HETEROLOGOUS PROTEIN EXPRESSION OF TPA AND ELP

LIMIN XIE

HETEROLOGOUS PROTEIN EXPRESSION: PRODUCTION OF TISSUE PLASMINOGEN ACTIVATOR IN *PICHIA PASTORIS* AND PROBING INTEIN ACTIVITY ON ELASTIN-LIKE POLYPEPTIDE AGGREGATES

By

LIMIN XIE, M. A. SC.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Applied Science

McMaster University

MASTER OF APPLIED SCIENCE (2005) McMaster University

(Chemical Engineering) Hamilton Ontario

TITLE: Heterologous Protein Expression: Production of Tissue Plasminogen Activator in *Pichia Pastoris* and Probing Intein Activity on Elastin-Like Polypeptide Aggregates

AUTHOR: Limin Xie, M.A. S. (Chinese Academy of Sciences)

SUPERVISOR: Dr. Carlos Filipe

NUMBER OF PAGES: xi, 76

ABSTRACT

Tissue plasminogen activator (tPA), is commonly used as thrombolytic agent for the treatment of various cardiovascular diseases. This thesis constitutes the first report on cloning and expression of tPA in the methylotrphic yeast *Pichia pastoris*.

The tPA gene was first cloned into an *E. coli/Pichia* shuttle plasmid and then integrated into the *Pichia* genome. The recombinant *Pichia* was able to express tPA intracellularly, under methanol induction. The tPA produced by the *Pichia* had a similar molecular weight as native tPA and it had serine protease activity. At the shake flask scale, the recombinant *Pichia* strain was able to produce twice as much tPA as reported for *E. coli*.

Elastin-like polypeptides (ELP) are proteins that have a peculiar characteristic: they are able to undergo a reversible inverse phase transition temperature within a very narrow temperature range. On a second aspect of heterologous protein, a construct composed of thioredoxin-intein-ELP was used to provide direct evidence, for the first time, that protein folding and activity, in this case the intein, was maintained when the tripartite fusion was present in the aggregated state. These results are important, since they provide the necessary degree of confidence to stimulate future work directed towards expression and maintenance of proper folding of aggregation-prone proteins when expressed *in-vivo E. Coli* as ELP directed inclusion bodies. It is also shown that the intein-ELP system may be a very interesting system to be used as a drug delivery vehicle.

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere appreciation to my supervisor, Dr. Carlos Filipe for everything he has done for me during the last 20 months Thanks for his guidance on my research work, his encouragement and understanding, as well as his great effort on my thesis revision. I have benefited a lot from him, both his knowledge and his personality. I don't think I can possibly make it without his guidance and support. I also want to thank my previous supervisor, Dr. Lisa Crossley for her support of my work.

I also want to thank all the people who contributed to the completion of this thesis: Dr. William Sheffield and Varsha Bhakta in the Department of Pathology and Molecular Medicine for their help in tPA cloning and providing access to their lab; Glenn McClung for providing technical assistance and sharing of their resources; Xin Ge for valuable discussion and suggestions; Dong Yang for helping me on some of my lab work. I just want to expression my whole-hearted appreciation to these people. There are so many people in this department that I want to thank, for their kindness and friendship that made the past two years a great experience in my life.

Finally, I would like to thank my husband, Ralph Shi, for his selfless support, understanding, encouragement, and love. I also want to say thank you for my parents for taking care of my children when I am away. I want to say sorry to my two little kids, my son Nathan and my daughter Tanya, for my absence during the past 11 months. I love them so much and I am very proud of them. So I hereby dedicate this thesis to my family.

TABLE OF CONTENTS

TITLE PAGE.....i

DESCRIPTIVE NOTE ii

ABSTRACT.....iii

ACKNOWLEDGEMENTS.....iv

TABLE OF CONTENTS.....v

LIST OF FIGURES.....viii

LIST OF TABLES x

LIST OF ABBREVIATIONS xi

1. Introduction.....1

2. Cloning and Heterologous Expression of Tissue Plasminogen Activator in the Methylotrophic Yeast *Pichia pastoris*.....6

2.1. Introduction.....6

2.1.1. Tissue Plasminogen Activator (tPA) 6

2.1.2. Methylotrophic Strain Pichia pastoris 13

2.2. Materials and Methods.....16

2.2.1. tPA Cloning into *Pichia Pastoris*.....16

2.2.2. Growth and Expression.....20

2.2.3. Analytical Methods.....20

2.3. Results and Discussions.....21

2.3.1. Induction of tPA Expression by Methanol 21

2.3.2. Improving tPA Secretion by Changing Temperature of Incubation 27

2.3.3. tPA Expression Using MM Medium 29

2.3.4. Increasing tPA Expression by Optimization of the BMMY Medium 31

2.4. Conclusions 34

3. Probing the In-Vitro Activity of an Intein in ELP Based Aggregates 36

3.1. Introduction 36

3.2. Materials and Methods 39

3.2.1. Expression of the Thioredoxin-inten-ELP fusion protein 39

3.2.2. Purification of ELP-fused Protein 40

3.2.3. Monitoring of the Inverse Phase Transition and Thioredoxin Activity Test 41

3.2.4. Particle Size Measurement by Mastersizer 41

3.3. Results and Discussion 42

3.3.1. Purification of ELP Fused Protein by Phase Transition 42

3.3.2. Phase Transition Behavior of Trx-Intein-ELP Fusion Protein....43

3.3.3. Protein Activity of Trx-Intein-ELP Protein in Aggregated Form

.....45

3.3.4. Particle Size of ELP Aggregation Matrix 50

3.4. Conclusions 51

4. Conclusions and Recommendations for Future Work 53

5. References 55

6. Appendices 63

Appendix I tPA DNA Sequences 63

Appendix II: Map of pETPER 65

Appendix III: TPA Sequencing of pUC-tPA4 66

Appendix IV: TPA Sequencing of pZamp-tPA4 68

Appendix V: Medium and Recipes 70

Appendix VI: Solutions and Buffers 71

Appendix VII: PCR of the tPA Gene 75

AppendixVIII: Western Blotting Analysis of tPA 76

LIST OF FIGURES

Figure 2.1.1 Function of tPA in Blood Fibrin Degradation.....6

Figure 2.1.2 Schematic Visualization of the Molecular Interactions Regulating Physiological Fibrinolysis 7

Figure 2.1.3 3D Rendering Structure of Human Tissue Plasminogen Activator Binding a Small Molecule Target 9

Figure 2.2.1 Map of pZamp-tPA Recombinant Plasmid 18

Figure 2.3.1 Growth of Pichia in BMMY Media 22

Figure 2.3.2 SDS-PAGE of tPA Expression after Methanol Induction.....24

Figure 2.3.3 SDS-PAGE of Protein Samples.....25

Figure 2.3.4 Western Blotting of tPA 25

Figure 2.3.5 Growth of Pichia S2 at Different Temperatures 28

Figure 2.3.6 Endocellular tPA Expression in BMMY at Different Temperatures.....29

Figure 2.3.7 tPA Expression in MM and BMMY Medium 31

Figure 2.3.8 Growth of the S4 Strain in Different Induction Medium 32

Figure 2.3.9 SDS-PAGE of Endocellular Proteins of Recombinant *Pichia* S4 in Different Induction Medium.....33

Figure 3.2.1 Map for Plasmid pTME 40

Figure 3.2.2 Structure Scheme of Mastersizer 42

Figure 3.3.1 Purification of Trx-Intein-ELP Fusion Protein by NaCl Triggered Phase Transition 43

Figure 3.3.2 Phase Transition Profiles for Samples of Trx-Intein-ELP at Different NaCl Concentrations.....44

Figure 3.3.3 DTT-induced Intein Cleavage in Thioredoxin-intein-ELP90 Aggregation Matrix.....45

Figure 3.3.4 DTT- induced Intein Cleavage of Thioredoxin in Aggregation Matrix...47

Figure 3.3.5 Thioredoxin Distribution after Intein-cleavage in the Aggregation Matrix 48

Figure 3.3.6 Thioredoxin Activity Tests on the Aggregation Matrix 49

Figure 3.3.7 Particle Size Distribution of Aggregation Matrix 51

LIST OF TABLES

Table 2 -1 Total Protein Concentration before and after Methanol Induction 23

Table 2 -2 Comparison of Cell Growth and pH in MM and BMMY Medium 30

Table 2 -3 Cell Concentration and tPA Activity Test in Different Batches 33

Table 3 -1 Thioredoxin Activity Distribution on the Aggregation Matrix.....49

LIST OF ABBREVIATION

DNA	Deoxyribonucleic acid
ELP	Elastin-like polypeptide
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate - poly-acrylamide gel electrophoresis
tPA	Tissue plasminogen activator

1. Introduction

Heterologous protein expression is one of the cornerstones of bioprocess engineering. In its simplest form, this process is started by isolating a gene from the DNA of a particular species (e.g. *Homo Sapiens*), cloning this gene into a DNA vector that can be transferred to a different organism called the host, with the most common host being *E. Coli*. It is possible then to use the protein production machinery of the host to produce the protein of interest in elevated quantities. This approach is an essential research tool, since it allows researchers to rapidly obtain a particular protein of interest. It is also a common-place approach in large scale applications, where a protein of commercial interest can be produced in large scale reactors using genetically modified organisms. Following production, the protein of interest needs to be recovered in a highly pure form, which is a substantial challenge on its own.

Unfortunately, heterologous protein expression does not have 100% success rate, resulting sometimes in the formation of a protein that does not have exactly the same conformation as the protein expressed in the original organism. One of most common source of problems is that not all organisms are capable of catalyzing the formation of disulfide bonds (a covalent bond between two sulfhydryl groups of two cysteine residues that is formed under oxidizing conditions). Disulfide bonds are important in defining and maintaining the tertiary structure of proteins and hence, are essential for proper protein activity. Mammalian cells do have the ability to form disulfide bonds with great precision,

even in proteins that contain several such bonds. *E. Coli* is not able to catalyze the formation of disulfide bonds, due to fact that its cytoplasm is maintained at a reduced redox state as well as due to the fact that it lacks the proper enzymes necessary to catalyze this reaction. In addition, mammalian cells can glycosylate proteins in a very specific manner. Glycosylation may be an important factor for proper protein function, as well as a possible cause of an immunogenic response when administered to an organism which glycosylates the same protein, but in a different manner (Higgins and Cregg, 1998). Disulfide bond formation and glycosylation occur after the protein is formed and hence are called posttranslational modifications.

A common strategy for heterologous protein expression is to identify the simplest and fastest growing organism that is able to produce the protein in the largest possible quantity, with a correct set of disulfide bonds being formed, and with a similar glycosylation pattern as the protein expressed in the native organism. For the first part of this research project, tissue plasminogen activator (tPA) was selected as the target protein for heterelogous expression. This protein was chosen since it has high commercial value, it has high demand for therapeutic purposes and that is notoriously difficult to express in a correct conformation (Mattes, 2001). *Pichia Pastoris*, a methylotrophic yeast, was selected as the host organism for tPA expression, since it is capable to carry posttranslational modifications to proteins, such as disulfide bond formation and glycosylation. This organism has several attractive features from a practical point of view, such as: high growth rate (Higgins and Cregg, 1998; Cereghino and Gregg, 2000), protein expression can be tightly controlled and driven to high levels; it has the ability to secrete

proteins to the media, if the protein contains a small leading sequence that targets the protein for secretion (Higgins and Cregg, 1998); growth conditions can be manipulated to minimize the quantity of proteases which could destroy the product (Higgins and Cregg, 1998).

Since tPA is a highly complex molecule and very difficult to express in a variety of hosts (Collen and Lijnen, 2004), it was decided to conduct a series of experiments, , using an unrelated expression system, but also in the area of heterologous expression. In this second set of experiments a simpler expression host was used (*E. Coli*). This study was motivated by a set of disparate observations in the literature associated with expression of elastin-like polypeptides (ELP) in *E. Coli* that may have important consequences on a problem that plagues much of heterelogous protein expression in *E. Coli*, the formation of inclusion bodies (aggregated and inactive protein that is deposited as insoluble entities). ELP are polypeptides consisting of tandem repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa can be any amino acid except proline (Urry *et al.*, 1985; Urry, 1992). A peculiar characteristic of ELP is their ability to undergo an inverse and reversible phase transition within a very narrow temperature range (Urry, 1988; Meyer and Chilkoti, 1999). At a temperature below the inverse phase transition temperature (T_t) temperature, ELP is soluble but it becomes insoluble if the temperature exceeds T_t. It has been shown that this feature of ELP is retained when fused to a variety of proteins.

A study done by Guda et al. (1995), focused on expressing ELP in *E. Coli* in a T7 based system (a T7 system uses the RNA polymerase from the T7 virus, which is able to

achieve high protein expression levels, Studier et al., 1990). A key aspect of this study was that the culture was grown for a period of 24 hours without the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to induce protein expression. The leaky nature of T7 based systems, where protein expression is not totally repressed in the absence of inducer (IPTG), allowed the accumulation of extremely high concentrations of ELP and all of it was deposited in the cytoplasm as "soluble inclusion" bodies. The aggregates were observed with TEM and occupied more than 90% of the cytoplasm of the cell. In recent years, it has been reported by several researchers (Trabicc-Carlson et al., 2004; Shimazu et al., 2003) that when ELP is expressed as a fusion partner to a protein targeted for purification, and when expression is done in the absence of IPTG, the target protein is expressed as a fusion partner to an ELP tag, this results in unprecedented high levels of expression. More importantly, the target proteins were recovered in their functional form, indicating that these soluble inclusion bodies are not formed via aggregation of the target protein.

Protein aggregation is due in large part to that the cytoplasm of a cell is a crowded environment. Local concentrations of proteins are extremely high. Over-expression of a target protein will severely increase the potential for aggregation of protein and formation of inclusion bodies. A sometimes successful strategy to prevent the formation of inclusion bodies is to fuse the target protein to be produced with a highly soluble fusion partner, such as thioredoxin (Meyer et al., 2001). Results on this approach have been mixed. We hypothesized that the ELP tag will undergo an inverse phase transition in the cytoplasm of the cell (as demonstrated by Guda et al., 1995), acting to actually decrease

the solubility of the fusion protein. The result should be the formation of ELP directed aggregates and not target protein directed inclusion bodies. If this hypothesis is true, then it should be possible to have the target deposited as a reversible inclusion body, due to the high tendency of the ELP tags to aggregate, with this aggregation being reversed when the cells are disrupted at low temperature. If this hypothesis turns out to be confirmed, then the potential implications are quite significant: it may be possible to over-express sparingly soluble proteins, which is not possible with currently available systems; it may be possible to generate a dual expression system, where free ELP tags are first produced and accumulated in the cytoplasm of the cell and then using these reversible inclusion bodies as a capture matrix for a second round of induction, but now of a membrane based protein fused to an ELP tag, preventing it from reaching the cell membrane and killing it.

All the possibilities presented above are exciting and if confirmed would make for very valuable contributions. However, it is important to ask first a very basic question: when a target protein is fused to an ELP tag and an inverse phase transition is induced, does the target protein retain the correct folding during the aggregation process or is the folding lost. We provide a set of data from in vitro experiments that show that proteins retain folding during aggregation.

2. Cloning and Heterologous Expression of Tissue Plasminogen Activator in the Methylotrophic Yeast *Pichia pastoris*

2.1. Introduction

2.1.1. Tissue Plasminogen Activator (tPA)

Tissue plasminogen activator, also known as tPA, is a protein that exists naturally in human plasma. As a physiological activator, tPA converts the inactive proenzyme, plasminogen, to its active form, plasmin, which can then degrade the fibrin of clots in blood vessels into small soluble fragments (Collen and Lijnen, 2004). Figure **2.1.1** shows the physiological role that tPA plays in anti-coagulation.

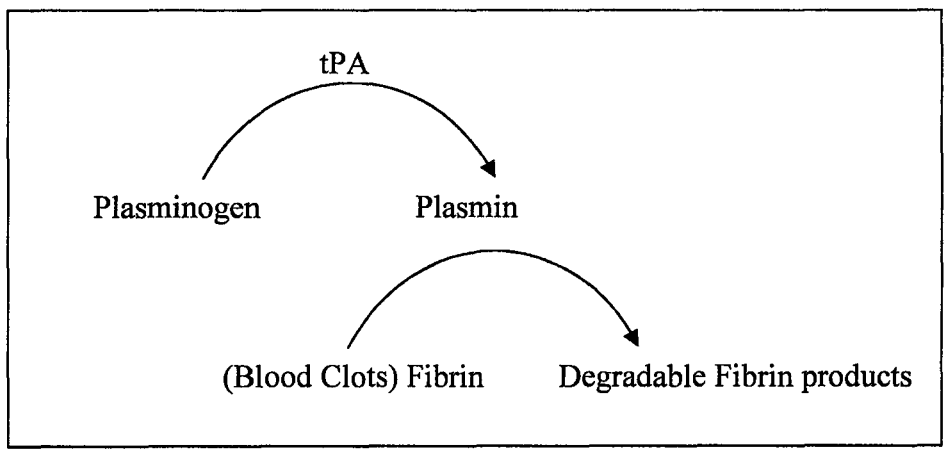


Figure 2.1.1 Function of tPA in blood fibrin degradation

tPA is currently used as a thrombolytic agent for the treatment of various cardiovascular diseases, such as acute myocardial infarction (AMI), massive pulmonary embolism, deep vein thrombosis and peripheral arterial occlusion (Collen and Lijnen, 2004). As a fibrinolytic drug, tPA has one advantage over other drugs, such as urokinase and streptokinase: its high fibrin-specificity. tPA only activates plaminogen when it associates itself with the fibrin clot (Figure 2.1.2) and only the clotting fibrins are degraded. This high level of specificity significantly reduces the risk of haemorrhage of patients under treatment (Collen and Lijnen, 2004; Pennica et al., 1983).

