ENHANCING THE CAPABILITIES OF THE METHYLOTROPHIC YEAST *Pichia pastoris* FOR RECOMBINANT PROTEIN EXPRESSION

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ENHANCING THE CAPABILITIES OF THE METHYLOTROPHIC YEAST *Pichia pastoris* FOR RECOMBINANT PROTEIN EXPRESSION

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NY-ESO-1 is an important cancer immunotherapy target that has proven to be impossible to produce in soluble form in *Escherichia coli*. Targeting this protein for secretion in *Pichia pastoris* results in its retention within the cell. By utilizing the downstream purification methods developed for *E. coli* inclusion bodies, we are able to purify NY-ESO-1 aggregates from *P. pastoris* lysate. The his-tag is cleaved off by unknown cellular processes preventing the purification of NY-ESO-1 by metal affinity chromatography. Removal of one C-terminal arginine residue destroys a potential Kex2 protease site and results in the retention of the his-tag.

The A33 scFv protein is efficiently secreted by *P. pastoris* to the culture supernantant at levels approaching 4 g/L. An attempt was made to further enhance this yield by overexpressing two proteins involved in the last stage of protein secretion, the *Saccharomyces cerevisae* SNARE proteins Sso1 and Snc2. Overexpression of Snc2 or Sso1 have no impact on cell growth, and Sso1 cooverexpression leads to the enhancement of A33 scFv yields by up to 81% in high cell density fermentor cultures. Snc2 cooverexpression has no effect, and cooverexpression of either of these proteins with A33 scFv and the ER resident chaperone BiP led to a defect in cell growth and a decrease in A33 scFv yield.

There is a growing body of evidence showing that certain proteins in a wide range of cell types are secreted by means not involving the classical secretory

pathway. One of these proteins is enolase, and it has been shown in *S. cerevisae* to be capable of driving the secretion of enolase-GFP and enolase-invertase fusions to the extracellular space independent of the ER and golgi. A fusion protein between enolase and the cancer differentiation antigen MelanA was generated. In shake flask cultures, fusion protein could be detected in the supernatant by immunoblot, and additional protein was able to be liberated from the cell wall by gentle washing with buffer containing 2-mercaptoethanol. Fermentor cultures, however, accumulated very little fusion protein in the culture supernatant, but significant degradation could be detected when blotting with an anti-enolase polyclonal antibody.

BIOGRAPHICAL SKETCH

Kyle Adam Anderson was born on October 23, 1980 to Rodney and Faye Anderson. The first few years of his life were spent in Winterport, Maine. In 1986 the family relocated to neighboring town, Hampden, where Kyle enjoyed the rest of his childhood and adolescent days. He attended the local public school system where he early on excelled at academics while being active in Church, Boy Scouts, Music and Track. In 1995 Kyle entered Hampden Academy. He maintained many activities throughout high school including Concert Band, Concert Choir, Cross Country, Dollars for Scholars, and National Honors Society. He also worked as a lifeguard and swim instructor at the Memorial Pool. He continued to find academic success and began to realize his strong interest in the Biological Sciences. His participation in an advanced placement biology class solidified his love for the field and the direction in which his life would steer.

At a college fair, Kyle was introduced to the Rochester Institute of Technology. He knew immediately that this would be his first choice for obtaining his undergraduate degree. Kyle received numerous academic scholarships upon acceptance at RIT. He entered the college in the fall of 1999 with a major in Biotechnology. While at RIT he continued lifeguarding and teaching swim lessons. Kyle maintained his academic consistency, making the Dean's List each quarter of his undergraduate career. He also earned the prestigious David M. Baldwin Award. Entering his second year at RIT,

Kyle became a Resident Advisor. He enjoyed the interaction with his younger peers and chose to keep his RA position through not only the summer, but also his entire third year. With all the responsibilities of school and work Kyle was still able to obtain a desirable summer internship at Wyeth. He remained involved with biology upon the

start on his third year by taking a position as a laboratory technician with the Biological Sciences department. He began to see his future in the laboratory, specifically in earning a Doctorate degree.

By Kyle's senior year, he was confident that obtaining a Ph.D. in the field of Microbiology was the next step in his academic career. In the winter of 2003 he was accepted to the Microbiology department at Cornell University. In May of 2003 Kyle graduated from RIT with highest honors. After graduation he returned to Maine where he married his high school sweetheart, Kelly. Following a beautiful wedding and relaxing honeymoon, the newlyweds set up their first “home” in Ithaca.

In July of 2003 Kyle began his graduate school education at Cornell University. He was enthusiastic about the unique opportunities that working in the Batt laboratory would provide to him. He was able to participate in industrial scale protein production and purification from microbial expression systems, the products of which are being used for human clinical trials. In addition to his success as a student, Kyle experienced many personal milestones during his time at Cornell. In July of 2004 he and Kelly became homeowners. The new house and huge yard wasn't complete without the addition of a puppy, Toby, to share it with. In the years that followed, Kyle continued to thrive in the GMP protein production facility. All of the science took a backseat, however, to the biggest life changing event in Kyle and Kelly's life: the birth of their first daughter, Nora Elyse in July of 2006. Kyle's role as a father would be both challenging and rewarding. He balanced fatherhood with laboratory responsibilities amazingly well. The joy on Nora's face when Daddy returned home from work would make the long days in the lab worth while.

Kyle's young family added motivation to the progression of his graduate degree. He faced his laboratory responsibilities with a focus on the future, specifically providing for his family. During the ups and downs of Kyle's last couple of years as a graduate student, it is that dedication to his family that has lead him through. He is confident that the hard work he has put forth and the solid education he has received will be rewarded. As Kyle awaits the birth of his second child in the fall, he also looks forward to his future as a Doctor of Philosophy.

This thesis is dedicated to my beautiful wife Kelly, without whom I would not have been able to complete this journey. I must also dedicate this to my parents, Rod and Faye, without whom I wouldn't have been able to start this journey to begin with.

ACKNOWLEDGMENTS

There are so many people to acknowledge that it should be its own chapter. I must first acknowledge Carl Batt for accepting me into this unique and entertaining lab. What we are able to accomplish on the third floor of Stocking with such a small, tightly knit crew is truly amazing. I must also acknowledge my committee (or "Team" as my mom likes to say), Linda Nicholson and Ted Clark, for sitting through my incredibly long and boring A-exam defense and for being there when I needed them. To all the other faculty both at Cornell and at RIT who helped me along the way, I thank you.

One individual for whom "acknowledgment" isn't nearly enough is my wife Kelly. Her name should be on this dissertation as much as mine, for the help and support she gives me day in and day out. I can't stress enough that her being there was pivotal in keeping me on track. To my little Princess Nora, I can't thank you enough for all the smiles and laughs you put on my face after even the hardest of days. And lest any future children feel left out when they read this (yea right!) the thought my future family fills me with happiness. To my parents, you instilled in me the importance of family, and it is for my family that I do this work. Dad, I miss you so much, and I know you are there watching over us every day. I wish I could have finished this up before you had to go.

Special thanks to Lloyd Old and Gerd Ritter. Without your dedication to helping cancer patients and not simply turning a profit, you made this lab a reality and I know we will be helping many people with these proteins. To the Batt Lab crew, past and present, I thank you for your enthusiasm and commitment to keeping the lab a fun place to be. We have our whole lives to wear suits, it is nice to be able to keep

wearing shorts and laughing a little bit longer. To the LICR team, Leo, Jack, Rishard, Deuce, Adam, Brian, Denise, Rachel, Todd, and even Frank, it was all of you who made those hours watching filters bearable.

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Chapter 1

Introduction

Expression of full length recombinant proteins for therapeutic or industrial uses in microbial expression systems is becoming increasingly popular as more useful proteins are isolated and characterized. As larger and increasingly complex proteins are desired, more sophisticated expression platforms are also needed to address the specific challenges associated with their production. Attention must also be paid to how the specific choice of expression platform and its treatment will impact the eventual downstream purification.

Escherichia coli is often one of the first choices for recombinant protein expression because of its facile and well known genetics, high growth rate, and ability to express large quantities of protein rapidly. Often, bacteria are incapable of properly folding and modifying many proteins isolated from higher eukaryotes. As a result the protein is incorporated into large insoluble masses that collect at the poles of the cell called inclusion bodies (Villaverde and Mar Carrió 2003). While inclusion bodies are highly enriched with the protein of interest and can be isolated from the host cell contaminants by centrifugation or filtration, the protein is often useless for its intended purpose and will require refolding. Coupling this with the need to use high concentrations of denaturants and detergents to keep the misfolded protein in solution during purification, downstream processing can often be difficult and expensive when using bacterial expression systems (Marston 1986).

At the other end of the spectrum lies the expression of recombinant proteins in mammalian cell lines. Immortal cell lines such as Chinese Hamster Ovary (CHO) and Human Embryonic Kidney (HEK) have been developed and used for the expression of a number of recombinant proteins (Wurm 2004). The main advantage with this

expression system is the ability of the cells to perform all of the posttranslational modifications that are required. The protein is extracted from the cells ready to use once purified. Genetic manipulation of these cell lines is not as simple as with bacteria, and growth media can be expensive and complex. Cell density is far lower than that achieved with bacteria, and total growth times can be much longer.

Yeast expression systems provide some of the advantages of both bacterial fermentation and mammalian cell culture. *Saccharomyces cerevisiae* has been used for thousands of years in the manufacturing of bread and alcohol, and its physiology and genetics are very well understood. The transformation of *S. cerevisiae* and related yeasts is simple and rapid, and fermentation media is simple and well defined. Cell densities can be very high, and fermentation can be completed quickly, yielding high concentrations of protein. One important difference between yeast based expression versus bacterial expression is in the ability of yeast to perform many post translational modifications, such as disulfide bond formation, glycosylation, and phosphorylation. Furthermore, yeast are sometimes able to secrete the target protein to the exterior of the cell, removing the need for cell lysis and greatly facilitating downstream purification. While the post-translational modifications performed by the yeast cells are not always the same as performed in mammalian systems, this is often outweighed by the speed of growth and the far less complex and expensive growth requirements, particularly for proteins with a non-clinical use.

Pichia pastoris

The yeast *Pichia pastoris* was originally isolated and studied for its ability to consume methanol. Phillips Petroleum was trying to develop methods to better utilize the large volumes of methanol they would produce as a waste product. *P. pastoris*

fermentations on methanol were developed to produce single cell protein, intended as a feed additive (Cereghino and Cregg 2000). Ultimately, the oil crisis of the late 1970's prevented this process from being cost effective, and the program was canceled. Dr. James Cregg began working with *P. pastoris* as an expression system and was the first to isolate the promoter for the alcohol oxidase gene AOX1, the first protein involved in the consumption of methanol.

From a recombinant protein production standpoint, the AOX1 promoter is ideal because it is very tightly regulated in the presence of most carbon sources such as glycerol and glucose, but is very strongly induced upon exposure to methanol as the sole carbon source, when alcohol oxidase levels can reach 30% of total cellular protein (Cregg et al. 1989). Alcohol oxidase is delivered to the peroxisome, and the peroxisomes can get very large and constitute up to 80% of the cell volume, with each of these full of large crystal aggregates of alcohol oxidase (Subramani 1998). Subsequent work showed that *P. pastoris* was capable of processing secretion signals commonly used in *S. cerevisae* recombinant expression systems, in particular the α-mating factor (Cregg et al. 1993). These experiments opened the door to the use of *P. pastoris* as a recombinant expression platform, and later work expanded upon the library of plasmids and promoters available to researchers (Cregg et al. 2000).

The mode of transformation is particularly well suited to protein expression as the expression cassette is incorporated into the genome through homologous recombination into the AOX1 promotor locus (Cregg et al. 1985). Initial transformant selection can be done through auxotrophic or antibiotic resistance markers, and with the case of antibiotics, they need not be included in downstream media recipes as the cassette integration is very stable. From a regulatory standpoint, the lack of antibiotics

is very beneficial, and strain characterization is simpler as the stability of plasmids does not have to be demonstrated. Numerous different strains are available with a variety of knock-out mutations, allowing transformation with five or more different expression cassettes (Daly and Hearn 2005). While the AOX1 promoter remains the preferred promoter, the weaker PEX8 promoter has recently been isolated and utilized for weaker, methanol induced expression of proteins for which the levels of expression using AOX1 prove detrimental to cell growth (Johnson et al. 2001). Constitutive expression using the GAP promoter has also gained in popularity, as it induces to a lesser extent than AOX1 as well, but constitutive expression is not desirable for toxic proteins (Waterham et al. 1997).

Selection of transformants using Zeocin or Geneticin has the added benefit of easily selecting for multi-copy integration events (Vassileva et al. 2001). In a low percentage of transformants, the expression cassette will be integrated in multiple copies (Clare et al. 1991). These transformants can grow on higher levels of antibiotic, and this provides a quick and easy method to screen for those transformants that may produce larger quantities of recombinant protein. Hohenblum et. al. demonstrated that a strain with one copy of the human trypsinogen gene showed a 1.5 fold increase over a constitutive control, two copies led to a two fold increase, while three copies or more had low trypsinogen levels in the cell supernatant (Hohenblum et al. 2004).

While the advantages of *P. pastoris* certainly argue for the increased use of this organism as an expression platform, the main advantage clearly lies in the secretory ability. As the culture supernatant contains very few host proteins, protein purity is already very high with no purification steps whatsoever (Tschopp et al. 1987). Many proteins arrive in the culture supernatant fully active, and it is possible that a simple

buffer exchange is all that is required to prepare the protein for many uses. Coupled with a simple, defined medium, the act of removing the cell mass can lead to impressive purities with no additional steps. Secretion can, however, be difficult to achieve, a trait that seems to be very protein specific. In *P. pastoris*, secretion of gelatin has been achieved at levels of 14.8 gL^{-1} (Werten et al. 1999), and single chain antibody fragments (scFv) at 4 gL^{-1} (Damasceno et al. 2004) while the proteins MAGE-A3 and MelanA, among others, fail to be secreted at all. If secretion is achieved, scale up from shake flask to high cell density fermentor cultures is typically straightforward, and the most difficult step is often generating a strain that produces any protein whatsoever (Cregg et al. 2000).

Cancer Vaccination and Immunotherapy

The treatment of cancer through vaccination is currently in its infancy. The discovery of tumor associated antigens has led to the realization that it may be possible to coerce the immune system into recognizing tumors as non-self entities, resulting in their elimination. Indeed, it is likely that regular immunosurveillance by the body detects and eliminates potentially cancerous cells all the time, preventing their growth and formation into tumors. Cells that are able to escape initial immunosurveillance and form small tumors may enter into a period of equilibrium with the immune system, where the tumor growth is kept in check but is unable to be completely eradicated. During this time, tumor cells will continue to accumulate mutations and develop the ability to escape detection, and it is these cells that go on to form clinically relevant tumors.

The continual evolution of the tumor to evade the immune system makes cancer vaccination a difficult endeavor. One of the more difficult steps is the identification of

markers on the tumor cell which are not present, or present in very low concentrations, on normal tissue. These tumor associated antigens (TAA) consist of mutated proteins such as p53 (Ito et al. 2007) and K-ras (Gjertsen et al. 1997), cancer testis (CT) antigens such as NY-ESO-1 (Wang et al. 1998) and MAGE-A1 (Traversari et al. 1992), differentiation antigens such as MelanA (Kawakami et al. 1994) and NY-BR-1 (Wang et al. 1998), and overexpressed proteins such as Her-2 (Fisk et al. 1995) and

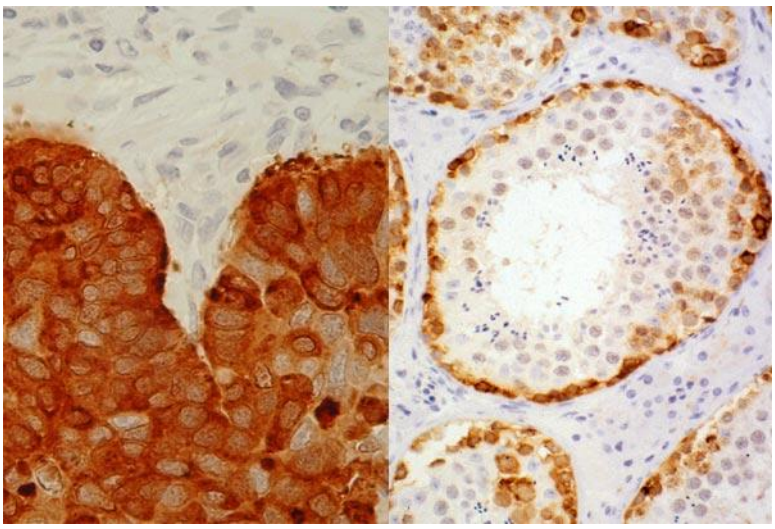

Figure 1-1-Immunohistochemical staining of NY-ESO-1 in lung cancer (left) and normal testis (right). Image from http://www.licr.org/D_programs/d4c1_AbCharacterization.php

PSMA (Horiguchi et al. 2002). These proteins are what will differentiate a tumor from the surrounding normal tissue. Many vaccines consist of peptides from these proteins or the full length protein itself in an attempt to initiate an immune response against cells bearing these markers. Other treatments target these proteins directly, such as the monoclonal antibody herceptin used to target Her-2 overexpressing breast cancer cells (Hudis 2007).

Vaccination with CT antigens has been the subject of numerous clinical trials. NY-ESO-1 protein has been shown to be highly expressed in a wide variety of tumor types (Figure 1-1), and its expression is restricted in normal tissue to testis (Chen et al. 1997). Since testicular tissue does not express major histocompatibility complex, NY-ESO-1 vaccination will not target these cells. One study vaccinated 12 patients with advanced, NY-ESO-1 positive metastatic disease with three overlapping NY-ESO-1 peptides. Of the 12 patients, seven exhibited disease stabilization that correlated with an anti-NY-ESO-1 immune response (Jäger et al. 2000). Another study used to evaluate the safety of the full length NY-ESO-1 protein in combination with the adjuvant ISCOMATRIX provided evidence that the full length protein confers protection against relapse of fully resected melanoma. Of 16 relapsed patients, five of seven received placebo, nine of 16 received NY-ESO-1 protein alone, and only two of 19 received NY-ESO-1 with ISCOMATRIX adjuvant (Chen et al. 2004; Davis et al. 2004). More phase 1 and 2 clinical trials are ongoing around the world targeting a wide array of different tumor types to study safety, adjuvants, and vaccination schedules (Tabi and Man 2006). These data provide exciting evidence that NY-ESO-1 vaccination, with the right adjuvants, can help many cancer patients.

P. pastoris *Derived NY-ESO-1 as a Potential Skin-Testing Antigen*

One of the critical assays for evaluating the vaccines effectiveness is determining the specificity of any observed immune responses, and also to evaluate the presence of memory T-cells to the specific vaccine antigen. One way to quickly and accurately measure this is through the delayed type hypersensitivity skin test (Figure 1-2).

The skin test is commonly used in humans to detect previous exposure to the pathogen *Mycobacterium tuberculosis* (Vukmanovic-Stejic et al. 2006). This test, called the

Mantoux test, involves the intradermal injection of tuberculin purified protein derivative (PPD). Damage to epithelial cells during the injection and the introduction of antigen leads to the release of pro-inflammatory cytokines and activation of dermal dendritic cells and langerhans cells through toll-like receptors. These cells phagocytose antigen and emigrate to the lymphatic system. Here, antigen is presented to naïve and memory T cells, which undergo clonal expansion. Antigen specific CD4+ T-cells travel to the site of injection, where they mediate effector responses such as macrophage activation to clear the antigen (Vukmanovic-Stejic et al. 2006). This response causes induration and swelling at the site of injection that peaks at 48-72 hours.

The skin testing of cancer vaccine responses would first and foremost test for the presence of memory T-cells specific to the cancer vaccine protein. This is vital to generating long term immunity and equilibrium between the immune system and the tumor. Secondly, skin testing provides a means of evaluating the specificity of the immune response to the cancer protein. NY-ESO-1 used in human clinical trials is derived from *E. coli* fermentation. As a result, there will always be host specific contaminants within the vaccine preparation at low concentrations. These can be host proteins, endotoxin, DNA, etc., and are able to elicit an immune response within the patient (Davis et al. 2004).

If the same reagent used to vaccinate is used for skin testing, one cannot tell if a positive response is due to a reaction to the intended antigen or merely a response to a contaminant. If, however, the skin test protein is generated from a different expression platform the resulting contaminants will inherently be different. In this case, a positive response to the skin test is a good indication that the patient is mounting an

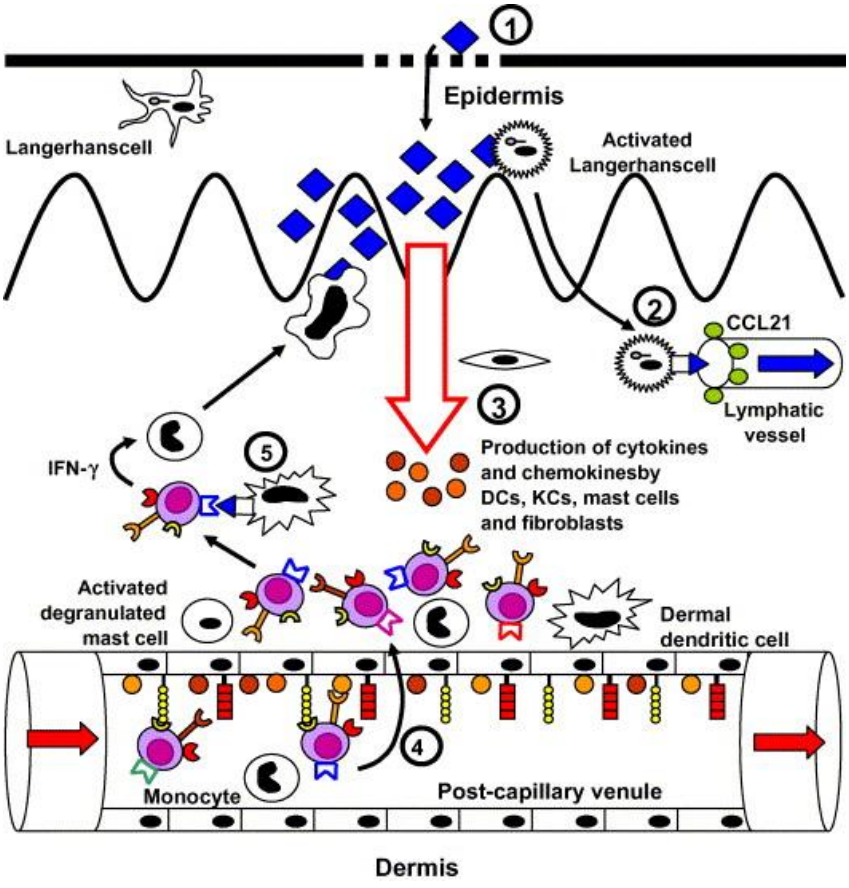

Figure 1-2-The immune response generated by a skin test antigen. See text for details. Image from Vukmanovich-Stejic et al. 2006.

effective cellular response to the vaccine itself. Neither vaccination nor skin testing are possible, however, without the necessary reagents. In the case of full length protein based therapies, secretion of the target molecule by yeast can be an effective means to generate a steady, cost effective supply.

Potential Bottlenecks to Protein Secretion

Translocation into the Endoplasmic Reticulum

While the capacity is present for tremendous secretion yields in *P. pastoris*, there are

numerous checkpoints in the secretory pathway that must be passed before a protein is released. The first is the import of the target protein into the endoplasmic reticulum. This can be accomplished with one of two different types of secretory signals; co-translational signal peptides or post-translational signal peptides (Rapoport 2007). Cotranslational signal peptides are typically composed of very hydrophobic residues early in the protein sequence that are recognized by the multi-subunit protein complex signal recognition particle, or SRP (Egea et al. 2005). SRP in mammals is composed of a 7S RNA and six different proteins (Halic and Beckmann 2005).

