract, a His trap HP column was chosen. The column is immobilized with Nickel atoms which have a high affinity to the histidine tag. This method is called Immobilized metal affinity chromatography. The total volume of extract was around100 ml. The volume of extracts was divided into 40 ml in two tubes and the rest was kept at 4° C.

For the first run through the column, three buffers were used named as buffer A, buffer B and buffer C. Buffer A contains 10mM imidazole, buffer B has 50 mM imidazole and buffer C has 500 mM imidazole. The entire buffer has a pH of 7.5, at this pH the bonding of the histidine tag and the metal is at maximum.

The lower concentration of imidazole used in the buffer is mainly used to wash the non-specific binding of the protein that have histidine clusters. The higher concentration of the imidazole (500 mM) is used to elute the recombinant protein with the histidine tag.

The His Trap HP column is put in a stand connected to a motor that pumps buffer, extract from the bottle at the constant rate. The constant rate chosen here is 2ml/min. All the bottles containing buffers and extracts were put in ice.

Two types of buffer B were used. One had a concentration of 50 mM imidazole and other had a 150 mM imidazole. All the other ingredients of the buffer were same for both. Buffer B was mainly used for the purpose of washing the unwanted protein which has a histidine cluster.

3.9.1 Washing with buffer B (50 mM imidazole):

The procedure is as follows:

a) The column was washed with 20% ethanol at the rate of 2ml/min.

b) The column was again washed with 10 ml dH_2O at a constant rate.

c) The column was washed with 10 ml of buffer A (10 mM imidazole)

d) The column was now supplied with 40 ml of extract.

e) The column was again washed with 10 ml of buffer A. All the flow through of steps d and e were stored in a bottle and named as flow through 1 (FT 1).

f) The column was now washed with10 ml of buffer B (50 mM imidazole). This time the fraction was collected in 10 different eppendorf tubes. Each tube had a volume of approximately 1 ml. The tubes were labeled B1, B2 to B10

g) The final step was the use of 10 ml of buffer C (500 mM imidazole) to elute the protein from the His column. Just like above, the eluted samples were collected in 10 different tubes. Each tube had a volume of 1 ml and the tubes were labeled C1, C2 to C10.

3.9.2 Washing with buffer B (150 mM imidazole):

h) The column was washed with 10 ml of buffer A.

i) The column was applied with 40 ml of remaining extract.

j) The column was applied with 10 ml of buffer A. The flow through from step i and j were stored in a bottle and marked flow through 2 (FT 2).

k) The column was washed with 10 ml of buffer B (150 mM imidazole). This time the fractions were collected in 10 different tubes. Each tube had a volume of 1 ml. the tubes were labeled as B11, B12 to B20.

l) The final step was the use of 10 ml of buffer C (500 mM imidazole) to elute the protein from the His column. Just like above, the eluted samples were collected in 10 different tubes. Each tube had a volume of 1 ml and the tubes were labeled C11, C12 to C20.

m) The column was washed with 10 ml of dH$_2$O.

n) The final step was the washing of the column with 20% ethanol.

All the samples were stored at 4 ^{o} C and subjected to SDS-PAGE and western blot.

All the samples were mixed with 2X SDS sample buffer and a resolving gel concentration of 8% was used this time.

3.10 Mass Spectrometry Analysis:

The bands seen in the coomassie staining of the SDS-PAGE were investigated for protein identification. The sequence of the proteins in the bands were investigated by Q-TOF (LC-MSMS. 2 bands from the gel obtained after washing with buffer B (50 mM imidazole) and 1 band from the elution fraction (buffer C) were excised by clean scapel (1 scapel per band was used), placed in clean eppendorf tubes, tightly packed and sent to Institutt for medisinsk

biologi Det helsevitenskapelige fakultet, Universitetet i Tromsø, Norway. The sequence for the protein of interest was also sent along to compare it to the protein in the band.

The samples were trypsinated and analyzed on the Q-TOF (LC-MSMS).

4. RESULTS:

From a cDNA library from Atlantic cod (*Gadus morhua*); two genes encoding transglutaminase have been isolated. These two genes were cloned into pET151/D-Topo vector and found to be correctly inserted by sequencing. In this study these two recombinant constructs encoding TG-1 and TG-2 proteins were tested for recombinant expression in *Echerichia coli (E.coli).*

**Small scale recombinant production of the TG1 and TG2 in *E.Coli* at 37° C.**

Five different expression plasmids containing transglutaminase genes were made. These recombinant vectors were named as 44, 46, 73, 76 and 77. Vectors 44 and 46 have the gene for transglutaminase 1 (TG1) and rest have the gene for transglutaminase 2 (TG2). An additional vector containing the gene for flagellin D (Fla D) was used as a positive control (Hynes, Furnes et al. 2011) and a vector without an insert was used as a negative control. In addition to the his tag that is already present in the vector, an additional myc tag is cloned just downstream at the C- terminal of the TG1 and Fla D, which allowed the detection of the protein using anti-myc as an antibody .

A simplified figure of the different vectors used is shown below.
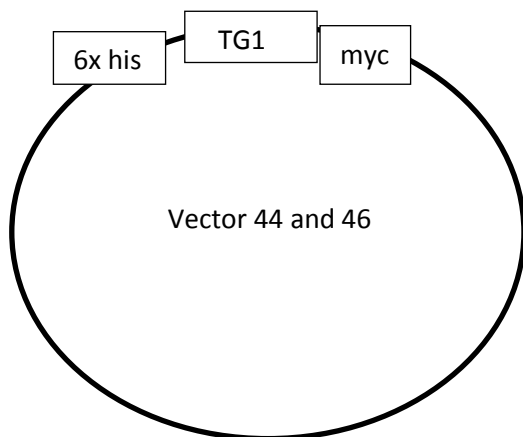
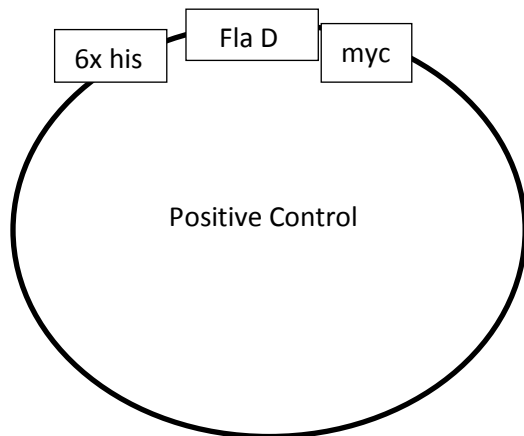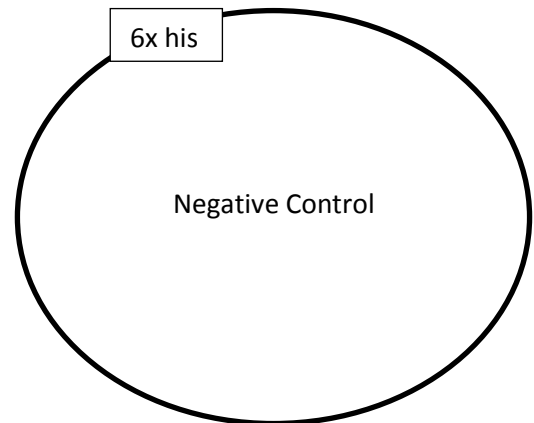A)   B)
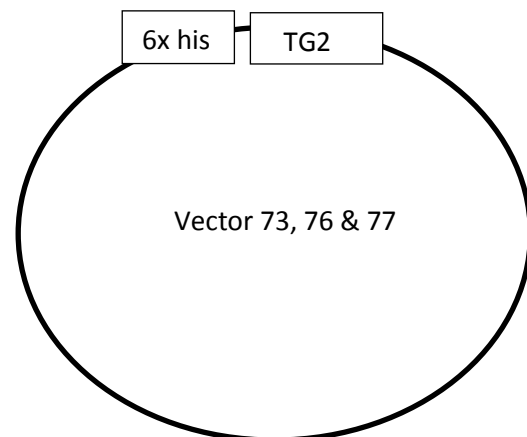
C)   D)