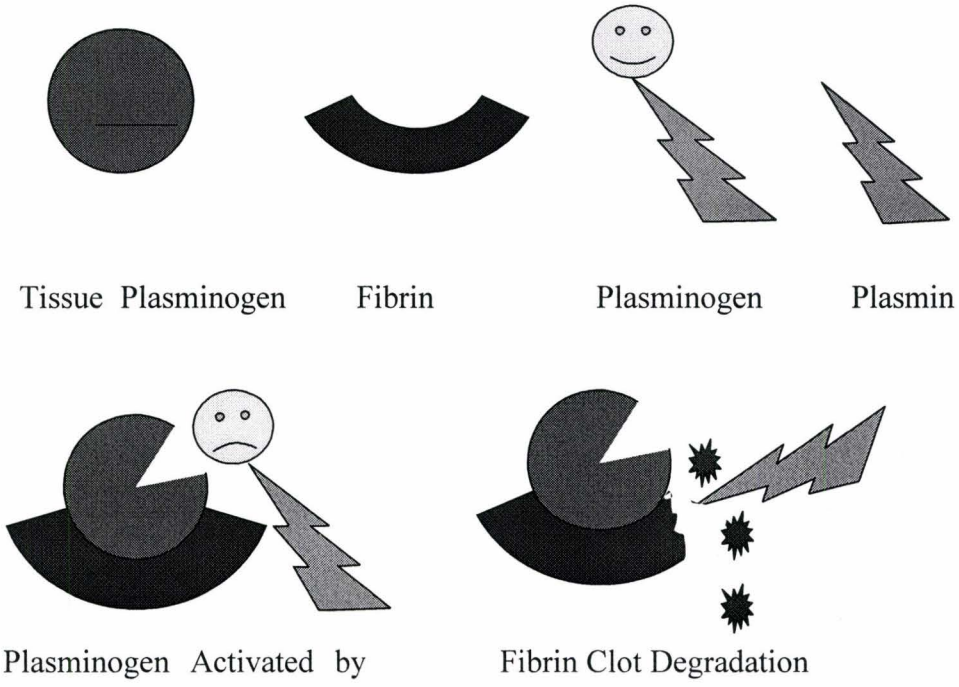


Figure 2.1.2 Schematic visualization of the molecular interactions regulating physiological fibrinolysis. (Based on Collen and Lijnen, 2004)

Human tPA consists of a single-chain glycosylated protein. It contains 527 amino acids with molecular weight of about 68 KDa. It has 35 cysteines, resulting in the formation of 17 pairs of disulfide bonds. When partially hydrolyzed by plasmin at the Arg275-Ile276 peptide bond, tPA converts to a double-chain molecule which is held by an inter-chain disulfide bond.

Studies of the structure of tPA (Figure 2.1.3) showed that the molecule can be divided into 5 distinct domains: a finger domain, which is important for the high level of affinity towards fibrin; one epidermal growth factor (EGF) domain, which is homologous with EGF and contains the structure of the liver cell receptor; two kringle regions K1 and K2, which are highly homologous with plasminogen; and a serine protease domain that contains the active sites responsible for the serine protease activity. tPA is glycosylated at three different sites, (Collen and Lijnen, 2004; Mattes, 2001). Glycosylation is believed to regulate the clearance of tPA from the circulation system. There are discrepancies reported on the role that glycosylation plays in the fibrinolytic activity of tPA. Research shows that glycosylation is not required for the fibrinolytic activity tPA expressed by *E. coli* but is required for tPA activity when produced in mammalian hosts (Mattes, 2001). It has also been reported (Mattes, 2001) that un-glycosylated variants of tPA have a longer half –life as compared to glycosylated tPA, which is highly desirable from treatment point of view. On the other hand, it is thought that un-glycosalyted tPA may cause a response from the immune system (Mattes, 2001).

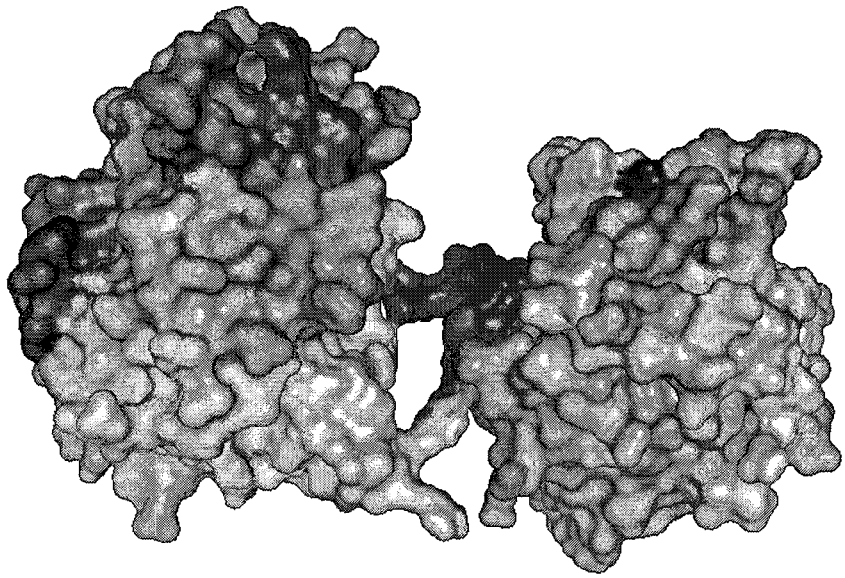


Figure 2.1.3 3D Rendering Structure of Human Tissue Plasminogen Activator Binding a Small Molecule Target (coordinates for structure were downloaded from the Protein Database using accession code 1A5H and the rendering of the molecule was done using Pymol, DeLano 2002)

Pennica *et al.* (1982) were the first group to obtain the complementary DNA (cDNA) encoding the full length gene of human tPA and to clone it in *E. coli*. The cDNA was obtained from mRNA from human melanoma cells that could synthesize tPA. The plasmid containing the tPA gene was transformed into *E. coli* and the protein was expressed under the control of the tryptophan promoter. The cell lysate from the culture was assayed for tPA activity using the fibrin plate assay and using the chromogenic

substrate assay (Pennica *et al.,* 1983; Granelli-Piperno and Reich, 1978). It was shown that tPA with fibrinolytic activity was present and the expression level was about 50-80 μg/L (Pennica *et al.,* 1983; Collen and Lijnen, 2004).

This initial study caused a flurry of research activity on tPA production in *E. coli* expression systems. In addition to the expression of the full length tPA molecule, other tPA variants were expressed in *E. coli* (Mattes, 2001). The K2 domain and the protease domain were expressed as a fusion protein with the staphylococcal protein A signal peptide that could bind IgG (Waldenstrom, *et al.*, 1991). The fusion protein was secreted into the medium and fibrinolytically activity was observed. Another tPA variant containing the kringle 2 domain and the serine protease domain was expressed using phage display in *E. coli* (Manosroi *et al,* 2001). They found that the recombinant tPA variant was partially secreted to the medium. The secreted tPA variant showed amidolytic activity.

Because of the degeneracy of the genetic code and because not all organisms have the same ability to produce all tRNAs in non-limiting quantities, the cDNA encoding a human protein was redesigned to allow optimal expression, from a tRNA availability point of view in *E. coli* (Hale and Thompson, 1998). Another approach is to endow *E. Coli* with the ability to synthesize these rare tRNAs, by supplementing the cells with helper plasmids that encode for their production (Mattes, 2001).

The use of *E. Coli* expression systems has intrinsic problems that it are very difficult to overcome, with respect to tPA expression. *E. coli* is a prokaryotic organism and as such, it lacks the ability to perform posttranslational modifications to proteins,

such as glycosylation and disulfide-bond formation. These "extra" transformations are required for defining and maintaining the function of a protein such as tPA, mainly formation of the correct set of disulfide-bonds. It has been shown that full-length tPA produced in *E. Coli* is unstable (Mattes, 2001). Some progress has been made towards production of tPA in *E. coli* by co-overexpression of heterologous cysteine oxidoreductases and disulfide isomerase. This allows disulfide-bonds to be formed in *E. Coli*, and hence production of a stable form of the protein (Qiu, *et al.*, 1998). Another significant problem, this one being associated with economics, is that when *E. Coli* is used as an expression system the majority of the tPA is accumulated as inclusion bodies. These inclusion bodies need to be separated, denatured and refolded *in vitro*, a process with very low efficiency in terms of recovery of active tPA and unfeasible from an economic point of view.

In several parallel efforts, researchers reported on the expression of tPA in a variety of hosts: *S. cerevisiae* (Lemontt *et al.*, 1985), fungi *A. nidulans* (Upshall et al, 1987), and *A. niger* (Wiebe *et al*, 2001), as well as in Chinese hamster ovary (CHO) cell lines (Choi *et al.*, 1995). Expression of tPA in *S. cerevisiae* resulted in a glycosylated tPA protein with fibrinolytic activity (Lemontt *et al.*, 1985), but no protein secretion was observed. Although several signal peptides were tried to direct tPA for secretion, the protein remained being accumulated intracellularly, with most of the tPA activity being associated with cell debris after cells disruption. Another potential problem with *S. cerevisiae* is that the glycosylation pattern is different from that observed in higher eukaryotic cells. tPA expressed by *S. cerevisiae* was over-glycosylated and the

glycosylation had a different pattern than that observed in its native form (Lemontt *et al.,* 1985). Some results indicate that glycosylation is not required for fibrinolytic activity and that un-glycosylated tPA has longer half-life as compared to the glycosylated forms (Mattes, 2001). However, there are also reports that show that un-glycosylation, or over-glycosylation could give rise to serious immune reactions when the molecule is used for medical treatment (Mattes, 2001).

tPA has also been expressed in the filamentous fungus *A. nidulans.*, resulting in secretion of the active molecule to the culture media (Upshall *et al*, 1987). Additional results indicate that the molecule was not hyperglycosylated. *A. niger* was also used as a host for tPA expression (Wiebe *et al*, 2001). It was found that only 1% of the total tPA expressed was in the active form. Although a protease- deficient strain was chosen, the active tPA produced by this fungus disappeared very rapidly from the culture medium (Wiebe *et al,* 2001).

Currently, recombinant tPA is commercially produced in CHO cells (Activase, Genetech Inc.; Actilyze, Boehringer Ingelheim GmbH). CHO cells can produce active tPA with the correct structure and with exactly the same glycosylation pattern as its natural form. Although production can be carried out in large scale, the cost is high and the process is complex. The high cost of the expression and purification procedures results in an extremely high price for tPA, about USD2000/100mg (Collen and Lijnen, 2004).

2.1.2. Methylotrophic Strain *Pichia pastoris*

Little work has been done on expressing tPA in the methylotrophic yeast *Pichia pastoris*. As far as we know, all the work that has been done with this host and tPA is on expression of the krinngle 2 domain of tPA (Nilsen et al., 1997). No work has been reported on the expression of full length tPA in *Pichia*.

Methanol utilization by *P. pastoris* requires two unique alcohol oxidases: *AOX1* and *AOX2*. *AOX1* plays the major in the overall alcohol oxidase activity. *AOX1* catalyzes the first step in the methanol metabolism pathway, converting methanol to formaldehyde and hydrogen peroxide. To prevent the toxic effect of hydrogen peroxide, this reaction occurs in the peroxisome, which is separated from the rest of the cell. The hydrogen peroxide is further degraded to oxygen and water. Part of the formaldehyde produced by *AOX1* leaves the peroxisome and is further oxidized to formate and carbon dioxide, providing energy for cell growth. The rest of the formaldehyde is converted to other compounds and they are further metabolized through the cyclic pathway (Cino, 2002; Higgins and Cregg, 1998). *AOX2* is a weaker alcohol oxidase compared with *AOX1* and its methanol utilization rate is much slower (Higgins and Cregg, 1998).

The expression of alcohol oxidase *AOX1* is controlled by its own promoter at the transcription level. *AOX1* expression is regulated by two mechanisms, a repression/depression mechanism and an induction mechanism. On one hand, the existence of any carbon resource other than methanol in the culture media will repress the expression of *AOX1*. On the other hand, *AOX1* is expressed at high level only through induction using methanol (Higgins and Cregg, 1998). There is virtually no basal leakage

in terms of expression if methanol is not present. Various *P. pastoris* expression vectors carrying the AOX1 promoter and various selection markers are currently available.

All *P. pastoris* expression strains are derivatives of the wild type strain Y-11430 (ATCC No. 76273). Most strains have a mutation in the HIS4 gene that encodes for the synthesis of histidine. This histidine auxotrophic phenotype allows for selection of successful integration of HIS4+ vector upon transformation (Higgins and Cregg, 1998). According to the methanol metabolic phenotype, three types of *P. pastoris* strains can be found:

1. Mut+ - methanol utilization plus type. It has the same methanol metabolic rate as the wild type strain.
2. Mut ^{S}- methanol utilization slow type. The AOX1 gene is partially deleted and the strain must rely on the weaker AOX2 gene for metabolizing methanol. This strain grows slower on methanol than Mut^{+}.
3. Mut^{-} - methanol utilization minus type. In this strain, both AOX1 and AOX2 genes are deleted so that the strain cannot use methanol at all (Higgins and Cregg, 1998).

In order to maximize the expression levels of *Pichia Pastoris* based systems, protease-deficient strains have been constructed. Upon deletion of the genes encoding for the protease, these strains had reduced proteolytic activity towards heterologous proteins (Higgins and Cregg, 1998). This is very important in large-scale fermentation because the combination of high cell densities and large reactor volumes, results in a high concentration of proteases.

Various P. pastoris vectors are now commercially available. All these vectors are *P. Pastoris / E. coli* shuttle vectors, which means that they carry origins of replication of both *P. pastoris* and *E. coli* (Higgins and Cregg, 1998). These plasmids usually contain the *AOX1* promoter followed by a multi-cloning site (MCS) sequence that allows simple insertion of foreign genes. The MCS is then followed by the transcriptional termination sequence for the *AOX1* gene. Some plasmids also have a signal peptide sequence following the *AOX1* promoter that can direct the secretion of foreign proteins to either the periplasm or to the culture medium (Higgins and Cregg, 1998; Invitrogen). Commonly used signal include the *P. pastoris* acid phosphatase (PHO1) signal and the *S. cerevisiae* α-factor prepro- peptide. If a foreign protein is naturally secreted, its own signal peptide can be used in *P. pastoris*. For convenient selection of positive transformants, selectable markers are also included in the plasmids. Usually, biosynthetic pathway genes such as HIS4 and ARG4 are used. Transformants can be selected by complementary growth of auxotrophic phenotype host strains. Another gene, *Sh ble* gene from *S. hindustans*, which confers the resistance to the drug Zeocin, has been widely used in recent years as a new selectable marker for positive transformants recognition (Higgins and Cregg, 1998; Invitrogen on-line Manual Version G 122701). In order to select tranformants in *E. coli*, these shuttle plasmids also have anti-drug genes that are commonly used in *E. coli* system, such as the ampicillin-resistance gene ((Higgins and Cregg, 1998).

Natural tPA exists in mammalian plasma at a very low concentration, which is normally in the order of 5-10 ng/ml. Obtaining tPA from these sources is impractical. Using molecular cloning techniques, tPA gene recombination and heterogeneous

expression, it is now possible to obtain adequate amounts of tPA using CHO mammalian cells. However, the cost of production is very high and there is a very strong demand for this drug. A large number of patients potentially could, or already do benefit from this drug. This demand has served as a very strong motivation for scientists scattered around the world to find ways to produce tPA with a high level of quality, in large quantities while doing it in a cost –efficient manner. This study was done to determine if all these goals could be achieved using *Pichia Pastoris* as the host expressing tPA.

2.2. Materials and Methods

2.2.1. tPA Cloning into *Pichia Pastoris*

The gene for tPA (full sequence including native leading sequence is presented Appendix I), was obtained using PCR from plasmid pETPFR (ATTC number 40403, vector map in appendix II), using the following primers:

Forward Primer:

↓

5' GCAT CTC GAG AAG AGA TCT TAC CAA GTG ATC TG 3'

XhoI

Reverse Primer:

↓

5' GCAT GCG GCC GCT CAC GGT CGC ATG TTG TCA CG 3'

NotI

The primers were designed to obtain the tPA gene without its native leading sequence. The two endonuclease cutting sites (XhoI and NotI) were built into the primers so that the PCR products after purification could be digested and inserted by ligation into the complementary sites of XhoI and NotI double digested pUC19 and pPicz9ss amp (herein referred to as pZamp for convenience). pZamp is a shuttle plasmid that can proliferate in both *E. coli* and *Pichia* and it was kindly donated by Dr. W. Sheffield's, from the Department of Pathology, McMaster University. This plasmid is an improved version of the original plasmid available from Invitrogen. It contains double antibiotic resistances, anti-Zeocin and anti-ampicillin. This special property makes it possible to grow the plasmid in *E. coli* only in the presence of ampicillin. Since Zeocin is very expensive, using ampicillin is a more cost-efficient and facilitated manipulation. Similarly to the regular pPICz plasmids, this plasmid contains the *AOX1* promoter that allows methanol-inducible, high-level expression of target protein in *Pichia.* Furthermore, the plasmid contains the native *S. cerevisiae* α-factor that is frequently used to direct the secretion of target protein to the extracellular space (Higgins and Cregg, 1998).