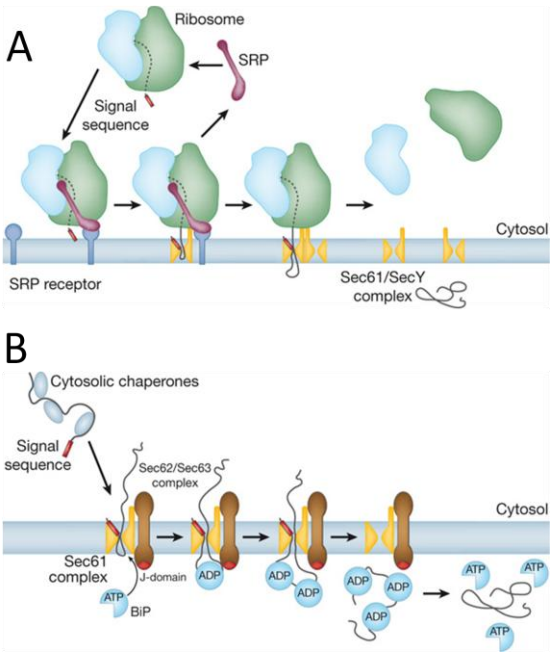

Figure 1-3-Translocation of nascent polypeptides into the ER can occur either co-translationally (**A**) or post-translationally (**B**). Figures were adapted from Rapoport 2007.

The order of events in cotranslational translocation can be seen in Figure 1-3, A. As the protein sequence exits the ribosome, SRP binding to the nascent polypeptide

causes translation to stall while the SRP-ribosome-polypeptide complex moves to the ER membrane. At the ER membrane, SRP interacts with the SRP receptor composed of two subunits, SRα and SRβ, followed by an interaction between the ribosome and the translocon channel formed by Sec61α and Sec61γ (Van den Berg et al. 2004). SRP is released, and the hydrophobic signal peptide is bound within the translocon with its N-terminus remaining in the cytosol. Translation is resumed and the protein is threaded into the ER lumen. Upon completion of translocation, the hydrophobic stretch of the signal peptide bound within the translocon channel is cleaved by signal peptidase, and the protein proceeds to fold and resume its journey to the cell exterior (Tuteja 2005).

Posttranslational signal peptides (see Figure 1-3, B), such as the *S. cerevisae* α-mating factor which is in wide spread use in both *S. cerevisiae* and in *P. pastoris*, are less hydrophobic than their cotranslational cousins, and as a result SRP is not recruited to the nascent chain and translation proceeds unchecked (Ng et al. 1996). The newly formed protein is fully translated into the cytoplasm, and the polypeptide chain is stabilized by cytosolic chaperones. The signal peptide is bound to the translocon, and part of the protein is threaded through the pore (Plath and Rapoport 2000). The peptide can travel in both directions through the pore, but on the lumenal side of the ER membrane, the chaperone BiP binds to the substrate, inhibiting its backwards movement (Matlack et al. 1999). BiP is recruited to the translocon through its interaction with the J domain of the protein complex Sec62/63, which interacts with the translocon. Interaction with the J domain lowers the binding specificity of BiP so that it can bind virtually any stretch of amino acids (Misselwitz et al. 1998). Once the protein has been fully ratcheted into the ER, signal peptidase again cleaves the signal peptide and further post translational modifications can take place. In this scenario, it

is vital that the protein does not fold in such a way within the cytoplasm as to obscure the signal peptide, else translocation will not occur. Regardless of which route is chosen, once the protein is within the ER, there are still yet many checkpoints to pass before secretion is successful.

Protein Folding and ER Quality Control

Likely one of the biggest hurdles to successful secretion is the ability of the protein to be properly folded and modified within the endoplasmic reticulum. Evolution has necessitated that cells have very strict control mechanisms to prevent improperly folded protein from traveling to a cellular compartment or cell exterior where its incorrect folding may elicit a harmful activity. Numerous chaperones are present in the ER at high concentrations to enable this folding to occur. One of the primary chaperones is BiP. BiP is recruited to the translocon where it actively binds polypeptide chains as they are threaded through the pore (Brodsky et al. 1995). In this fashion it prevents premature folding and aggregation until the entire chain is present. Through a cycle of binding and releasing from hydrophobic stretches, it acts to allow the protein to fold slowly and correctly. Other chaperones assist in certain post-translational modifications, in particular protein disulfide isomerase (PDI) which assists in the correct formation of disulfide bonds in the tertiary structure (Freedman et al. 1994).

If proteins are unable to fold successfully, the cell initiates an unfolded protein response (UPR) (Figure 1-4). The unfolded protein response has three goals (Görlach et al. 2006). The first is to reduce the amount of protein entering the ER, while the second is to enhance the ability of the ER to effectively process the unfolded protein backlog. The third process is initiated when the first two are unable to successfully

rectify the unfolded protein situation, and the result is cell death (Ron and Walter 2007). These processes are put in motion when ER membrane proteins sense the presence of unfolded proteins. The proteins IRE1 and PERK contain ER lumen domains that have been shown to bind the chaperone BiP. BiP maintains these proteins in their monomeric state. As unfolded protein accumulates, BiP is titrated away from IRE1 and PERK in an effort to complete proper folding. The titration of BiP leads to the production of IRE1/IRE1 and PERK/PERK dimers (Harding et al. 2003). Dimerization results in the phosphorylation of each protein**,** which in turn leads to the activation of pathways designed to rectify the ER stress. Dimerization of PERK leads to the phosphorylation of eukaryotic translation initiation factor-2 (eIF2a), which causes it to be locked in its inactive GDP bound form (Harding et al. 1999). The decreased amount of active eIF2a lowers the level of translation in the cell, decreasing the amount of new protein being introduced into the already stressed ER. eIF2a phosphorylation also leads to the upregulation of genes involved in the unfolded protein response, such as chaperones (Harding et al. 2003).

IRE1 functions in much the same way as PERK, however its phosphorylation leads to the activation of an endoribonuclease activity on the cytoplasmic tail of the protein. This activity removes an intron from the yeast mRNA HAC1 (XBP1 in mammals). The processed HAC1 mRNA is translated and this protein is a transcription factor that activates UPR genes (Cox and Walter 1996). This response can also be seen during high levels of protein overexpression commonly seen in recombinant protein manufacturing. Terminally misfolded proteins are eventually tagged with ubiquitin and translocated out of the ER, where they are then degraded by cytoplasmic proteases and the proteasome, which are also upregulated during the UPR.

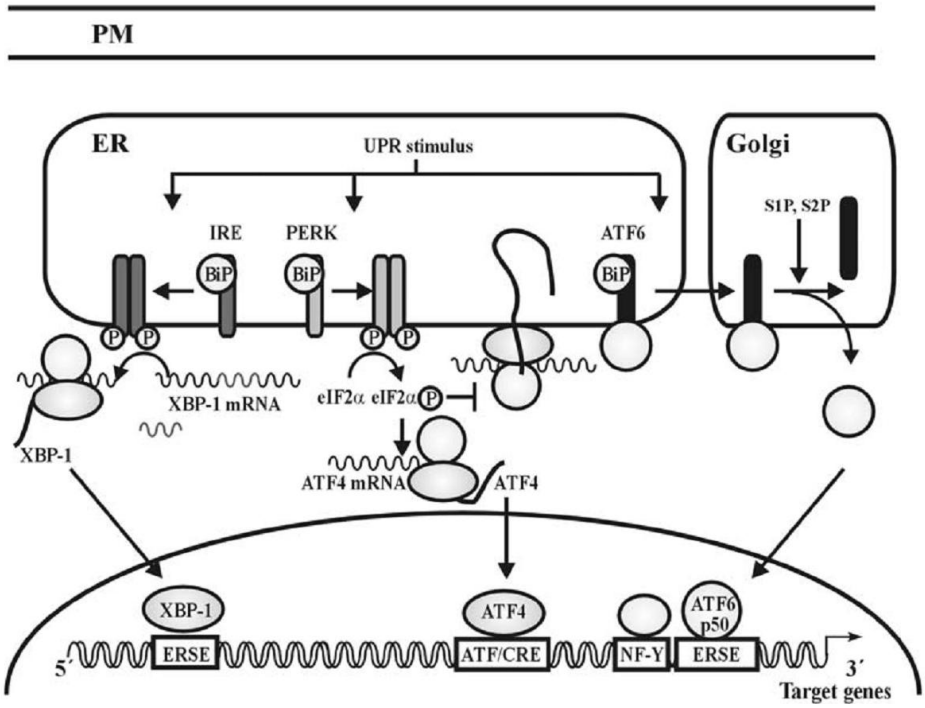

Figure 1-4-An overview of the unfolded protein response pathways. Figure adapted from Görlach et al. 2006.

A third mechanism for initiating the UPR is through the protein activating transcription factor 6 (ATF6) (Haze et al. 1999). ATF6 is a transmembrane protein that is retained in the ER through its interaction with BiP (Shen et al. 2002). Upon titration of BiP away from ATF6 due to unfolded protein accumulation, ATF6 traffics to the golgi where it is cleaved by the golgi resident proteases site 1 protease and site 2 protease. These cleavage events lead to the release of the cytosolic effector portion of ATF6 which traffics to the nucleus to upregulate UPR target genes (Ye et al. 2000).

Protein folding, especially in recombinant production strains, is a major bottleneck to successful protein secretion. Overexpression of BiP in *P. pastoris* has been shown to enhance the secretory yield of A33 scFv (Damasceno et al. 2007) and trypsinogen

(Hohenblum et al. 2004), while overexpression of protein disulfide isomerase (PDI) in *S. cerevisae* results in enhanced levels of human platelet derived growth factor B homodimer and *Schizosaccharomyces pombe* acid phosphatase (Robinson et al. 1994). This is clearly an important area to optimize when trying to maximize secretory yields in industrial protein production.

ER Exit and Golgi Processing

Once a protein is folded correctly, the next checkpoint it must bypass is exit from the ER. This phenomenon is well studied in *S. cerevisae,* and involves COPII coated vesicles (Figure 1-5). Transmembrane proteins destined for the plasma membrane or other cellular compartments are recognized by a subunit of COPII by their cytoplasmic tails and are thus sequestered together at the trans-ER face. This was first described for the ER exit of the vesicular stomatitis virus G protein (VSVG) (Nishimura and Balch 1997). Its cytoplasmic tail contains a di-acidic motif Asp-X-Glu, which is recognized by the combination of activated Sar1p and Sec23p/24p, components of the COPII assembly. The Sar1p/Sec23p/Sec24p complex at the ER exit site recruits two subunits each of Sec31p and Sec13p (Lederkremer et al. 2001; Sato and Nakano 2007). The recruitment of multiple COPII complexes causes the curvature of the membrane and eventual budding and vesicle formation. These vesicles are tagged by the small GTPase Ypt1 and travel to the cis-golgi, where the vesicles fuse and the contents are released into the golgi (Morsomme and Riezman 2002).

How soluble cargo is selected is not quite as clear. It is likely that there are transmembrane proteins that bind to ER cargo through non-specific means, possibly hydrophobic or ionic interactions. In yeast, this has been shown that the soluble protein pro-α-factor is recognized by the multispanning membrane protein Erv29p (Belden and Barlowe 2001). This membrane protein binds a hydrophobic region in the pro region of the precursor molecule and sequesters it at the trans-ER face. The cytosolic tail of Erv29p then interacts with COPII, leading to exit of the soluble proteins. These signals that direct exit from the ER have been shown to be able to

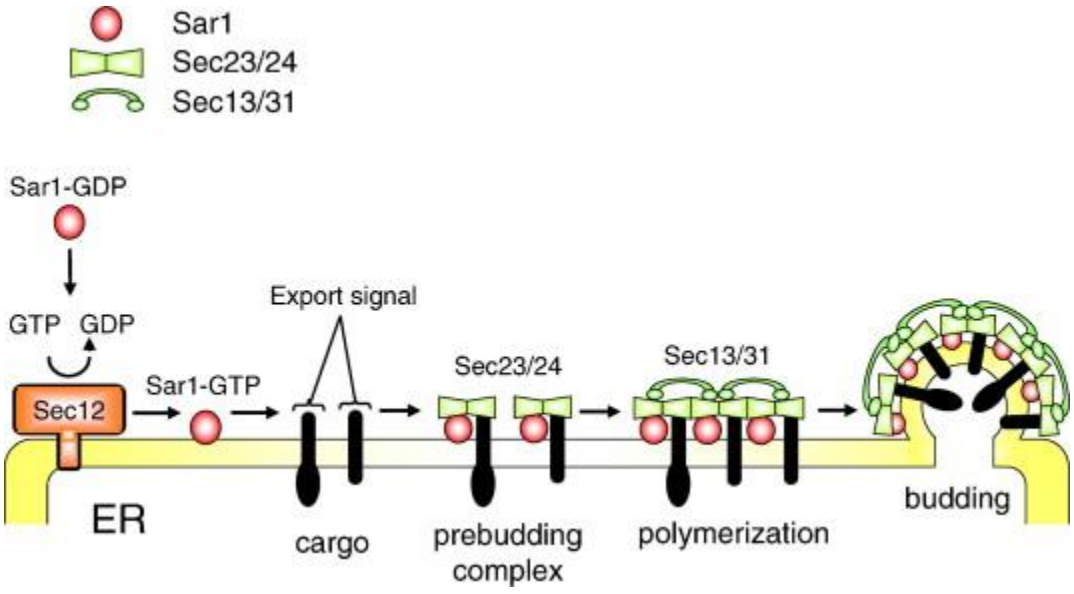

Figure 1-5-The mechanism for vesicle budding from the ER. See text for details. Figure from Sato and Nakano 2007.

override ER retention signals and lead to the export of typically ER resident proteins (Otte and Barlowe 2004).

Once within the golgi apparatus, proteins undergo final post translational modifications. Misfolded molecules that manage to escape ER quality control are either transported back to the ER to undergo ER associated degradation, or are

directed to the endosomal system for degradation (Arvan et al. 2002). The details of how misfolded proteins are detected in the golgi are still being elucidated. One protein involved in quality control in yeast is the golgi membrane protein Rer1p (Sato et al. 2001). It has been shown to interact with other transmembrane domains and detect the presence of polar residues introduced by misfolding or mutation. Rer1p then interacts with COPI, which is responsible for the retrograde transport of cargo from the golgi to the ER. These misfolded proteins are then allowed to properly fold or are degraded.

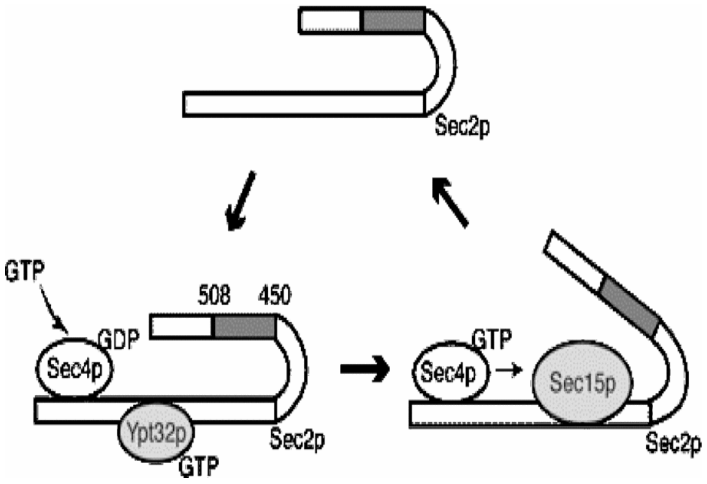

Figure 1-6-A Rab cascade is essential for secretory vesicle trafficking and tethering at the exocyst. See text for details. Image from Novick et. al 2006.

Trafficking and Docking With the Plasma Membrane

From the golgi, cargo must be correctly targeted to a number of cellular locations, such as the vacuole, nucleus, and the cell exterior. This requires the activation of a number of proteins at different steps, called a Rab cascade (Figure 1-6). For secretion, newly formed vesicles are tagged with the small GTPase Ypt31/32, which travel along

actin filaments towards the plasma membrane (Benli et al. 1996). GTP bound Ypt31/32 recruits the protein Sec2, which activates the GTPase activity of Ypt31/32 and its eventual release from the vesicle surface (Ortiz et al. 2002). Sec2 then recruits another GTPase Sec4 in its GTP bound state and vesicle traffic proceeds towards the plasma membrane (Novick et al. 2006). Sites of active secretion are differentiated by the presence of a large complex of proteins called the exocyst. Sec4 binds to Sec15, an exocyst component, which displaces Sec2 and initiates the GTPase function of Sec4, leading to its release as well (Novick et al. 2006). The Rab cascade serves to bring the secretory vesicle in close proximity to the plasma membrane and its tethering to the exocyst, a necessity for vesicle fusion and cargo release.

The overexpression of molecules required for protein trafficking has been successful in enhancing protein secretion in both *P. pastoris* and *S. cerevisae*. Sec4 overexpression in *P. pastoris* led to the increased secretion of glucoamylase (Liu et al. 2005) and in *S. cerevisae* to the increased secretion of α-amylase (Toikkanen et al. 2003). As with chaperone overexpression, enhancement of the secretory pathway by elevating the levels of essential trafficking proteins is an important step to reducing and eliminating protein secretion bottlenecks.

SNARES

For vesicle fusion to occur, it is necessary that the vesicle and the target membrane are able to be brought together very tightly. The class of proteins responsible for this critical step are called SNARE proteins (Soluble NSF Attachment protein REceptor). The discovery of SNARE proteins depended in large part on the study of the neuronal synaptic cleft, where vesicle fusion and release of neurotransmitters is critical for nervous system function. In this system, the first proteins identified in vesicle fusion

were syntaxin (STX1), SNAP-25 (25 kDa synaptosome-associated protein), and VAMP (vesicle-associated membrane protein), also called syaptobrevin. Initial classification systems divided SNARE proteins into v-SNAREs and t-SNAREs, depending on their location on the vesicle or target membrane (Chen and Scheller 2001). Refinements in understanding led to the renaming of the SNAREs as R-SNAREs or Q-SNAREs, depending on the identity of a specific amino acid (arginine or glutamine) at a highly conserved region (Fasshauer et al. 1998).

SNARE proteins are anchored to the membrane either as integral membrane proteins or are modified to contain lipid chains at their C-terminus (Burri L. and Lithgow T. 2004). Insertion of the integral membrane SNARE proteins proceeds through a pathway that has only recently begun to be defined in yeast. SNARE proteins are recognized as they come off of the ribosome by the protein GET3, which scans all nascent polypeptide chains for the presence of C-terminal hydrophobic stretches. GET1 and GET2 recruit the GET3-SNARE protein complex which results in the insertion of the SNARE protein into the ER membrane (Schuldiner et al. 2008). Sorting of the SNARE proteins to the proper final destination takes place after this step, through currently unknown means.

Vesicle fusion requires the assembly of a four membered coiled coil, called a SNAREpin, containing 3 Q-SNAREs and 1 R-SNARE (Sutton et al. 1998). After docking of the vesicle at the exocyst, SNAREs present on both the vesicle and the target begin to intertwine and "zip" together, pulling the vesicle and target membrane tightly together (Figure 1-7) (Südhof and Rothman 2009). The energy generated by this zipping up process provides the energy necessary to overcome repulsive ionic forces between the membranes, and also to dissipate the hydration layer between the two membranes (Chen et al. 2001). The amount of energy released by SNAREpin formation has been shown to be ~35 k_bT, while the energy required to fuse the bilayers

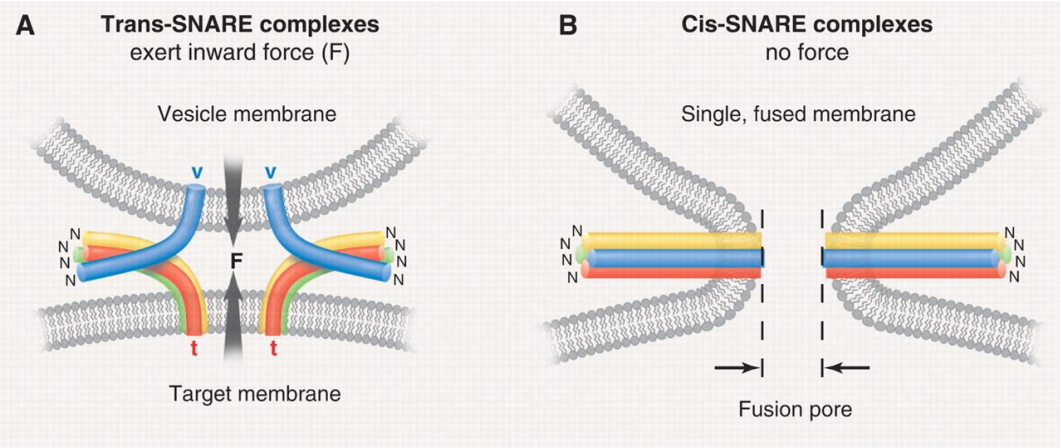

Figure 1-7-Membrane fusion occurs when three SNARE molecules on the target membrane interact with one SNARE on the vesicle membrane. **A**-The coiled coil structure that forms as the SNARE proteins assemble pulls the two membranes together to overcome repulsive ionic forces. **B**-The fully formed SNAREpin and resultant fused membrane. Image adapted from Südhof and Rothman 2009.

is 50-100 k_bT, indicating that each vesicle fusion event will require the formation of three stable SNAREpin structures (Li et al. 2007).

It is apparent that there are a number of steps where proteins can get retained on their path out of the cell, and consequently specific areas that can potentially be

manipulated to enhance the secretory capacity of the expression system. Proper signal peptide selection is important to ensure a steady flow of protein into the ER. If a protein quickly aggregates in the cytoplasm and obscures a post-translational signal peptide, no amount of downstream manipulation will lead to high secretion yields.

Furthermore, if a protein is unable to fold in the unique environment of the ER, it will simply face retrograde transport out of the ER and be degraded. Assuming translocation and folding are possible, then manipulation of the accessory proteins involved in the remainder of the secretory pathway is an attractive target for enhancing secreted protein yields.

Unconventional Protein Secretion

Because of the advantages to downstream purification offered by protein secretion, it is worth the time and energy required to generate strains that secrete protein with high yields. Certain proteins, however, seem to be completely resistant to secretion by traditional methods. Recent work has indicated that there are a number of proteins that are secreted by cells through routes that do not involve the traditional ER-golgi pathway. Work is currently being done to elucidate the pathways involved, but it seems clear that the pathways are highly specific for a particular protein. Multiple proposals have been put forward to explain how each of these molecules reaches the extracellular space, and can be seen in Figure 1-8. These proposals fall into four primary categories: direct translocation of proteins across the membrane; lysosomal secretion; secretion by fusion of multivesicular bodies with the plasma membrane; and secretion by plasma membrane blebbing and vesicle shedding (Nickel and Rabouille 2009). At this early stage, however, it appears that there is not one conserved pathway involved, but that each protein that secretes by unconventional methods has evolved

its own unique secretion method.

Of the proteins identified to undergo this process, the most well studied is fibroblast growth factor 2 (FGF-2). The current model for secretion begins first with the finding that FGF-2 is capable of binding to the phosphoinositide phosphatidylinositol 4,5 bisphosphate (PIP PI(4,5)P2). This molecule is known to form islands in the plasma membrane, so it is currently thought that these islands recruit FGF-2 to the inner leaflet of the plasma membrane (Engling et al. 2002). What happens after recruitment to result in extracellular FGF-2 is still under debate. There are three proposed routes out of the cell (Nickel and Seedorf 2008). The first requires a transport channel to allow the passive diffusion of FGF-2 across the membrane to the extracellular space. The second proposal requires a conformational change upon binding to PI(4,5)P2 to a conformation that would readily pass through the membrane. This is unlikely, as fusion proteins between FGF-2 and GFP are also able to secrete with no apparent loss of efficiency (Engling et al. 2002; Florkiewicz et al. 1995), and it is difficult to rationalize how the GFP fusion protein would participate in this process. The third possibility is that FGF-2 multimers are able to adopt a conformation that allows their passage through the membrane (Facchiano et al. 2003; Nickel et al. 2008). Detailed secretion pathways for FGF-2 and other unconventionally secreted proteins are currently being elucidated.