The different types of vectors used in the experiments. A) vector used for TG1 with "his" and "myc" tag. B) vector used for TG2 with "his" tag. C) vector used for FlaD myc (positive control) with "his" and "myc" tag. D) vector used for negative control (no insert).

Seven different eppendorf tubes were labeled 44, 46, 73, 76, 77, Positive control (+ve C) and Negative control (–ve C). The vectors were used to transform chemically competent BL21 cells. Both cell growth and induction of the cells with IPTG were done at 37° C. In addition, 7 cell cultures were grown but not induced with IPTG. Since the protein of interest is expressed inside the host, the cells were lysed with lysozyme, freeze and thaw and sonication. The pellet and supernatant were separated by centrifugation.

The pellet and supernatant fractions were analyzed for the presence of recombinant protein. Both samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie blue. Protein band with a molecular weight of about 80 kDa was observed at the pellet fraction (Figure 1; lane 7, 9, 11, 13 and 15). From the amino acid sequence of the protein, the molecular weight of the protein was found to be 78.3 kDa. The molecular weight of the recombinant protein fused to its tag was about 80.3 kDa. This band was absent in the lane containing the sample from *E. coli* with empty vector (Figure 1; lane 1 and 3; Figure 2; lane 2 and 3). In supernatant fraction, there were no indication of bands for TG1 and TG2 (Figure 1 and 2; lane 7, 9, 11, 13 and 15)
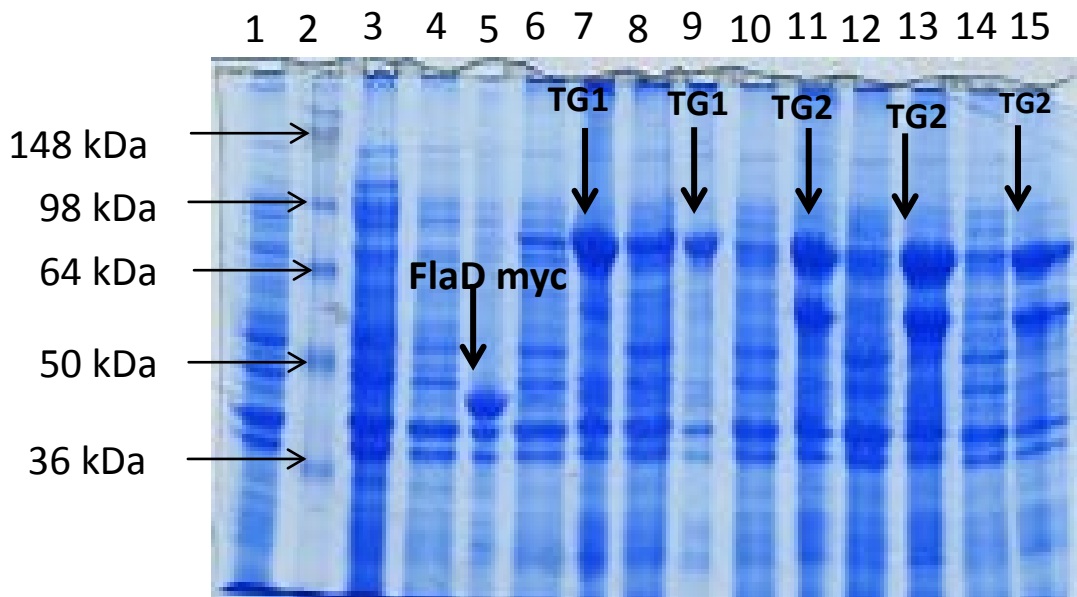
Figure 1: SDS-PAGE analysis of the pellet fraction from the small scale production of TG1 and TG2 in *E.coli* at 37° C. Following harvesting, the pellet fraction were run on a 12% SDS-PAGE, 170 V for 1 hour and stained with Coomassie Brilliant Blue. The numbers at the top indicate lanes and the molecular weights of the standards are indicated at the left margins. Lane 1: Empty vector without IPTG (Negative control), Lane 2: Protein ladder (Seeblue prestained), Lane 3: Empty vector with IPTG (Negative control),; Lane 4: Positive control without IPTG; Lane 5: Positive control (Fla D myc) with IPTG; Lane 6: TG1 (44) without IPTG; Lane 7: TG1 (44) with IPTG; Lane 8: TG1 (46) without IPTG; Lane 9: TG1 (46) with IPTG; Lane 10: TG2 (73) without IPTG; Lane 11: TG2 (73) with IPTG; Lane 12: TG2 (76) without IPTG; Lane 13 TG2 (76) with IPTG; Lane 14 TG2 (77) without IPTG; Lane 15: TG2 (77) with IPTG.

Figure 1 shows the SDS-PAGE analysis of the pellet fraction from the small scale production of TG1 and TG2 in *E. coli* at 37° C. As seen in the picture, the protein of interest TG1 and TG2 are produced quite well. Also the positive control (FlaD myc) is produced quite well as can be seen in lane 5. The protein of interest TG1 can be seen expressed and produced, as shown in the lane 7 and 9. The band is distinct as compared to other bands in the same lane. The comparison of the molecular weight of the band with the ladder suggests that the weight of the protein is nearly 80 kDa which is the size of the expected recombinant protein. Lane 6 and 8 contain the samples which were not induced (without IPTG) and we clearly see from the figure that there are no distinct bands, which suggest that the protein was not produced. Lane 11, 13 and 15 also shows distinct band at about 80 kDa, which corresponds to TG2. Lane 10, 12 and 14 were left uninduced (without IPTG) and no distinct bands are seen in the molecular range of 80kDa.