After inserting the tPA gene into the pUC19 an pZamp plasmids, the sequence of the inserted tPA fragment was checked by DNA sequencing. Results of sequencing can be found in appendices III and IV. Site mutations occurred both in pUC19-tPA and pZamp-tPA1, but at different points of the gene. An additional round of cloning was done to correct these mutations by removing the correct tPA fragments from the two recombinant plasmids, and introducing them into the pZamp plasmid to form a new recombinant pZamp-tPA. plasmid for *Pichia* transformation and tPA expression.

The map of the resulting pZamp-tPA recombinant plasmid is shown in Figure 2.2.1. The plasmid was double digested with XhoI and NotI (Fermentas) to further verify the size of the inserted tPA gene. The result of the DNA electrophoresis can be found in Figure 2.2.2. Details of PCR can be found in Appendix VII.

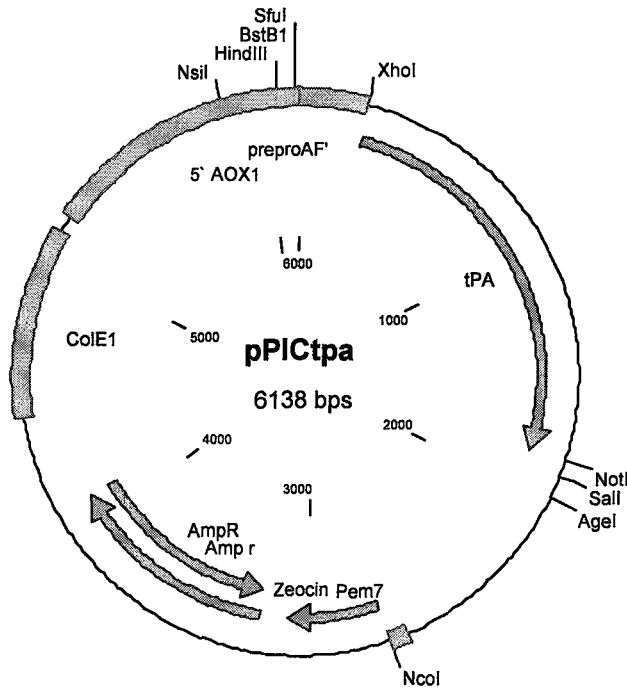


Figure 2.2.1 Map of pZamp-tPA Recombinant Plasmid

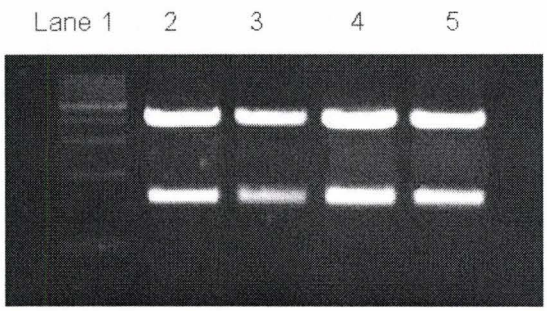


Figure 2.2.2 Digestion of pZam-tPA Clones from Three-partial Ligation with XhoI and NotI. Lane 1, 1Kb step ladder; Lane 2 – Lane 5, clones No. 1-No.4 digested by XhoI and NotI.

Since plasmids tend to integrate randomly into the *Pichia* genome and the copy number can not be maintained constant, plasmids have to be integrated into the *Pichia* genome by homologous recombination to ensure stable heterologous gene expression. This integration is done between the target-DNA carrying plasmid to be transformed and regions of homology in the *Pichia* genome. The resulting integrants have high stability in the absence of selection pressure. The most commonly used homologous region for recombination is the *AOX1* locus in the *Pichia* gonome. To increase the efficiency of homologous recombination, vector plasmids should first be linearized by one of the unique restriction enzymes in the 5' *AOX1* region. To obtain stable tPA-containing *Pichia* by homologous recombination, four recombinant plasmids pZamp-tPA3-1,2,3,4 were first linearized by the restriction enzyme PmeI (Fermentas), which generates unique cutting sites on the plasmids. After PmeI digestion, the samples were checked on a DNA agarose gel to make sure that all plasmids were linearized completely. The linearized

plasmids were precipitated in 100% alcohol. After recovery by centrifugation, the pellet was resuspended in 10 μl MilliQ water. Electroporation (1.5 kV, 25 μF, 200 Ω) was chosen to transform the *Pichia*, which was spread on YPDS plates containing 100μg/ml Zeocin.

2.2.2. Growth and Expression

Growth and induction of the recombinant *Pichia* was carried out using baffled shake-flasks. Recipes for all mentioned medium can be found in Appendix V. Cells were allowed to grow at 30°C. BMGY media was used for cell growth and BMMY or MM media was used for induction of tPA expression. After induction with methanol, the cells were harvested by centrifugation at 4°C, 5000-7000 rpm for 5-10 minutes.

2.2.3. Analytical Methods

Total protein concentration was determined using the Bradford method in a 96 well-plate in a plate reader (MRX Revelation TC). A calibration curve was constructed using standards with known concentrations of Bovine Serum Albumin (BSA). Protein was analyzed by SDS-PAGE, using Coomassie Brilliant Blue G250 for staining.

Western Blotting was done by first running the sample in SDS-PAGE. The protein bands on the gel were transferred to a PVDF membrane under an electric field, at 80v for 1.5 hours. After transfer, the membrane was treated with blocking buffer for 1 hour with gentle shaking to block blank binding sites. The membrane was incubated in antibody

binding buffer with 1: 1000 primary sheep anti-tPA antibodies (Cedarlane Lab. Ltd.) for 1 hour with gentle shaking. Then the membrane was allowed to contact with the rabbit-anti-sheep secondary antibodies (Sigma). The membrane was washed three times with washing buffer. This helped to remove the non-specific binding between the first antibodies and other proteins in the sample. Finally, the specific binding of tPA and its antibody was visualized by incubation in detecting buffer with NBC (nitroblue tetrazolium, Biorad) and CNIP (5-bromo-4-chloro-3-indolyl phosphate, Biorad). The membrane was stored in plastic bag. Details can be seen in Appendix Ⅷ.

The tPA activity was determined by measuring the OD_{405} using a plate reader (MRX Revelation TC). 100 μl of tPA activity analysis buffer was loaded and the plate was incubated at 37°C for 2-4 minutes. 15 μl samples were added to each well afterwards and incubated at 37°C for 2-4 minutes. 100 μl of the chromogenic substrates S-2288 was added to each well. The plate was incubated at 37°C and the OD_{405} at different time intervals was recorded. The tPA activity can be calculated by the following formula:

$$U/ml = \Delta A/min \times 3.99$$

2.3. Results and Discussions

2.3.1. Induction of tPA Expression by Methanol

Expression of tPA in *Pichia* was investigated using shake flask cultivation. First, recombinant *Pichia* cells were proliferated in BMGY medium at 30°C, 250 rpm for 12 to 16 hours until the OD_{600} reached a value between 4.0 and 6.0. During this period, the

glycerol in the medium repressed the AOX1 promoter, with the cells growing without expressing tPA. The cells were centrifuged to remove the BMGY medium and resuspended in fresh BMMY or MM medium and the methanol induction period was started. With methanol as the only carbon source available, the AOX1 promoter was triggered and tPA expression was induced. The induction was done at 28-30°C, 250 rpm in a baffled shake flask. Samples were taken at different time intervals to measure the OD_{600}. The total concentration of both extracellular and endocellular protein was also measured by Bradford method. The results are shown in Figure 2.3.1 and Table 2-1. In both cases, the wild type *Pichia* strain without tPA gene was used as control.

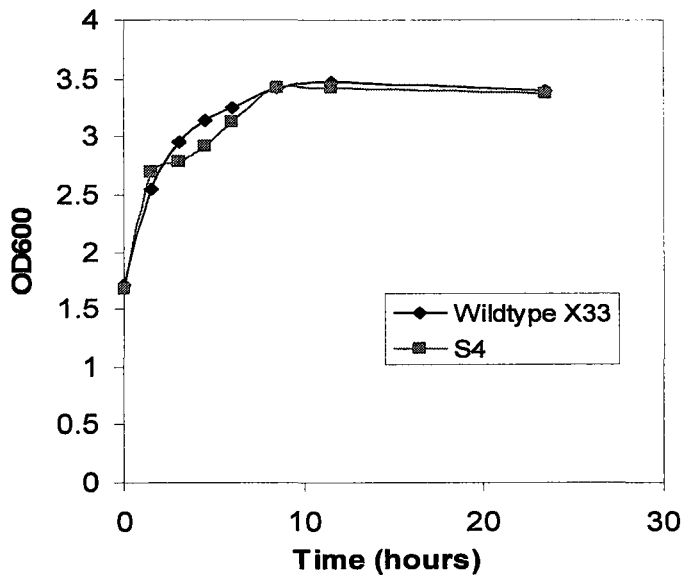


Figure 2.3.1 Growth of *Pichia* in BMMY Media (Results from shake flask culture at 30°C, 250rpm)

Table 2-1 Total Protein Concentration before and after Methanol Induction

Samples	Before Induction		After 22 hours of Induction	
	Lysate	Supernatant	Lysate	Supernatant
X33(mg/ml culture)	0.0886	0.0057	0.093	-0.02
S4 (mg/ml culture)	0.0833	0.004	0.1904	-0.02

From these results, it can be seen that the expression of tPA does not have much of a negative effect on the host cells. Recombinant *Pichia* cells grew at almost the same rate as the wild type strain. Based on the total protein concentrations of the recombinant *Pichia* culture before and after methanol, there is a 100% increase of the intracellular protein concentration. This indicates that the AOX1 promoter worked properly and protein expression was efficiently induced by methanol.

Cells obtained after 24 hours of induction, from both wild type strain and recombinant *Pichia,* were first disrupted using glass beads. The supernatant containing the extracellular protein and the cell lysate containing the intracellular proteins were analyzed by SDS-PAGE. The gel (**Figure 2.3.2**) shows that the samples containing the intracellular protein fractions for all the recombinant strains (lanes 4, 6 and 8), had a very strong protein band corresponding to a molecular weight close to 70 KDa, which corresponds to the molecular weight of tPA. Moreover, the sample from the wild type X33 cells did not show any significant band at this molecular weight, which indicates, as expected, that the band identified in lanes 4, 6 and 8 corresponds to the over-expression of tPA. The gel also shows no obvious band in the samples containing the extracellular

proteins, for all three recombinant *Pichia* cultures, which indicates that either the protein was not secreted, or its concentration in the media is so low that it cannot be detected. The SDS-PAGE result is shown in Figure 2.3.2.

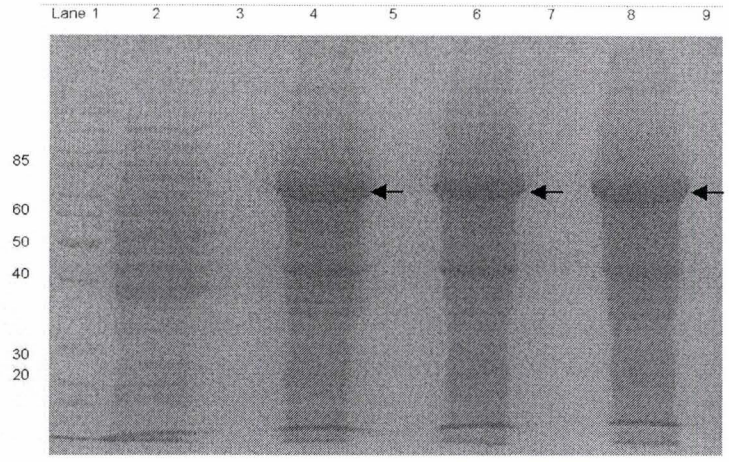


Figure 2.3.2 SDS-PAGE of tPA Expression after Methanol Induction. All strains were induced in BMMY medium at 30°C for 24hours. Lane 1, protein molecular weight markers (MWM); Lanes2 and 3 are cell lysate and supernatant from wild-type *Pichia* X33; Lanes 4 and 5, cell lysate and supernatant from tPA-expressing strain S1; Lanes 6 and 7, cell lysate and supernatant from tPA-expressing strain S2; Lanes 8 and 9 8, cell lysate and supernatant from tPA-expressing strain S4.

A Western blot was done to determine if the ~70 kDa protein generated after methanol induction is indeed tPA protein. Pictures of the SDS-PAGE and PVDF membrane after primary antibody binding, secondary antibody binding and NBC/CNIP detection are shown in Figure 2.3.3 and Figure 2.3.4, respectively.

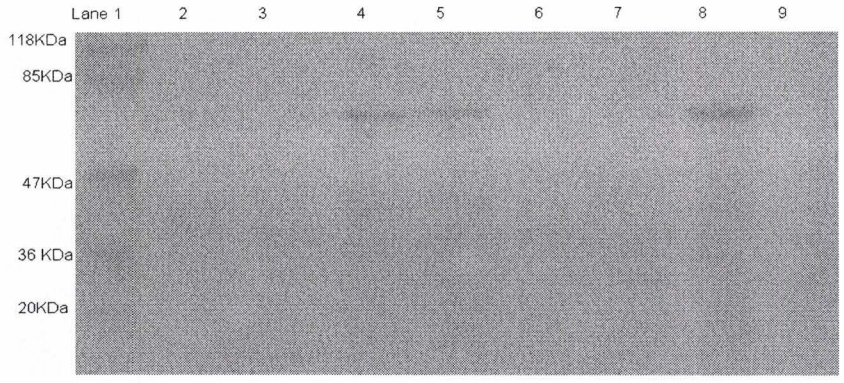


Figure 2.3.3 SDS-PAGE of Protein Samples. Lane 1, molecular weight markers; Lanes 2, 3, 4, 5 and 6 represent the intracellular protein fractions for strains X33, S1, S2, S3 and S4, respectively after a 24-hour induction period; Lane7, extracellular protein fraction from recombinant *Pichia* S4 before induction; Lanes 8 and 9, intracellular and extracellular protein fractions of strain S4 after a 24-hour induction period.

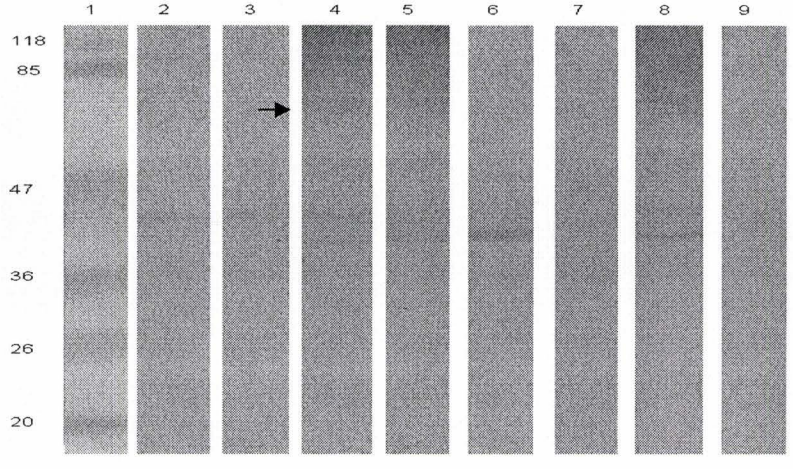


Figure 2.3.4 Western Blotting of tPA Lane 1, molecular weight markers; Lanes 2, 3, 4, 5 and 6 represent the intracellular protein fractions for strains X33, S1, S2, S3 and S4, respectively after a 24-hour induction period; Lane7, extracellular protein fraction from recombinant *Pichia* S4 before induction; Lanes 8 and 9, intracellular and extracellular protein fractions of strain S4 after a 24-hour induction period.

A faint band, occurring at 70 KDa in the intracellular protein fractions of post-induction culture of recombinant *Pichia* S1, S2 and S4 (lanes 4, 5 and 8) suggests the presence tPA. In contrast, there was no band with the same molecular weight in the sample from S4-endocellular protein before induction, which is a proof that AOX1 promoter is tightly regulated by methanol. Additionally, the absence of a band at 70kDa for the sample containing the extracellular protein fraction of strain S4 after induction (lane 9), indicates that the vast majority of the tPA produced remained inside cells, or that its concentration in the media was below the detection limit.. It should be noticed that there are other bands in the lanes corresponding to the samples containing the intracellular proteins. These bands might correspond to tPA fragments that may still maintain their ability to bind to the antibody. The bands larger than 70 KDa might be explained as oligomers of tPA formed endocellularly during the over expression.