Enolase as an Unconventionally Secreted Protein

There are multiple examples of other, well studied cytoplasmic proteins that are often found in the extracellular space, however at this point their classification as "unconventionally secreted" proteins is still up for debate. Many of these proteins are highly expressed in the cytoplasm, and it is thought that their localization in the

extracellular space is simply due to small amounts of cell lysis (Klis et al. 2007). One of these proteins is enolase, a highly expressed cytoplasmic protein that is involved in the conversion of 2-phosphoglycerate to phosphoenolpyruvate. It has been found in the extracellular space in a number of organisms, including gram positive bacteria such as *Lactobacillus crispatus* (Antikainen et al. 2007) and *Listeria monocytogenes* (Schaumburg et al. 2004), the trematodes *Schistosoma mansoni* (Knudsen et al. 2005) and *Echinostoma caproni* (Marcilla et al. 2007), and in the yeast *Candida albicans* (Pitarch et al. 2004). Many of these studies have attempted to control for cell lysis in a variety of ways and have concluded that the presence of enolase in the extracellular space is not simply an artifact. Until the export mechanism is fully defined, its identity as an unconventionally secreted protein will be questioned.

In *C. albicans* and other pathogenic organisms, enolase expression and display on the cell surface has a role in disease progression. It has been shown that enolase is capable of binding to plasminogen, aiding in tissue invasion and increasing pathogenicity (Jong et al. 2003). Furthermore, an immune response against *C. albicans* enolase is indicative of survival and recovery from systemic candidiasis (Pitarch et al. 2004).

Enolase, along with a number of other proteins involved in glycolysis, has been shown to have a variety of different functions within the cell. These proteins have been termed moonlighting proteins (Gancedo and Flores 2008). Enolase has been ascribed a number of different activities in addition to its main role in the conversion of 2-phosphoglycerate to phosphoenolpyruvate. Eno2 has been shown to be required in the import of mitochondrial tRNA into the mitochondrion, while Eno1 was shown to perform this function as well, although with reduced efficiency (Entelis et al. 2006).

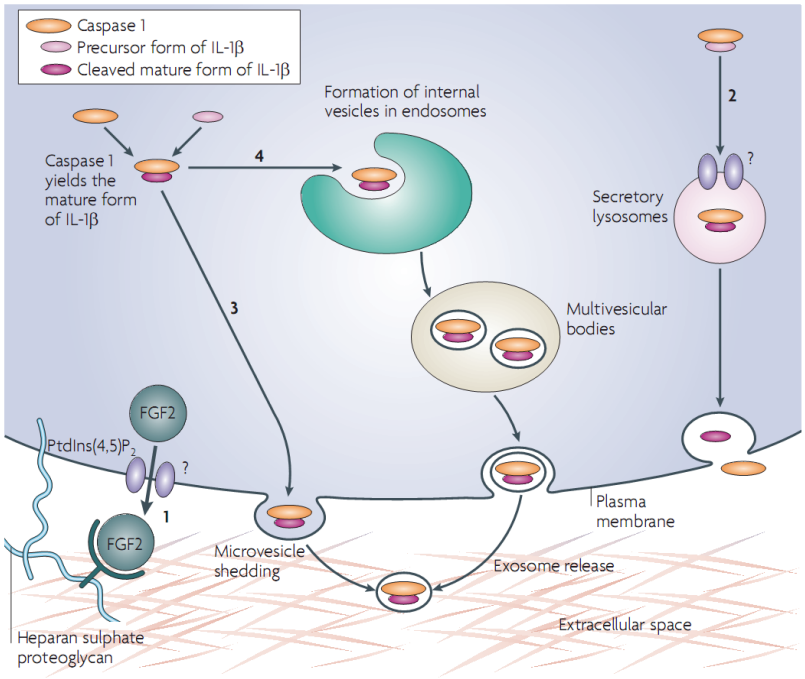

Figure 1-8-Potential mechanisms of unconventional cytoplasmic protein export. See text for details. Image from Nickel and Rabouille 2009.

This function was shown to be independent of the enzymes function in glycolysis, as a mutant with 1% glycolytic activity was still able to direct tRNA import.

A second activity that has been demonstrated for enolase is the homotypic fusion of vacuoles in *S. cerevisae*. The addition of recombinant, purified Eno1 or Eno2 in an *in vitro* vacuole fusion assay showed that enolase was able to stimulate fusion in a dose dependent manner (Decker and Wickner 2006). Enolase knockout strains also demonstrated a fragmented vacuole phenotype. As with mitochondrial tRNA import, this function was demonstrated to be independent of enzyme function (Decker et al. 2006). Other studies have shown that enolase is capable of binding to phospholipids (Zhu et al. 2001), an ability which could be crucial in both its moonlighting functions

and also for its ability to be secreted.

Enolase has been used in *S. cerevisae* to direct the secretion of both invertase and GFP to the extracellular space (López-Villar et al. 2006). In these experiments, invertase expressed intracellularly was unable to allow the growth of invertase deficient mutants on sucrose plates, while enolase fusions grew effectively, indicating that the fusion was being specifically released to the extracellular space and was not simply released by lysis. Furthermore, the ability to direct the extracellular localization was determined to reside within the N-terminal 169 amino acids. This data was confirmed with GFP fusions, which were shown to be localized to the plasma membrane through confocal microscopy. Neither fusion protein showed evidence of glycosylation, further indicating that secretion did not rely on the ER-golgi pathway. More research is needed into the unconventional secretion of enolase and other proteins to define their secretory pathway and to understand how and why this has evolved.

Conclusion

The impact of recombinant proteins in our everyday lives is only now starting to be realized. Although industrial applications such as enzymatic cleaners have been around for many years, new markets such as biofuel production are quickly emerging that will benefit from the fast and efficient production of purified proteins. The market for therapeutic proteins such as monoclonal antibodies is young and continues to grow at a fast pace. Purified proteins are needed in many aspects of basic scientific research to advance our current knowledge and to develop new applications for biologically derived material. All of these endeavors stand to benefit from increased research and understanding of the microbial factories central to protein production. Knowledge of the secretory pathway of *P. pastoris* will enable researchers to select

strains and growth conditions optimized to effectively secrete many difficult proteins.

BIBLIOGRAPHY

Antikainen, J., Kupannen, V., Lähteenmäki, K., and Korhonen, T. K. (2007). pH-Dependent Association of Enolase and Glyceraldehyde-3-Phosphate Dehydrogenase of Lactobacillus crispatus with the Cell Wall and Lipoteichoic Acids. J Bacteriol. *189*, 4539–4543.

Arvan, P., Zhao, X., Ramos-Castaneda, J., and Chang, A. (2002). Secretory pathway quality control operating in Golgi, plasmalemmal, and endosomal systems. Traffic *3*, 771-80.

Belden, W. J., and Barlowe, C. (2001). Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. Science *294*, 1528-31.

Benli, M., Döring, F., Robinson, D. G., Yang, X., and Gallwitz, D. (1996). Two GTPase isoforms, Ypt31p and Ypt32p, are essential for Golgi function in yeast. EMBO J *15*, 6460-75.

Brodsky, J. L., Goeckeler, J., and Schekman, R. (1995). BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum. Proc Natl Acad Sci U S A *92*, 9643-6.

Burri L., and Lithgow T. (2004). A Complete Set of SNAREs in Yeast. Traffic *5*, 45-52.

Cereghino, J. L., and Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast Pichia pastoris. FEMS Microbiol Rev *24*, 45-66.

Chen, Q., Jackson, H., Parente, P., Luke, T., Rizkalla, M., Tai, T. Y., Zhu, H., Mifsud, N. A., Dimopoulos, N., Masterman, K., et al. (2004). Immunodominant CD4+ responses identified in a patient vaccinated with full-length NY-ESO-1 formulated with ISCOMATRIX adjuvant. Proc Natl Acad Sci U S A *101*, 9363-8.

Chen, Y. A., and Scheller, R. H. (2001). SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol *2*, 98-106.

Chen, Y. T., Scanlan, M. J., Sahin, U., Türeci, O., Gure, A. O., Tsang, S., Williamson, B., Stockert, E., Pfreundschuh, M., and Old, L. J. (1997). A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci U S A *94*, 1914-8.

Clare, J. J., Rayment, F. B., Ballantine, S. P., Sreekrishna, K., and Romanos, M. A. (1991). High-level expression of tetanus toxin fragment C in Pichia pastoris strains containing multiple tandem integrations of the gene. Biotechnology (N Y) *9*, 455-60.

Cox, J. S., and Walter, P. (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell *87*, 391-404.

Cregg, J. M., Barringer, K. J., Hessler, A. Y., and Madden, K. R. (1985). Pichia pastoris as a host system for transformations. Mol Cell Biol *5*, 3376-85.

Cregg, J. M., Cereghino, J. L., Shi, J., and Higgins, D. R. (2000). Recombinant protein expression in Pichia pastoris. Mol Biotechnol *16*, 23-52.

Cregg, J. M., Madden, K. R., Barringer, K. J., Thill, G. P., and Stillman, C. A. (1989). Functional characterization of the two alcohol oxidase genes from the yeast Pichia pastoris. Mol Cell Biol *9*, 1316-23.

Cregg, J. M., Vedvick, T. S., and Raschke, W. C. (1993). Recent advances in the expression of foreign genes in Pichia pastoris. Biotechnology (N.Y.) *11*, 905-910.

Daly, R., and Hearn, M. T. W. (2005). Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production. J Mol Recognit *18*, 119-38.

Damasceno, L. M., Anderson, K. A., Ritter, G., Cregg, J. M., Old, L. J., and Batt, C. A. (2007). Cooverexpression of chaperones for enhanced secretion of a single-chain antibody fragment in Pichia pastoris. Appl Microbiol Biotechnol *74*, 381-9.

Damasceno, L. M., Pla, I., Chang, H., Cohen, L., Ritter, G., Old, L. J., and Batt, C. A. (2004). An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in Pichia pastoris. Protein Expr Purif *37*, 18-26.

Davis, I. D., Chen, W., Jackson, H., Parente, P., Shackleton, M., Hopkins, W., Chen, Q., Dimopoulos, N., Luke, T., Murphy, R., et al. (2004). Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans. Proc Natl Acad Sci U S A *101*, 10697-702.

Decker, B. L., and Wickner, W. T. (2006). Enolase activates homotypic vacuole fusion and protein transport to the vacuole in yeast. J Biol Chem *281*, 14523-8.

Egea, P. F., Stroud, R. M., and Walter, P. (2005). Targeting proteins to membranes: structure of the signal recognition particle. Curr Opin Struct Biol *15*, 213-20.

Engling, A., Backhaus, R., Stegmayer, C., Zehe, C., Seelenmeyer, C., Kehlenbach, A., Schwappach, B., Wegehingel, S., and Nickel, W. (2002). Biosynthetic FGF-2 is targeted to non-lipid raft microdomains following translocation to the extracellular surface of CHO cells. J Cell Sci *115*, 3619-3631.

Entelis, N., Brandina, I., Kamenski, P., Krasheninnikov, I. A., Martin, R. P., and Tarassov, I. (2006). A glycolytic enzyme, enolase, is recruited as a cofactor of tRNA targeting toward mitochondria in Saccharomyces cerevisiae. Genes Dev *20*, 1609-20.

Facchiano, A., Russo, K., Facchiano, A. M., De Marchis, F., Facchiano, F., Ribatti, D., Aguzzi, M. S., and Capogrossi, M. C. (2003). Identification of a novel domain of fibroblast growth factor 2 controlling its angiogenic properties. J Biol Chem *278*, 8751-60.

Fasshauer, D., Sutton, R. B., Brunger, A. T., and Jahn, R. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proceedings of the National Academy of Sciences of the United States of America *95*, 15781-15786.

Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. (1995). Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med *181*, 2109-17.

Florkiewicz, R. Z., Majack, R. A., Buechler, R. D., and Florkiewicz, E. (1995). Quantitative export of FGF-2 occurs through an alternative, energy-dependent, non-ER/Golgi pathway. J Cell Physiol *162*, 388-99.

Freedman, R. B., Hirst, T. R., and Tuite, M. F. (1994). Protein disulphide isomerase: building bridges in protein folding. Trends Biochem Sci *19*, 331-6.

Gancedo, C., and Flores, C. (2008). Moonlighting Proteins in Yeasts. Microbiol. Mol. Biol. Rev. *72*, 197-210.

Gjertsen, M. K., Bjorheim, J., Saeterdal, I., Myklebust, J., and Gaudernack, G. (1997). Cytotoxic CD4+ and CD8+ T lymphocytes, generated by mutant p21-ras (12Val) peptide vaccination of a patient, recognize 12Val-dependent nested epitopes present within the vaccine peptide and kill autologous tumour cells carrying this mutation. Int J Cancer *72*, 784-90.

Görlach, A., Klappa, P., and Kietzmann, T. (2006). The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxid Redox Signal *8*, 1391-418.

Halic, M., and Beckmann, R. (2005). The signal recognition particle and its interactions during protein targeting. Curr Opin Struct Biol *15*, 116-25.

Harding, H. P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature *397*, 271-4.

Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., et al. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell *11*, 619-33.

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell *10*, 3787-99.

Hohenblum, H., Gasser, B., Maurer, M., Borth, N., and Mattanovich, D. (2004). Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant Pichia pastoris. Biotechnol Bioeng *85*, 367-75.

Horiguchi, Y., Nukaya, I., Okazawa, K., Kawashima, I., Fikes, J., Sette, A., Tachibana, M., Takesako, K., and Murai, M. (2002). Screening of HLA-A24-restricted epitope peptides from prostate-specific membrane antigen that induce specific

antitumor cytotoxic T lymphocytes. Clin Cancer Res *8*, 3885-92.

Hudis, C. A. (2007). Trastuzumab -- Mechanism of Action and Use in Clinical Practice. N Engl J Med *357*, 39-51.

Ito, D., Visus, C., Hoffmann, T. K., Balz, V., Bier, H., Appella, E., Whiteside, T. L., Ferris, R. L., and DeLeo, A. B. (2007). Immunological characterization of missense mutations occurring within cytotoxic T cell-defined p53 epitopes in HLA-A*0201+ squamous cell carcinomas of the head and neck. Int J Cancer *120*, 2618-24.

Jäger, E., Gnjatic, S., Nagata, Y., Stockert, E., Jäger, D., Karbach, J., Neumann, A., Rieckenberg, J., Chen, Y. T., Ritter, G., et al. (2000). Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. Proc Natl Acad Sci U S A *97*, 12198-203.

Johnson, M. A., Snyder, W. B., Cereghino, J. L., Veenhuis, M., Subramani, S., and Cregg, J. M. (2001). Pichia pastoris Pex14p, a phosphorylated peroxisomal membrane protein, is part of a PTS-receptor docking complex and interacts with many peroxins. Yeast *18*, 621-41.

Jong, A. Y., Chen, S. H. M., Stins, M. F., Kim, K. S., Tuan, T., and Huang, S. (2003). Binding of Candida albicans enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. J Med Microbiol *52*, 615-622.

Kawakami, Y., Eliyahu, S., Sakaguchi, K., Robbins, P. F., Rivoltini, L., Yannelli, J. R., Appella, E., and Rosenberg, S. A. (1994). Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J Exp Med *180*, 347-52.

Klis, F. M., Jong, M. D., Brul, S., and Groot, P. W. J. D. (2007). Extraction of cell surface-associated proteins from living yeast cells. Yeast *24*, 253-258.

Knudsen, G. M., Medzihradszky, K. F., Lim, K., Hansell, E., and McKerrow, J. H. (2005). Proteomic Analysis of Schistosoma mansoni Cercarial Secretions. Mol Cell Proteomics *4*, 1862-1875.

Lederkremer, G. Z., Cheng, Y., Petre, B. M., Vogan, E., Springer, S., Schekman, R., Walz, T., and Kirchhausen, T. (2001). Structure of the Sec23p/24p and Sec13p/31p complexes of COPII. Proc Natl Acad Sci U S A *98*, 10704-9.

Li, F., Pincet, F., Perez, E., Eng, W. S., Melia, T. J., Rothman, J. E., and Tareste, D. (2007). Energetics and dynamics of SNAREpin folding across lipid bilayers. Nat Struct Mol Biol *14*, 890-896.

Liu, S., Chou, W., Sheu, C., and Chang, M. (2005). Improved secretory production of glucoamylase in Pichia pastoris by combination of genetic manipulations. Biochemical and Biophysical Research Communications *326*, 817-824.

López-Villar, E., Monteoliva, L., Larsen, M. R., Sachon, E., Shabaz, M., Pardo, M., Pla, J., Gil, C., Roepstorff, P., and Nombela, C. (2006). Genetic and proteomic evidences support the localization of yeast enolase in the cell surface. Proteomics *6 Suppl 1*, S107-18.

Marcilla, A., Pérez-García, A., Espert, A., Bernal, D., Muñoz-Antolí, C., Esteban, J. G., and Toledo, R. (2007). Echinostoma caproni: Identification of enolase in excretory/secretory products, molecular cloning, and functional expression. Experimental Parasitology *117*, 57-64.

Marston, F. A. (1986). The purification of eukaryotic polypeptides synthesized in Escherichia coli. Biochem J *240*, 1-12.

Matlack, K. E., Misselwitz, B., Plath, K., and Rapoport, T. A. (1999). BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane. Cell *97*, 553-64.

Misselwitz, B., Staeck, O., and Rapoport, T. A. (1998). J proteins catalytically activate Hsp70 molecules to trap a wide range of peptide sequences. Mol Cell *2*, 593-603.

Morsomme, P., and Riezman, H. (2002). The Rab GTPase Ypt1p and tethering factors couple protein sorting at the ER to vesicle targeting to the Golgi apparatus. Dev Cell *2*, 307-17.

Ng, D., Brown, J., and Walter, P. (1996). Signal sequences specify the targeting route

to the endoplasmic reticulum membrane. J Cell Biol *134*, 269-78.

Nickel, W., and Rabouille, C. (2009). Mechanisms of regulated unconventional protein secretion. Nat Rev Mol Cell Biol *10*, 148-155.

Nickel, W., and Seedorf, M. (2008). Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. Annu Rev Cell Dev Biol *24*, 287-308.

Nishimura, N., and Balch, W. E. (1997). A di-acidic signal required for selective export from the endoplasmic reticulum. Science *277*, 556-8.

Novick, P., Medkova, M., Dong, G., Hutagalung, A., Reinisch, K., and Grosshans, B. (2006). Interactions between Rabs, tethers, SNAREs and their regulators in exocytosis. Biochem Soc Trans *34*, 683-6.

Ortiz, D., Medkova, M., Walch-Solimena, C., and Novick, P. (2002). Ypt32 recruits the Sec4p guanine nucleotide exchange factor, Sec2p, to secretory vesicles; evidence for a Rab cascade in yeast. J Cell Biol *157*, 1005-15.

Otte, S., and Barlowe, C. (2004). Sorting signals can direct receptor-mediated export of soluble proteins into COPII vesicles. Nat Cell Biol *6*, 1189-94.

Pitarch, A., Abian, J., Carrascal, M., Sánchez, M., Nombela, C., and Gil, C. (2004). Proteomics-based identification of novel Candida albicans antigens for diagnosis of systemic candidiasis in patients with underlying hematological malignancies. Proteomics *4*, 3084-106.

Plath, K., and Rapoport, T. A. (2000). Spontaneous release of cytosolic proteins from posttranslational substrates before their transport into the endoplasmic reticulum. J Cell Biol *151*, 167-78.

Rapoport, T. A. (2007). Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. Nature *450*, 663-9.

Robinson, A. S., Hines, V., and Wittrup, K. D. (1994). Protein Disulfide Isomerase Overexpression Increases Secretion of Foreign Proteins in Saccharomyces cerevisiae. Nat Biotech *12*, 381-384.

Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol *8*, 519-29.

Sato, K., Sato, M., and Nakano, A. (2001). Rer1p, a retrieval receptor for endoplasmic reticulum membrane proteins, is dynamically localized to the Golgi apparatus by coatomer. J Cell Biol *152*, 935-44.

Sato, K., and Nakano, A. (2007). Mechanisms of COPII vesicle formation and protein sorting. FEBS Lett *581*, 2076-82.

Schaumburg, J., Diekmann, O., Hagendorff, P., Bergmann, S., Rohde, M., Hammerschmidt, S., Jänsch, L., Wehland, J., and Kärst, U. (2004). The cell wall subproteome of Listeria monocytogenes. Proteomics *4*, 2991-3006.

Schuldiner, M., Metz, J., Schmid, V., Denic, V., Rakwalska, M., Schmitt, H. D., Schwappach, B., and Weissman, J. S. (2008). The GET Complex Mediates Insertion of Tail-Anchored Proteins into the ER Membrane. Cell. *134*, 634–645.

Shen, J., Chen, X., Hendershot, L., and Prywes, R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. Dev Cell *3*, 99-111.

Subramani, S. (1998). Components involved in peroxisome import, biogenesis, proliferation, turnover, and movement. Physiol Rev *78*, 171-88.

Südhof, T. C., and Rothman, J. E. (2009). Membrane fusion: grappling with SNARE and SM proteins. Science *323*, 474-7.

Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature *395*, 347-53.

Tabi, Z., and Man, S. (2006). Challenges for cancer vaccine development. Advanced Drug Delivery Reviews *58*, 902-915.

Toikkanen, J., Miller, K., Soderlund, H., Jantti, J., and Keranen, S. (2003). The beta subunit of the Sec61p endoplasmic reticulum translocon interacts with the exocyst complex in Saccharomyces cerevisiae. Journal of Biological Chemistry *278*, 20946-20953.

Traversari, C., van der Bruggen, P., Luescher, I. F., Lurquin, C., Chomez, P., Van Pel, A., De Plaen, E., Amar-Costesec, A., and Boon, T. (1992). A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. J Exp Med *176*, 1453-7.

Tschopp, J. F., Sverlow, G., Kosson, R., Craig, W., and Grinna, L. (1987). High-level secretion of glycosylated invertase in the methylotrophic yeast, Pichia pastoris. Bio/technology *5*, 1305-1308.

Tuteja, R. (2005). Type I signal peptidase: an overview. Arch Biochem Biophys *441*, 107-11.

Van den Berg, B., Clemons, W. M., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., and Rapoport, T. A. (2004). X-ray structure of a protein-conducting channel. Nature *427*, 36-44.

Vassileva, A., Chugh, D. A., Swaminathan, S., and Khanna, N. (2001). Expression of hepatitis B surface antigen in the methylotrophic yeast Pichia pastoris using the GAP promoter. J Biotechnol *88*, 21-35.

Villaverde, A., and Mar Carrió, M. (2003). Protein aggregation in recombinant bacteria: biological role of inclusion bodies. Biotechnology Letters *25*, 1385-1395.

Vukmanovic-Stejic, M., Reed, J. R., Lacy, K. E., Rustin, M. H. A., and Akbar, A. N.

(2006). Mantoux Test as a model for a secondary immune response in humans. Immunol Lett *107*, 93-101.

Wang, R. F., Johnston, S. L., Zeng, G., Topalian, S. L., Schwartzentruber, D. J., and Rosenberg, S. A. (1998). A breast and melanoma-shared tumor antigen: T cell responses to antigenic peptides translated from different open reading frames. J Immunol *161*, 3598-606.