For the negative controls (vector without an insert), we do not see any distinct band. The size of the bands at lane 7, 9, 11, 13 and 15 shows that the protein is well expressed and is produced in the insoluble fraction.
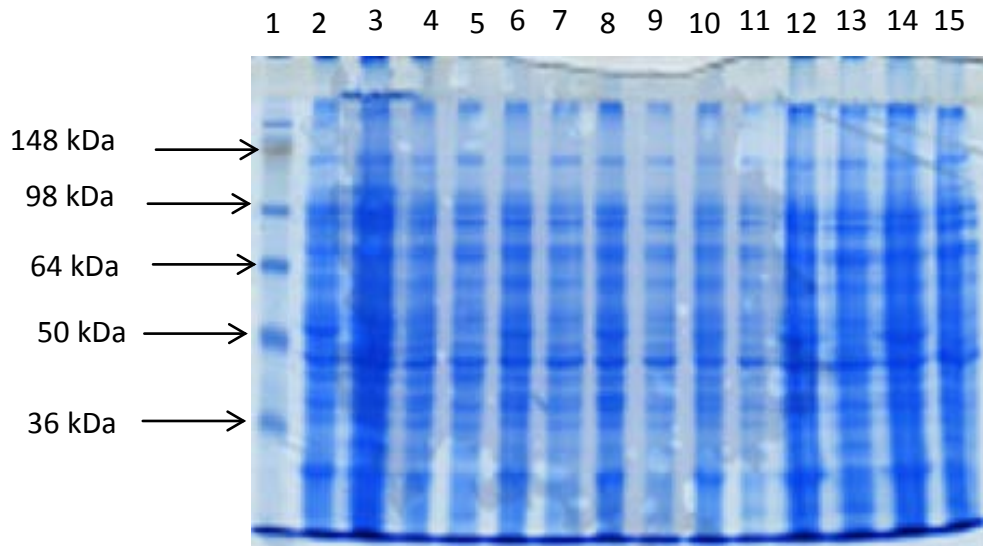
Figure 2: SDS-PAGE analysis of the supernatant fraction from the small scale production of TG1 and TG2 in *E.coli* at 37° C. Following harvesting, the supernatant fraction were run on a 12% SDS-PAGE, 170 V for 1 hour and stained with Coomassie Brilliant Blue. The numbers at the top indicate lanes and the molecular weights of the standards are indicated at the left margins. Lane 1: Protein ladder (Seeblue prestained); Lane 2: Negative control without IPTG, Lane 3: negative control with IPTG; Lane 4: Positive control without IPTG; Lane 5: Positive control (Fla D myc) with IPTG; Lane 6: TG1 (44) without IPTG; Lane 7: TG1 (44) with IPTG; Lane 8: TG1 (46) without IPTG; Lane 9: (TG1) with IPTG; Lane 10: TG2 (73) without IPTG; Lane 11: TG2 (73) with IPTG; Lane 12: TG2 (76) without IPTG; Lane 13 TG2 (76) with IPTG; Lane 14 TG2 (77) without IPTG; Lane 15: TG2 (77) with IPTG.

Figure 2 shows the SDS-PAGE analysis of the supernatant fraction from the small scale production of TG1 and TG2 in *E. coli* at 37° C. The bands that we are looking for in the lane 7, 9, 11, 13 and 15 at about 80 kDa is not distinct and cannot be distinguished from other bands in the lane. Lane 5 also does not visibly shows the expression of the positive control (FlaD myc). As expected the uninduced (without IPTG) and negative controls do not show any bands at the desired place. This result shows that the protein of interest may or may not have been produced in the soluble fraction at 37°C. If it was produced, it was not produced at a concentration detected or distinguished by coomassie staining.

Immunological confirmation of the identity of the TG bands found in the pellet fractions.

A primary antibody against the myc epitope was utilized in order to confirm the identity of the recombinant band. Immunoblotting was used for identification of the 80 kDa protein. After binding of the primary antibody with the proteins bound to the membrane, the specifically bound antibody was detected with horseradish peroxidase conjugated secondary antibody.

The result from the immunoblotting of the pellet fraction showed the protein was present in the pellet fraction (results not shown). The immunoblotting of the supernatant fraction was not carried out.

Since the coomassie staining of the SDS-PAGE showed protein in the pellet fraction, when expressed at 37° C, the production of recombinant protein was also carried out at 16° C to test if more protein would end up in the soluble fraction.

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The results from the expression of the recombinant protein at 37° C shows that the protein was mostly expressed in the insoluble fraction (pellet). Since the aim of this study was to produce the recombinant protein in the soluble fraction (supernatant). We chose to lower the temperature of the induction, so that the protein is produced in the soluble fraction.

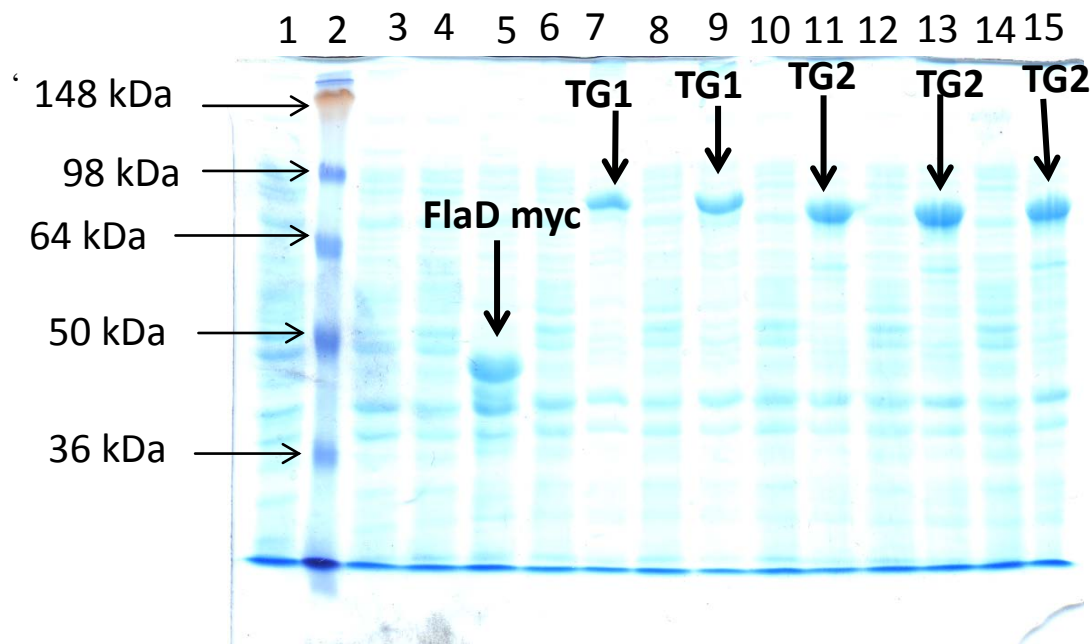**Small scale recombinant production of the TG1 and TG2 in *E. coli* at 16° C.**

Figure 3: SDS-PAGE analysis of the pellet fraction from the small scale production of TG1 and TG2 in *E. coli* at 16° C. Following harvesting, the pellet fractions were run on a 12% SDS-PAGE, 170 V for 1 hour and stained with Coomassie Brilliant Blue. The numbers at the top indicate lanes and the molecular weights of the standards are indicated at the left margins. Lane 1: Negative control without IPTG, Lane 2: Protein ladder, Lane 3: negative control with IPTG; Lane 4: Positive control without IPTG; Lane 5: Positive control (FlaD myc) with IPTG; Lane 6: TG1 (44) without IPTG; Lane 7: TG1 (44) with IPTG; Lane 8: TG1 (46) without IPTG; Lane 9: (TG1) with IPTG; Lane 10: TG2 (73) without IPTG; Lane 11: TG2 (73) with IPTG; Lane 12: TG2 (76) without IPTG; Lane 13 TG2 (76) with IPTG; Lane 14 TG2 (77) without IPTG; Lane 15: TG2 (77) with IPTG.