The activity of the expressed tPA was determined using the chromogenic substrate S2288 (Diapharma). This method tests the serine protease activity of the tPA to release p-nitroaniline from the substrate. Since *Pichia* may generate some serine proteases endogenously, the wild-type strain X33 was used as "blank" by determining how the absorbance increased as a function of time. For all other samples, the measured values of absorbance as a function of time were corrected with the values obtained from the measurement of the "blank". Using this method, the samples containing the intracellular proteins from the recombinant tPA-expressing *Pichia* strains S1, S2, and S4 showed tPA-like activity with a value close to 12U/mL culture. There was no detectable tPA activity in the extracellular samples. This productive is about twice as high as that

previously reported for *E.coli* (Pennica, 1983). However, fermentation using *Pichia* can be carried out using a much higher cell density as compared to *E. Coli* cultures (Higgins and Cregg, 1998). Additionally, very limited protein productivity can be obtained by growing *Pichia* in shake flasks. These two aspects, when taken together, suggest that *Pichia* has a significantly higher potential for production of tPA as compared to *E. Coli.*

2.3.2. Improving tPA Secretion by Changing Temperature of Incubation

As described in the Materials and Methods section, tPA was cloned into plasmid pZamp downstream of the yeast α-factor signal peptide to promote secretion of the protein. However, the previous results show that tPA was absent from the media leading us to conclude that the total amount of tPA produced was very low, resulting in non-detectable concentrations in the media, and/or secretion did not occur. Some reasons that are associated with no secretion are: the signal peptide fails to direct the tPA to the extracellular space; tPA may be actually secreted but it may be degraded as it leaves the cell and if this is the case, then a protease-deficient strain should be used; the conditions used for cell cultivation also have an effect on the ability of *Pichia* to secrete proteins It has been reported that in some cases, the level of secretion of heterologous from *Pichia* can be improved by decreasing the temperature during the methanol induction period (Woo *et al.*, 2004). To evaluate this last possibility, *Pichia* strain S2 was first cultivated in BMGY medium over-night and then inoculated in to two bottles containing fresh BMMY medium. One bottle was incubated at 30°C, while the other was maintained at

room temperature (22°C). Samples were taken from both bottles at different time intervals and the cell concentration and tPA expression were measured.

Cell density measurements at OD_{600} (Figure 2.3.5) indicate that *Pichia* grows much slower at lower temperatures. At the lower temperature, there was no obvious sign of tPA in extracellular fraction, as seen from the SDS-PAGE gel (Figure 2.3.6) or from the activity test (data not shown). Furthermore, it can be seen in the SDS-PAGE image in Figure 2.3.6, the *AOX1* promoter was not effectively induced at room temperature. It was not only until about 22 hours had elapsed after induction, that tPA could be detected intracellularly. After 28 hours, there was a substantial amount of tPA inside the cells. There was comparably more tPA protein generated at 30°C then at room temperature at all time intervals. The decreased temperature of incubation and induction did not improve the ability of *Pichia* to secrete the protein.

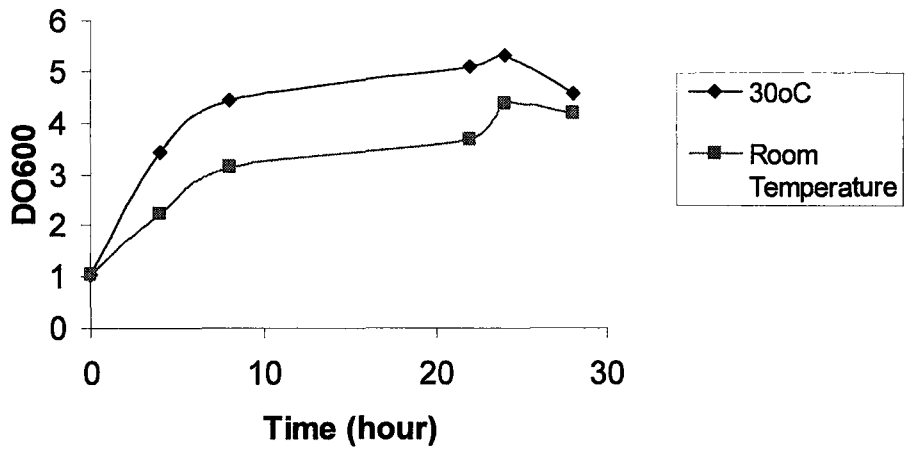


Figure 2.3.5 Growth of *Pichia* S2 at Different Temperature

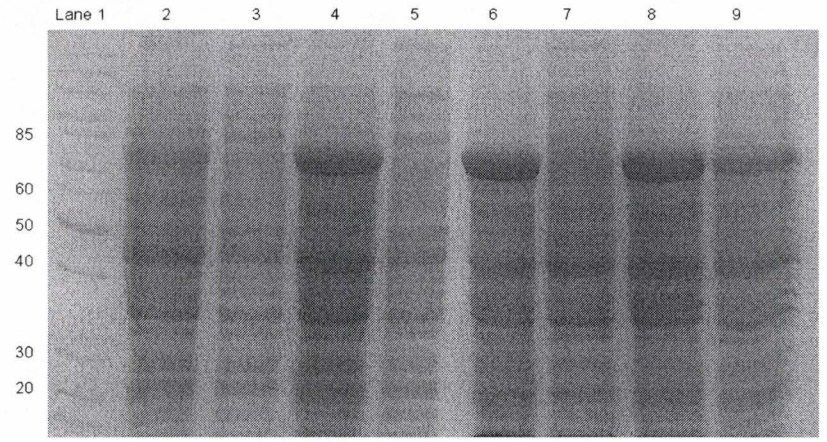


Figure 2.3.6 Endocellular tPA Expression in BMMY at Different Temperature. Lane 1, Protein MW markers; Lane 2, 30oC, t=4 hours; Lane 3, room temperature, t=4 hours; Lane 4, 30oC, t=8 hours; Lane 5, room temperature, t= 8 hours; Lane 6, 30oC, t=22 hours; Lane 7, room temperature, t=22 hours; Lane 8, 30oC, t=28 hours; Lane 9, room temperature, t= 28 hours.

2.3.3. tPA Expression Using MM Medium

Changing induction medium is another option to improve foreign protein expression in *Pichia*. An alternative medium for methanol induction for *Pichia* is MM (minimal medium), which is made of yeast extract, biotin and methanol. Since there are no inorganic salts serving as the pH buffer, the pH of the medium changed as the cells grew. Recombinant *Pichia* strains were inoculated in both BMMY and MM. Data on cell density (Table 2-2) and visual inspection of the SDS-PAGE gel (Figure 2.3.7), show that *Pichia* grew much slower in MM than in BMMY. By the end of the induction phase, which lasted for about 24 hours, the cell density, as measured by OD_{600}, of BMMY

culture was three times higher than that of the MM culture (Table 2-2). Since MM does not contain buffering salts, the pH dropped from 6.0 to about 3.0, after 24 hours of cultivation (Table 2-2). On the other hand, the pH of culture growing in BMMY, which is a buffered medium, remained constant at 6.5 throughout the entire procedure (Table 2-2). The potential benefit of using un-buffered medium like MM for protein secretion is that the low pH may inhibit most of the proteases generated by the host (Higgins and Cregg, 1998). However, this benefit has to be based on the assumption that the target protein is stable at that specific pH value.

Table 2-2 Comparison of Cell Growth and pH in MM and BMMY Medium

Methanol Medium	MM			BMMY		
Pichia Strain	S1	S2	S4	S1	S2	S4
OD_{600} at 24- hr	1.562	1.771	1.364	4.257	4.246	4.51
pH at 24-hour	2.74	2.68	2.97	7.39	6.66	6.49

There was no evidence on the production of tPA, either from the SDS-PAGE gel or from the activity test, for the extracellular proteins protein fraction in the MM medium. Electrophoresis of the endocellular protein samples showed bigger and darker band of tPA in the BMMY induced culture as compared to the MM culture induced culture,

which further demonstrates that BMMY is a more suitable medium for tPA expression in *Pichia.*

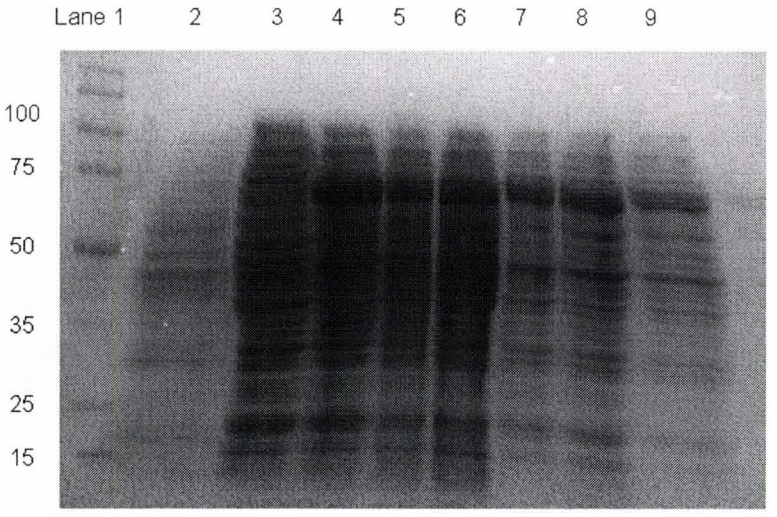


Figure 2.3.7 tPA Expression in MM and BMMY Medium. Lane 1, Protein MWM; Lane 2, X33 strain, after 24- hour induction; Lane 3, strain S1 before induction; Lanes 4-6, strain S1 in BMMY after 7, 24 and 30 hours of induction; Lanes 7-9, strain S1 in MM after 7, 24 and 30 hours of induction

2.3.4. Increasing tPA Expression by Optimization of the BMMY Medium

The effect of the methanol concentration present in the BMMY medium on the expression of tPA was evaluated by using four different methanol concentration: 0.5%, 1%, 1.5% and 2%. OD_{600} readings at 24hours showed no noticeable difference in cell density and tPA activity (data not shown).

An attempt to increase cell density and tPA expression was made by feeding fresh medium to the cell culture during the middle of the induction phase. Three induction strategies were carried out and the results were compared. The first strategy was based on the use of 50 ml of BMMY containing 0.5% methanol, with the cultivation being done at 30°C, 250 rpm. The second strategy was also based on the use of 0.5% methanol BMMY, but 10 ml fresh BMMY medium was added to the culture at 9.5 hours after induction. In the third strategy, the same approach was used as for the previous scheme, but the medium contained 1% methanol instead of 0.5%. The cell density, SDS-PAGE, and tPA–like activity results are shown in Figure 2.3.8, Figure 2.3.9, and Table 2-3, respectively.

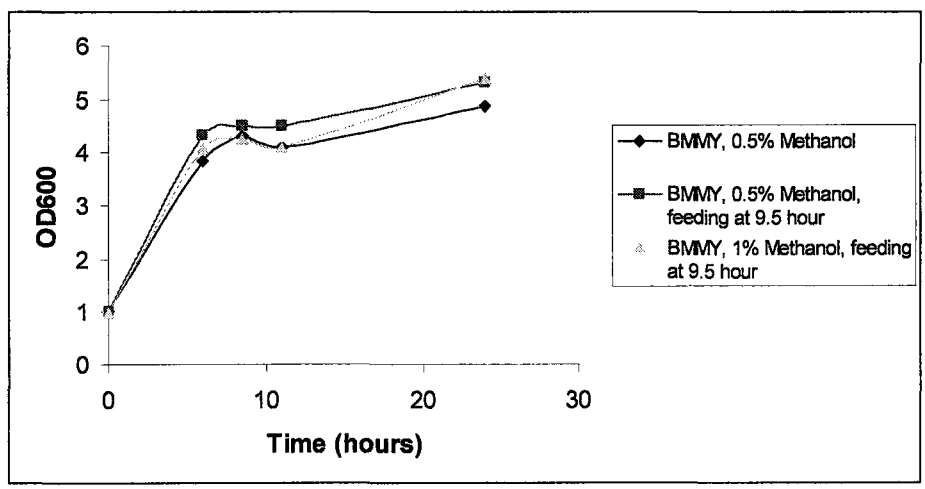


Figure 2.3.8 Growth of the S4 Strain in Different Induction Medium

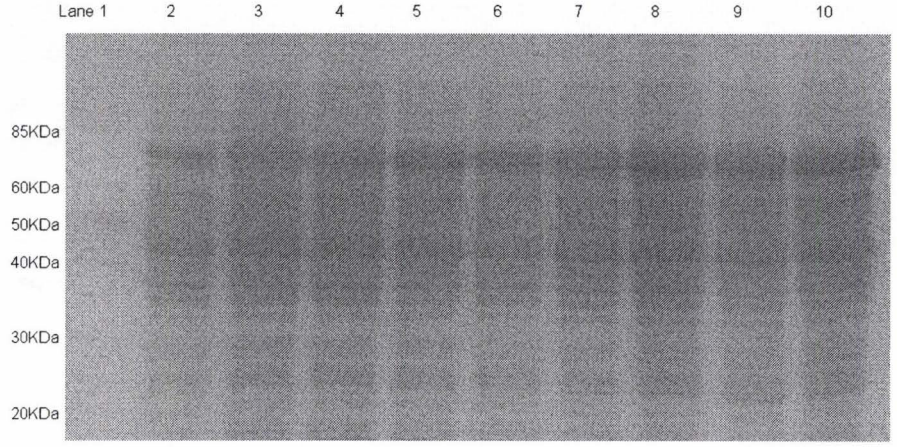


Figure 2.3.9 SDS-PAGE of Endocellular Proteins of Recombinant *Pichia* S4 in Different Induction Medium. Lane1, protein MWM; Lanes 2,3, and 4, 0.5%- methanol BMMY w/o feeding, 6, 11, and 24 hours of induction, respectively; Lane 5,6, and 7, 0.5%-methanol BMMY with fresh feeding, 6, 11, and 24 hours of induction, respectively; Lane 8, 9, and 10, 1%-methanol BMMY with fresh feeding, 6, 11, and 24 hours of induction, respectively.

Table 2-3 Cell Concentration and tPA Activity Test in Different Batches (Batch 1, BMMY/0.5% methanol; Batch 2, BMMY/0.5% methanol+ 10 ml feeding; Batch 3, BMMY/1% methanol + 10 ml feeding. Note- Fresh feeding medium was added to culture at 9.5 hours after induction)

Batch	1	2	3
OD_{600}, 24 hours	4.84 ± 5%	5.31 ± 8%	5.39 ± 2%
Total Volume (ml)	40	50	50
tPA Activity (U/mL)	12.6	11.5	13.2

Pichia grows very fast in BMMY. From the growth profiles in Figure 2.3.8, it is clear that the cells proliferated at a very high rate during the first 8 hours after inoculation. After that, the growth slowed down and the cell concentration was basically steady during the rest of the induction period. Since the fast-proliferating cells consumed most of the substrate during the first period, a supplement of fresh medium should provide the cells with sufficient substrate to grow to a higher density, but no noticeable difference in the tPA activity was observed for the three fed- batches.

2.4. Conclusions

As a natural anti-coagulation protein, tPA has been used to treat many critical cardiovascular diseases. The limited availability and the extremely high price of tPA hinders its general use as a life saving medicine.

In this work, we were able, for the first time, to successfully clone the gene encoding the full length tPA for expression in the methylotrophic yeast *Pichia pastoris*. tPA protein expression could be triggered by using the *AOX1* promoter of *Pichia Pastoris,* using methanol as the inducer. By visual inspection of various SDS-PAGE gels, it was estimated that tPA accounted for about 30% of the total intracellular protein. Western blotting and tPA activity tests suggested the production of tPA and fibrinolytic activity. Further investigations, using the recombinant strains generated in this work, showed that a higher methanol percentage in the BMMY medium and supplementation of substrate improved the growth of the recombinant *Pichia* strain.

Notwithstanding, there are several problem with this tPA expression system that need to be further investigated. First, the tPA expression levels were low and potentially not secreted. Although there is relatively large quantity of tPA inside the cells, from a processing point of view, it would be highly desirable to have the protein being secreted in significant quantity. The necessity to disrupt the cells adds significantly to the complexity and cost of protein purification, as well as to lower yields. To address this issue, different modes of cultivation should be explored, namely using fermenters for high-density cultivation and if secretion is not observed to an appreciable extent, other signal peptide, preferably the native signal peptide of tPA itself, should be tried. The glycosylation pattern of *Pichia* expressed tPA also needs to be identified, since the glycosalation patterns of *Pichia*, a lower eukaryotic species, is different from those associated with other higher eukaryotic species. As a glycoprotein, the length of the sugar chain of tPA generated in *Pichia*, as well as the composition of sugar chain needs to be identified and compared with native tPA. This is necessary to prevent an immune reaction of the human body to the recombinant tPA.

Based on the confirmation of the ability to produce tPA in *Pichia Pastoris*, a more detailed parametric study, aiming towards the optimization of operational parameters, should be conducted. These parameters could nonexclusively be the composition of medium, the methanol concentration, the pH value, the oxygen supply rate, and rate of fed-batch cultivation, etc.