Waterham, H. R., Digan, M. E., Koutz, P. J., Lair, S. V., and Cregg, J. M. (1997). Isolation of the Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene *186*, 37-44.

Werten, M. W., van den Bosch, T. J., Wind, R. D., Mooibroek, H., and de Wolf, F. A. (1999). High-yield secretion of recombinant gelatins by Pichia pastoris. Yeast *15*, 1087-96.

Wurm, F. M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol *22*, 1393-8.

Ye, J., Rawson, R. B., Komuro, R., Chen, X., Davé, U. P., Prywes, R., Brown, M. S., and Goldstein, J. L. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell *6*, 1355-64.

Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., et al. (2001). Global analysis of protein activities using proteome chips. Science *293*, 2101-5.

Chapter 2-The Expression of the Cancer Testis Antigen NY-ESO-1 in *Pichia pastoris*

Abstract

NY-ESO-1 is currently one of the most attractive proteins for cancer immunotherapy. Expressed highly in a wide array of cancers, and limited to expression in testicular tissue in normal cells, it is an excellent marker for the immune system to use to differentiate between normal and cancerous cells. The expression of NY-ESO-1 in Escherichia coli has led to the production of protein that is currently being used in clinical trials, but there is a need for a secondary source of NY-ESO-1 for use in skin testing of vaccine recipients. The yeast *Pichia pastoris* was engineered to secrete NY-ESO-1 to the culture supernatant, but during its passage through the cell, it gets retained and is only seen when the cells are disrupted and solubilized with detergents or urea. Furthermore, the protein extracted from the cells in this fashion is not processed and is ~10 kDa larger than the wild type protein due to the retention of the α-mating factor secretion signal. This protein was prepared in a high cell density fermentation, and attempts to purify the protein by metal affinity chromatography were performed. It was discovered that the C-terminal 6X his-tag included to aid in purification was being removed within the cell by unknown processes. The C-terminal arginine was removed by site directed mutagenesis to delete a putative Kex2 protease site, and the new construct did indeed retain the his-tag. These results suggest that NY-ESO-1 is inserted into the membrane upon translocation into the ER. The topology of the protein is such that the C-terminus is within the ER/golgi lumen, where the his-tag is cleaved by Kex2 protease.

Introduction

An important aspect of vaccination is determining that the immune response seen in the patient is indeed in response to the vaccine itself and not any contaminants present. When vaccinating with a recombinant protein, it is vital that the patient elicit a

response to the recombinant protein and not contaminating proteins from the expression host. One way to measure the specificity of the response is by administering a delayed-type hypersensitivity (DTH) test (Davis et al. 2004). In this test, a small amount of protein derived from a different source than the original vaccine is injected directly under the dermis. A patient who has elicited a prior cellular immune response to the protein will demonstrate a response within 24-72 hours. Patients who have not been vaccinated will not respond. This test is commonly used to test for prior exposure to tuberculosis (Vukmanovic-Stejic et al. 2006).

Treatment of cancer using the patient's own immune system is showing promise, with new vaccination strategies being developed to stimulate the immune system to recognize tumors as non-self. To do this, it is necessary to identify markers that exist on the surface of tumors that are not present on normal tissue. One such marker that has been identified in a number of different tumor types is the protein NY-ESO-1 (Wang et al. 1998). This protein was originally discovered in esophageal cancer, and has shown promise as a vaccine in a number of Phase I clinical trials at extending the survival of cancer patients (Jäger et al. 2000; Chen et al. 2004; Davis et al. 2004). To date, NY-ESO-1 used in these trials has been derived from *Escherichia coli* expression systems, and it is important to verify that the immune response is to the NY-ESO-1 protein and not residual *E. coli* host protein. To achieve this, it is necessary that a source of NY-ESO-1 be developed that is produced in a different expression strain.

The methylotrophic yeast *Pichia pastoris* is an attractive protein expression system as it is simple to transform with a gene of interest, grows to very high cell densities, and its ability to secrete many proteins facilitates downstream purification (Cereghino and Cregg 2000). NY-ESO-1 derived from *P. pastoris* would make an ideal DTH reagent.

Responses to the *P. pastoris* DTH test would be a good indication that the immune response to NY-ESO-1 is real and not an artifact due to responses to *E. coli* derived contaminants. To that end, *P. pastoris* was transformed with NY-ESO-1 containing a C-terminal his-tag, the protein produced in a high-cell-density fermentation, and the protein purified using methods developed for *E. coli* derived NY-ESO-1 purification.

Materials and Methods

Plasmid Construction

The NY-ESO-1 gene was amplified by PCR from *E. coli* pET9a24a NY-ESO-1 plasmid previously prepared in our lab for *E. coli* NY-ESO-1 production. Primers were designed to add a 5' XhoI site and a 3' NotI site for cloning into pPICZαA (Invitrogen, Carlsbad, CA). After PCR amplification, the product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA) using the TOPO-TA cloning kit following manufacturer's instructions. Several successful transformants were selected and grown overnight in 2XYT medium and the plasmid DNA collected using the Qiaprep Spin Miniprep Kit (Qiagen, Valencia, CA). Sequencing was performed using T7 High Temp and M13 Reverse universal primers at the Cornell University BioResource Center. A plasmid with the proper sequence and the *P. pastoris* expression vector pPICZαA were digested with NotI and XhoI, run on a 1% (w/v) agarose gel, and the bands purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Ligation was carried out using the Quick Ligation Kit (New England Biolabs, Ipswich, MA) following manufacturers instructions. Ligation products were used to transform *E. coli* TOP10 and plated on LB supplemented with 25 µg/ml Zeocin (Invitrogen, Carlsbad, CA). A resistant transformant was again grown overnight and the plasmid collected.

P. pastoris Transformation

All *P. pastoris* transformations were carried out using the condensed transformation protocol described previously (Lin-Cereghino et al. 2005). Briefly, a 5 ml YPD (1% yeast extract, 2% peptone, 2% dextrose) overnight culture was used to inoculate 50 ml YPD to an OD_{600} of 0.1-0.2. After growth at 30°C to an OD_{600} of 0.8-1, the cells were harvested by centrifugation and resuspended in 9 ml BEDS buffer (10 mM bicine-NaOH, pH 8.3, 3% (v/v) ethylene glycol, 5% (v/v) dimethyl sulfoxide, 1 M sorbitol) supplemented with 1 ml 1 M dithiothreitol (DTT). After incubation for 5 min with gentle shaking at 30°C, the cells were again harvested by centrifugation and resuspended in 0.5 ml BEDS buffer. 40 µl aliquots of competent cells were mixed with 100 ng of plasmid that had been linearized with ClaI (New England Biolabs, Ipswich, MA) and purified with the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Cells were electroporated using a GenePulser II (BioRad Laboratories, Hercules, CA) with settings of 1500 V, 200 Ω, and 25 µF in a 2 mm gap electroporation cuvette, and immediately resuspended in 1 ml YPD supplemented with 1 M sorbitol. After 1-2 hr at 30°C, cells were plated on YPDS with 100 µg/ml Zeocin.

Fermentation

Expression was carried out as described, with modifications (Damasceno et al. 2004). Briefly, a 50 ml YPD culture of each strain was grown to an OD_{600} of 8-10 and used to inoculate a 2.5 L working volume New Brunswick BioFlo 3000 fermentor (New Brunswick Scientific Company, Edison, NJ) containing 1 L of modified basal salts medium (0.23 $g \cdot L^{-1}$ $CaSO_4$-$2H_2O$, 4.55 $g \cdot L^{-1}$ K_2SO_4, 3.73 $g \cdot L^{-1}$ $MgSO_4$-$7H_2O$, 10.3 $g \cdot L^{-1}$ KOH, 6.68 $ml \cdot L^{-1}$ H_3PO_4, 5% (v/v) glycerol) with 0.5 ml Antifoam 204. 15% (v/v) ammonium hydroxide was used to maintain the pH at 3.0 and also to serve as the nitrogen source. Dissolved oxygen (DO) was controlled at 40% with a combination of

increasing agitation and pure oxygen supplementation. After glycerol consumption during the batch phase, marked by a sharp increase in dissolved oxygen, a decreasing glycerol feed was initiated while the methanol probe was calibrated to transition the cells to methanol consumption. Methanol feeding was controlled using a closed-loop PID scheme to maintain the methanol level at 1.0 $g \cdot L^{-1}$ (Damasceno et al. 2004) or a methanol limited fed batch using methanol feed to control DO (Jahic et al. 2003). At this point, the temperature of the fermentation was also decreased to 15°C. Samples were taken periodically to determine OD and wet cell weight, as well as protein concentration. Clarified supernatant was prepared for SDS-PAGE by dilution with 4X LDS loading buffer (Invitrogen, Carlsbad, CA).

Cell Lysis and Tangential Flow Filtration

Fermentation broth was centrifuged at 4000 rpm in a swinging bucket centrifuge for 30 minutes and the supernatant discarded. The cell mass was resuspended in yeast breaking buffer (50 mM sodium phosphate pH 7.4, 1 mM EDTA, 5% (v/v) glycerol) in a Waring blender, and the cells lysed by five passes at 19000 psi in an Emulsiflex C3 microfluidizer (Avestin, Ottowa, ON). The lysate was washed with five volumes of 100 mM sodium phosphate, pH 7.4, by tangential flow filtration using a 0.2 µM Centramate Cassette (Pall, East Hills, NY).

Purification

One volume of washed lysate was diluted into 3 volumes of 8.75 M urea, 100 mM phosphate pH 7.5 and allowed to mix at room temperature for four hr. The solubilized material was then filtered through a 0.5 micron filter (Millipore, Billerica, MA) prior to chromatography. Immobilized metal affinity chromatography (IMAC) was carried out using an AKTA Explorer FPLC equipped with a 5 ml HisTrap FF column (GE Healthcare, Waukesha, WI). The column was equilibrated with IMAC Buffer A (4 M

urea, 100 mM phosphate pH 7.5). A step gradient with IMAC Buffer B (4 M urea, 100 mM phosphate pH 7.5, 0.5 M imidazole) was used for elution, with two steps at 75 mM imidazole (low imidazole) and 500 mM imidazole (high imidazole).

Analytical Methods

Cell lysates were run on SDS-PAGE and stained with Simply Blue Safe Stain (Invitrogen, Carlsbad, CA). Duplicate gels were blotted to nitrocellulose and probed with either monoclonal anti-NY-ESO-1 or anti-6X his-tag, alkaline phosphatase

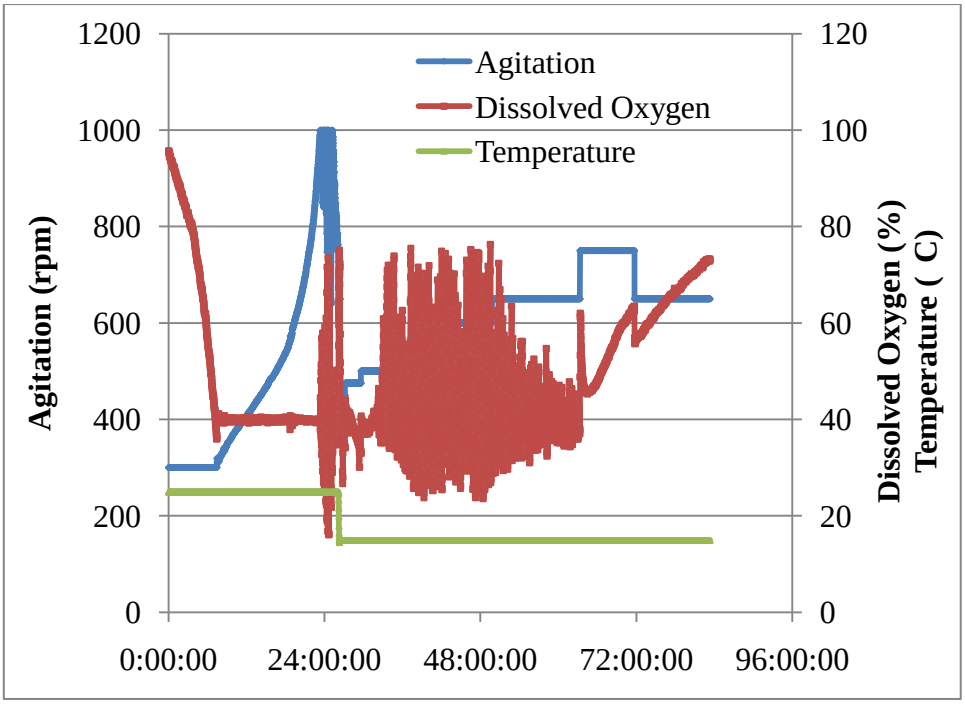

Figure 2-1-A fermentation profile for a methanol limited fed batch of *P. pastoris*. In this strategy, the methanol feed rate was adjusted to maintain dissolved oxygen concentration at 40%. After the initial DO spike at 24 hours, the temperature of the culture was dropped to 15°C, a glycerol feed was begun, and the methanol probe calibrated once 15°C was reached. After calibration, the glycerol feed was terminated and the methanol feed began. The pH of the culture was maintained at 3.0 by the addition of NH_4OH. The fermentation was terminated at 96 hours, at which point methanol consumption had slowed dramatically.

tagged antibodies (Sigma, St. Louis, MO). For LC-MS/MS, the band corresponding to NY-ESO-1 was visualized by coomassie staining of SDS-PAGE gels, excised and sent to the Cornell University BioResource Center for digestion with chymotrypsin and subsequent LC-MS/MS.

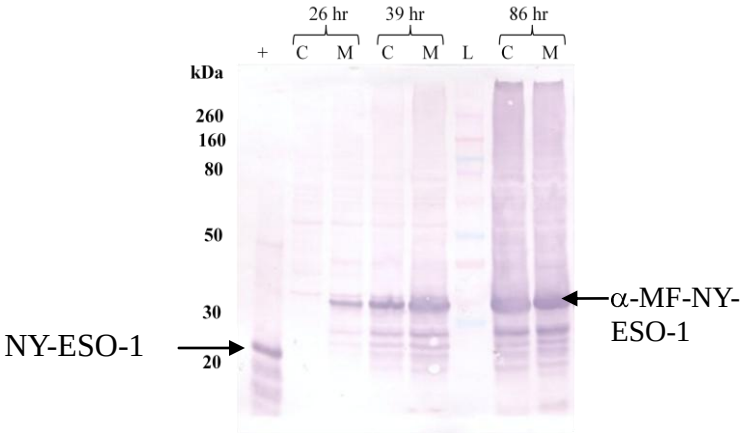

Figure 2-2-A methanol limited fed batch and control process were run concurrently and the intracellular, insoluble protein fraction collected at different time points to look for NY-ESO-1 expression. Protein fractions were run on SDS-PAGE and blotted to nitrocellulose membranes. The membranes were probed with anti-NY-ESO-1 monoclonal antibodies. Wild type NY-ESO-1 from *E. coli* migrates at ~22 kDa, while the NY-ESO-1 from *P. pastoris* migrates at ~32 kDa, reflecting the lack of α-mating factor (α-MF) signal peptide cleavage. **L**-Protein ladder. **+**-NY-ESO-1 standard. **C**-Control fermentation samples. **M**-MLFB fermentation samples.

Results

NY-ESO-1 Fermentation

In order to maximize NY-ESO-1 production in *P. pastoris* and minimize degradation, the fermentation process incorporates a limiting glycerol feed immediately after the

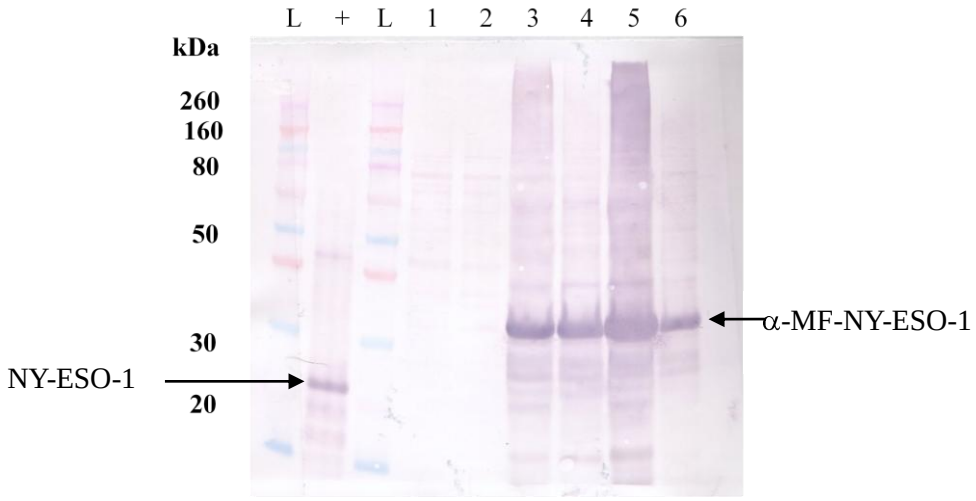

Figure 2-3-Initial downstream processing of NY-ESO-1 from *P. pastoris*. Cells from a high cell density fermentation were collected by centrifugation and resuspended in yeast breaking buffer. Lysis was done by five passes through a microfluidizer at 19,000 PSI, and the insoluble aggregates washed with 100 mM phosphate buffer (pH 7.4) by tangential flow filtration with a 0.2 µm PES filter. The lysate was solubilized in three volumes of 8.75 M urea overnight before filtration through a 0.5 µM filter prior to chromatography. Samples were run on SDS-PAGE, blotted to nitrocellulose, and probed with anti-NY-ESO-1 antibodies. **L**-Protein Marker. **+**-NY-ESO-1 positive control. **1**-Early TFF permeate. **2**-Late TFF permeate. **3**-Pre-solubilized lysate. **4**-Urea-solubilized lysate. **5**-SDS-solubilized lysate. **6**-Post-filtration.

initial batch phase to transition the cells to methanol consumption. This is done in combination with a decrease in temperature from 25°C to 15°C. These two changes minimize cell death upon methanol induction while enhancing the NY-ESO-1 content within the cells (data not shown). A typical fermentation profile is shown in Figure 2-1.

Despite the presence of the α-mating factor secretion signal on its N-terminus, NY-ESO-1 fails to secrete from the cell. Low levels of protein can be detected within the intracellular soluble fraction by western blotting, while the majority of the protein remains within the cell in an insoluble form. Previous data has shown that NY-ESO-1

colocalizes with the ER resident chaperone BiP, indicating that it enters the secretory pathway but is unable to be properly folded and secreted (data not shown). Removing the signal peptide prevents NY-ESO-1 accumulation in all cellular compartments. This could indicate that the insertion of NY-ESO-1 into the secretory pathway sequesters and protects the protein from degradation by intracellular proteases.

A time course study shows that NY-ESO-1 protein accumulates in the insoluble fraction over time, and the protein is larger than expected due to the lack of α-mating factor cleavage (Figure 2-2). Two different fermentation strategies were employed for the production of NY-ESO-1 in *P. pastoris* that differ in methanol feeding. The control method, in which methanol concentration is maintained at 1 gL^{-1}, has been very successful for the production of A33 scFv (Damasceno et al. 2004). The NY-ESO-1 yields from the intracellular insoluble fraction over time can be seen in Figure 2-2, Lane C. The second fermentation strategy uses the methanol feed to maintain the DO of the fermentation at 40%, and protein yields from this strategy can be seen in Figure 2-2, Lane M. This feeding strategy limits the concentration of methanol in the culture, which has been demonstrated to result in less cell death (Jahic et al. 2003). Both fermentation strategies yielded similar levels of NY-ESO-1 in the intracellular insoluble fraction at 86 hours. The MLFB strategy, however, had a more rapid accumulation of protein in the early stages of induction (Figure 2-2, 26 and 39 hours). Using the MLFB strategy, we were able to get sufficient quantities of protein for downstream purification studies.

Cell Lysis and Insoluble Fraction Washing

The location of the bulk NY-ESO-1 in the insoluble phase led to using a purification method developed for *E. coli* derived inclusion bodies. Cells were lysed with a microfluidizer, and the lysate was washed via tangential flow filtration using a 0.2 µM filter to remove soluble protein and other contaminants. Analysis of the filter

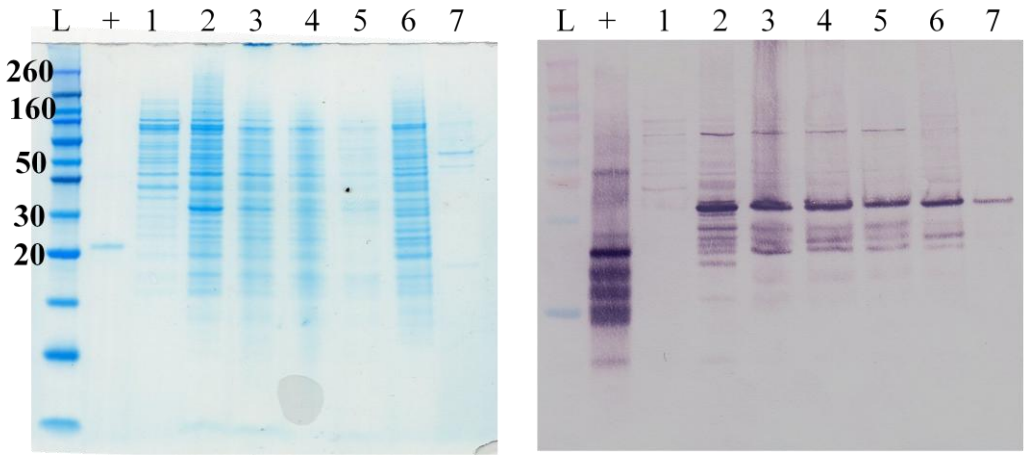

Figure 2-4-NY-ESO-1 purification by metal affinity chromatography. A 5 ml IMAC column was equilibrated with a 100 M phosphate, 4 M urea solution and 10 ml solubilized lysate loaded onto the column. The flow through, low imidazole, and high imidazole elutions were run on duplicate SDS-PAGE gels and stained with coomassie (left) or blotted to nitrocellulose and probed with anti-NY-ESO-1 antibodies. **L**-Protein Marker. **+**-NY-ESO-1 standard. **1**-TFF permeate. **2**- Pre-solubilized lysate. **3**-Post-solubilized lysate. **4**-Post-0.5 µM filtration/IMAC load. **5**-IMAC flow through. **6**-Low imidazole elution. **7**-High imidazole elution.

permeate by Western blotting shows that NY-ESO-1 does not pass through the membrane, as is seen with *E. coli* inclusion bodies (Figure 2-3, Lanes 1 and 2). Washing of the NY-ESO-1 aggregates by TFF removes many soluble contaminants (Figure 2-4, Lane 1), and also concentrates the protein to facilitate further downstream purification steps.

Solubilization and Immobilized Metal Affinity Chromatography

The washed lysate was mixed with three volumes of an 8.75 M urea, 100 mM phosphate buffer to solubilize NY-ESO-1 aggregates. After mixing for at least 4 hours, the solution was filtered through a 0.5 µM filter to remove any large particles. Filtration did result in a loss of NY-ESO-1 as shown by immunoblotting (Figure 2-3, lane 6), suggesting that lysis and solubilization methods are not sufficient to fully disrupt the cells and NY-ESO-1 aggregates. The inclusion of SDS in the solubilization of a small aliquot of the washed lysate did seem to support this conclusion as it appears to have higher yields of NY-ESO-1 (Figure 2-3, lane 5), however this material was clarified by centrifugation and not filtration, making this comparison difficult. The filtered, solubilized lysate was loaded onto a 5 ml HisTrap FF column, and the flow through and elution fractions analyzed. NY-ESO-1 was present in the flow through, as well as in the low imidazole fractions (Figure 2-4, Lanes 5 and 6). Trace amounts of NY-ESO-1 were seen in the high imidazole fraction (Figure 2-4, Lane 7). Binding to the IMAC was poor, and its gradual elution from the column in all fractions suggests that any interaction was non-specific.