Figure 3 shows the SDS-PAGE analysis of the pellet fraction from the small scale production of TG1 and TG2 in *E .coli* at 16° C. The gel shows the production of the protein in the lane 5, 7, 9, 11, 13 and 15. The bands are quite distinct and visible at their respective molecular weight. TG1 is shown in lane 7 and 9, TG2 is shown in the lane 11, 13 and 15 and FlaD myc is shown in the lane 5. The negative controls (lane 1 and 3) and the uninduced (lane 4, 6, 8, 10, 12) shows bands but not at the desired length which was quite expected. The bands in the lane 5, 7, 9 11, 13 and 15 are quite distinct and bigger than the rest of the band in their respective lanes. This result clearly shows   that the recombinant protein was expressed when induced with IPTG and not produced when left uninduced.
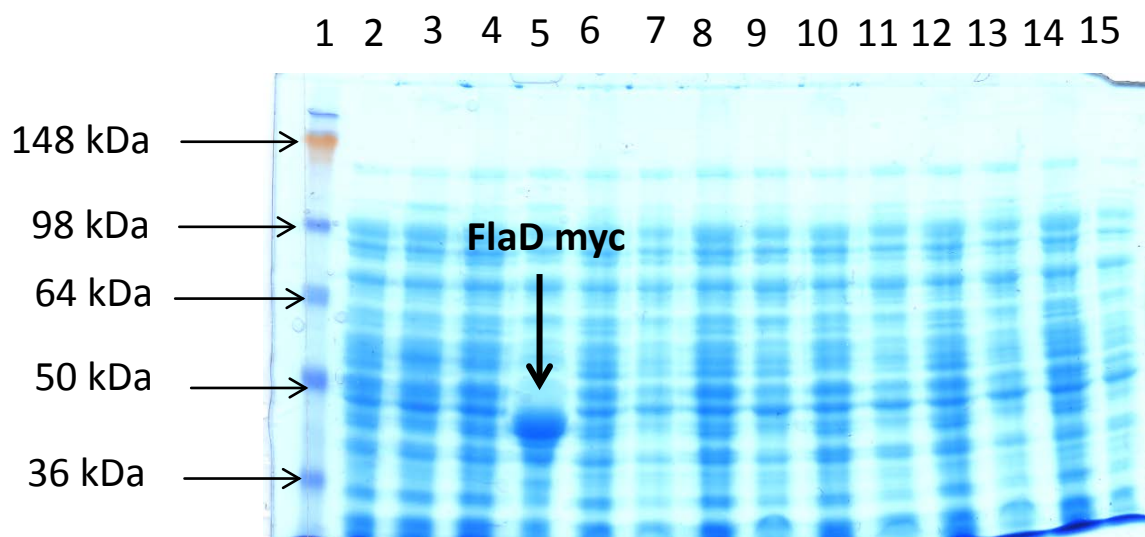
Figure 4: SDS-PAGE analysis of the supernatant fraction from the small scale production of TG1 and TG2 in *E. coli* at 16° C. Following harvesting, the supernatant fraction were run on a 12% SDS-PAGE, 170 V for 1 hour and stained with Coomassie Brilliant Blue. The numbers at the top indicate lanes and the molecular weights of the standards are indicated at the left margins. Lane 1: Protein ladder; Lane 2: Negative control without IPTG, Lane 3: negative control with IPTG; Lane 4: Positive control without IPTG; Lane 5: Positive control (FlaD myc)with IPTG; Lane 6: TG1 (44) without IPTG; Lane 7: TG1 (44) with IPTG; Lane 8: TG1 (46) without IPTG; Lane 9: (TG1) with IPTG; Lane 10: TG2 (73) without IPTG; Lane 11: TG2 (73) with IPTG; Lane 12: TG2 (76) without IPTG; Lane 13 TG2 (76) with IPTG; Lane 14 TG2 (77) without IPTG; Lane 15: TG2 (77) with IPTG.

Figure 4 shows the SDS-PAGE analysis of the supernatant fraction from the small scale production of TG1 and TG2 in *E. coli* at 16° C. The result shows that the positive control FlaD myc (lane 5) is produced well enough to be detected in the gel. The band for the positive control at about 40 kDa is quite distinct and separable from other bands. The positive control without IPTG shows no band at all at the same molecular weight. There are a lot of bands in lanes 7, 9, 11, 13 and 15 but are not distinct as was seen in the pellet fraction and no band at 80kDa could be seen. The bands for the sample uninduced are also not seen at their respective positions which were quite expected. This result shows that the recombinant protein FlaD myc was still produced, but seems to be more in the insoluble fraction than in the soluble fraction. The production of the protein of interest in soluble fraction was further investigated with western blotting.

Immunological confirmation of the identity of the TG bands found in the pellet fractions.

A primary antibody against the myc epitope was utilized in order to confirm the identity of the recombinant band. Immunoblotting was used for identification of the 80 kDa protein.
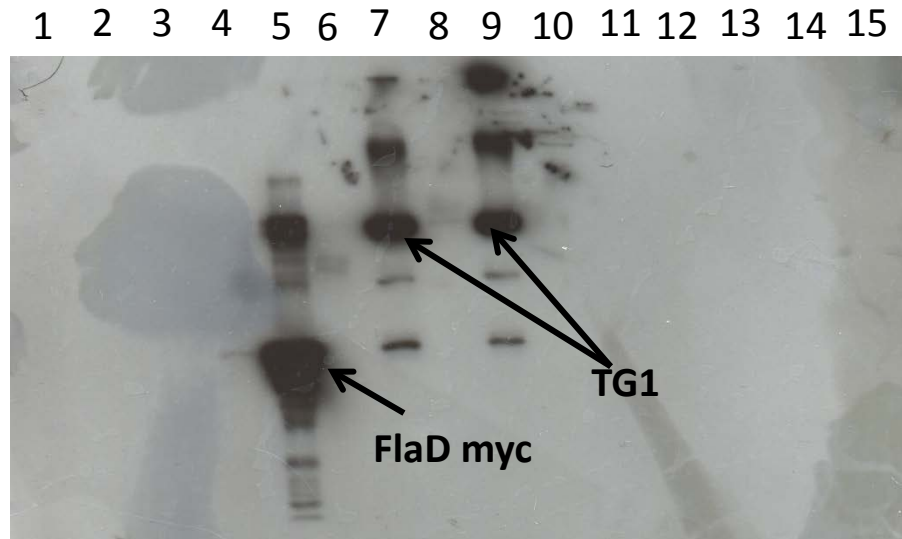

Figure 5: Immunoblot testing of TG1 using anti myc antibody (1:3000) as primary antibody and HRP conjugated goat anti mouse (1:5000) as a secondary antibody. Lane 1: Negative control without IPTG, Lane 2: Protein ladder (Seeblue presatined), Lane 3: negative control with IPTG; Lane 4: Positive control without IPTG; Lane 5: Positive control (FlaD myc) with IPTG; Lane 6: TG1 (44) without IPTG; Lane 7: TG1 (44) with IPTG; Lane 8: TG1 (46) without IPTG; Lane 9: (TG1) with IPTG; Lane 10: TG2 (73) without IPTG; Lane 11: TG2 (73) with IPTG; Lane 12: TG2 (76) without IPTG; Lane 13 TG2 (76) with IPTG; Lane 14 TG2 (77) without IPTG; Lane 15: TG2 (77) with IPTG.