3. Probing the In-Vitro Activity of an Intein in ELP Bases Aggregates

3.1. Introduction

A very common problem associated with heterologous protein expression, especially in *E. Coli*, is the formation of inclusion bodies. Inclusion bodies consist of insoluble deposits in the cytoplasm of cells, which are formed due to aggregation of over-expressed proteins. The cytoplasm of a cell is a crowded environment and local protein concentrations are extremely high. Over-expression of a target protein will severely increase the potential for aggregation of protein and formation of inclusion bodies. The main problem associated with inclusion bodies is the fact that the proteins contained in them are not functional due to loss of folding during the aggregation process. To recover functionally active protein, it is first necessary to isolate the inclusion bodies, denature them under very harsh conditions and refold the protein using an appropriate buffer. The yield and the economics of this process are not ideal. A sometimes successful strategy to prevent the formation of inclusion bodies is to fuse the target protein to be produced with a highly soluble fusion partner. Results on this approach have been mixed

A variety of papers have been published in the last few years, regarding over-expression of elastin-like polypeptides (ELPs) in *E. Coli* (Guda *et al.* 1995; Meyer and Chilkoti, 1999; Trabicc-Carlson et al., 2004; Shimazu et al., 2003) ELP are polypeptides consisting of tandem repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa can be any amino acid except proline (Urry *et al.*, 1985; Urry, 1992). ELPs are able undergo an

inverse and reversible phase transition within a very narrow temperature range (Urry, 1988; Meyer and Chilkoti, 1999). At a temperature below the inverse phase transition temperature (T_t) temperature, ELP is soluble but it becomes insoluble if the temperature exceeds T_t. It has been shown that this feature of ELP is retained when fused to a variety of proteins.

Guda *et al.* (1995) observed that when *E. Coli* was used to express an ELP in a T7 based system, but without IPTG induction, an ELP matrix was accumulated in the cytoplasm, occupying close to 90% of its volume. Using ELP as a fusion partner to a variety of target protein, different groups (Trabicc-Carlson et al., 2004; Shimazu et al., 2003), were able to produce unprecedented large quantities of these fusion protein in the cytoplasm of the cells and that the target proteins were fully functional after cell disruption.

To explain these very high levels of expression previously reported, we hypothesized that the ELP tag will undergo an inverse phase transition in the cytoplasm of the cell (as demonstrated by Guda *et al.,* 1995), acting to actually decrease the solubility of the fusion protein. The result should be the formation of ELP directed aggregates and not target protein directed inclusion bodies, which explains the fact that the proteins produced always showed activity after cell disruption. If this hypothesis holds, the implications can be significant and can be particularly important for over-expression of membrane based proteins. Membrane based proteins are the least well studied group of proteins, mainly because it is very difficult to obtain them in large

quantities, due to the fact that when over-expressed in a host, they will cause the cell membrane to loose its integrity, resulting in cell death.

As an initial step towards evaluating the use of ELP tags for difficult to express proteins, we asked a very simple, but critical question: when a target protein is fused to an ELP tag and an inverse phase transition is induced, does the target protein retain the correct folding or is the folding lost? If that is not the case, then the usefulness of ELP tags will be much less. We directly addressed this initial question by using a recently reported ELP based construct (Ge *et al.*, 2005). This construct encodes for a tripartite fusion protein, consisting of (from the N-terminus of the fusion to the C-terminus of the fusion), thioredoxin, an intein, followed by an ELP tag. Inteins are proteins that have the ability to splice themselves out of the fusion, through activation by a small molecule (dithiothreitol, DTT). For the intein to be active and its activity to result in splicing, the intein must maintain a specific conformation (Telenti, 1997). The use of a small molecule, such as DTT, allows probing the activity of the intein in aggregates formed with the tripartite fusion, since the small molecule should be able to access the interior zones of the aggregates. If intein cleavage can be activated through the addition of DTT to a solution containing the tripartite fusion in the aggregated state, than this will constitute evidence that the intein maintains its activity, and hence its folding, inside the ELP based particles. We show that activity is indeed maintained.

3.2. Materials and Methods

3.2.1. Expression of the thioredoxin-inten-ELP fusion protein

The plasmid pTME (Ge *et al.,* 2005), based on a pET-32b vector (Novagen), was used to expresses a tripartite fusion protein (Figure 3.2.1): thioredoxin (Trx) was fused at its C-terminus with a mini-intein from *Mycobacterium xenopi* GyrA gene (Mxe) (Evans *et al.* 1998) and an ELP, consisting of 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3 respectively . The intein contains an Asn –> Ala mutation at the C-terminus of the intein for N-terminal cleavage (Evans *et al.,* 1998). The ELP was separated from the intein by using a flexible linker.

E. Coli BL21 harboring the plasmid pTME was inoculated in 50ml of LB media, supplemented by ampicillin of 100μg/ml and grown overnight at 37°C and 140 rpm. 5 ml of the overnight culture was inoculated to a baffled flask containing 300 ml of fresh LB media supplemented with ampicillin at final concentration of 100μg/ml, incubated at 37°C, 120rpm until OD_{600}= 1.0. After the culture was cooled down to 18°C, IPTG was added to a final concentration of 0.5 mM. Cells are grown at 18°C, 120rpm for 18 to 20 hours. The cells were harvested by centrifugation at 7000 g, 4°C for 5 -10 minutes.

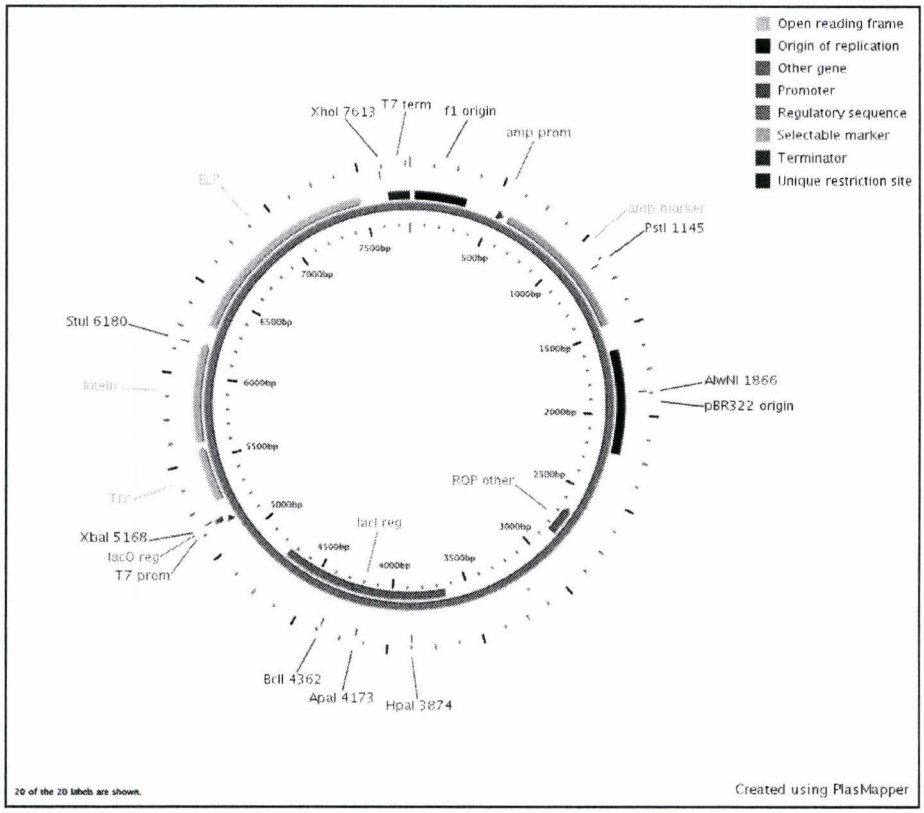


Figure 3.2.1 Map for Plasmid pTME

3.2.2. Purification of ELP-fused Protein

After harvesting the cells, the supernatant was discarded and the pellet was re-suspended in 15 ml PBS buffer. This solution was placed on ice and the cells were lysed using intermittent 20 second ultrasonic pulses, cooling without sonication for 2 minutes. These two steps were repeated for a total of 6 cycles. This solution was centrifuged and the supernatant was transferred to a 50 ml tube with 15 ml of 5M NaCl. After the solution became turbid, it was centrifuged at ~30°C, 18,000 g for 10 minutes. After discarding the

supernatant, the pellet was washed with 5ml 2.5 M NaCl. The pellet was then dissolved in 5ml of MilliQ water. The solution was centrifuged again at 18,000g, 4°C for 5minutes. The supernatant was transferred to a 15ml tube. The protein solution could be stored at 4°C for one week.

3.2.3. Monitoring of the Inverse Phase Transition and Thioredoxin Activity Test

Temperature-dependent aggregation of the ELP protein, Trx-ELP fusion protein, or mixtures of both proteins, was characterized on a Cary 100 Ultraviolet-Visible spectrophotometer equipped with a Peltier multicell temperature controller (Varian Instruments). The turbidity of the samples was monitored as a function of solution temperature at a wavelength of 350nm. The phase transition temperature (T_t), was defined as the temperature at which the turbidity of the sample attained 50% of its maximum value. The temperature increase ramp of the measurement was set at a constant rate of 0.5°C/min. Thioredoxin enzyme activity, by insulin precipitation activity assay (Holmgren, 1979)

3.2.4. Particle Size Measurement by Mastersizer

The particle size of ELP-tagged fusion protein aggregates was measured by Mastersizer (Malvern, UK). A schematic of the Mastersizer is shown in Figure 3.2.2. Particle size is measured by the diffraction of a laser beam. During the laser diffraction measurement, particles are passed through a focused laser beam. These particles scatter

light at an angle that is inversely proportional to their size. The angular intensity of the scattered light is then measured by a series of photosensitive detectors. The number and positioning of these detectors in the Mastersizer has been optimized to achieve maximum resolution across a broad range of sizes. (Malver Instruments website http://www.malvern.co.uk).

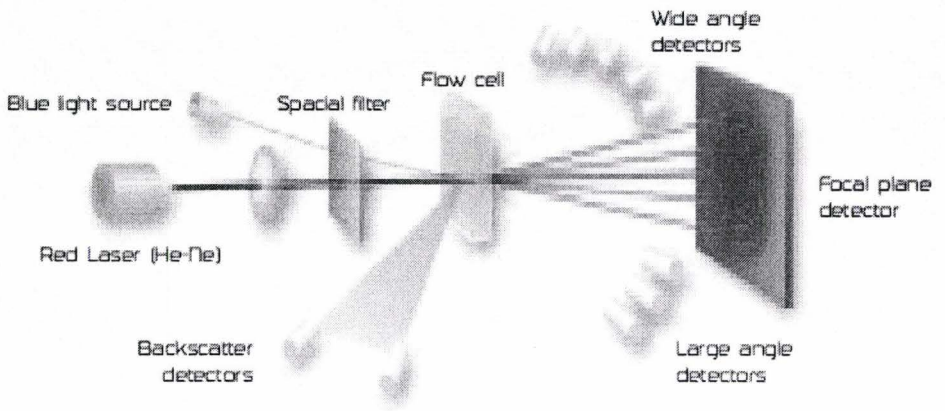


Figure 3.2.2 Structure Scheme of Mastersizer (Image from Malvern Instruments Online Brochure, **http://www.malvern.co.uk**)

3.3. Results and Discussion

3.3.1. Purification of ELP Fused Protein by Phase Transition

ELP-tagged fusion proteins undergo an inverse phase transition at a well defined temperature and salt concentration. When expressing the fusion protein in *E.Coli,* the protein can be purified simply by adding an equal volume of 5M NaCl and using

centrifugation to separate the ELP based aggregates. These aggregates can be readily dissolved in water. This procedure can be repeated to achieve a high degree of protein purity, as shown in the SDS-PAGE gel in Figure 3.3.1.

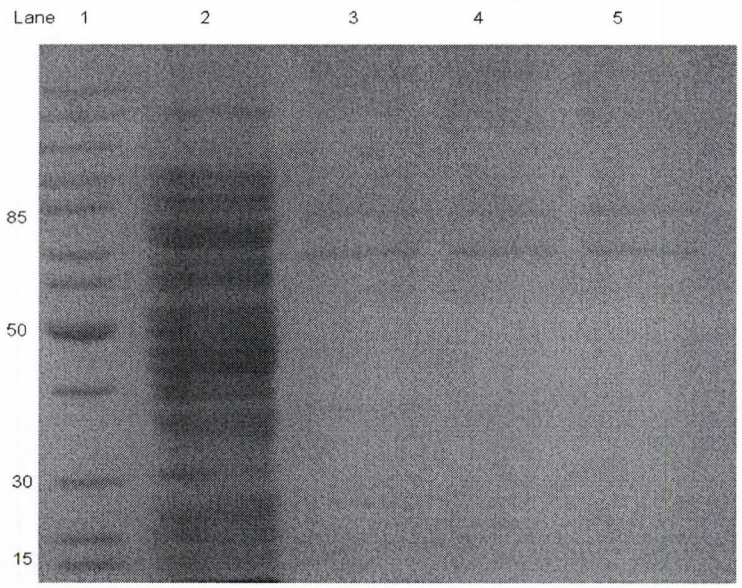


Figure 3.3.1 Purification of Trx-Intein-ELP Fusion Protein by NaCl Triggered Phase Transition. Lane 1, Protein Marker; Lane2, cell lysate; Lane 3, Protein after 1st round of phase transition; Lane4, Protein after 2nd round of phase transition; Lane 5, Protein after 3rd round of Phase transition.

3.3.2. Phase Transition Behavior of Trx-Intein-ELP Fusion Protein

The phase transition temperature, Tt, of ELP-tagged protein is highly dependent on the salt concentration. Phase transition is detected through monitoring the change of absorbance of the solution at 440 nm. Phase transition profiles were obtained for samples containing the fusion Trx-Intein-ELP at a concentration of 14 μM. The protein

concentration was measured by determining the absorbance at 280nm and using an extinction coefficient of 33,000 $M^{-1}cm^{-1}$. Figure 3.3.2 shows that the phase transition temperature, changes dramatically with salt concentration. When NaCl is present, the tendency of water molecules to hydrate the hydrophobicaly based ELP decreases solution, As a result, the ELP molecules undergo a rapid conformational change to β-helices and these β-helices are able to interact with each other resulting in aggregation (Urry, 1992)

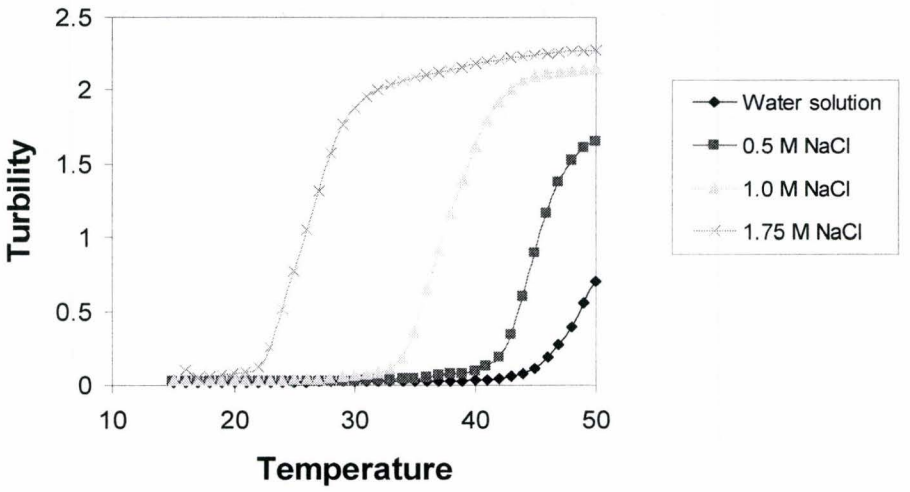


Figure 3.3.2 Phase Transition Profiles for Samples of Trx-Intein-ELP at Different NaCl Concentrations

3.3.3. Protein Activity of Trx-Intein-ELP Protein in Aggregated Form

The critical question we wanted to answer from this part of the work was to determine if the intein maintains its activity when its present in the ELP based aggregates. The activity of the intein was detected by its ability to cleave itself off from the tripartite construct in the presence of DTT, leading to the release of the thrioredoxin component of the fusion (which is present at the N-terminus of intein). Aggregation was achieved by incubating a sample with the fusion, suspended in 2.5M NaCl at 37°C. After the solution became cloudy, DTT was added to a final concentration of 20mM. A sample, containing the same fusion protein and salt concentration, but without DTT being added, was used as the control experiment. Samples were collected from both reaction mixtures at different times and SDS-PAGE was run to see the cleavage of thioredoxin. The results are shown in Figure 3.3.3.

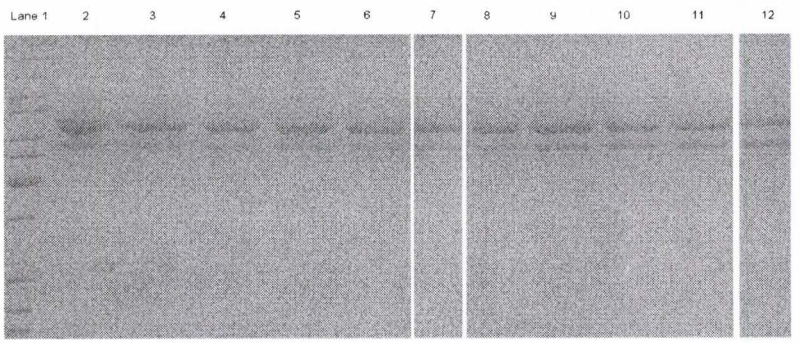


Figure 3.3.3 DTT-induced Intein Cleavage in Thioredoxin-intein-ELP90 Aggregation Matrix. Lane 1, Protein Marker; Lane 2, Purified Trx-Mxe-ELP protein before cleavage; Lanes 3, 4, 5 6, 7, Cleavage w/o DTT at t=20min,50 min ,100 min, 3hr, and 6 hrs; Lanes 8, 9, 10, 11, 12, Cleavage w/ 20mM DTT at t= 20min, 50 min; 100min, 3 hr, and 6hr.