NY-ESO-1 Does Not Contain the Histidine Tag

The NY-ESO-1 construct includes a C-terminal 6X his-tag to facilitate purification. IMAC chromatoagraphy shows that NY-ESO-1 binds to the column but simply washes out for the remainder of the run. This suggests that the his-tag is either not present in the final protein product or is obscured by the folding of the protein, and the

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVA

VLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKR↓EAEAMQAEGRGTGGS

TGDADGPGGPGIPDGPGGNAGGPGEAGATGGRGPRGAGAARASGPGG

GAPRGPHGGAASGLNGCCRCGARGPESRLLEFYLAMPFATPMEAELAR

RSLAQDAPPLPVPGVLLKEFTVSGNILTIRLTAADHRQLQLSISSCLQQLS

LLMWITQCFLPVFLAQPPSGQRRHHHHHH

Figure 2-5-LC-MS/MS analysis of *P. pastoris* derived NY-ESO-1. The NY-ESO-1 sequence including the N-terminal secretion signal and the C-terminal 6X histidine tag. The Kex2 cleavage site is indicated by ↓. Peptides detected by LC-MS/MS are highlighted.

binding seen is nonspecific in nature (Figure 2-4). Further evidence for this can be seen as NY-ESO-1 from *P. pastoris* not being detected by anti-6X his-tag antibodies (data not shown). Detection of NY-ESO-1 could also not be seen even with a 4X his antibody, suggesting that folding and aggregation of the protein may not be simply obscuring the his-tag. LC-MS/MS analysis of NY-ESO-1 detected no peptides containing the his-tag, but did detect a peptide that terminated directly upstream of the his-tag with the C-terminal –RR amino acids of the wild type NY-ESO-1 sequence (Figure 2-5).

Kex2 Processes the His-tag in the Golgi

LC-MS/MS results led to the hypothesis that the his-tag is getting cleaved at the –RR motif by the golgi resident Kex2 protease. To test this, one arginine residue was removed from the NY-ESO-1 sequence by PCR to generate NY-ESO-1ΔR180, and the new construct was transformed into *P. pastoris* KM71H. Five ml cultures were grown in BMM pH 6.0 for 48 hours at 15°C, the cells lysed with glass beads, and the intracellular, insoluble lysate dot-blotted onto nitrocellulose. Duplicate membranes were screened with anti-NY-ESO-1 and anti-6X his-tag antibodies, and a clone was identified that was reactive to both (data not shown). This clone was allowed to express for another 24 hours, and the intracellular insoluble extract analyzed by SDS-PAGE and western blotting with anti-NY-ESO-1 and anti-6X his-tag antibodies (Figure 2-6). Probing with anti-NY-ESO-1 antibodies detects protein from both cultures, while NY-ESO-1ΔR180 migrates slightly higher (Figure 2-6, B). Degradation of the NY-ESO-1 can be seen in both protein extracts, although the level of background staining is high due to overloading of the gel. As has been demonstrated before, the control NY-ESO-1 protein is undetectable by anti-6X his-tag antibodies, however NY-ESO-1ΔR180 is effectively detected, clearly demonstrating the maintenance of the his-tag on the protein (Figure 2-6, C).

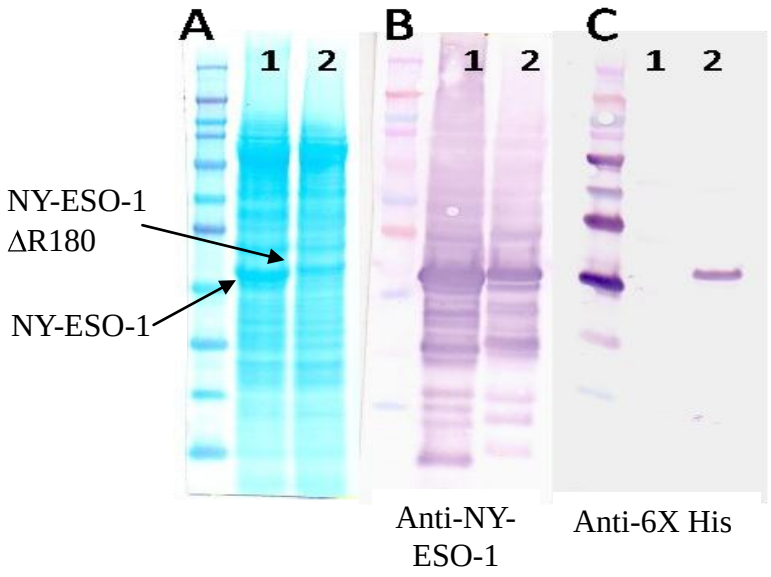

Figure 2-6-Five ml *P. pastoris* cultures of the (**1**) control NY-ESO-1 and (**2**) NY-ESO-1ΔR180 were grown in BMM pH 6.0 at 15°C for 72 hours. The intracellular insoluble lysates were run on SDS-PAGE and blotted onto duplicate nitrocellulose membranes and probed with either anti-NY-ESO-1 or anti-6x his antibodies. **A**-SDS-PAGE. **B**-Anti-NY-ESO-1 immunoblot. **C**-Anti-6x his tag immunoblot.

Discussion

The cancer antigen NY-ESO-1 has the potential to be an effective weapon in the fight against cancer. Its expression and purification from *E. coli* has been completed, and clinical trials are ongoing to determine the best vaccination strategy. While the secretion of this protein in *P. pastoris* has been unsuccessful, there is still potential for this expression system to produce a version of NY-ESO-1 that will be used as a skin test antigen. Skin testing will allow clinicians to quickly and easily identify responses to the *E. coli* derived vaccine.

Despite the presence of the α-mating factor secretion signal, NY-ESO-1 protein does not reach the extracellular space. It is also not found in the cytoplasm, but instead is

only liberated from the cells when they are disrupted with detergents, indicating it is either embedded in the membrane, in the lumen of intracellular organelles, or found as large insoluble aggregates within the cell, similar to bacterial inclusion bodies. Furthermore, N-terminal sequencing showed that the signal sequence is not removed, resulting in the final protein being ~10 kDa larger than expected (data not shown). Confocal microscopy had previously shown that NY-ESO-1 and the ER resident chaperone BiP colocalize within the cell (unpublished results). The resolution of this technique could not determine whether or not it was within the ER lumen or inserted within the membrane, however analysis of the amino acid sequence with the program SPLIT 4.0 predicts a strong transmembrane domain at the C-terminus of the protein (Juretić et al. 2002). Regardless of its localization, the α-mating factor is essential for the production of the protein, as its removal for intracellular expression prevented the accumulation of the protein (data not shown). Secretion signal recognition leads to the full length protein being sequestered and protected from degradation within the cell.

Previous work with NY-ESO-1 in *P. pastoris* showed that the culture must be maintained at 15°C for maximal protein production. Temperatures higher than this lead to little NY-ESO-1 accumulation. With intracellular expression, it is important to generate high levels of biomass, and at this temperature *P. pastoris* grows well, resulting in wet cell weights >250 gL^{-1}. Another factor shown to impact NY-ESO-1 production in this system is the type of feeding strategy. The typical fermentation strategy has the methanol concentration in the vessel controlled by an external methanol probe and pump to keep the concentration constant at 1 gL^{-1}. With NY-ESO-1, another strategy was implemented to maintain the methanol at the lowest possible concentration and further slow production of NY-ESO-1. A methanol limited fed batch (MLFB) was performed where methanol feeding was controlled by

dissolved oxygen (DO) concentration (Jahic et al. 2003). As methanol was consumed, dissolved oxygen would rise as the cells ran out of carbon. This initiated the feed pump, and as DO dropped, the pump slowed and eventually stopped. While this control was crude and resulted in noisy control of dissolved oxygen and agitation (Figure 2-1), NY-ESO-1 production was higher at 26 and 39 hours EFT (Figure 2-2). While the amount of NY-ESO-1 seen at the end of the fermentation was nearly the same in both the MLFB and control process, the use of the MLFB strategy would allow earlier termination of growth while maintaining high yield.

The location of NY-ESO-1 in the insoluble intracellular fraction was not unexpected, as the C-terminal portion of the protein is highly enriched with hydrophobic residues. In *E. coli* expression, the protein is found exclusively within inclusion bodies, and the purification requires high concentrations of urea to keep the protein soluble. As the final protein is used as a vaccine and does not require functional NY-ESO-1, the entire purification is carried out with 4 M urea. We chose to explore the possibility of adapting this purification strategy for use with this system. The first critical step is the washing of the insoluble fraction by tangential flow filtration. Experience with *E. coli* shows that the NY-ESO-1 inclusion bodies are unable to pass through a 0.2 µM PES filter, allowing the wash of inclusion bodies with Tris and phosphate buffers. This process removes many soluble proteins and concentrates the insoluble fraction. This method was shown to be effective for *P. pastoris* lysate, and no NY-ESO-1 was found in the TFF permeate (Figure 2-3, lanes 1 and 2).

Solubilization of the *P. pastoris* lysate was done as with *E. coli*, by diluting the washed lysate in 3 volumes of 8.75 M urea. Any remaining large particles after this step are removed by filtration through a 0.5 µM filter. Lysis and solubilization are two areas

where yields could be improved, as filtration did seem to remove NY-ESO-1 from the final preparation (Figure 2-3, Lane 6). This suggests that lysis could be inefficient, and visualization of *P. pastoris* lysate after five passes through the microfluidizer showed that the cells were still largely intact but dead as shown by staining with propidium iodide. The use of detergents in the lysis step could help to alleviate this problem, however this could lead to foaming during microfluidization.

Metal affinity chromatography is the primary purification step with *E. coli* derived NY-ESO-1, but with *P. pastoris* NY-ESO-1, binding to the IMAC column was poor, with only non-specific interactions occurring and the protein bleeding out throughout the column wash and elution. This observation prompted us to ensure the presence of the his-tag on the protein. Detection of the protein by western blotting with anti-his antibodies is very effective in *E. coli*, but in this work no detection occurred, however the use of anti-NY-ESO-1 efficiently detected the protein. An example in the literature with erythropoietin (EPO) suggested that the his-tag may be buried within the protein and undetectable with antibodies specific for a 6X his-tag (Debeljak et al. 2006). The use of antibodies specific to only four histidines was shown to detect EPO, however with this antibody there was still no detection of NY-ESO-1. Sequencing of the expression cassette from the *P. pastoris* genome confirmed that the his-tag was present in the coding sequence. The protein was sent for LC-MS/MS, which showed that the his-tag was indeed not present in the final protein, however a fragment directly upstream of the his-tag was detected.

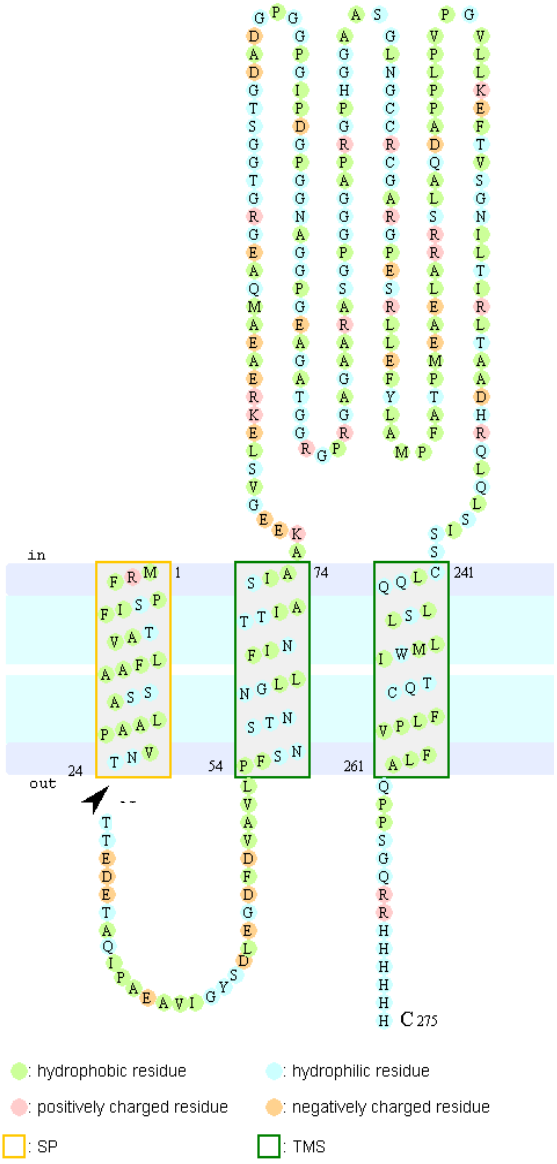

Figure 2-7-Membrane protein topology analysis of NY-ESO-1 from *P. pastoris*. The full length NY-ESO-1 sequence containing the α-mating factor signal peptide was analyzed by CONPRED II to determine membrane topology. The cytoplasmic side of the membrane is designated by "in." The location of the –RR-6XHis motif is predicted to be on the "out" side of the membrane, or within the ER/golgi lumen, where it would be susceptible to processing by Kex2 protease. The black arrow designates the predicted cleavage site for signal peptidase. N terminal sequencing of the wild type NY-ESO-1 indicates that signal peptidase is unable to recognize and remove the pre region of the α-mating factor. http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/

The final two amino acids of the wild type NY-ESO-1 protein are both arginine. We speculated that the his-tag is being cleaved after these residues by the golgi resident protease Kex2, which will recognize dibasic sequences such as –RR, albeit with 2-fold lower efficiency than with the consensus –KR sequence (Bevan et al. 1998). It is possible that the protein is being inserted into the membrane in such a way that the C-terminal his-tag is exposed to Kex2 within the golgi, but the N-terminal α-mating factor is not. The full length NY-ESO-1 sequence, with the attached α-mating factor, was analyzed by the web based topology prediction program CONPRED II (Figure 2-7) (Arai et al. 2004). The output from this program shows that NY-ESO-1 has two transmembrane spanning domains, one within the signal peptide and the other at the C-terminal end. The predicted orientation for this protein has the C-terminal end within the ER/golgi lumen, and thus susceptible to Kex2 degradation. The Kex2 sequence located within the signal peptide in this program is shown to be located within the cytoplasm, possibly explaining the lack of signal peptide cleavage. To test this, one arginine was removed by site directed mutagenesis and the new construct transformed into *P. pastoris* KM71H. A clone expressing NY-ESO-1, again in the intracellular insoluble fraction, was isolated and expressed in shake flask cultures. As with the previous strains, there was no processing of the signal peptide, however the protein lacking the C-terminal arginine residue did indeed contain the 6X his-tag as determined by immunoblotting, supporting the hypothesis that Kex2 protease is removing the his-tag. This strongly suggests that instead of forming insoluble aggregates, NY-ESO-1 is inserted into the ER membrane. It is interesting to note that in humans, NY-ESO-1 localizatoin has been shown to be cytoplasmic in both tumor and testis tissue (Gnjatic et al. 2006). Lacking any retention sequence, it likely traffics throughout the cell, coming in contact with Kex2 within the golgi. Further experiments with confocal and electron microscopy to better characterize the

localization of this protein within the cell are warranted. Future work to scale up the expression of this molecule and to adapt the *E. coli* based NY-ESO-1 purification scheme to this molecule is necessary to produce a viable reagent for skin testing.

BIBLIOGRAPHY

Arai, M., Mitsuke, H., Ikeda, M., Xia, J., Kikuchi, T., Satake, M., and Shimizu, T. (2004). ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. Nucleic Acids Res *32*, W390-393.

Bevan, A., Brenner, C., and Fuller, R. S. (1998). Quantitative assessment of enzyme specificity in vivo: P2 recognition by Kex2 protease defined in a genetic system. Proc Natl Acad Sci U S A. *95*, 10384–10389.

Cereghino, J. L., and Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast Pichia pastoris. FEMS Microbiol Rev *24*, 45-66.

Chen, Q., Jackson, H., Parente, P., Luke, T., Rizkalla, M., Tai, T. Y., Zhu, H., Mifsud, N. A., Dimopoulos, N., Masterman, K., et al. (2004). Immunodominant CD4+ responses identified in a patient vaccinated with full-length NY-ESO-1 formulated with ISCOMATRIX adjuvant. Proc Natl Acad Sci U S A *101*, 9363-8.

Damasceno, L. M., Pla, I., Chang, H., Cohen, L., Ritter, G., Old, L. J., and Batt, C. A. (2004). An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in Pichia pastoris. Protein Expr Purif *37*, 18-26.

Davis, I. D., Chen, W., Jackson, H., Parente, P., Shackleton, M., Hopkins, W., Chen, Q., Dimopoulos, N., Luke, T., Murphy, R., et al. (2004). Recombinant NY-

ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans. Proc Natl Acad Sci U S A *101*, 10697-702.

Debeljak, N., Feldman, L., Davis, K. L., Komel, R., and Sytkowski, A. J. (2006). Variability in the immunodetection of His-tagged recombinant proteins. Anal Biochem *359*, 216-23.

Gnjatic, S., Nishikawa, H., Jungbluth, A. A., Güre, A. O., Ritter, G., Jäger, E., Knuth, A., Chen, Y., and Old, L. J. (2006). NY-ESO-1: review of an immunogenic tumor antigen. Adv. Cancer Res *95*, 1-30.

Jäger, E., Gnjatic, S., Nagata, Y., Stockert, E., Jäger, D., Karbach, J., Neumann, A., Rieckenberg, J., Chen, Y. T., Ritter, G., et al. (2000). Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. Proc Natl Acad Sci U S A *97*, 12198-203.

Jahic, M., Wallberg, F., Bollok, M., Garcia, P., and Enfors, S. (2003). Temperature limited fed-batch technique for control of proteolysis in Pichia pastoris bioreactor cultures. Microbial Cell Factories *2*, 6.

Juretić, D., Zoranić, L., and Zucić, D. (2002). Basic charge clusters and predictions of membrane protein topology. J Chem Inf Comput Sci *42*, 620-632.

Lin-Cereghino, J., Wong, W. W., Xiong, S., Giang, W., Luong, L. T., Vu, J., Johnson,

S. D., and Lin-Cereghino, G. P. (2005). Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris. Biotechniques. *38*, 44–48.

Vukmanovic-Stejic, M., Reed, J. R., Lacy, K. E., Rustin, M. H. A., and Akbar, A. N. (2006). Mantoux Test as a model for a secondary immune response in humans. Immunol Lett *107*, 93-101.

Wang, R. F., Johnston, S. L., Zeng, G., Topalian, S. L., Schwartzentruber, D. J., and Rosenberg, S. A. (1998). A breast and melanoma-shared tumor antigen: T cell responses to antigenic peptides translated from different open reading frames. J Immunol *161*, 3598-606.

Chapter 3-The Cooverexpression of A33 scFv and the *S. cerevisae* SNARE proteins Sso1 and Snc2 in *Pichia pastoris*

Abstract

The secretion of A33 scFv by *Pichia pastoris* was shown to be enhanced by the cooverexpression of the *Saccharomyces cerevisae* SNARE protein Sso1. *P. pastoris* has proven to be capable of secreting large quantities of heterologous proteins to the culture medium in its active state. The simple medium and low levels of host proteins that are secreted into the supernatant facilitate downstream purification. Efforts to improve secretory yields have mainly focused on optimizing culture conditions, but more recent work has focused on modulating the levels of proteins involved in the secretory pathway. The *S. cerevisae* SNARE proteins Snc2 and Sso1 were cloned and expressed in *P. pastoris* GS115 that is also expressing the single chain antibody fragment targeting the colonic epithelium surface protein A33. Neither Snc1 or Sso1 impaired growth, however Snc2 overexpression was shown to inhibit A33 scFv secretion in 25 ml shake flask cultures. In a high cell density fermentation, Snc2 expression did not have any effect on A33 scFv yields. Sso1 overexpression improved the secretion of A33 scFv by 31% in shake flask cultures, while in a high cell density fermentation, Sso1 enhanced secretion by 80% after 36 hours of fermentation. These two SNARE proteins were then expressed in a *P. pastoris* strain which expresses both the A33 scFv and also the ER resident chaperone BiP. The growth of triple transformants with A33 scFv, BiP and either Snc2 or Sso1 was impaired, and they secreted 43% and 34% less A33 scFv on a per cell basis, respectively.

Introduction

The expression of heterologous proteins in the methylotrophic yeast *Pichia pastoris* continues to gain in popularity due to its ease of use and high protein yields. One of the more attractive aspects of the system is the ability to secrete large quantities of active protein, which becomes even more desirable when one considers the low

secretion levels of host proteins. Once the cells are removed, the product is highly enriched, facilitating further downstream purification. Success in terms of a viable method for producing a heterologous protein highlights the importance of studying and improving the secretion of heterologous proteins in this expression platform.

There have been a number of strategies implemented to enhance protein production in a variety of microbial hosts. In yeast, there has been a focus on the overexpression of chaperones in addition to the recombinant protein (Damasceno et al. 2007; Shusta et al. 1998; Inan et al. 2006). This strategy is based on the assumption that the basal level of chaperones normally present in the cell is insufficient to properly fold the high levels of protein present when overexpressing a heterologous molecule. This can especially be true in *P. pastoris*, where the most common promoter used to drive expression, AOX1, is highly induced when exposed to methanol (Tschopp et al. 1987; Ellis et al. 1985). The overexpression of the chaperone Immunoglobulin Binding Protein (BiP) has successfully enhanced the secretion yield of A33 scFv from *P. pastoris* (Damasceno et al. 2007), and has also been helpful in the expression of hirudin in *S. cerevisae* (Kim et al. 2003). Other strategies to improve secreted protein yield have focused on the overexpression of proteins involved at critical bottlenecks in the protein trafficking pathway. In *P. pastoris*, the overexpression of the Rab GTPase Sec4, involved in secretory vesicle docking at the plasma membrane, has been shown to enhance the secretion of glucoamylase (Liu et al. 2005). Other proteins acting throughout the secretory pathway could also have a role in enhancement of the secretory capacity, and we chose to test two SNARE proteins critical for membrane fusion with the plasma membrane, Snc2 and Sso1.

SNARE proteins form a complex called a SNAREpin, with four individual SNARE

proteins involved in the generation of a coiled coil structure. SNAREpin formation is thought to provide the energy required to overcome the repulsive forces generated when the membranes fuse (Chen and Scheller 2001). In *S. cerevisae*, the SNAREpin at the plasma membrane is composed of one molecule each of Snc2 and Sso1, and two copies of Sec9 (Burri L. and Lithgow T. 2004). Overexpression of the *Kluyveromyces lactis* SNARE protein Sso1 in *S. cerevisae* has been shown to enhance the secretion of α-amylase more than two fold (Toikkanen et al. 2004). For this work, the *S. cerevisae* Snc2 and Sso1 genes were cloned and placed under the control of the Pex8 methanol inducible promoter and transformed into *P. pastoris* secreting A33 scFv, an antibody fragment useful for targeting colon cancer cells, to examine their ability to enhance secretion of this clinically useful molecule. Sso1 cooverexpression was shown to enhance A33 scFv yields in both shake flask cultures and high cell density fermentation, while Snc2 impaired protein secretion yields in shake flask cultures. The cooverexpression of both of these proteins concurrently with the ER resident chaperone BiP and the A33 scFv led to a decrease in growth rates, and a decrease in A33 scFv yield on a per cell basis.

Materials and Methods

Strains and growth conditions

All cloning was carried out using *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) according to manufacturers specifications. The Snc2 and Sso1 genes were amplified by PCR from *S. cerevisae* genomic DNA. *P. pastoris* GS115 carrying the A33 scFv gene in a Mut+ background were prepared as described (Pla et al. 2006). GS200 cells

Table 3-1-Primers used in vector construction. EcoRI and NotI restriction sites are underlined.