The western blot shows that the positive control (figure 6, lane 5), TG1 were produced and detected (figure 7, lane 7 and 9). TG2 were not seen in the blot because TG2 lacks myc epitope and this detection was done with the primary antibody against myc epitope.

Immunological confirmation of the identity of the TG bands found in the supernatant fractions.

A primary antibody against the myc epitope was utilized in order to confirm the identity of the recombinant band. Immunoblotting was used for identification of the 80 kDa protein.
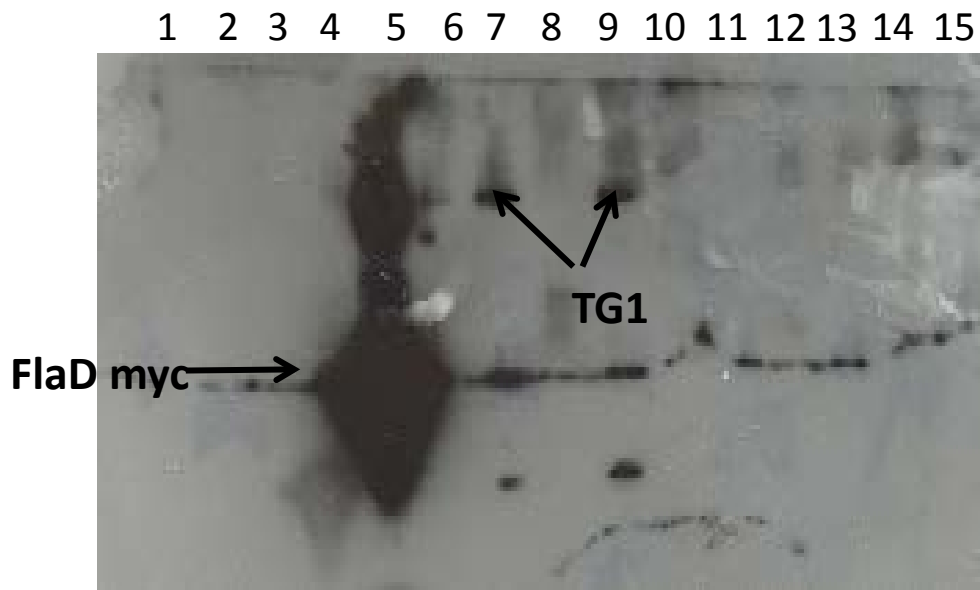

Figure 6: Immunoblot testing of TG1 using anti myc antibody (1:3000) as primary antibody and HRP conjugated goat anti mouse (1:5000) as a secondary antibody. Lane 1: Protein ladder; Lane 2: Negative control without IPTG, Lane 3: negative control with IPTG; Lane 4: Positive control without IPTG; Lane 5: Positive control (FlaD myc)with IPTG; Lane 6: TG1 (44) without IPTG; Lane 7: TG1 (44) with IPTG; Lane 8: TG1 (46) without IPTG; Lane 9: (TG1) with IPTG; Lane 10: TG2 (73) without IPTG; Lane 11: TG2 (73) with IPTG; Lane 12: TG2 (76) without IPTG; Lane 13 TG2 (76) with IPTG; Lane 14 TG2 (77) without IPTG; Lane 15: TG2 (77) with IPTG.

The western blot shows that the positive control (figure 6, lane 5), TG1 were produced and detected (figure 6, lane 7 and 9). TG2 were not seen in the blot because TG2 lacks "myc" epitope and this detection was done with the primary antibody against "myc" epitope. The bands seen in the blot seems distinct and the when compared to the positive control (40 kDa), the molecular weight of the spot falls around 80 kDa, which is the desired weight. Positive control seems to be produced in excess amount.

Isolation of TG-1 plasmid (44)

Large amount of TG-1 plasmid was isolated for expression purposes.Plasmid (44) was amplified and purified for the purpose of the large scale production of the recombinant protein. DNA concentration was determined by both UV spectrophotometry and quantitative analysis on an agarsose gel. The size of the plasmid was also checked by agarose gel electrophoresis. (results not shown)

Determination of Plasmid concentration:

After the isolation and purification, the concentration of the plasmid was determined by NANODROP spectrophotometer. The concentration was found to be 23.4 ng/µl.

Verification of recombinant vector by sequencing

Sequencing was carried out to verify the sequence of the pET151/D-TopoTG-1 plasmid. The junction between the vector and the transglutaminase insert was confirmed by sequence analysis. Furthermore sequencing showed that no mutations were introduced during the plasmid amplification of the TG-1 plasmid. (results not shown)

Large scale recombinant production of the TG1 in *E.Coli* at 16° C.

After the results from the small scale production at 37° C and 16^{0} C, the most consistent and appropriate TG was found to be TG1 and the construct number 44 was chosen to be used in the large scale recombinant production.

The cells were harvested in a larger volume for the large scale. The supernatant were harvested while the pellets were discarded.

Optimizing, the washing and the eluting conditions for the His Trap column:

The supernatant fraction of the large scale production was utilized for the purification of the protein. The supernatant fraction was run through His Trap Column. The protein of interest and other non-specific protein of interest attach to the column. Washing buffer (buffer B) was used to wash the non-specific protein out of the column and elution buffer (buffer C) was used to elute the protein of interest. Imidazole in the buffer helps to wash and elute the specific and non-specific proteins from the column. So, it was necessary to determine the concentration of imidazole in the buffers which elutes contamination, non-specific bound proteins while leaving the protein of interest still attached to the column. Since we want to elute the protein of interest with 500 mM, we had to optimize the concentration of imidazole in buffer B.

Different concentrations of imidazole in buffer B were tested with different samples. First the 250mM of imidazole was used to wash the column. 250mM imidazole was too high for the washing since it eluted the protein of interest. 150 mM of imidazole and 100 mM of imidazole was tried for washing with the same results. Finally, as we decreased the concentration of imidazole to 50 mM in washing buffer B, it showed positive results. It only washed unwanted protein and the protein of interest was eluted with 500 mM of imidazole (buffer C).

The supernatant was run in His Trap column and was first washed by the buffer containing 50 mM imidazole and then eluted with buffer containing 500 mM imidazole.