From the SDS-PAGE we can see that the there was no thioredoxin cleavage over a period of 6 hours in the aggregation matrix when no DTT was present. With the presence of DTT, thioredoxin cleavage occurred and after 6 hours about 50% of the thioredoxin in the original protein was released due to the intein activity. This demonstrates that the intein activity remained, either totally, or at least partially, when the fusion protein was in the aggregated state. These results also indicate that the intein was able to maintain folding, even when it was present in the ELP based aggregates. The consequence of these results is that it is not necessary to refold the fusion protein after phase transition takes place, since folding was never lost during the aggregation process. From an application point of view, these results suggest that Intein-ELP based systems should be ideal for protein-based drug delivery system, because the proteins trapped within the ELP matrix are maintained in active form, at a very high local concentration and can be released only in the presence of a triggering agent.

An additional set of experiments was done to determine if the cleaved thioredoxin stays within the ELP based aggregates or if it is released to the liquid phase. Purified Trx-Intein-ELP fusion protein was aggregated in 2.5M NaCl at 37°C and then DTT was added to a final concentration of 20mM to induce intein cleavage. After 20 hours of incubation, the reaction mixture was centrifuged at 37°C, 14,000g to separate the aggregates from the liquid phase. The pellet was re-suspended in water. Both the supernatant and the solubilized aggregates were separately loaded to 10KDa MWCO mini ultrafiltration column and centrifuged at 10000 g for 20 minutes for 4 times to remove the DTT. The material retained by the membranes was resuspended in water,

using the same volume of water as the volume of the sample prior to centrifugation. These two samples were analyzed using SDS-PAGE, to determine what portion of the cleaved thioredoxin remained with the aggregates and what portion of the thioredoxin was released to the solution. These results are shown in Figure 3.3.4 and Figure 3.3.5. It can be clearly seen that most of the thioredoxin was cleaved after 20 hours of incubation with DTT. This cleavage rate is comparable with that of the tripartite fusion protein in water under conditions at which no phase transition takes place. From the gel we can also see that the aggregates consisted of Intein-ELP fusion, while the cleaved thioredoxin was only present in the supernatant.

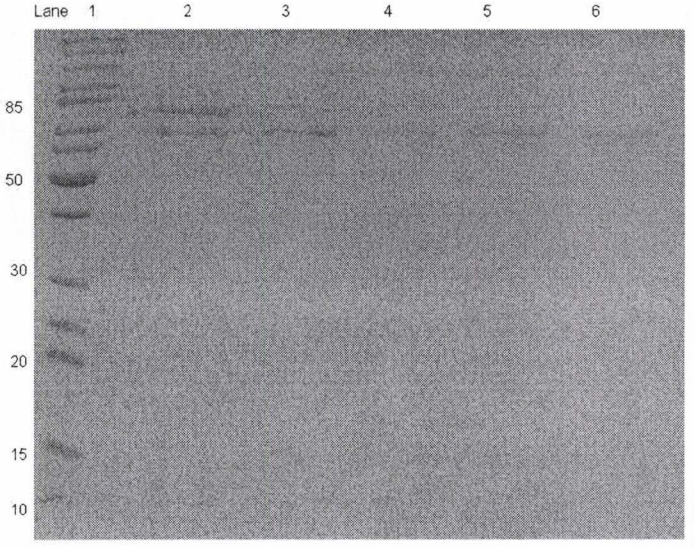


Figure 3.3.4 DTT- induced Intein Cleavage of Thioredoxin in Aggregation Matrix. Lane 1, Protein marker; Lane 2, t=1 hr; Lane 3, t=3 hrs; Lane 4, t=5 hrs ; Lane5, t=10 hr ; Lane 6, t= 20 hrs.

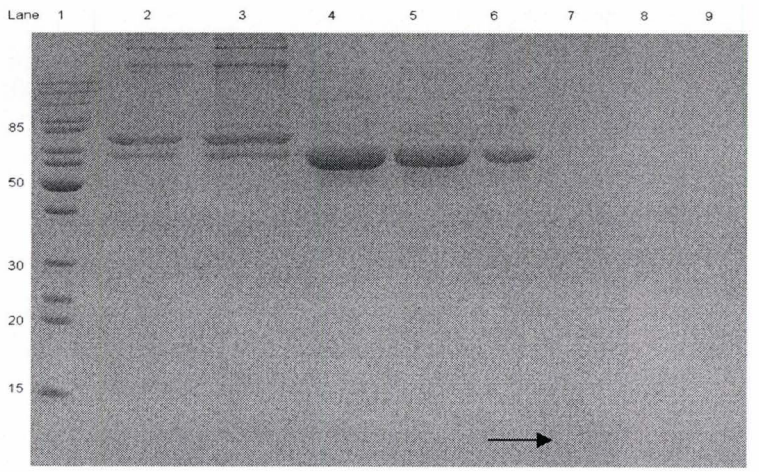


Figure 3.3.5 Thioredoxin Distribution after Intein-cleavage in the Aggregation Matrix. Lane 1, Protein Marker; Lane2 & Lane 3, purified protein before DTT-induced intein cleavage 5μl and 10 μl of samples, respectively; Lane 4, 5 and 6, pellet of aggregation after cleavage, 15,10 and 5 μl of samples, respectively; Lane 7, 8 and 9, supernatant of aggregation after cleavage, 15,10 and 5μl of samples, respectively.

To obtain quantitative information on the distribution of the thioredoxin, activity assays were done using the insulin disulfide-bond reduction method (Holmgren, 1979). This method is based on the fact that thioredoxin catalyzes the reduction of disulfide bonds in insulin through the utilization DTT. After reduction, the B chain of insulin B aggregates causing the solution to become turbid. The reaction rate can be quantitatively measured by recording the change of spectral absorbance of the sample at 650 nm. Thus the concentration of thioredoxin can be calculated by determining the rate at which the DO_{650} increases. The assays were done at 25°C in sodium phosphate buffer. The insulin reduction rate curves for 50μl samples from both the supernatant and the solubilized

aggregates were recorded after the addition of 2mM DTT. The results are shown in Figure 3.3.6. The calculation of the thioredoxin concentration is shown in Table3.2.

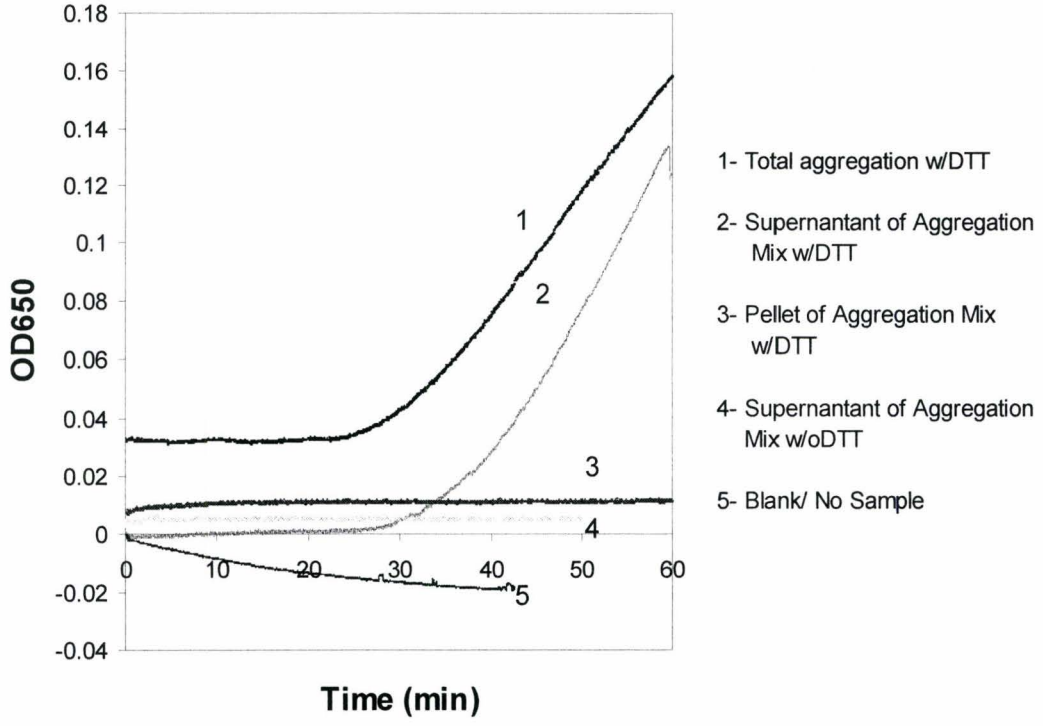


Figure 3.3.6 Thioredoxin Activity Tests of the Aggregation Matrix

Table 3-1 Thioredoxin Activity Distribution of the Aggregation Mix

Fusion Protein before Intein Cleavage	0.095 mg/ml
Supernatant after Cleavage	0.117 mg/ml
Pellet after Cleavage	0.0006 mg/ml

Most of the thioredoxin was released into the liquid phase after intein cleavage. This result reveals several important facts:

1. The intein activity is maintained when the fusion protein is presented in the aggregated state.

2. The ELP based aggregates constitute a porous matrix The small DTT molecule can easily enter the aggregates, as demonstrated by a high level of intein activity when the fusion protein was maintained in the aggregated form, as well as due to the fact that a small protein, such as thioredoxin (~12KDa), can be transported across the matrix and into solution..

These facts confirm the potential of using ELP based aggregates as vehicles for drug delivery, since both the substrate, as well as the product can be easily transferred into and from the matrix respectively.

3.3.4. Particle Size of ELP Aggregation Matrix

To further investigate the properties of the ELP aggregates, an experiment was done to determine the particle size distribution associated with the population of ELP based aggregates. A Mastersizer was used to detect the particle size distribution of the Trx-Intein-ELP fusion protein, at a concentration of 8 μM in 2.5 M NaCl. The particles had an average particle size of 1.0 μm in diameter (Figure 3.3.7) and experiments performed with difference protein concentrations, ranging from 4.3 to 12 μM, showed that particle diamter remained relatively constant for this protein concentration range.

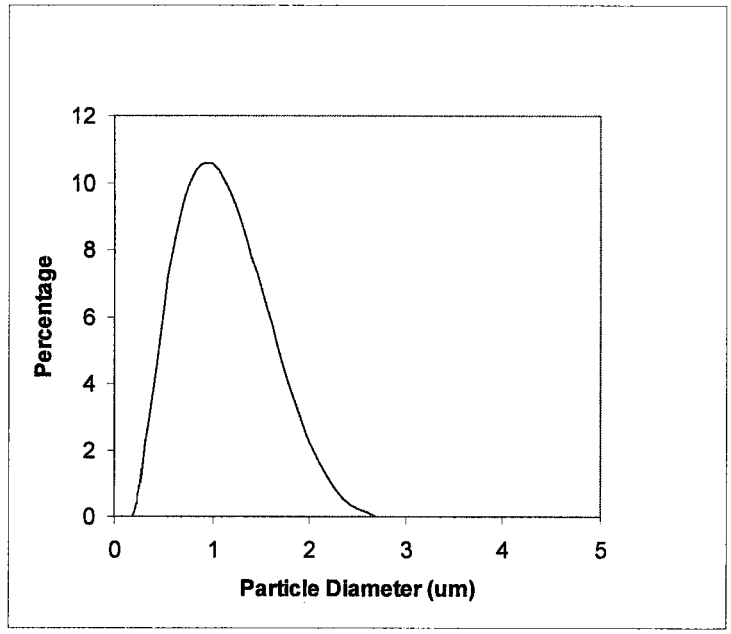


Figure 3.3.7 Particle Size Distribution of Aggregation Matrix (result of protein concentration of 8.0 mM)

3.4. Conclusions

A protein fusion consisting of Trx-Intein-ELP has the ability to undergo an inverse phase, which can be triggered by NaCl at room temperature. It was shown that the intein component of the fusion maintains its correct folding and self-cleaving ability, when the fusion protein was present in its aggregated form, with a particle size of about 1.0 μm. This result provides direct evidence that protein folding is not lost when ELP tagged proteins undergo phase transition. This is an important observation that allows us to proceed with some degree of confidence on the use of ELP tags to form highly ordered

ELP based aggregates *in-vivo* in *E. Coli*, for expression of aggregation prone proteins. The Intein-ELP based system is potentially interesting as a vehicle for drug delivery.

4. Conclusions and Recommendations for Future Work

Two separate projects were done as part of this thesis. In the first part, the gene encoding for the full-length tissue plasminogen activator was cloned and expressed in the methylotrophic yeast *Pichia pastoris*. In the second project, the activity of an intein, as part of a tripartite fusion protein, containing an ELP tag, was probed when the fusion was present in the aggregated form.. From these two studies, several conclusions can be drawn.

- *Pichia pastoris* can be used as a host for the heterologous expression of tPA, a fibrinolytic agent with a complex structure. The protein expressed using this system exhibited tPA-like activity and the protein productivity using this system is higher than when *E. coli* is used. As an eukyatic organism, *Pichia* can perform posttranslational modifications on protein, such as formation of disulfide bonds and glycosylation, which will result in a protein that will resemble much more closely the form in which tPA is expressed in mammalian cells, as compared to *E. Coli*.
- The work with the Trx-Intein-ELP fusion protein provided direct evidence that the activity of the intein was maintained when the fusion protein is maintained in the aggregated state. Small-molecules, such as DTT and thioredoxin, can move into the aggregation matrix and larger molecules, such as thioredoxin, can move out of the matrix. The intein-ELP based system shows some promise as a drug delivery agent.

Based on the results of the work reported in this thesis, the following recommendations are made for future work:

The most critical problems associated with the recombinant *Pichia* strains developed in this project are that tPA can only be expressed at low levels and/or is not secreted in an efficient manner. To solve the former problem, a systematic study on optimal cultivation conditions should be done using fermenters. Parameters to be optimized could be: the composition of medium, the optimal methanol concentration, the optimal pH condition, the oxygen supply rate, and rate of fed-batch cultivation.

Regarding tPA secretion, a relatively high percentage of tPA was observed in the intracellular protein fraction, so the protein was indeed produced, but the cell disruption process that is required will inevitably increase the complexity of protein separation and purification. A possible way to circumvent this problem is through the use of other signal peptide, including the native signal peptide of tPA.

The glycosylation pattern of *Pichia* expressed tPA needs to be identified. As a lower eukaryotic organism, the glycosalation pattern of *Pichia* is different from that of other higher eukaryotic species. As a glycoprotein, the length and composition of the sugar chain of the tPA generated in *Pichia* needs to be identified and compared with native tPA. This is necessary to provide an initial indication of potential immunological problems associated with the use of this tPA.

ELP are proteins and as such they are biodegradable. This fact makes ELP quite an interesting material with potential application in the field of biomaterials. As a potential material for drug delivery, it would be desirable to accurately control the particle size of the aggregates.

5. References

D. Collen and H.R. Lijnen (2004) Tissue-Type Plasminogen Activator: a Historical Perspective and Personal Account. *Journal of Thrombosis and Haemostasis,* 2: 541-546.

Christa Heussen and Eugene B. Dowdle (1980). Electrophoretic Analysis of Plasminogen Activators in Polyacrylamide Gels Containing Sodium Dodecyl Sulfate and Copolymerized Substrates. *Analytical Biochemistry,* 102, 196-202.

Diane Pennica, William E. Holmes, William J. Kohr, Richard N. Harkins, Gordon A. Vehar, Carole A. Ward, William F. Bennett, Elizabeth Yelverton, Peter H. Seegurg, Herbert L. Heyneker and David V. Goeddel (1983). Cloning and Expression of Human Tissue-Type Plasminogen Activator cDNA in *E. coli. Nature,* Vol. 301 20 January, 214-221.

Margarete Waldenström, Erik Holmgren, Anneli Attersand, Christina Kalderén, Björn Löwenadler, Benny Rådén, Lennart Hansson and Gunnar Pohl (1991). Synthesis and Secretion of a Fibrinolytically Active Tissue-Type Plasminogen Activator Variant in *Escherichia coli. Gene,* 99 243-248.

Ji Qiu, James R. Swartz, and George Georgiou (1998). Expression of Active Human Tissue-type Plasimogen Activator in *Escherichia coli. Applied and Environmental Microbiology,* P4891-4896.

Jiradej Manosroi, Chatchai Tayapiwatana, Friedrich Götz, Rolf G. Werner, and Aranya Manosroi (2002). Serection of Active Recombinant Human Tissue Plasimogen Activator Derivatives in *Escherichia coli. Applied and Environmental Microbiology,* June 2002, P 2657-2664.

Ralf Mattes (2001), The Production of Improved Tissue-Type Plasminogen Activator in *Escherichia coli. Seminars in Thrombosis and Hemostasis,* Vol 27, No. 4, , P325-335.