Primer	Sequence
Sso1 Forward	5'-GAATTCACGATGAGTTATAATAATCCGTAC-3'
Sso1 Reverse	5'-GCGGCCGCTTAACGCGTTTTGACAACGGCTGGGACAA-3'
Snc2 Forward	5'-GAATTCACGATGTCGTCATCAGTGCCATAC-3'
Snc2 Reverse	5'-GCGGCCGCTTAGCTGAAATGGACGACGATAGGAACGAT-3'

harboring the chaperone BiP under the control of the AOX1 promoter were prepared as described (Damasceno et al. 2007).

Plasmid Construction

The Snc2 and Sso1 genes (GenBank accession numbers L16243.1 and X67729.1, respectively) were amplified by PCR from *S. cerevisae* genomic DNA. Primers were designed to add a 5' EcoRI site and a 3' NotI site for cloning into pJS1 (Johnson et al. 2001). Primers used are indicated in Table 3-1. After PCR amplification, the product was cloned into pCR2.1 (Invitrogen) using the TOPO-TA cloning kit following manufacturers instructions. Several successful transformants were selected and grown

overnight in 2XYT medium and the plasmid DNA collected using the Qiaprep Spin

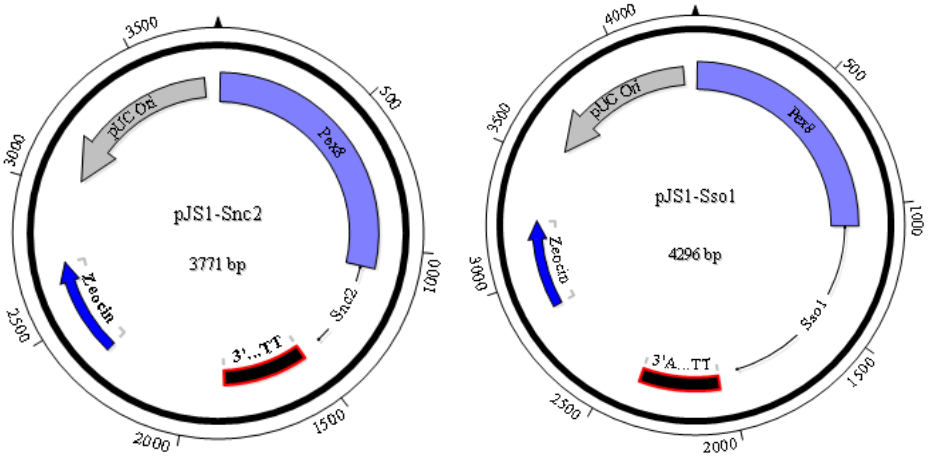

Figure 3-1-Plasmids used in this study.

Miniprep Kit (Qiagen, Valencia, CA). Sequencing was performed using T7 High Temp and M13 Reverse universal primers at the Cornell University BioResource Center. A plasmid with the proper sequence and the *P. pastoris* expression vector pJS1 were digested with EcoRI and NotI, run on a 1% (w/v) agarose gel, and the bands purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Ligation was carried out using the Quick Ligation Kit (New England Biolabs, Ipswich, MA) following manufacturers instructions. Ligation products were used to transform *E. coli* TOP10 and plated on Luria broth supplemented with 25 µg/ml Zeocin (Invitrogen, Carlsbad, CA). A Zeocin resistant transformant was again grown overnight and the plasmid DNA collected.

***P. pastoris* Transformation**

All *P. pastoris* transformations were carried out using the condensed transformation protocol described previously (Lin-Cereghino et al. 2005). Briefly, a 5 ml YPD (1% yeast extract, 2% peptone, 2% dextrose) overnight culture was used to inoculate 50 ml

YPD to an OD_{600} of 0.1-0.2. After growth at 30°C to an OD_{600} of 0.8-1, the cells were harvested by centrifugation and resuspended in 9 ml BEDS buffer (10 mM bicine-NaOH, pH 8.3, 3% (v/v) ethylene glycol, 5% (v/v) dimethyl sulfoxide, 1 M sorbitol) supplemented with 1 ml 1 M dithiothrietol (DTT). After incubation for 5 min with gentle shaking at 30°C, the cells were again harvested by centrifugation and resuspended in 0.5 ml BEDS buffer. 40 µl aliquots of competent cells were mixed with 100 ng of the appropriate plasmid that had been linearized with Cla1 (New England Biolabs, Ipswich, MA) and purified with the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Cells were electroporated using a GenePulser II (BioRad Laboratories, Hercules, CA) with settings of 1500 V, 200 Ω, and 25 µF in a 2 mm gap electroporation cuvette, and immediumtely resuspended in 1 ml YPD supplemented with 1 M sorbitol. After 1-2 hr at 30°C, cells were plated on YPDS with 100 µg/ml Zeocin.

Small Scale Expression

After 2-3 days, several transformants were picked and analyzed for A33 scFv secretion. For small scale expression, 5 ml YPD cultures were grown overnight in YPD at 25°C and diluted the next day in 25 ml MD medium (1.34% yeast nitrogen base, $4x10^{-5}$% biotin, 2% dextrose) to an OD_{600} of 0.1-0.5. Growth was allowed to proceed to an OD_{600} of 1-5, the cells harvested by centrifugation, and resuspended in the same volume of MM medium (1.34% yeast nitrogen base, $4x10^{-5}$% biotin, 0.5% methanol) and transferred to 250 ml baffled Erlenmeyer flasks. Cultures were supplemented with 100% methanol to 0.5% (v/v) every 12 hr. Samples were centrifuged at 13000 rpm and the supernatants and cell pellets frozen at -20°C for further analysis.

Fermentation

High cell density fermentation was carried out as described, with minor modifications (Damasceno et al. 2004). Briefly, a 50 ml YPD culture of each strain was grown to an OD_{600} of 8-10 and used to inoculate a 2.5 L working volume New Brunswick BioFlo 3000 fermentor (New Brunswick Scientific Company, Edison, NJ) containing 1 L of modified basal salts medium (0.23 $g \cdot L^{-1}$ $CaSO_4$-$2H_2O$, 4.55 $g \cdot L^{-1}$ K_2SO_4, 3.73 $g \cdot L^{-1}$ $MgSO_4$-$7H_2O$, 10.3 $g \cdot L^{-1}$ KOH, 6.68 $ml \cdot L^{-1}$ H_3PO_4, 5% (v/v) glycerol) with 0.5 ml Antifoam 204. 15% (v/v) ammonium hydroxide was used to maintain the pH at 3.0 and also to serve as the nitrogen source. Dissolved oxygen (DO) was controlled at 40% with a combination of regulating impeller speed and pure oxygen supplementation. After glycerol consumption during the batch phase, which was marked by a sharp increase in dissolved oxygen, methanol feeding was initiated using a closed-loop PID control scheme to maintain the methanol level at 1.0 $g \cdot L^{-1}$ (Damasceno et al. 2004). Samples were taken periodically to determine OD_{600} and wet cell weight, as well as protein concentration.

Protein Methods

Clarified supernatant was prepared for SDS-PAGE by dilution with 4X LDS loading buffer (Invitrogen, Carlsbad, CA). Ten µl samples were loaded on 12% bis-tris gels and run at 100 V in MES buffer for 90 minutes. Gels were stained either using coomassie (SimplyBlue Safe Stain, Invitrogen, Carlsbad, CA) or by silver staining (SilverQuest Staining Kit, Invitrogen, Carlsbad, CA) following manufacturers instructions. Gels were dried using a DryEase gel drying kit (Invitrogen, Carlsbad, CA). Gels were scanned, and the images were analyzed for image densitometry analysis using ImageJ (NIH, Bethesda, MD).

Real Time RT-PCR

An aliquot of cells corresponding to an OD_{600} of 1.0 was centrifuged and the mRNA was harvested using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer's instructions. Cells were lysed through mechanical disruption using acid washed glass beads. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA). A total of approximately100 ng of RNA was applied to each well of a 96 well rtPCR plate and a 2X stock of SYBR Green Master Mix (Sigma, St. Louis, MO) and the appropriate primers was added to each well. Primer sequences are listed in Table 3-2. Amplification was carried out using an ABI Prism 7000 Real Time Thermocycler (Life Technologies, Carlsbad, CA). Data analysis was performed using ABI Prism 7000 SDS Software.

Results

Table 3-2-Primers used for rtPCR.

Primer	Sequence
AOX1 Reverse	5'-AAGTCCTTGGTTTTCCAGCCCTCGGC-3'
AOX1 Forward	5'-GAGCCATTGTTCCATGTGCTAACGTCTTGG-3'
Act1 Forward	5'-CCCACACACAGTGTTCCCATCGGTCGT-3'
Act1 Reverse	5'-GGTGCCAGATCTTTTCCATATCGTCCCAGT-3'
Snc2 Forward	5'-TATGTGCCTCCAGAGGAGAGTAA-3'
Snc2 Reverse	5'-CTCAATGGATGTTAGCCTTTCAC-3'
scFv Forward	5'-TGGAGATCAAAGGTGGTTCC -3'
scFv Reverse	5'-GACTGAAGCCGATTCCAGAG-3'

Overexpression of Snc2 in a Mut+ scFv Strain

A Mut+ strain of *P. pastoris* expressing A33 scFv was transformed with the *S. cerevisae* gene encoding the V-SNARE Snc2 under the control of the methanol

inducible promoter Pex8. This promoter allows induction on methanol, but at levels far less than the AOX1 promoter that was used to drive the A33 scFv expression. Image densitometry analysis of silver stained SDS-PAGE gels demonstrated that

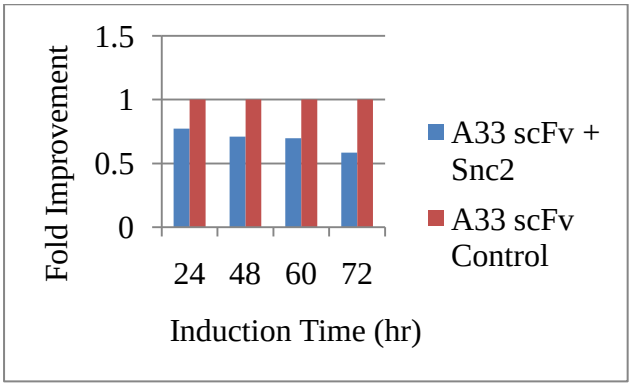

Figure 3-2-The effect of Snc2 cooverexpression on A33 scFv yields. Mut+ strains of *P. pastoris* harboring the A33 scFv gene with and without the *S. cerevisae* Snc2 gene were grown in 25 ml MM medium for 72 hours. Supernatants were collected, run on SDS-PAGE, and silver stained. Gels were analyzed for scFv yield using image densitometry and the band intensities compared to the control to determine fold improvement.

overexpression of Snc2 led to a decrease over time in A33 scFv yield in the supernatant (Figure 3-2). After only 24 hours of induction, there was a 23% decrease in A33 scFv yield in the culture supernatant. This value decreased further over time, with the 72 hour sample showing a 49% loss of A33 scFv compared to the control. Growth curves of all cultures showed that the decrease was not due to difference in cell growth.

High Cell Density Fermentation

An Snc2 overexpressing strain was chosen for high cell density fermentation. Previous work with the A33 scFv demonstrated that the protein is highly susceptible to degradation at pH 6, so all fermentations were done at pH 3.0 and 25°C (Damasceno

et al. 2004). When analyzed by SDS-PAGE, both the Snc2 overexpressing strain and the control strain had nearly identical A33 scFv yields after 36 hours of induction. As was seen in the shake flask experiments, the growth of the Snc2 overexpressing strain was not impaired by the presence of the additional *S. cerevisae* gene. OD_{600} values for

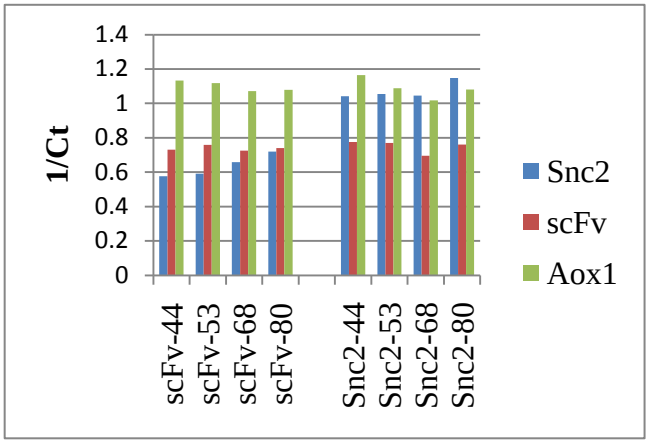

Figure 3-3-The expression of Snc2, A33 scFv, and AOX1 in strains of *P. pastoris*. RNA samples from various time points during the fermentation of *P. pastoris* GS115 expressing A33 scFv with and without cooverexpression of Snc2 were analyzed by SYBR Green real-time rtPCR. Primers against Snc2, A33 scFv, Aox1, and Act1 were designed, and Ct values were standardized against Act1.

both the Snc2 and control strain peaked at ~400, and the wet cell weight for both cultures reached ~450 gL^{-1}.

Real time RT-PCR

As the inhibition seen in the shake flask cultures was not seen during fermentation, real time RT-PCR was performed to determine Snc2 expression levels in the fermentation. Amplification was carried out using primers specific to Snc2 and A33 scFv, with control primers for Aox1 and Act1. Expression levels for scFv, Act1, and

Aox1 were similar in both cultures, and only the Snc2 culture had expression of the Snc2 gene, as expected (Figure 3-3). Despite the efficient use of methanol for the

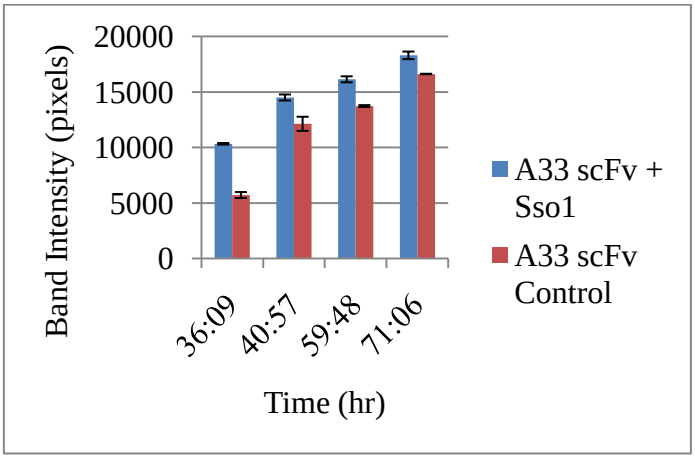

Figure 3-4-The effect of Sso1 on A33 scFv yield. *P. pastoris* GS115 cooverexpressing A33 scFv with Sso1 was grown in high cell density fermentation. Aliquots of culture supernatant were adjusted for culture OD_{600} and prepared for SDS-PAGE. Coomassie stained gels were scanned and analyzed using image densitometry.

duration of the fermentation, however, expression yields of A33 scFv and alcohol oxidase decreased over time. Actin expression also decreased. Snc2 expression levels in the Snc2 culture remained high throughout. When normalized for Actin Ct, however, the expression levels of A33 scFv and Aox1 appear to be constant.

Sso1 Overexpression

Sso1 overexpression in shake flasks was performed to ascertain its effect on A33 scFv secretory yields. 25 ml shake flask cultures of several transformants showed that A33 scFv yields were improved by the cooverexpression of Sso1 (data not shown). One representative clone was selected for analysis in small scale fermentation. In contrast with Snc2, overexpression of Sso1 in a high cell density fermentation resulted in enhancement of A33 scFv yield by 81% at 36 hours elapsed fermentation time as

determined by comparison of coomassie stained gels by image densitometry(Figure 3-4). The enhancement remained throughout the course of the fermentation, dropping to 10% at the end of the fermentation at 71 hours.

Sso1 or Snc2 Cooverexpression with BiP

As Sso1 and Snc2 operate at the terminal end of the secretory pathway, it was hypothesized that any enhancements in secretory vesicle trafficking would not be apparent if there was a bottleneck earlier in the pathway. To test this, Sso1 or Snc2 were used to transform a strain of *P. pastoris* GS200 harboring the ER resident chaperone BiP under the control of the AOX1 promoter. Earlier work demonstrated that this strain had an approximately three fold increase in secreted A33 scFv levels compared to controls (Damasceno et al. 2007).

Strains expressing both BiP and either of the SNARE proteins exhibited slower growth than a control with neither BiP or SNARE proteins (Figure 3-5). A GS200 strain

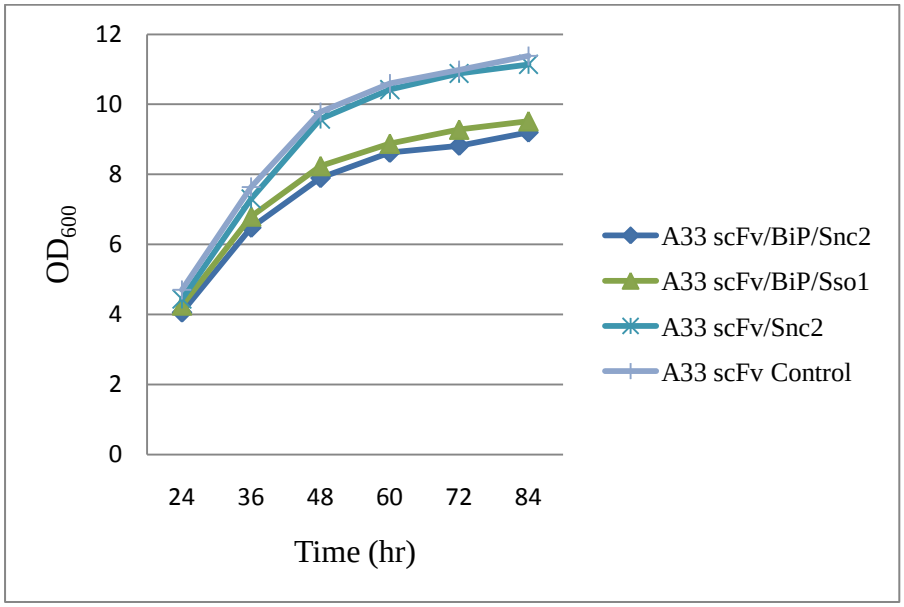

Figure 3-5-Growth curves of SNARE and chaperone overexpressing strains of *P. pastoris*. *P. pastoris* GS200 strains harboring A33 scFv were transformed with BiP and Snc2 or Sso1 (A33 scFv/BiP/Snc2 and A33 scFv/BiP/Sso1, respectively), Snc2 alone (A33 scFv/Snc2), or empty vector controls (A33 scFv Control) and were grown in 25 ml MM cultures at 25°C for 84 hours. OD_{600} values were taken every 12 hours starting at 24 hours.

without BiP but with Snc2 did not show this same defect in growth, indicating that the growth defect was due to the overexpression of all three proteins (A33 scFv, BiP, and either Snc2 or Sso1). Despite the fact that the SDS-PAGE samples were adjusted for this difference in culture OD_{600}, image densitometry analysis showed that the triple transformants still showed a marked decrease in scFv yields of 45% for A33 scFv/BiP/Snc2 and 55% for A33 scFv/BiP/Sso1 (Figure 3-6). As with the previous small scale expressions without BiP (Figure 3-2), Snc2 overexpression in *P. pastoris* GS200 led to a decrease in A33 scFv yield when compared with the control of 27% (Figure 3-6, A33 scFv/Snc2).

Discussion

Many strategies are available to increase the yield of secreted recombinant proteins. The overexpression of ER resident chaperones in both *S. cerevisae* and in *P. pastoris* has been shown to improve the secretory yield of numerous scFv molecules (Damasceno et al. 2007; Shusta et al. 1998). Overexpression of the Rab GTPase Sec4 has led to the improvement of glucoamylase secretion in *P. pastoris* (Liu et al. 2005). This work focused on enhancing the capacity of the terminal end of the secretory pathway by the overexpression of SNARE proteins involved in the fusion of secretory vesicles with the plasma membrane.

Overexpression of the *K. lactis* SNARE Sso1 in *S. cerevisae* has previously been shown to increase the yields of α-amylase 2.2 fold (Toikkanen et al. 2004). Using this work as a guide, we isolated the *S. cerevisae* genes for Sso1 and one of its partner, vesicle bound SNARE proteins Snc2. Together with Sec9, the assembly of a SNAREpin complex at the vesicle-plasma membrane interface drives fusion of the membranes and the release of their contents into the extracellular space. We hypothesized that increasing the available numbers of SNARE proteins would improve secretory yields in *P. pastoris*.

Sso1 overexpression in *P. pastoris* was not detrimental to cell growth. Sso1 overexpression had a positive effect on A33 scFv secretion, increasing yields by 31% in shake flask cultures. In high cell density fermentations, scFv yields were increased by 81% after 8 hours of induction and 10% after 43 hours. Snc2 overexpression in *P. pastoris* was shown to not impact the growth of the organism. At the small scale, however, the cooverexpression of Snc2 and A33 scFv was shown to result in lower yields of scFv when compared with the control. High cell density fermentations of the

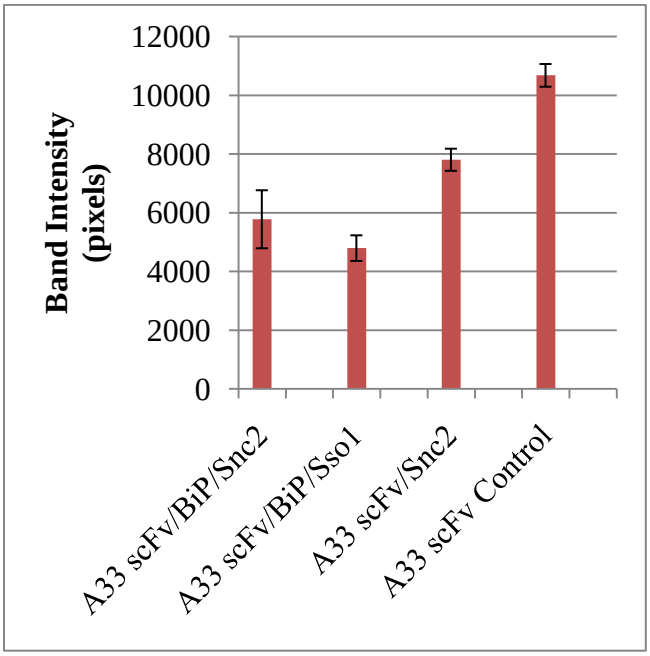

Figure 3-6-The effect of SNARE and BiP cooverexpression on A33 scFv yield. *P. pastoris* GS200 cultures expressing A33 scFv with different combinations of the chaperone BiP and the SNARE proteins Snc2 and Sso1 were grown in 25 ml MM at 25°C for 24 hr. Gel samples were prepared from culture supernatant at 60 hours and adjusted for culture OD_{600}. Ten µl samples were run in duplicate on SDS-PAGE gels, the gels dried, and analyzed by image densitometry.

same strains showed no appreciable defect in scFv yields, however there was no evidence of enhancement. RT-PCR confirmed that transcription of the Snc2 gene was indeed occurring, suggesting that the protein product itself is inhibiting secretion of A33 scFv.