10 fractions of 1 ml each was collected after wahing with buffer B. The fractions were analysed by SDS-PAGE and coomassie staining for the presence of the recombinant protein.
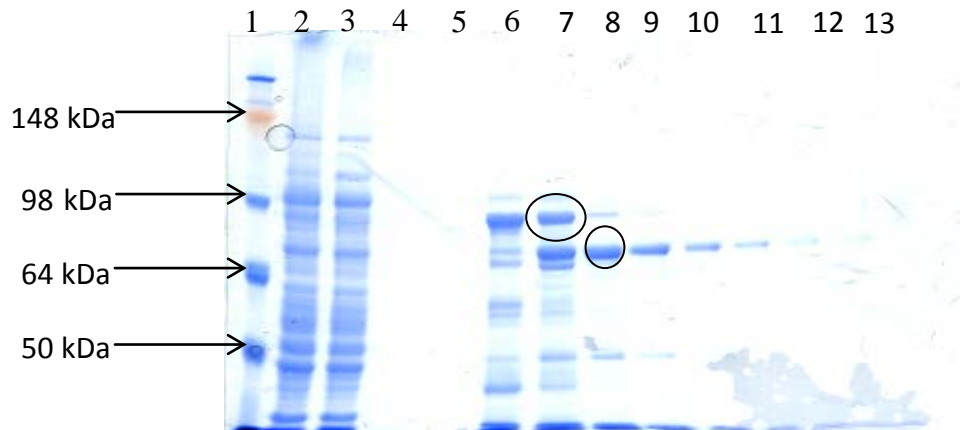

Figure 7: SDS-PAGE analysis of the fractions, collected from the His Trap column after washing with the buffer B (50 mM imidazole) from the large scale production of TG1 in *E.coli* at 16° C. Following harvesting, the supernatant fraction were run through His column, washed with buffer B (50 mM imidazole) and collected in 10 fractions of 1 ml each. The fractions were run on a 8% SDS-PAGE, 170 V for 1 hour and stained with Coomassie Brilliant Blue. The numbers at the top indicate lanes and the molecular weights of the standards are indicated at the left margins. Lane 1: Protein ladder; Lane 2: Extract; Lane 3: Flow through; Lane 4: B1; Lane 5: B2; Lane 6: B3; Lane 7: B4; Lane 8: B5; Lane 9: B6; Lane 10: B7; Lane 11: B8; Lane 12: B9; Lane 13: B10.

Figure 7 shows the SDS-PAGE analysis of the fractions, collected from the His Trap column after washing with the buffer B (50 mM imidazole) from the large scale production of TG1 in *E.coli* at 16° C. Two of the bands were sent to Tromsø for the MSMS analysis. The bands sent for the mass spectrometry analysis is shown in circle in the figure 7. The analysis showed that the bands did not have the amino acid sequence of our protein of interest and were of bacterial origin. This results show that the washing procedure was able to wash proteins that are non-specifically attached to the His column.

After washing with 10 ml of washing buffer (Buffer B), the His Trap column was eluted with buffer C (500 mM imidazole). 10 ml fractions of 1 ml each were collected and analyzed by SDS-PAGE and coomassie staining for the presence of the recombinant protein.
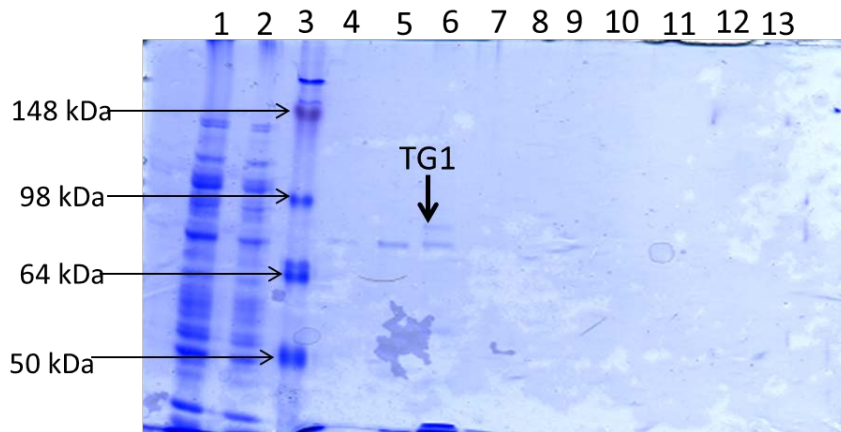

Figure 8: SDS-PAGE analysis of the fractions, collected from the His column after eluting with the buffer C (500 mM imidazole) from the large scale production of TG1 in *E.coli* at 16° C. Following harvesting, the supernatant fraction were run through His column, washed with buffer B (50 mM imidazole), eluted with buffer C (500 mM) and collected in 10 fractions of 1 ml each. The fractions were run on a 8% SDS-PAGE, 170 V for 1 hour and stained with Coomassie Brilliant Blue. The numbers at the top indicate lanes and the molecular weights of the standards are indicated at the left margins. Lane 1: Extract; Lane 2: Flow Through; Lane 3: Protein Ladder; Lane 4: E1; Lane 5: E2; Lane 6: E3; Lane 7: E4; lane 8: E5; Lane 9: E6; Lane 10: E7; Lane 11: E8; Lane 12: E9 and Lane 13: E10.

Figure 8 shows the SDS-PAGE analysis of the fractions, collected from the His column after eluting with the buffer C (500 mM imidazole) from the large scale production of TG1 in *E.coli* at 16° C. This gel shows lesser bands than earlier fractions (washing fractions). Only two bands can be seen in lane 6, which was where we expected to see our protein of interest. The upper and lower band was excised from the gel with fresh blade, put in a new eppendorf tube and were sent for Q-TOF (LC- MSMS) analysis in Tromsø. The sequence of the protein was also sent along for the verification of the protein. Results from Tromsø showed positive results. The sequence of the protein in the upper band showed 100% similarity with the sequence of our protein of interest. (results not shown)

Immunological confirmation of the identity of the recombinant protein band.

(Elution fraction)

The eluted fraction were futhur analysed by immunoblotting for the presence of the recombinant protein. The gels from SDS-Page were transferred to a nitrocellulose paper and the paper was blocked with blocking solution, washed with primary antibody, again washed with seconadary antibody and a film was developed to see the presence of the recombinant protein. Primary antibody against his epitope was used to detect the recombinant protein and HRP conjugated seconadry antibody against the primary antibody was used. The time given for the transfer of the protein bands from the gel to the paper was incresed by 30 minutes as compared to earlier immunoblotting.
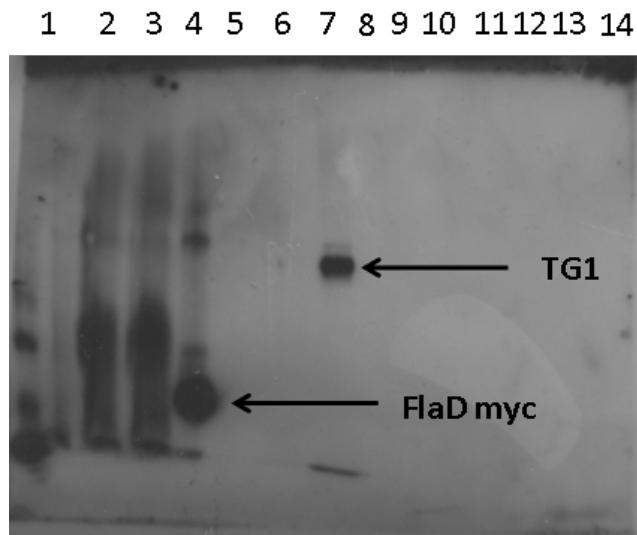

Figure 9: Immunoblot testing of TG1 using anti his antibody (1:3000) as primary antibody and HRP conjugated goat anti mouse (1:5000) as a secondary antibody. Lane 1: Magic Marker; Lane 2: Extract; Lane 3: Flow Through; Lane 4: FlaD myc; Lane 5: E1; Lane 6: E2; Lane 7: E3; Lane 8: E4; Lane 9: E5; Lane 10: E6; Lane 11: E7; Lane 12: E8; Lane 13: E9; Lane 14: E10.