Jeffrey F. Lemontt, Cha-Mer Wei, and William R. Dackowski (1985). Expression of Active Human Uterine Tissua Plasminogen Activator in Yeast. *DNA,* Vol. 4, No. 6, , P 419-428

A. Upshall, A.A. Kumer, M. C. Bailey, M.D. Parker, M.A. Favreau, K. P. Lewison, M. L. Joseph, J.M. Maraganore and G.L. McKnight (1987). Secretion of Active Human Tissue Plasminogen Activator from the Filamentous Fungus *Aspergillus Nidulans. Bio/Technology,* Vol. 5, Dec., P1301-1304

M.G. Wiebe, A. Karandikar, G.D. Robson. A.P. J. Trinci, J-LFlores Candia, S. Trappe, G. Wallis, U Rinas, P. M. F. Derkx, S.M. Madrid, H. Sisniega, I. Faus, R. Montijn, C. A. M. J. J. van den Hondel, P. J. Punt (2001). Production of Tissue Plasminogen Activator (t-PA) in *Aspergillus niger. Biotechnology and Bioengineering,* Vol. 76, No.2, Sep., P 164-174

Linda Hansen, Yitzhak Blue, Karen Barone, Desire Collens, and Glenn **R.** Larsen(1988). Functional Effects of Asparagine-linked Oligosaccharide on Natural and Variant Human Tissue-type Plasminogen Activator. *The Journal of Biological Chemistry,* Vol. 263, No. 30, Issue of October 25, pp. 15713-15719.

Steven R.Deitcher,MD, Michael R. Jaff, DO, FACP, FACC 920020. Pharmacologic and Clinical Characteristics of Thrombolytic Agents. *Reviews in Cardiovascular Medicine,* Vol 3 Suppl 2, P25-33.

Cavit BOZ, Abdurrahman AKBAŞ, Zekeriya ALİOĞLU, Mehmet OZMENOĞLU (2003). Tissue Plasminogen Activator Mass Concentration in Ischemic Acute Stroke Patients. *Turk J Med Sci,* 33 (2003) 155-159.

Laurence M. Melnick, Brian G. Turner, Patricia Puma, Bonnie Price-Tillotson, Kathleen A. Salvato, Dennis R. Dumais, Donald T. Moir, Robert J. Broeze, and George C. Avgerinos (1990) Characterization of a Nonglycosylated Single Chain Urinary Plasminogen Activator Secreted from Yeast. *The Journal of Biological Chemistry,* Vol. 265, No. 2, Issue of January 15,1990, pp. 801~807.

A. J. Jaques, G. Opdenakker, T. W. Rademacher, R. A. Dwek and S. E. Zamze (1996). The Glycosylation of Bowes Melanoma Tissue Plasminogen Activator : Lectin Mapping, Reaction with Anti-L2/HNK-1 Antibodies and the Presence of Sulphated/glucuronic Acid Containing Glycans. *Biochem. J.* 316, 427-437.

Joan Lin Cereghino, James Gregg (2000). Heterologous Protein Expression in the Methylotrophic Yeast *Pichia pastoris. FEMS Microbiology Reviews* 24 45-66.

Julia Cino (2002) High Yield Production of Pichia pastoris Yeast: A Protocol for Bench-top Fermentation. *Invitrogene Corporation.*

Emma D. Thorpe, Marc C. d'Anjou and Andrew J. Daugulis (1999). Sorbitol as a Non-Repressing Carbon Source for Fed-Batch Fermentation of Recombinant *Pichia pastoris. Biotechnology Letters* 21: 669-672.

Angela Granelli-Piperno, and E. Reich (1978). A Study of Proteases and Protease-Inhibitors Complexes in Biological Fluids. *J. Exo. Med.*147 223-234.

20. David R. Higgins and James M. Cregg *Pichia Protocols*, Humana Press, ISBN: 0-89603-421-6.

Thomas Kjeldsen1, Annette Frost Pettersson and Morten Hach (1999). Secretory Expression and Characterization of Insulin in *Pichia pastoris*. *Biotechnol. Appl. Biochem.* 29, 79–86.

Cornelia Marty, Patrick Scheidegger, Kurt Ballmer-Hofer, Roman Klemenz, and Reto A. Schwendener (2001). Production of Functionalized Single-Chain Fv Antibody Fragments Binding to the ED-B Domain of the B-isoform of Fibronectin in *Pichia pastoris*. *Protein Expression and Purification,* 21, 156–164.

J. C. Goodrick, M. Xu, R. Finnegan, B. M. Schilling, S. Schiavi, H. Hoppe, N. C. Wan (2001). High-Level Expression and Stabilization of Recombinant Human Chitinase Produced in a Continuous Constitutive *Pichia pastoris* Expression System. *Biotechnology and Bioengeering,* VOL. 74, NO. 6, 492-497

Jung Hee Woo, Yuan Yi Liu, Scott Stavrou, and David M. Neville, Jr (2004). Increasing Secretion of a Bivalent Anti-T-Cell Immunotoxin by *Pichia pastoris*. *Applied and Environmental Microbiology*, p. 3370–3376 Vol. 70, No. 6.

Antje Eiden-Plach, Tatjana Zagorc, Tanja Heintel, Yvonne Carius, Frank Breinig, and Manfred J. Schmitt (2004). Viral Preprotoxin Signal Sequence Allows Efficient Secretion of Green Fluorescent Protein by *Candida glabrata, Pichia pastoris, Saccharomyces cerevisiae,* and *Schizosaccharomyces pombe*. *Applied and Environmental Microbiology*, p. 961-966 Vol. 70, No. 2.

Johanna C. Rotticci-Mulder, Malin Gustavsson, Mats Holmquist, Karl Hult, and Mats Martinelle (2001). Expression in *Pichia pastoris* of Candida antarctica Lipase B and

Lipase B Fused to a Cellulose-Binding Domain. *Protein Expression and Purification 21, 386–392*

D. M. Soden, J. O'Callaghan2 and A. D. W. Dobson (2002). Molecular Cloning of a Laccase Isozyme Gene from *Pleurotus sajor-caju* and Expression in the Heterologous *Pichia pastoris* Host. *Microbiology, 148, 4003–4014 Printed in Great Britain.*

C. M. J. Sagt, B. Kleizen,1R. Verwaal, M. D. M. De Jong, W. H. MU" Ller, A. Smits, C. Visser, J. Boonstra, A. J. Verkleij, and C. T. Verrips (2000).Introduction of an N-Glycosylation Site Increases Secretion of Heterologous Proteins in Yeasts. *Applied and Environmental Microbiology, p. 4940–4944 Vol. 66, No. 11*

Marc W. T. Werten, Tanja J. van den Bosch, Richele D. Wind, Hans Mooibroek and Frits A. de Wolf (1999). High-yield Secretion of Recombinant Gelatins by *Pichia pastoris. Yeast 15, 1087–1096.*

Rainer Fischer, Jürgen Drossard, Neil Emans, Ulrich Commandeur and Stephan Hellwig(1999). Towards Molecular Farming in the Future: *Pichia pastoris*-based Production of Single-chain Antibody Fragments. *Biotechnol. Appl. Biochem. 30, 117–120 (Printed in Great Britain)*

Pichia Fermantation Process Guidelines, Invitrogen Co., Version B 053002.

EasySelect *Pichia* Expresion Kit, Invitrogen Version G 122701.

S.L. Nilsen, M. E. Deford, M. Prorok, B. A. K. Chibber, R. K. Brethaurer, and F. J. Castellino, High-level secretion in *Pichia pastoris* and biochemical characterization of the recombinant kringle 2 domain of tissue plasminogen activator. *Biotechnology and Applied Biochemistry* (1997) 25, 63-74.

Jung Hee Woo, Yuan Yi Liu, Scott Stavrou, and David M. Neville, Jr., Increasing secretion of a Bivalent anti-T-cell immunotoxin by *Pichia pastoris*. Applied and Environmental Microbiology, June 2004, p. 3370–3376 Vol. 70, No. 6.

.

Dan E. Meyer and Ashutosh Chilkoti, Purification of recombinant proteins by fusion with thermally-responsive polypeptides. *Nature Biotechnology* Vol. 17, Nov. 1999 1112-1115.

Dan E. Meyer, K. TRabbic-Carlson, and A.Chilkoti, Protein purification with an environmentally responsive elastin-like polypeptide: Effect of Polypeptide length on the purification of thioredoxin. *Biotechnol. Prog.* 2001, 17,720-728.

C. J. Noren, J. Wang, and F. B. Perler, Dissecting the chemistry of protein splicing and its applications. *Angew. Che. Int. Ed.* 2000, 39, 450-466.

D. W. Wood, Simplified protein purification using engineered self-cleavage affinity tags. *Journal of Chemical Technology and Biotechnology*. 78, 2003, 103-110.

Ge, X., Yang, D.S., Trabbic-Carlson, K., Kim, B., Chilkoti, A., Filipe, C.D.M. 2005. Self-cleavable stimulus responsive tags for protein purification without chromatography. *J. Am. Chem. Soc.* 127(32):11228-11229

F. B Perler, and E. Adam, Protein splicing and its applications. *Current Opinion in Biotechnology* 2000, 11, 377-383.

J. P. Gangopadhyay, S. Jiang, and H. Paulus, An in vitro screening system for protein splicing inhibitors based on green fluorescent protein as indicator. *Anal. Chem.* 2003, 75, 2458-2462.

W. Wu, D. W. Wood, G. Belfort, V. Derbyshire, and M. Belfort, Intern-mediated purification of cytotoxic endonuclease I-TevI by insertional inactivation and pH-controllable splicing. *Nucleic Acids Research*, 2000, Vol 30 No. 22, 4864-4871.

A. Telenti, M. Southworth, F. Alcaide, S. Daugelat, W. R. Jacobs, Jr, and F. B. Perler, The Mycobacterium GyrA protein splicing element: Characterization of a minimal intein. *Journal of Bacteriology*, Oct. 1997, 6378-6382.

A. Holmgren, Thioredoxin catalyzes the reduction of insulin disulfides by the dithiothreitol and dihydrolipoamide. *The Journal of Biological Chemistry*, Vol. 254, No. 19, Oct.10, 1979, 9627-9632.

Evans J.T.C., Benner J. and Xu M.Q. Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Protein Sci* 7: 2256-2264 (1998).

DeLano, W.L. (2002). The PyMOL User's Manual. DeLano Scientific, San Carlos, CA, USA.

Guda, C., Zhang, X., Mcpherson, D.T., Xu, J., Cherry, J.H., Urry, D.W, Daniell H. (1995). Hyper Expression of an Environmentally Friendly Synthetic-Polymer Gene. Biotechnol Lett 17:745–750.

Shimazu, M., Mulchandani, A., Chen, W. 2003. Thermally triggered purification and immobilization of elastin-OPH fusion. Biotehnol. Bioeng. 81: 74-79

Studier, F.W. Rosenberg, A.H., Dunn J.J., Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. Methods in Enzymol. 185, 60-89

Urry, D.W. 1988. Entropic elastic processes in protein mechanisms. I. Elastic structure due to an inverse temperature transition due to internal chain dynamics. J. Protein. Chem. 7: 1-34

Urry, D.W., Trapane, T.L., Prasad, K.U. 1985. Phase-structure transitions of the elastin polypentapeptide-water system within the framework of composition-temperature studies Biopolymers 24: 2345–2356

Urry, D.W. 1992. Free energy transduction in polypeptides and proteins based on inverse temperature transitions. Prog. Biophys. Mol. Biol. 57: 23-57

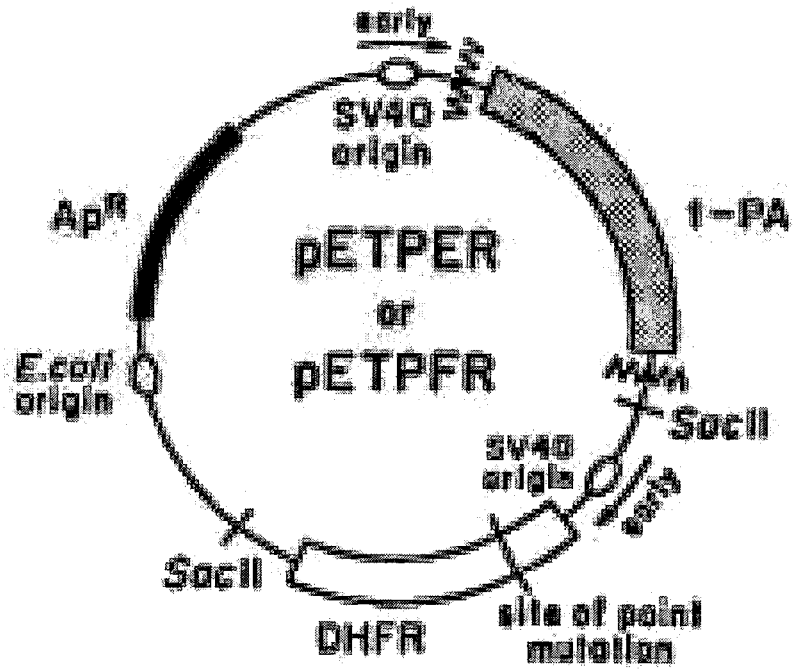
Appendices

Appendix I: tPA DNA Sequences

(**ATG** GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT GCC CGA TTC AGA AGA GGA GCC AGA TCT TAC CAA GTG ATC TGC AGA GAT GAA AAA ACG CAG)**ATG** ATA TAC CAG CAA CAT CAG TCA TGG CTG CGC CCT GTG CTC AGA AGC AAC CGG GTG GAA TAT TGC TGG TGC AAC AGT GGC AGG GCA CAG TGC CAC TCA GTG CCT GTC AAA AGT TGC AGC GAG CCA AGG TGT TTC AAC GGG GGC ACC TGC CAG CAG GCC CTG TAC TTC TCA GAT TTC GTG TGC CAG TGC CCC GAA GGA TTT GCT GGG AAG TGC TGT GAA ATA GAT ACC AGG GCC ACG TGC TAC GAG GAC CAG GGC ATC AGC TAC AGG GGC ACG TGG AGC ACA GCG GAG AGT GGC GCC GAG TGC ACC AAC TGG AAC AGC AGC GCG TTG GCC CAG AAG CCC TAC AGC GGG CGG AGG CCA GAC GCC ATC AGG CTG GGC CTG GGG AAC CAC AAC TAC TGC AGA AAC CCA GAT CGA GAC TCA AAG CCC TGG TGC TAC GTC TTT AAG GCG GGG AAG TAC AGC TCA GAG TTC TGC AGC ACC CCT GCC TGC TCT GAG GGA AAC AGT GAC TGC TAC TTT GGG AAT GGG TCA GCC TAC CGT GGC ACG CAC AGC CTC ACC GAG TCG GGT GCC TCC TGC CTC CCG TGG AAT TCC ATG ATC CTG ATA GGC AAG GTT TAC ACA GCA CAG AAC CCC AGT GCC CAG GCA CTG GGC CTG GGC AAA CAT AAT TAC TGC CGG AAT CCT GAT GGG GAT GCC AAG CCC TGG TGC CAC GTG CTG AAG AAC CGC AGG CTG ACG TGG GAG TAC TGT GAT GTG CCC TCC TGC TCC ACC TGC GGC CTG AGA CAG TAC AGC CAG CCT CAG TTT CGC ATC AAA GGA GGG CTC TTC GCC GAC ATC GCC TCC CAC CCC TGG CAG GCT GCC ATC TTT GCC AAG CAC AGG AGG TCG CCC GGA GAG CGG TTC CTG TGC GGG GGC ATA CTC ATC AGC TCC TGC TGG ATT CTC TCT GCC GCC CAC TGC TTC CAG GAG

AGG TTT CCG CCC CAC CAC CTG ACG GTG ATC TTG GGC AGA ACA TAC CGG GTG GTC CCT GGC GAG GAG GAG CAG AAA TTT GAA GTC GAA AAA TAC ATT GTC CAT AAG GAA TTC GAT GAT GAC ACT TAC GAC AAT GAC ATT GCG CTG CTG CAG CTG AAA TCG GAT TCG TCC CGC TGT GCC CAG GAG AGC AGC GTG GTC CGC ACT GTG TGC CTT CCC CCG GCG GAC CTG CAG CTG CCG GAC TGG ACG GAG TGT GAG CTC TCC GGC TAC GGC AAG CAT GAG GCC TTG TCT CCT TTC TAT TCG GAG CGG CTG AAG GAG GCT CAT GTC AGA CTG TAC CCA TCC AGC CGC TGC ACA TCA CAA CAT TTA CTT AAC AGA ACA GTC ACC GAC AAC ATG CTG TGT GCT GGA GAC ACT CGG AGC GGC GGG CCC CAG GCA AAC TTG CAC GAC GCC TGC CAG GGC GAT TCG GGA GGC CCC CTG GTG TGT CTG AAC GAT GGC CGC ATG ACT TTG GTG GGC ATC ATC AGC TGG GGC CTG GGC TGT GGA CAG AAG GAT GTC CCG GGT GTG TAC ACC AAG GTT ACC AAC TAC CTA GAC TGG ATT CGT GAC AAC ATG CGA CCG **TGA**