It is interesting that Sso1 but not Snc2 enhanced secretory capacity. Despite there being no defect in cell growth in these strains, the decreased secretory yields could be due to a slight metabolic burden on the organism deriving from the need to overexpress the SNARE protein in addition to the A33 scFv. This is not likely, as the overexpression of chaperone molecules using the same promoter in *P. pastoris* has shown a marked increase in A33 scFv yields (Damasceno et al. 2007). It is likely that the Snc2 sequence isolated from *S.cerevisae* is too divergent from the *P. pastoris* homolog to participate in the generation of a stable SNAREpin. The genome sequence of *P. pastoris* GS115 was recently published and the *P. pastoris* Snc2 and Sso1 homologs were identified by searching with the *S. cerevisae* protein sequences used in this study. An alignment of the Snc2 protein sequences shows that the *S. cerevisae* Snc2 sequence contains a five residue insertion directly upstream of the SNARE domain that begins at residue 28 (Figure 3-7). The residues that are buried within the hydrophobic core of the SNAREpin are well conserved, but it is possible that this insertion and other differences at the N terminal end (residues 20-40) of the SNAREpin may be preventing initiation of the coiled coil domain formation. The Sso1 homologs show no such insertions within or around the SNARE domain (Figure 3-8). While there are amino acid differences within the SNARE domain, the hydrophobic core residues are well conserved, providing further evidence that the *S. cerevisae* Sso1 homolog is functional in *P. pastoris*.

There has been a great deal of research looking at the specificity of SNARE interactions and the role they play in determining protein flow in the cell. Out of 275 possible SNARE combinations in *S. cerevisae*, only nine of them result in successful membrane fusion, and eight of those correspond to known pathways (Varlamov et al.

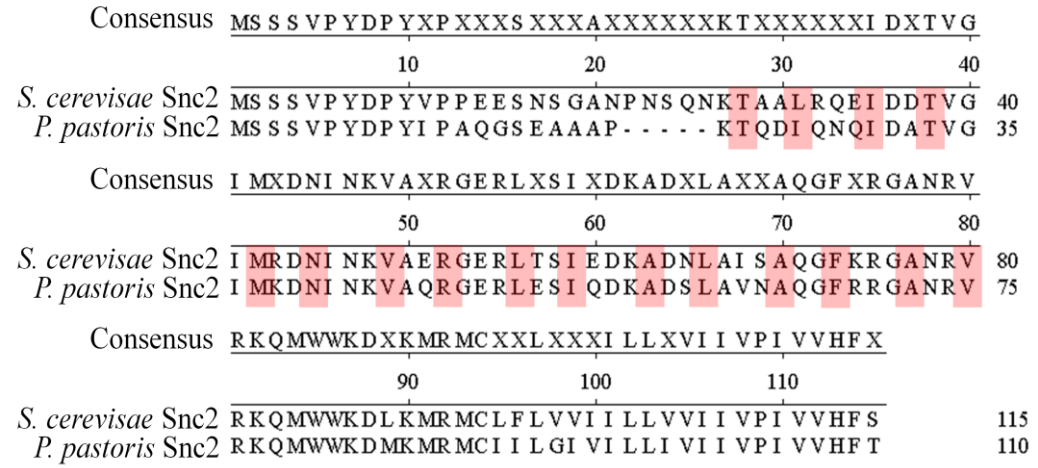

Figure 3-7-An alignment of the *P. pastoris* and *S. cerevisae* Snc2 protein sequences. The protein sequences for the *S. cerevisae* Snc2 (GenPept Accession #CAA89974) was used to BLAST for the *P. pastoris* GS115 homolog (GenPept Accession #XP_002490822). The sequences were aligned using DNAStar Lasergene 8.0 with the ClustalW method. The residues predicted to reside in the hydrophobic core of the SNAREpin domain are highlighted in red.

2004). This suggests that differences between *S. cerevisae* Snc2 and its *P. pastoris* V-SNARE homolog would prevent the successful fusion of vesicles in *P. pastoris* that harbor the *S. cerevisae* Snc2 SNARE protein. This would function as an inhibitory SNARE, lowering overall secretion efficiency by preventing the formation of successful SNAREpins.

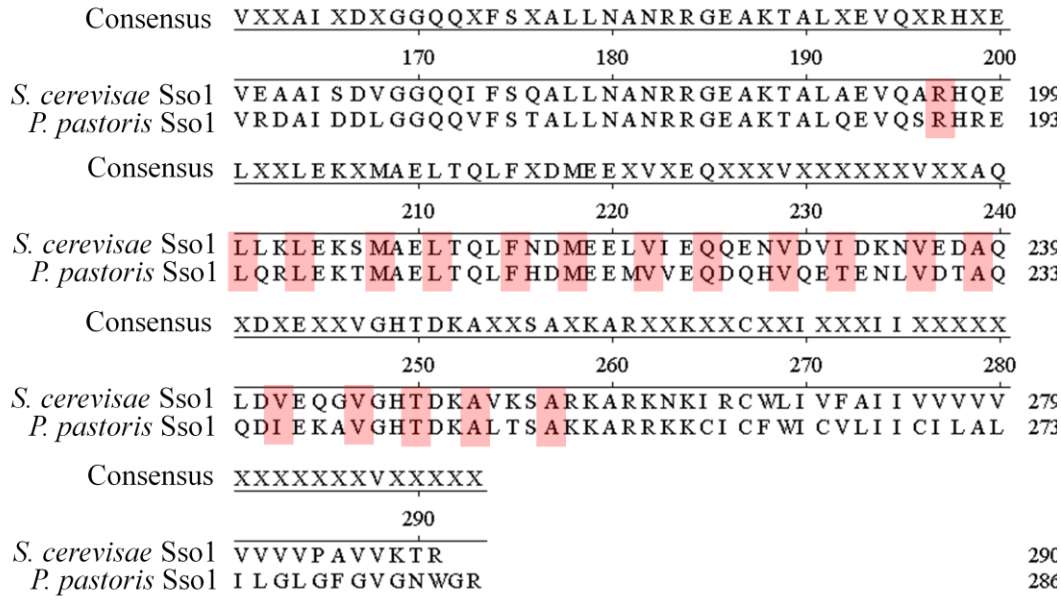

Figure 3-8- An alignment of the *P. pastoris* and *S. cerevisae* Sso1 SNARE domain protein sequences. The protein sequences for the *S. cerevisae* Sso1 (GenPept Accession #CAA97949) was used to BLAST for the *P. pastoris* GS115 homolog (GenPept Accession #XP_002490413). The sequences were aligned using DNAStar Lasergene 8.0 with the ClustalW method. The residues predicted to reside in the hydrophobic core of the SNAREpin domain are highlighted in red.

The cooverexpression of BiP by our group in *P. pastoris* clearly showed that higher amounts of secretion are possible without the overexpression of machinery involved in the movement of secretory vesicles throughout the cell (Damasceno et al. 2007). We hypothesized that these strains would derive greater benefit from SNARE overexpression, as these strains would be moving more protein through the secretory pathway. The strains cooverexpressing A33 scFv, BiP, and either Sso1 or Snc2 displayed a defect in growth that led to a corresponding decrease in scFv yield. It is likely that the metabolic burden associated with the overexpression of three proteins, two of which are under the control of the strong, methanol inducible AOX1 promoter impairs cell growth. Future work coupling the overexpression of chaperones and

SNARE proteins using weaker, possibly constitutive promoters such as GAP could reveal a synergistic effect. Furthermore, any enhancement at this step may have been limited by similarity differences between the *S. cerevisae* proteins used and their *P. pastoris* homologs, particularly in the case of Snc2. Future work in this area should focus on first isolating the native *P. pastoris* sequences.

BIBLIOGRAPHY

Burri L., and Lithgow T. (2004). A Complete Set of SNAREs in Yeast. Traffic *5*, 45-52.

Chen, Y. A., and Scheller, R. H. (2001). SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol *2*, 98-106.

Damasceno, L., Anderson, K., Ritter, G., Cregg, J., Old, L., and Batt, C. (2007). Cooverexpression of chaperones for enhanced secretion of a single-chain antibody fragment in Pichia pastoris. Applied Microbiology and Biotechnology *74*, 381-389.

Damasceno, L. M., Pla, I., Chang, H., Cohen, L., Ritter, G., Old, L. J., and Batt, C. A. (2004). An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in Pichia pastoris. Protein Expr Purif *37*, 18-26.

Ellis, S. B., Brust, P. F., Koutz, P. J., Waters, A. F., Harpold, M. M., and Gingeras, T. R. (1985). Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast Pichia pastoris. Mol Cell Biol *5*, 1111-21.

Inan, M., Aryasomayajula, D., Sinha, J., and Meagher, M. M. (2006). Enhancement of protein secretion in Pichia pastoris by overexpression of protein disulfide isomerase. Biotechnol. Bioeng *93*, 771-778.

Johnson, M. A., Snyder, W. B., Cereghino, J. L., Veenhuis, M., Subramani, S., and Cregg, J. M. (2001). Pichia pastoris Pex14p, a phosphorylated peroxisomal membrane protein, is part of a PTS-receptor docking complex and interacts with many peroxins. Yeast *18*, 621-41.

Kim, M., Han, K., Kang, H., Rhee, S., and Seo, J. (2003). Coexpression of BiP increased antithrombotic hirudin production in recombinant Saccharomyces cerevisiae. Journal of Biotechnology *101*, 81-87.

Lin-Cereghino, J., Wong, W. W., Xiong, S., Giang, W., Luong, L. T., Vu, J., Johnson, S. D., and Lin-Cereghino, G. P. (2005). Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris. Biotechniques. *38*, 44–48.

Liu, S., Chou, W., Lin, S., Sheu, C., and Chang, M. D. (2005). Molecular genetic manipulation of Pichia pastoris SEC4 governs cell growth and glucoamylase secretion. Biochem Biophys Res Commun *336*, 1172-80.

Pla, I. A., Damasceno, L. M., Vannelli, T., Ritter, G., Batt, C. A., and Shuler, M. L. (2006). Evaluation of Mut+ and MutS Pichia pastoris phenotypes for high level extracellular scFv expression under feedback control of the methanol concentration. Biotechnol Prog *22*, 881-8.

Shusta, E. V., Raines, R. T., Pluckthun, A., and Wittrup, K. D. (1998). Increasing the secretory capacity of Saccharomyces cerevisiae for production of single-chain antibody fragments. Nat Biotech *16*, 773-777.

Toikkanen, J. H., Sundqvist, L., and Keränen, S. (2004). Kluyveromyces lactis SSO1 and SEB1 genes are functional in Saccharomyces cerevisiae and enhance production of secreted proteins when overexpressed. Yeast *21*, 1045-55.

Tschopp, J. F., Brust, P. F., Cregg, J. M., Stillman, C. A., and Gingeras, T. R. (1987). Expression of the lacZ gene from two methanol-regulated promoters in Pichia pastoris. Nucleic Acids Res *15*, 3859-76.

Varlamov, O., Volchuk, A., Rahimian, V., Doege, C. A., Paumet, F., Eng, W. S., Arango, N., Parlati, F., Ravazzola, M., Orci, L., et al. (2004). i-SNAREs: inhibitory SNAREs that fine-tune the specificity of membrane fusion. J Cell Biol *164*, 79-88.

Chapter 4-The Unconventional Secretion of the Cancer Differentiation Antigen MelanA in *Pichia pastoris*

Abstract

Protein secretion by *Pichia pastoris* can result in protein yields in the gL^{-1} range. The tendency of *P. pastoris* to secrete few native proteins, coupled with a simple and well defined growth medium, result in high purities of recombinant protein simply by removing the cells by centrifugation or filtration. As some proteins are unable to be successfully secreted by the traditional secretory pathway, we chose to explore the use of unconventional secretion to lead to the extracellular localization of the cancer antigen MelanA. A plasmid was designed that would result in the production of a fusion protein between the unconventionally secreted protein enolase and MelanA. A similar fusion was shown to lead to the extracellular localization of GFP and invertase in *Saccharomyces cerevisae*. Shake flask cultures exhibited fusion protein in the culture supernatant as determined by Western blotting with anti-MelanA antibodies. Enolase-MelanA fusion protein was also found bound to the cell exterior and could be released by washing of the cells with a 0.1 M Tris buffer (pH 7.6) with 0.5% 2-Mercaptoethanol. High cell density fermentation failed to accumulate enolase-MelanA fusion in the culture supernatant, but did result in protein bound to the cell walls. Western blotting with anti-*Candida albicans* enolase indicates that degradation of the fusion protein is occurring in the culture supernatant, preventing accumulation.

Introduction

The secretion of recombinant proteins by the yeast *Pichia pastoris* is an attractive means to achieve high yield and purity of a desired target molecule. Current methods to achieve secretion utilize the *Saccharomyces cerevisae* α-mating factor signal sequence to direct the translocation of the target protein into the ER and the rest of the secretory pathway. This fusion protein is then packaged into secretory vesicles and released into the extracellular environment. For *P. pastoris*, secretion of recombinant

proteins is especially attractive considering its simple growth medium and the tendency of *P. pastoris* to secrete few native proteins. Removal of the cells yields a supernatant highly enriched in the target protein of interest. However, there are certain proteins that are unable to be secreted by this process. There could be a number of factors at play, such as poor translation, improper folding and retention in the ER, and targeting to other compartments of the cell.

Unconventional protein secretion is a general term used to describe the release of proteins to the culture supernatant by methods that circumvent the traditional ER-golgi secretory route (Nombela et al. 2006; Nickel and Seedorf 2008). A number of proteins have been described that undergo this type of secretion, including fibroblast growth factor 2 (Engling et al. 2002), interleukin-1B (Andrei et al. 1999), and a variety of glycolytic enzymes (Chaffin 2008). One of these glycolytic enzymes, enolase, has been shown to be secreted by both *S. cerevisae* and *Candida albicans* (Jong et al. 2003; Edwards et al. 1999). In *C. albicans*, enolase secretion is implicated in the progress of the infection by this yeast, as it promotes tissue invasion through binding to plasminogen and plasmin, providing *C. albicans* cells with a localized high concentration of enzymes required for the breakdown of extracellular matrix proteins (Jong et al. 2003). Once secreted, enolase remains associated with the cell wall. The method by which it is secreted is currently unknown.

López-Villar et. al. created enolase-GFP and enolase-invertase fusions, demonstrating the ability of enolase to lead to the extracellular localization of other proteins (López-Villar et al. 2006). This study provided the impetus for the creation of fusion proteins between enolase and the cancer differentiation antigen MelanA. MelanA is an attractive vaccine candidate for cancer immunotherapy, owing to its wide distribution

in various cancer types and its restricted expression in normal tissue. Secretion of MelanA by the traditional α-mating factor directed route has been unsuccessful, making this a good candidate for unconventional protein secretion directed by enolase. This paper will discuss the utilization of unconventional protein secretion as a new means to achieve extracellular localization of recombinant proteins by *P. pastoris*.

Materials and Methods

Enolase cloning

The Eno1 gene sequence was found by a BLAST search of the *P. pastoris* genome using the *S. cerevisae* Eno1 sequence homolog. Using this sequence, the gene was amplified from *P. pastoris* X33 genomic DNA that was isolated using the Yeastar genomic DNA kit (Zymo Research, Orange, CA). The primers were obtained from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA) and were designed to add a 5' BstBI site and a 3' XhoI site for cloning into pPICZαA (Invitrogen, Carlsbad, CA). The Eno1 PCR product was TA cloned into pCR2.1 and its sequence verified. A construct bearing the correct sequence and pPICZαA was digested with BstBI and XhoI (New England Biolabs, Ipswich, MA) and separated on a 1% agarose gel. Bands were purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA) and fragments were ligated using the QuickLigase Kit (New England Biolabs, Ipswich, MA). Chemically competent TOP10 cells were transformed using a heat shock protocol, and transformants selected on Luria broth with 50 µg/ml Zeocin (Invitrogen, Carlsbad, CA). The presence of the correct insert was confirmed by colony PCR using primers specific to the AOX1 promoter and transcription terminator. The new plasmid was termed pEZ and was used for further cloning experiments.

The MelanA gene was previously prepared for use with pPICZαA and contained a 5' XhoI and 3' NotI site. A pCR2.1 construct containing MelanA and the pEZ plasmid, were digested with XhoI and NotI, the bands separated and purified by gel electrophoresis, and ligated with QuickLigase, as before. *P. pastoris* KM71H (Invitrogen, Carlsbad, CA) was made competent using the condensed protocol (Lin-Cereghino et al. 2005). Briefly, a 50 ml culture of *P. pastoris* was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) to an OD_{600} of 0.8-1.0 and the cells collected by centrifugation at 500g. The pellet was resuspended in 9 ml BEDS buffer ((10 mM bicine-NaOH, pH 8.3, 3% (v/v) ethylene glycol, 5% (v/v) dimethyl sulfoxide, 1 M sorbitol) supplemented with 1 ml dithiothreitol and the cells were incubated with gentle shaking for 5 min. at 30°C. The cells were again collected by centrifugation, and resuspended in 0.5 ml BEDS buffer without DTT. One µg of plasmid was linearized by digestion with PmeI (New England Biolabs, Ipswich, MA), a 1% agarose gel run and stained with ethidium bromide, and the linearlized plasmid excised and purified with the MinElute DNA Cleanup Kit (Qiagen, Valencia, CA). A total of 100 ng of linearized DNA was mixed with 40 µl competent cells, incubated on ice for 5 minutes, and subjected to electroporation using a Gene Pulser II (BioRad Laboratories, Hercules, CA) set to 1.5 kV, 100 µW, 25 µF. Cells were quickly resuspended with 0.5 ml YPD and 0.5 ml 1 M sorbitol, allowed to incubate at 30°C for 1 hour, and plated on YPD with 1 M sorbitol and 100 µg/ml zeocin (Invitrogen, Carlsbad, CA). Transformants were screened for the presence of the insert using colony PCR as described before.

Expression Studies

P. pastoris strains expressing Enolase-MelanA were grown in 5 ml YPD overnight at 30°C. The cultures were diluted in 10 ml YPD to an OD_{600} of 0.2 and allowed to grow

up to an OD of 1 before being collected by centrifugation. The pellets were resuspended in 10 ml BMM (100 mM potassium phosphate pH 6.0, 1.34% (w/v) yeast nitrogen base, $4x10^{-5}$% (w/v) biotin, 0.5% (v/v) methanol) or BMMY (BMM + 1% (w/v) yeast extract and 2% (w/v) peptone) and allowed to grow at 25°C for up to 96 hours. Every 24 hours, a one ml sample was taken to determine OD_{600} and the cells were collected by centrifugation.

Protein Methods

Supernatants were filtered through a 0.2 µm Acrodisc™ syringe filter (Pall, East Hills, NY) and diluted with 4X LDS sample buffer (Invitrogen, Carlsbad, CA) and 5% 2-mercaptoethanol, followed by heating at 65°C for 10 minutes, to prepare the samples for SDS-PAGE. The cells were frozen at -20C until the cultures were terminated, at which point the pellets from each time point were lysed using yeast breaking buffer (50 mM sodium phosphate buffer pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol) and acid-washed glass beads as recommended by the manufacturer (Invitrogen, Carlsbad, CA). The lysate was centrifuged and the intracellular soluble material removed and prepared for SDS-PAGE. The remaining insoluble lysate was gently resuspended with yeast breaking buffer containing 1% SDS, and this material was prepared for SDS-PAGE as well.

A ten µl aliquot of each gel sample was loaded onto precast bis-tris gels (Invitrogen) and run in MES buffer for 40 minutes at 200V. Gels were stained with SimplyBlue Safe Stain (Invitrogen), or blotted to nitrocellulose membranes (Sambrook 2001). Immunoblotting was carried out using the WesternBreeze kit (Invitrogen, Carlsbad, CA). The primary antibodies were a monoclonal anti-MelanA antibody or a polyclonal anti-*C. albicans*-Enolase antibody (Takara Bio USA).

Extraction of cell associated protein was performed by resuspending cell pellets in pH 7.6 or 9.0 tris buffer supplemented with 0.5% 2-mercaptoethanol or 10 mM DTT. The samples were incubated at 37°C for 15 min before the cells were pelleted and the

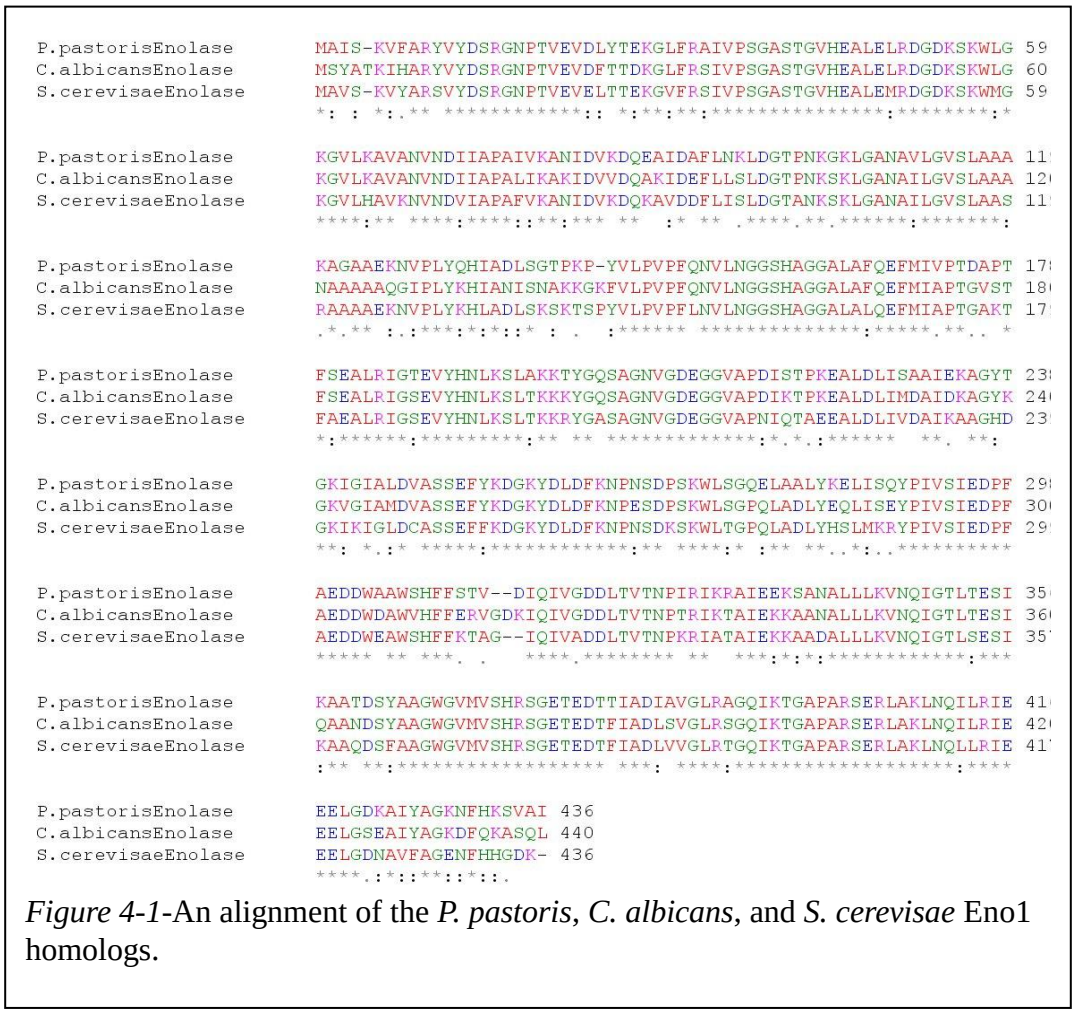

Figure 4-1-An alignment of the *P. pastoris*, *C. albicans*, and *S. cerevisae* Eno1 homologs.

supernatants taken for further analysis.