The blot shows that the recombinant protein was produced and only one spot can be seen in the elution fractions (figure 9, lane 7). Lane 7 shows the spot and the E3 was the sample that contained the recombinant protein. E3 is the same fraction that showed a band in SDS-PAGE gel and was sent for Q-TOF (LC-MSMS).

3 D structure prediction:

I-TASSER is an internet service system which is used for the prediction of protein structure and function (Roy, Kucukural et al. 2010) (Zhang 2008). This server predicts the structure of the protein based on the amino acid sequence of the protein. For the structure of our protein of interest, the amino acid sequence was provided in FASTA format to http://zhanglab.ccmb.med.umich.edu/I-TASSER/

The prediction of TG1 was predicted as shown in the figure below:

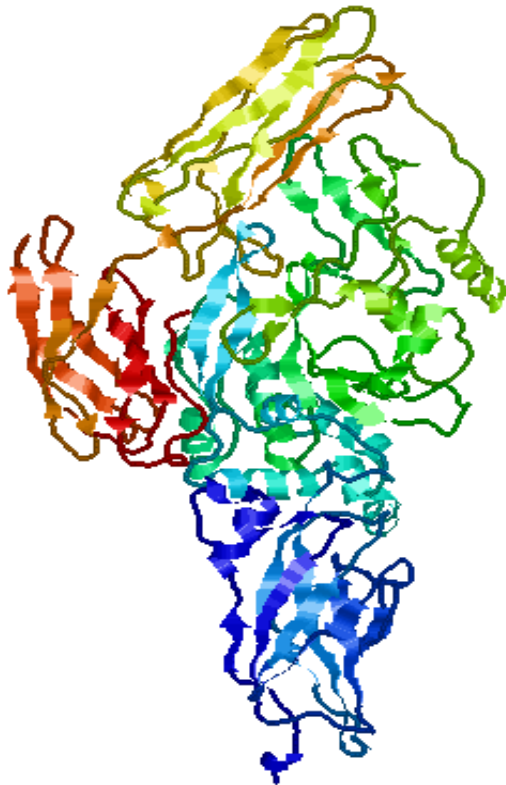Figure 10: **Prediction of 3D structure for TG1**.

The figure shows the 3D structure of TG1 predicted by I-Tasser (Roy, Kucukural et al. 2010) (Zhang 2008) . The prediction with the highest C-score was chosen prior to editing and visualization in Rasmol. Each chain is drawn as a smooth spectrum from blue through green, yellow and orangeto red. The N termini of proteins are colored blue and the C termini red.

5. Discussion:

5.1 Expression at 37° C vs. 16° C:

The demand for large amount of recombinant proteins is high for the purpose of its studies like structure, solubility, activity etc. The need to obtain the recombinant protein in soluble fraction is of great importance. Various factors such as choice of expression system, induction temperature, and fusion tags play important role in the solubility of the protein. This study was undertaken to find a suitable expression system, induction temperature and use of His tag (as a fusion peptide) for the production of transglutaminase from a pschyrophile organism (Atlantic cod).

The recombinant protein was expressed at two different temperatures (37° C and 16° C). The pellet and supernatant fractions were tested with both SDS-PAGE and immunoblotting. The fusion partner for the protein is hexa-histidine, which is cloned just upstream of the protein of interest. The effect of temperature in the solubility of the recombinant protein can be seen in this experiment. The positive control flagellin D (FlaD myc) is relatively small protein with respect to the protein of interest (TG). The SDS-PAGE analysis of the supernatant fraction at 37° C and 16° C shows that the FlaD myc was more soluble when expressed at 16° C then at 37° C (figure 2 and 4 of result). The coomassie staining of the gel shows a visible band at 40 kDa in figure 4 (16° C), but no visible band can be seen at 40 kDa in figure 2 (37° C). The decrease in temperature and the small size of FlaD myc may have been the reason for the increased solubility. Since lowering the temperature leads to lower general production rate, the nascent protein has more time to fold and inducing the culture at 16° C induces the expression of chaperones and cold shock protein which helps to maintain solubility and assist in folding (Niiranen, Espelid et al. 2007). The small size may also have aided in the solubility of FlaD myc because there is less possibility of a misfolding to occur when compared with larger protein. The solubility of the recombinant TGs is reduced when expressed at 37° C and 16° C. The recombinant TGs cannot be seen in the soluble fraction in the SDS-PAGE analysis but can only be detected by immunoblotting. This suggests that the solubilty of the protein is low at both temperatures. The reason for this may be a combination of the factors. The recombinant protein is bigger in size than FlaD myc (positive control). The bigger sized protein requires more time to fold which may introduce error and cause misfolding thus making it insoluble. There is optimal temperature for expression of particular protein with its fusion tag and these should be investigated when optimizing a protein expression system (MacDonald, Armson et al. 2003).

Another reason why the recombinant protein (TG) cannot be seen in the coomassie staining of SDS-PAGE gel is because of the action of the host's proteases which degrades the protein as it is not native. Premature termination, codon bias of the *E. coli* may also lead the protein to not translate properly, there by affecting the solubility of the recombinant protein (MacDonald, Armson et al. 2003). The introduction of a fusion protein such as maltose binding protein (MBP), N-utilizing substance A (Nus A), thioredoxin, cloned N –terminally to the protein of interest could be helpful in solubilizing the recombinant protein. These fusion protein also serves as a tag. It was suggested that MBP and Nus A are the best fusion proteins

for the recombinant production of a cold adpated protein, and among many other fusion proteins tested, Nus A increased the solubility of the protein at 16° C in AI strain and solubility was lowest when the recombinant protein was expressed with a hexa-histidine tag (Niiranen, Espelid et al. 2007). So to obtain a protein in soluble fraction, parameters such as expression strain, protein fusion tag, temperature, inducer concentration has to investigated thoroughly. Some reports also suggests that the solubility of the recombinant protein can be enhanced by introducing a dipeptide, glyclglycine in the range of 100mM to 1M in the medium which enhances the solubility by several folds (Ghosh, Rasheedi et al. 2004). All of these factors have to screened for increasing the solubility of the recombinant protein. In figure 5 and 6 (immunological analysis of pellet and supernatant fraction at 16^oC), two additional bands are seen in the insoluble fraction (pellet) of higher molecular weight but no additional bands are seen in the supernatant fraction. This may be because the insoluble form of the TG has made a complex with other bacterial proteins from the host. The bands which are smaller in size can be detected in the immunoblot. The reason may be because of the action of protease of the host which degraded the protein.