Appendix II: **Map of pETPER**



Appendix III: TPA Sequencing of pUC-tPA4

M13-forward:

TTNCG ACTNC GTACC CGCNN CTNCG NNAAN ANTNC TTACC AAGTG
ATNCT GCAGN AGNAT GAAAA AACGC AGNAT GATAT ACCAG CAACA
TCAGT CATGG CTGCG CCCTG TGCTC AGAAG CAACC GGGTG GAATA
TTGCT GGTGC AACAG TGGCA GGGCA CAGTG CCACT CAGTG CCTGT
CAAAA GTTGC AGCGA GCCAA GGTGT TTCAA CGGGG GCACC TGCCA
GCAGG CCCTG TACTT CTCAG ATTTC GTGTG CCAGT GCCCC GAAGG
ATTTG CTGGG AAGTG CTGTG AAATA GATAC CAGGG CCACG TGCTA
CGAGG ACCAG GGCAT CAGCT ACAGG GGCAC GTGGA GCACA GCGGA
GAGTG GCGCC GAGTG CACCA ACTGG AACAG CAGCG CGTTG GCCCA
GAAGC CCTAC AGCGG GCGGA GGCCA GACGC CATCA GGCTG GGCCT
GGGGA ACCAC AACTA CTGCA GAAAC CCAGA TCGAG ACTCA AAGCC
CTGGT GCTAC GTCTT TAAGG CGGGG AAGTA CAGCT CAGAG TTCTG
CAGCA CCCCT GCCTG CTCTG AGGGA AACAG TGACT GCTAC TTTGG
GAATG GGTCA GCCTA CCGTG GCACG CACAG CCTCA CCGAG TCGGG
TGCCT CCTGC CTCCC GTGGA ATTCC ATGAT CCTGA TAGGC AAGGT TTACA
CAGCA CAGAA CCCCA GTGCC CAGGC ACTGG GCCTG GGCAA ACATA
ATTAC TGCCG GAATC CTGAT GGGGA TGCCA AGCCC TGGTG CCACG
TGCTG AAGAACCGCA GGCTG ACGTG GGAGT ACTGT GATGT GCCCT
CCTGC TCCAC CTGCG GCCTG AGACA GTACA GCCAG CCTCA GTTTC
CATCA ANGAG GNTNT TCGCG NAATC GCCTC CACCC CTGGA GGCTG
CATTT TTGCA AGCAA AGAGG TCNCC GGAAA GCGTT CTGNG CGGGG
CAACT TATAA CTCTG GTGAT TNTTTT GCGCC ACTGT TCAGA A

M13-Reverse:

TGTTA CGCCA NCTTG CTGCC TGCAG GTNCG ACTCT AGNAG GNTCC
CCGCA TGCGG CCGCT CACGG TCGCA TGTTG TCACG AATCC AGTCT

AGGTA GTTGG TAACC TTGGT GTACA CACCC GGGAC ATCCT TCTGT
CCACA GCCCA GGCCC CAGCT GATGA TGCCC ACCAA AGTCA TGCGG
CCATC GTTCA GACAC ACCAG GGGGC CCTCC CGAAT CGCCC TGGCA
GGCGT CGTGC AAGTT TGCCT GGGGC CCGCC GCTCC GAGTG TCTCC
AGCAC ACAGC ATGTT GTCGG TGACT GTTCT GTTAA GTAAA TGTTG
TGATG TGCAG CGGCT GGATG GGTAC AGTCT GACAT GAGCC TCCTT
CAGCC GCTCC GAATA GAAAG GAGAC AAGGC CTCAT GCTTG CCGTA
GCCGG AGAGC TCACA CTCCG TCCAG TCCGG CAGCT GCAGG TCCGC
CGGGG GAAGG CACAC AGTGC GGGAC CACGC TGCTC TCCTG GGCAC
AGCGG GACGA ATCCG ATTTC AGCTG CAGCA NCGCA ATGTC ATTGT
CGTAA GTGTC ATCAT CGAAT TCCTT ATGGA CAATG TATTT TTCGA CTTCA
AATTT CTGCT CCTCC TCNCN AGGNA CACCC GTATG TTCTG CCNAN ATCAC
CGTCN GNGGG GGGCG NAACCTCNCT GNACA NTGGC GNCAA AAAAT
CACAG GACTA TAATT GCCCN NCAAG ACCCT TTCGG GAACT NTTGN
TGAAA ATGAN CTNCN GGTGG AGCAT TNNCA AANCC NTTTN CAAAT
AGNTG NTTCT NTNAN CCNGG NNNNG GGCAN NANTT CCCNN CCNGG
TTTAN NNGCC NGNTG NNCCN AGTCC GNNAT TTTNC NCCCN CCGCC

Appendix IV: TPA Sequencing of pZamp-tPA4

M13- forward:

ANNNC GTACC CGCNN CTNCG GNAAN ANNNC TTACC AAGTG ATNCT GCAGN AGNAT GAAAA AACGC AGNAT GATAT ACCAG CAACA TCAGT CATGG CTGCG CCCTG TGCTC AGAAG CAACC GGGTG GAATA TTGCT GGTGC AACAG TGGCA GGGCA CAGTG CCACT CAGTG CCTGT CAAAA GTTGC AGCGA GCCAA GGTGT TTCAA CGGGG GCACC TGCCA GCAGG CCCTG TACTT CTCAG ATTTC GTGTG CCAGT GCCCC GAAGG ATTTG CTGGG AAGTG CTGTG AAATA GATAC CAGGGCCACG TGCTA CGAGG ACCAG GGCAT CAGCT ACAGG GGCAC GTGGA GCACA GCGGA GAGTG GCGCC GAGTG CACCA ACTGG AACAG CAGCG CGTTG GCCCA GAAGC CCTAC AGCGG GCGGAGGCCA GACGC CATCA GGCTG GGCCT GGGGA ACCAC AACTA CTGCA GAAAC CCAGA TCGAG ACTCA AAGCC CTGGT GCTAC GTCTT TAAGG CGGGG AAGTA CAGCT CAGAG TTCTG CAGCA CCCCT GCCTG CTCTG AGGGA AACAG TGACT GCTAC TTTGG GAATG GGTCA GCCTA CCGTG GCACG CACAG CCTCA CCGAG TCGGG TGCCT CCTGC CTCCC GTGGA ATTCC ATGAT CCTGATAGGC AAGGT TTACA CAGCA CAGAA CCCCA GTGCC CAGGC ACTGG GCCTG GGCAA ACATA ATTAC TGCCG GAATC CTGAT GGGGA TGTCA AGCCC TGGTG CCACG TGCTG AAGAA CCGCA GGCTG ACGTG GGAGT ACTGT GATGT GCCCT CCTGC TCCAC CTGCG GCCTG AGACA GTACAGCCAG CCTCA GTTTC GCATC AAAGG AGGGC TCTTC GCCGA CATCG CCTCC ACCCC TGCAG GCTGC ATCTT TGCCA AGCAC AGGAG GTCGC CCGAG AGCGG TCCTG TGCGG GGCAT ACTAT AGCTC TGCTG ATTCT TCTGC GCCAC TGTTC AGAGA GTTCC GCCCA CACTG CGTNT CTGGA GAATC CGGTG TCTTG CAGAG ACAGA TTNAG TCAAA TCTTG CATAN GATCA TATAC CTCAA TAATG CTNTN ACTAA TCGAT CTCGT GGCAG AAGAC TGTCN ATGTC TCCGN GCTGA CTCGA TGNGA TGACT CGTCG ANTNG CTGTC TTTNN CGTAG GTTGA ATNCT CNCTG NTANT NTAA ATCGAT GTGGN

M13-reverse:

ATACG CCANC TTGCT GCCTG CAGGT NCGAC TCTAG NAGGN ATCCC CGCAT GCGGC CGCTC ACGGT CGCAT GTTGT CACGA ATCCA GTCTA GGTAG TTGGT AACCT TGGTG TACAC ACCCG GGACA TCCTT CTGTC CACAG CCCAG GCCCC AGCTG ATGAT GCCCA CCAAA GTCAT GCGGCCATCG TTCAG ACACA CCAGG GGGCC TCCCG AATCG CCCTG GCAGG CGTCG TGCAA GTTTG CCTGG GGCCC GCCGC TCCGA GTGTC TCCAG CACAC AGCAT GTTGT CGGTG ACTGT TCTGT TAAGT AAATG TTGTG ATGTG CAGCG GCTGG ATGGG TACAG TCTGA CATGA GCCTC CTTCA GCCGC TCCGA ATAGA AAGGA GACAA GGCCT CATGC TTGCC GTAGC CGGAG AGCTC ACACT CCGTC CAGTC CGGCA GCTGC AGGTC CGCCG GGGGA AGGCA CACAG TGCGG ACCAC GCTGC TCTCC TGGGC ACAGC GGGAC GAATC CGATT TCAGC TGCAG CAGCG CAATG TCATT GTCGT AAGTG TCATC ATCGA ATTCC TTATG GACAA TGTAT TTTTC GACTT CAAAT TTCTG CTCCT CCTCG CCAGG GACCA CCCGG TATGT TCTGC CCAAG ATCAC CGTCA GGTGG TGGGG CGGAA ACCTC TCCTG GAAGC AGTGG GCGGC AGAGA GAATC CAGCA GGAGC TGATG AGTAT GCCCC CGCAC AGGAA CCGCT CTCCG GGCGA CCTCC TGTGC TTGGC AAAGA TGGCA GCCTG CCAGG GGTGG GAGGC GATGT CGGCG AAGAG CCCTC CTTTG ATGCG AAACT GAGGC TGGCT GTACT GTCTC AGGCC GCAGG TGGAG CAGGA GGGCA CATCA CAGTA CTCCA CGTCA GCCTG CGGTT CTCAG CACGT GCACC AGGGC TGACA TCCCA TANGA TTCCG CAGTA ATATG TTTGC CAGGC CAGTG CTGGC ACTGG GTCTG TGCTG TGTAA CTTGC TATAG NTNTG AATTC ACGGA GCAGAGNACC GNTCG TAGCT GGCTG CACGT AGCTN CCATC CAAGA CATAT GTNCT AANAG AGGTN TNAAC TTACT GCTCC CTANC TAACN GTTNT TNTTG TNTNA NTTGG TCAGC ACTNG CNTGC CNCNT AGTNG CACCT TTCAT GGNTG CCTCG TGTCT CTATN NNGCN ANNGC GTNT

Appendix V: Medium and Recipes

Name	Recipe	Notes
LB (Luria –Bertani) Medium	1% tryptone, 0.5% yeast extract 1% NaCl, pH 7.0	*E. coli* cultivation
YPD (Yeast Extract Peptone Dextrose)	1% yeast extract 2% peptone (soytone peptone) 2% dextrose (D-glucose) Add 2% agar if making plates.	*Pichia* cultivation
BMGY (Buffered Glycerol-complex Medium)	1% yeast extract, 2% peptone, 100mM Potassium Phosphate, pH6.0 1.34% YNB 4 x 10-5% biotin 1% glycerol	*Pichia* cultivation
BMMY (Buffered Methanol-complex Medium)	1% yeast extract 2% peptone 100mM potassium phosphate, pH6.0 1.34% YNB 4 x 10-5% biotin 0.5% methanol	*Pichia* cultivation-methanol induction
MM (Minimal Methanol)	1.34% YNB 4 x 10-5% biotin 0.5% methanol	Un-buffered *Pichia* cultivation methanol induction

Appendix VI: Solutions and Buffers

Name	Recipe	Notes
DNA Electrophoresis Buffer	0.04mol/L Tris- acetic acid 0.001mol/L EDTA pH 8.0	Prepare 50 x solution as stock.
SDS-PAGE Separating Gel Buffer	Tris-base 36.3 g 1M HCl 48 ml Add MilliQ water to 100 ml. and . Filter via 0.2 µm paper store at 4°C	3.00M Tris-HCl pH 8.8
SDS-PAGE Stacking Gel Buffer	Dissolve 6.0 g tris-base in water Titrated to pH 6.8 with HCl Add water to final volume of 100ml Filtered via 0.2 µm paper Store at 4°C	0.50M Tris-HCl pH 6.8
10% Separating Gel	MilliQ water 4.21 ml Separating gel buffer 1.00 ml 10% SDS 80 ul 30% Acrylamide/Bis 2.67 ml 10% AP 40µl TEMED 4µl	Total volume: 8 ml
Stacking Gel	MilliQ water 1.20 ml Stacking gel buffer 0.50 ml 10% SDS 20 µl 30% Acrylamide/Bis 0.27 ml 10% AP 10 µl TEMED 2 µl	Total volume: 2 ml

Appendix VI: continued

SDS-PAGE Running Buffer (10x)	Tris-base 30.3 g Glycine 144g SDS 10g Dissolve in MilliQ water to 1L Store at 4°C	dilute to 1X before use
SDS-PAGE Sample buffer (with DTT)	0.5 M Tris-HCl(pH 6.8) 2.0 ml 10% SDS 2.0 ml 50% Glycerol 3.0 ml DTT 0.308g Bromophenol 5.0 mg 2.5 ml MilliQ water	2 X Store at -20°C
Isopropanol Fixing Solution	25% (v/v) isopropanol 10% (v/v) acetic acid 40% H2O	for rapid Coomassie blue staining
Rapid Coomassie Blue Staining Solution	10% (v/v) acetic acid 0.006% (w/v) Coomassie brilliant blue G-250 90% H2O	
De-staining Solution	5% methanol 10% acetic acid 85% H2O	for rapid Coomassie Blue staining
Membrane Transfer Buffer	3.03 g Tris-base 14.4g glycine 200ml methanol Add water to make 1liter.	Keep cold Use immediately

Appendix VI: Continued

TBS Buffer	8.76 g NaCl 6.05 g Tris-base Dissolve in water Adjust pH to 7.4 with 6.0N HCl Add water to final volume of 1.0 L	
Blocking Buffer for Western Blotting	5% w/v non-fat milk in TBS	
Antibody Binding Buffer	1% w/v non-fat milk 0.05% Tween 20 in TBS buffer	
Membrane Washing Buffer	0.5% w/v non-fat milk in TBS buffer	
Carbonate Buffer (10x)	200 mg $MgCl_2 \cdot 6H2O$ 8.4 g $NaHCO_3$ Add water and Adjust pH to 9.8 Add water to final volume of 100 ml	Store at 4 C.
Detecting Buffer (with NBT and BCIP)	Dissolve 15 mg NBT in 150 µl H2O and 350 µl DMF Dissolve 7.5 mg BCIP in 500 µl DMF Add above two reagents to 50 ml of 1x carbonate buffer	Use immediately
Thioredoxin Activity Testing Buffer	100mM Potassium phosphate 2 mM EDTA pH 7.0	

Appendix VI: Continued

Pichia Cell Breaking Buffer	Sodium phosphate (monobasic) 0.6 g EDTA 37.2 g Glycerol 5.0ml MilliQ water 90 ml Adjust pH to 7.4 with NaOH Bring volume to100 ml Store at 4°C Add PMSF to concentration of 1mM right before use	No PMSF for samples for tPA activity test
PBS Buffer	1.4 M NaCl 27 mM KCl 100 mM Na2HPO4 18 mM KH2PO4 pH 7.3	*E. coli* cell disruption

Appendix VII: PCR of the tPA Gene

The PCR reaction was catalyzed by the *KOD Hot Start* DNA polymerase (Novagen). The recipes are shown in following table:

Reaction No.	1	2	3	4	5
10x Buffer (μl)	2.5	2.5	2.5	2.5	2.5
2mM dNTP (μl)	2.5	2.5	2.5	2.5	2.5
25 mM MgSO4 (μl)	0	0.5	1	2	2
20μM Forward Primer (μl)	0.5	0.5	0.5	0.5	0.5
20μM Reverse Primer (μl)	0.5	0.5	0.5	0.5	0.5
tPA Template (μl)	1	1	1	1	0
ddH_2O (μl)	17.5	17	16.5	15.5	16.5
KOD (μl)	0.5	0.5	0.5	0.5	0.5

The reaction was carried out according to the following parameters: The PCR mix was first heated at 94°C for 2minutes. For each cycle, the PCR mix was heated at 94°C for 15 seconds, allowed to anneal at 55°C for 15 seconds. Chain extension was carried out at 72°C for 45 seconds. After 30 cycles, an extra 15 minutes was allowed for chain extension at 72° C.

After PCR reaction, the product was separated by 1% agarose gel.

Appendix VIII: Western Blotting Analysis of tPA

This method is used to identify a protein by detecting the specific binding of this protein with its antibody.

Firstly, protein sample were separated by SDS-PAGE. Then the protein bands on the gel were transferred to a PVDF membrane in an electric field. The membrane transfer was done at 80v for 1.5 hours. After transfer, the membrane was first treated with blocking buffer for 1 hour with gentle shaking to block those blank binding sites. The membrane was incubated in antibody binding buffer with 1: 1000 primary antibodies for 1 hour with gentle shaking. Then the membrane was allowed to contact with secondary antibodies. The membrane was washed three times with washing buffer. This helped to remove the non-specific binding between first antibodies and other proteins in the sample. Finally, the specific binding a protein and its antibody was visualized by incubating in detecting buffer with NBC and CNIP. The membrane should be stored in plastic bag.