The small scale production of the recombinant TG1 and TG2 at both temperature (37° C and 16° C) showed that TG1 was consistent and was expressed well in comparison to TG2. The expression of TG1 was relatively high in soluble fraction when compared to TG2. The two TGs are quite similar as TG1 is 78.3 kDa in size whereas TG2 is 78.8 kDa. The difference between recombinant TG1 and TG2 is the presence of additional myc tag cloned C-terminally to TG1. TG2 lacks it. The influence of myc tag in the expression and solubility of TG1 has to be further investigated.

5.2 Temperature vs. solubility

There have been a lot of studies and experiments in expressing cold adapted enzymes in bacterial expression system. The relationship between temperature and folding of the recombinant protein plays an important role in obtaining the recombinant protein in soluble and active form. Different studies show different results for the relation between temperature and solubility for different cold adapted enzymes.

One study showed the expression of soluble cold adapted enzyme acetate kinase from *Shewanella* species AS-11 in *E. coli* BL21 (DE3) was maximum at 20° C when the induction was carried out for 16 hours (Md. Abul Kashem Tang 2012) . Different induction temperature and time of induction was tested for this experiment.

Similarly some other report suggests different induction temperature to obtain high yield of soluble protein. Different induction temperature was tested to determine the optimum temperature and induction time for the production of trypsin I from Atlantic cod in the soluble fraction (Jónsdóttir, Bjarnason et al. 2004) . The result suggests that the optimum temperature for the induction of expression of HP-thioredoxin trypsin I was found out to be 25° C and the optimum time of induction was 10 hours.

Hartinger et al reported the expression of a mesophilic mycotoxin fumonisin B(1) in *E. coli* Arctic Express (DE3) which co-expresses two cold adapted chaperons   at 11^0 C finally

resulted in the production of active enzyme (Hartinger, Heinl et al. 2010). This result was even better than the expression of the recombinant protein with MBP as fusion partner at 30° C.

These results suggest that induction temperature depends upon the protein of interest. Different proteins have their own optimum temperature at which they correctly fold and remain in the active form. So the optimum temperature for the protein of interest has to be optimized by running a pilot scale at different temperature and different induction time. And once it has been established, then only the large scale production and purification steps should be carried on.

5.3 Large Scale Production:

Large scale production of TG1 was carried at 16° C using the same expression system used for the small scale production. The supernatant fraction was analyzed for the presence of TG1 and the pellet was discarded. Immobilized Metal Affinity Chromatography (IMAC) was used for the purification of the recombinant protein. The supernatant fraction was run through a column comprising of nickel atom which has a high affinity for the polyhistidine present in the recombinant protein. The column was washed with varying concentration of imidazole for the purpose of displacing the recombinant protein from the column.

The objective of the purification was to obtain the recombinant protein in a non-denaturing state or in a native state. So, the use of imidazole to displace the recombinant protein was chosen. At first, the column was washed with a buffer containing imidazole concentration of 250 mM, with an aim to wash the proteins which were attached nonspecifically. The concentration of imidazole in the washing buffer proved too high as it not only washed the contamination but also the recombinant protein with it. The final concentration of imidazole for washing was determined to be 50mM. The recombinant protein was finally eluted with 500mM imidazole**.**

The hexa-histidine available in the recombinant protein to attach itself to the nickel atom present in the His Trap column may have posed little problem. The elution of the recombinant protein with even 100 mM imidazole suggests that the bond between the nickel and recombinant protein was weak. The histidine tag may have folded into the recombinant protein so that fewer histidine was available for the nickel atom in the column. The amount of histidine at the surface should be enough to attach itself to the column. The washing of the recombinant protein even with 100mM imidazole suggests that enough histidine may not have been present at the surface of the recombinant protein. One reason could be that the histidine tag was folded towards the core of the protein which hid the histidine for the interaction with the nickel in the column. The interaction between the nickel and histidine could be enchanced, if nano-histidine tag is used instead of hexa-histidine. If the number of histidine is nine or more, the bond between histidine and nickel might be strong and the washing process would be more efficient.

6. Future projects:

The future projects involving TGs could be very interesting. The first step of optimizing the expression and purification can be followed from this experiment. The question of low concentration of soluble TGs in the supernatant fraction could be addressed by choosing a fusion protein as a tag which helps in the solubilizing the recombinant protein. Fusion protein such as MBP, Nus A have shown be effective in this situation. The yield of recombinant TG could be increased in these ways.

The future analysis could be based on the activity of the enzyme. Its activity in catalyzing acyl-transfer between the glutamine and lysine residue could be investigated. Michaelis constant K_m and V_{max} of the enzyme's reaction can be assayed. The removal of myc epitope from the C-terminal of TG1 could also be done by cloning TG1 without the myc epitope. The myc epitope may lower the activity of the enzyme which can also be studied.

Finally, if the purpose of using TGs is solely based in the food industry, expressing TGs from psychrophilic (microorganism) origin would be more advantageous than from vertebrates. The calcium independency of the microorganisms combined with cold adapted nature of the enzyme is a big benefit for the food industry. Microorganisms from the cold habitats could be screened for the presence of TGs, and purification steps should be optimized. But the use of TGs from non-bacterial origin still has other benefits in different fields than food industry.

7. Conclusion:

The recombinant protein production of an enzyme called transglutaminase from Atlantic cod (*Gadus morhua*) was carried out in this study. The genes for two transglutaminase (TG1 and TG2) were cloned in a pET151/D-TOPO vector and expressed in BL21 strain of competent *E. coli*. The gene for Flagellin D (Fla D) was also cloned to a vector with an additional myc tag attached to the C terminal and Fla D myc was chosen as a positive control throughout the experiments. All of the vectors had his tag cloned N terminally to the gene of the protein. The expression of the recombinant protein was induced at two different temperatures (37° C and 16° C). The expression was analyzed by SDS-PAGE and immunoblotting. The immunoblotting analysis confirmed that a protein size of 80 kDa (TG1) was expressed in the soluble fraction at 16° C.

The large scale production was carried out at 16° C for the recombinant production of TG1. The supernatant was used for the purification of the recombinant protein using His Trap column. Immobilized metal ion in the His Trap column was used to trap the recombinant protein. The column was first washed by 50mM imidazole buffer and the attached protein was eluted with 500 mM imidazole buffer. The eluted fraction was analyzed by SDS-PAGE and immunoblotting. The immunoblotting showed a band at 80 kDa, which confirmed the recombinant protein, was produced and eluted. The SDS-PAGE also confirmed the same result. The band from the SDS-PAGE was analyzed for amino acid sequence by Q-TOF (LC-MS/MS) and the result showed that the protein in the band matched the sequence of TG1.

The tranglutaminase (TG1) was expressed in BL21 strain of *E. coli* at 16° C and the protein was purified with His Trap column. But the concentration of the soluble TG could still be improved and should be investigated further. In future, the activity of the enzyme should be studied.

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