Fermentation

P. pastoris clones exhibiting fusion protein production were chosen for scale up to a 2.5 L fermentation. Briefly, a 50 ml YPD culture of each strain was grown to an OD_{600} of 8-10 and used to inoculate a 2.5 L working volume New Brunswick BioFlo

3000 fermenter (New Brunswick Scientific Co., Edison, NJ) containing 1 L of modified basal salts medium (0.23 $g \cdot L^{-1}$ $CaSO_4$-$2H_2O$, 4.55 $g \cdot L^{-1}$ K_2SO_4, 3.73 $g \cdot L^{-1}$ $MgSO_4$-$7H_2O$, 10.3 $g \cdot L^{-1}$ KOH, 6.68 $ml \cdot L^{-1}$ H_3PO_4, 5% (v/v) glycerol) with 0.5 ml Antifoam 204 (Sigma, St. Louis, MO). The addition of 15% (v/v) ammonium hydroxide was used to maintain the pH at 6.0 and also to serve as the nitrogen source. Dissolved oxygen (DO) was controlled at 40% with a combination of increasing agitation and pure oxygen supplementation. After glycerol consumption during the batch phase, marked by a sharp increase in dissolved oxygen, methanol feeding was initiated using a closed-loop proportional, integral, derivative (PID) control scheme to maintain the methanol level at 1.0 $g \cdot L^{-1}$ (Damasceno et al. 2004). Samples were taken periodically to determine OD_{600} and wet cell weight, as well as to collect supernatant and insoluble and soluble cell extracts.

Results

Enolase Cloning

The sequence for the *P. pastoris* enolase gene was provided by Dr. James Cregg, and this sequence was used to design primers to amplify the gene from *P. pastoris* genomic DNA. DNA sequencing confirmed the identity of the sequence, and an alignment of the *P. pastoris*, *S. cerevisae*, and *C. albicans* Eno1 protein products can be seen in Figure 4-1, showing the highly conserved sequence of the enzyme. The primers were designed to add a 5' BstBI site and a 3' XhoI site for cloning into pPICZαA. Digesting pPICZαA with these enzymes removes the α-mating factor secretion signal.

Purified enolase and pPICZαA DNA fragments digested with BstBI and XhoI were

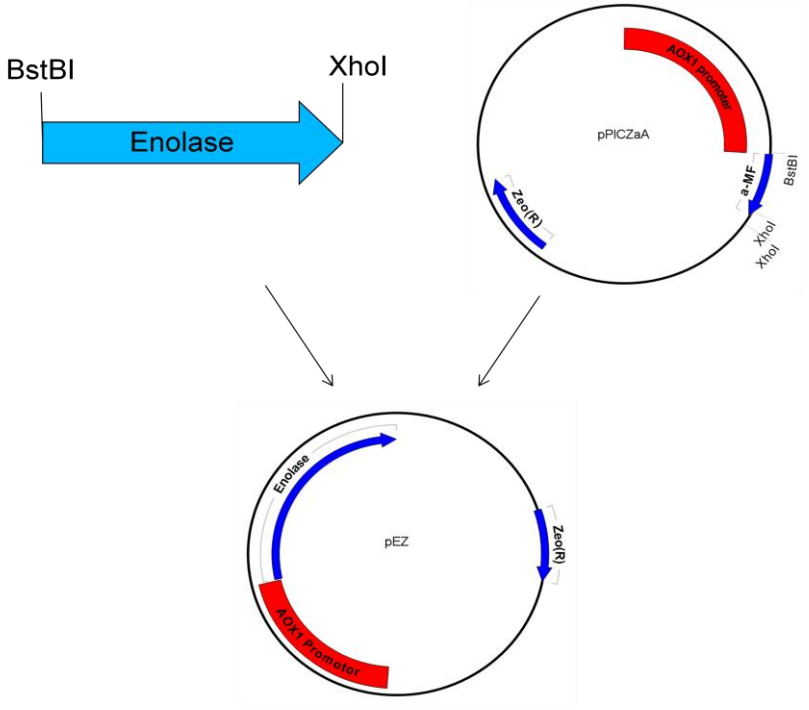

Figure 4-2-The enolase gene and pPICZαA were digested with BstBI and XhoI and the fragments purified by gel extraction. Religation generated the plasmid pEZ, in which the full length enolase takes the place of the α-mating factor secretion signal.

religated to generate the plasmid pEZ (Figure 4-2). In this construct, the multiple cloning site from pPICZαA is maintained, allowing the insertion of genes at the XhoI and NotI sites. Cloning of genes using these restriction enzymes in pPICZαA generates a fusion protein between the α-mating factor and the protein of interest, and this capability was maintained in pEZ. The cancer differentiation antigen MelanA was cloned into pEZ using XhoI and NotI, generating an enolase-MelanA fusion product. This construct was transformed into *P. pastoris* KM71H, and transformants were selected by zeocin resistance.

Small Scale Expression Studies

Three transformants (designated EnoMel1, EnoMel2, or EnoMel3) were picked and grown in 10 ml BMM shake flask cultures to look for fusion protein secretion. When compared with a control KM71H culture, all clones had similar growth rates. The

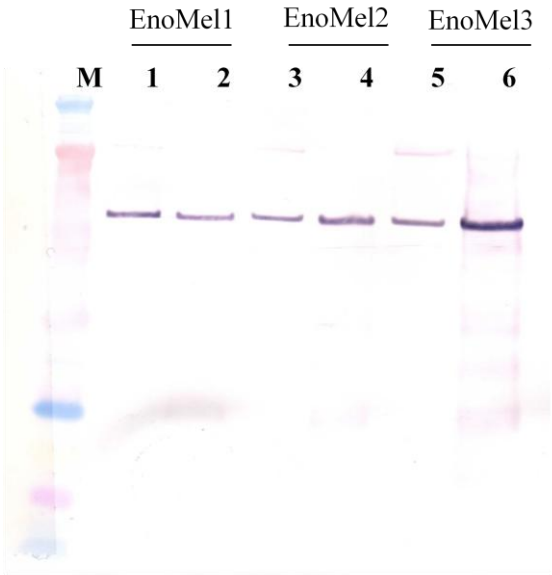

Figure 4-3-Three *P. pastoris* clones expressing the enolase-MelanA fusion protein were grown in shake flask cultures in BMM (pH 6.0). Their supernatants were collected and the cell pellet washed to remove cell wall-bound protein. **Lanes 1, 3, and 5-**Supernatant samples. **2, 4, and 6-**Cell wash samples.

culture supernatant for each clone contained fusion protein at the expected size of ~60 kDa when probed with anti-MelanA antibodies (Figure 4-3, Lanes 1, 3, and 5). Furthermore, each culture also contained enolase-MelanA bound to the cell walls and extracted by washing with 2 mM Tris (pH 8.0) with 0.5% 2-mercaptoethanol (Figure 4-3, Lanes 2, 4, and 6). There was no degradation seen in any of the cultures, and no apparent differences in protein yield in the supernatant. Clone EnoMel3, however, did

seem to yield more protein that was extractable from the cell surface (Figure 4-3, Lane 6), and this clone was selected for future experiments.

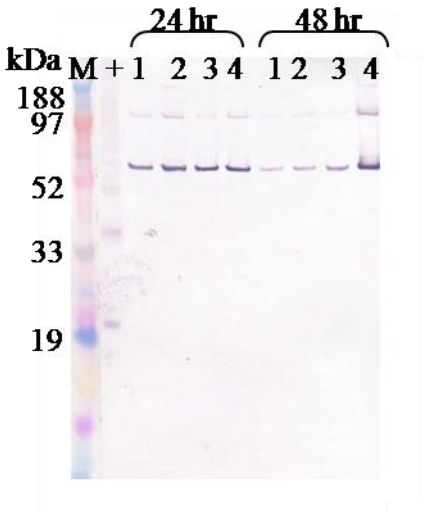

Figure 4-4-P. pastoris EnoMel3 was grown under four different conditions and the supernatants from 24 and 48 hours examined by immunoblotting with anti-MelanA antibodies. **M**-Protein ladder. **+**-MelanA control. **1**-BMMY pH 3.0. **2**-BMMY pH 6.0. **3**-BMM pH 3.0. **4**-BMM pH 6.0.

Next we decided to test the effects of medium composition and pH on protein yield. Cultures were prepared with either BMM or BMMY at either pH 3.0 or 6.0, and maintained at 25°C for 48 hours. In each of the four conditions tested, enolase-MelanA was seen in Western blots at 24 and 48 hours (Figure 4-4). A band was detected migrating at a higher molecular weight, which could correspond to dimerization of the fusion protein. At 48 hours, however, only the culture with BMM at pH 6.0 had an increase in enolase-MelanA yield. All other conditions tested resulted in a loss of protein over time, presumably due to proteolysis. When the cells

were washed with 2 mM tris pH 8.0/0.5% 2-mercaptoethanol only the cell pellet from BMM pH 6.0 had any anti-MelanA reactive protein (Figure 4-5, B), and this wash fraction had the lowest amount of contaminating host protein (Figure 4-5, A). From these results, minimal medium at pH 6.0 was chosen for all future experiments.

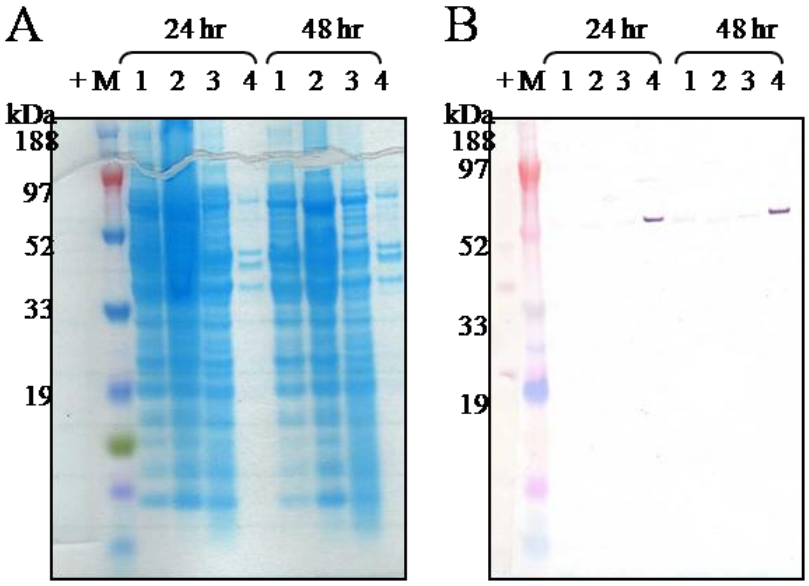

Figure 4-5- P. pastoris EnoMel3 was grown in either BMM or BMMY at pH 3.0 or 6.0. Cell pellets after 24 or 48 hr of induction were washed with 2 mM Tris pH 8.0 with 0.5% 2-mercaptoethanol. Proteins released from the cell walls were analyzed by SDS-PAGE gels and Western blotting. **A**-Coomassie stained SDS-PAGE. **B**-Immunoblot with anti-MelanA antibodies. **M**-Protein ladder. **+**-MelanA control. **1**-BMMY pH 3.0. **2**-BMMY pH 6.0. **3**-BMM pH 3.0. **4**-BMM pH 6.0.

Release of Cell Wall Bound Protein

The effect of temperature and different cell washing conditions on enolase-MelanA yield was also tested. Cell pellets were collected and normalized for culture OD_{600} at 48 hr post induction from cultures maintained at 20°C or 30°C, and washed with one

of four buffers: 0.1 M Tris (pH 7.6) with 0.5% 2-Mercaptoethanol; 0.1 M Tris (pH 7.6) with 10 mM DTT; 0.1 M Tris (pH 9.0) with 0.5% 2-Mercaptoethanol; or 0.1 M Tris (pH 9.0) with 10 mM DTT. The pH 9.0 buffers, regardless of reducing agent, released

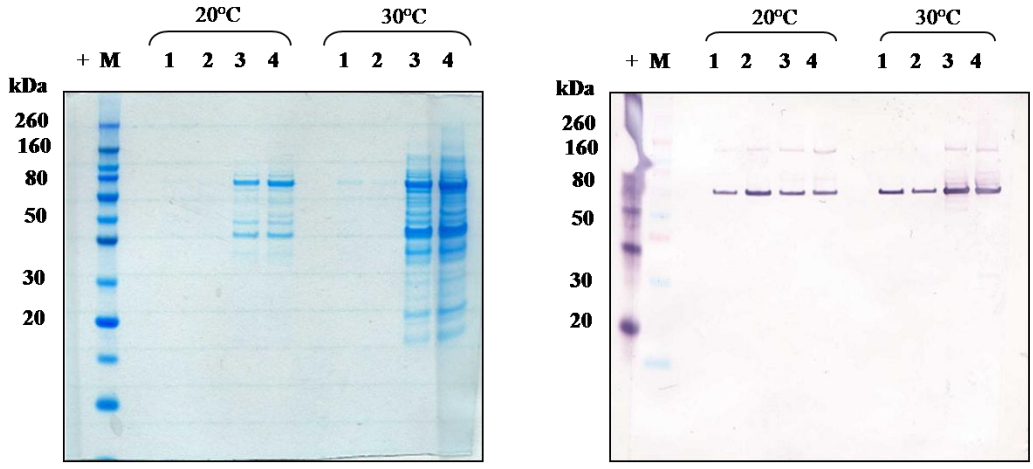

Figure 4-6-Cell pellets from *P. pastoris* EnoMel3 cultures grown in BMM pH 6.0 at either 20 or 30°C were normalized for cell number and washed with one of four buffers to test their effects on fusion protein release from the cell surface. Duplicate gels were stained with coomassie (left) or transferred to nitrocellulose membranes and blotted with anti-MelanA antibodies (right). +-MelanA control. **M**-Protein ladder. **1**-0.1 M Tris pH 7.6 + 0.5% 2-Mercaptoethanol. **2**-0.1 M Tris pH 7.6 + 10 mM DTT. **3**-0.1 M Tris pH 9.0 + 0.5% 2-Mercaptoethanol. **4**-0.1 M Tris pH 9.0 + 10 mM DTT.

more total protein as determined by coomassie staining of an SDS-PAGE gel (Figure 4-6, Lanes 3 and 4).

Growth at 30°C also led to higher amounts of extractable total protein. While a reducing agent is necessary for enolase-MelanA release, the choice of DTT or 2-Mercaptoethanol did not have any impact on protein totals. Western blotting with anti-MelanA antibodies however indicates that the level of fusion protein released from approximately the same number of cells was nearly the same regardless of washing conditions used (Figure 4-6, right).

High Cell Density Fermentation

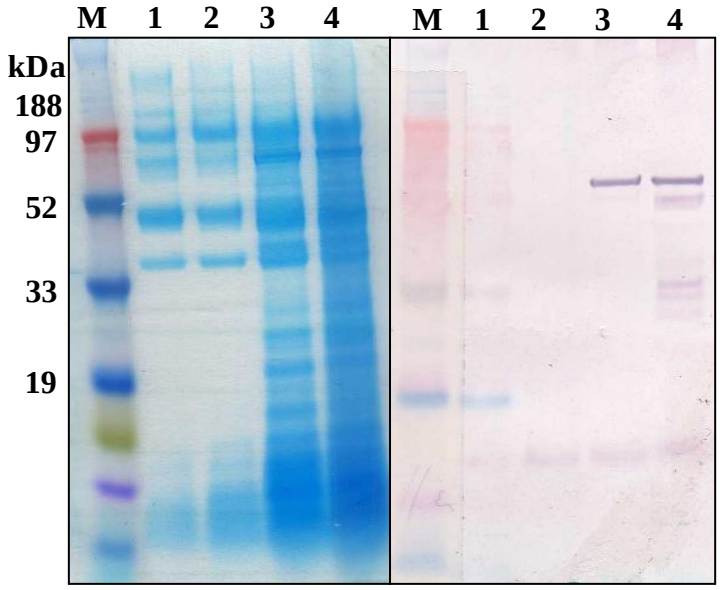

*Figure 4-7-*A *P. pastoris* EnoMel3 high cell density fermentation of with basal salts media at pH 6.0 was carried out, and the supernatants and cell pellets examined for the presence of enolase-MelanA fusion protein by coomassie staining (left) and immunoblotting with anti-MelanA antibodies (right). Cell pellets were washed with 0.1 M Tris (pH 7.6) + 0.5% 2-Mercaptoethanol. **1 and 2**-24 and 48 hour culture supernatants, respectively. **3 and 4**-24 and 48 hour cell washes, respectively.

Based on results obtained at the small scale, a fermentation was prepared using the ¼ basal salts medium that has been used successfully for high protein yields of a traditionally secreted protein, the A33 scFv (Damasceno et al. 2004). Enolase-MelanA was undetectable in the supernatant by immunoblotting, and the amount washed from the cell wall was also very low (data not shown).

Standard *P. pastoris* fermentations typically contain 1.34 mM $CaSO_4$, however calcium concentration has been shown to be an important factor for multivesicular body fusion with the plasma membrane (Savina et al. 2005), a possible route for

enolase export. We chose to increase the concentration of $CaSO_4$ four fold, to 5.34 mM. At 24 and 48 hr, higher calcium concentrations did increase fusion protein released from the cell wall (Figure 4-7, Lanes 3 and 4), but at these time points nothing was detected in the supernatant (Figure 4-7, Lanes 1 and 2). Fusion was found in the supernatant at 72 and 96 hours (Figure 4-8, Lanes 3 and 4). Western blotting with a polyclonal antibody raised against *C. albicans* Eno1 showed degraded forms of the fusion protein in the supernatant at all time points, with the degradation becoming more extensive at 72 and 96 hours (Figure 4-8). The primary band seen with the anti-enolase antibody does migrate lower than the fusion protein, at approximately ~50 kDa. This band could possibly be enolase itself, which has a molecular weight of ~47 kDa.

One ml fermentation samples were taken and protein extracted from the cell surface as with the shake flask cultures. A further goal of this experiment was to determine if starvation and/or temperature upshift led to an increase in extractable enolase-MelanA from the cell surface, as has been seen with glyceraldehyde-3-phosphate dehydrogenase (Delgado et al. 2003). Extraction of a pellet with no treatment yielded 1.17 mg/ml of total protein, while incubation of the pellet in prewarmed water at 30°C for one hr increased the level of extractable total protein to 1.69 mg/ml (Figure 4-9, A). Incubation of the pellet in water at 37°C resulted in no change. Incubation in YPDS at either 30 or 37°C for one hour decreased the extractable total protein concentration by 19.5 and 14.6 fold, respectively. This difference in total protein can be seen in SDS-PAGE (Figure 4-9, B, Lanes 3 and 5), as 7 µl of protein extract was loaded per lane, regardless of protein concentration. The difference in enolase-MelanA fusion yields as determined by immunoblotting is negligible for all treatments except 37°C YPDS (Figure 4-9,C, Lane 5), which exhibited a noticeable loss in band

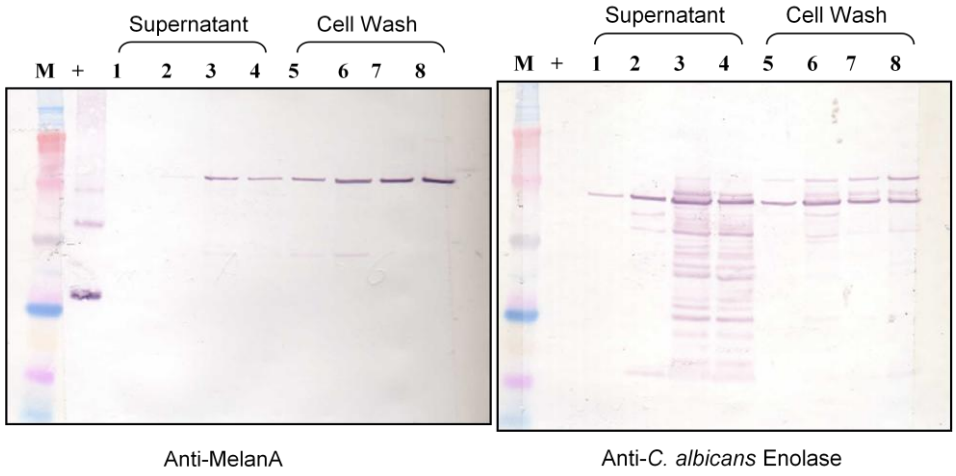

Figure 4-8- A *P. pastoris* EnoMel3 high cell density fermentation with basal salts media at pH 6.0 was carried out, and the supernatants and cell pellets examined for the presence of enolase-MelanA fusion protein by immunoblotting with anti-MelanA antibodies (Left) or anti-*C. albicans* enolase antibodies (Right). One ml cell pellets were washed with one ml of 0.1 M Tris (pH 7.6) + 0.5% 2-Mercaptoethanol. All times are post methanol induction. +-MelanA control. **M-**Protein ladder. **1 and 5-**24 hours. **2 and 6-**48 hours. **3 and 7-**72 hours. **4 and 8-**96 hours.

intensity. No fusion protein was found in the water or YPDS used for incubation (Figure 4-9, C, Lanes 6-9). The percentage of enolase-MelanA in these samples is very low, as no protein band can be seen in coomassie staining that corresponds to the immunoblot.

Discussion

The secretion of heterologous proteins by the traditional secretory pathway is a well-established method to aid in downstream purification. As we and others have seen, heterologous proteins are often unable to be properly folded and secreted in yeast systems (Agaphonov et al. 2002, 2005; Sagt et al. 2002). To try and circumvent the quality control checkpoints within the secretory pathway preventing secretion, we generated a fusion utilizing a protein that is secreted in a manner that bypasses the ER

altogether.

Unconventional protein secretion has only recently been reported, with the secretion routes for proteins such as fibroblast growth factor 2 and interleukin B 1 being the most well characterized (Engling et al. 2002; Andrei et al. 1999). In yeast, however, only one protein is known to be secreted by an unconventional route, the yeast a-factor. Yeast a-factor is a 12 amino acid peptide that is transported across the membrane by Ste6, a member of the ATP-binding cassette transporters (McGrath and Varshavsky 1989). Evidence for the secretion of other soluble proteins through unconventional methods is mounting and the pathways are being elucidated.

One unconventionally secreted protein found in a number of organisms is enolase. Fusions between enolase and either invertase or GFP results in the extracellular localization of the fusion protein in *S. cerevisae* (López-Villar et al. 2006). We chose to explore the possibility of using enolase to direct the extracellular localization of cancer antigens resistant to secretion by the traditional pathway in *P. pastoris*. The plasmids designed in this study were prepared so that the full length enolase gene would replace the α-mating factor secretion signal. The result is a fusion protein between enolase and the cancer differentiation antigen MelanA, with a Kex2 protease site and short linker between them. Unlike with α-mating factor directed secretion, the fusion protein should not be cleaved by Kex2 protease, as its secretion should completely bypass the traditional secretion pathway. The *P. pastoris* enolase sequence shares high homology between the *S. cerevisae* and *C. albicans* proteins, and fusion proteins between enolase and MelanA were detected in the culture supernatant by immunoblotting. At the small scale, the absence of host proteins in the culture supernatant suggests that lysis was not responsible for the extracellular localization of

the fusion protein. Culture conditions were varied in an attempt to maximize the amount of protein located in the supernatant and on the cell exterior, and we determined that the optimal yields were achieved minimal medium at pH 6.0. The use of pH 3.0, at which *P. pastoris* grows well and exhibits low proteolytic activity in the culture supernatant, had undetectable levels of the fusion protein bound to the cell wall, and the amount in the supernatant decreased from 24-48 hours.

Shake flask culture results indicate that the majority of the fusion protein remains associated with the cell pellet. This protein can be released by incubating the cells in buffers containing reducing agents. Interestingly, the pH of the buffer or the type of reducing agent, either DTT or 2-mercaptoethanol, did not result in any appreciable differences in fusion protein released from the cell pellet. These conditions did greatly impact the amount of other host proteins released from the cell. The pH 9.0 extracts yielded far more protein than did the pH 7.6 buffers. This is likely due to the release of protein bound by ionic interactions with negatively charged cell wall glycoproteins, as many yeast proteins have a pKa <8.0 (Klis et al. 2007). Despite the wide differences in total protein yields, there was little difference in enolase-MelanA released by the different buffers. This suggests that the interaction of the fusion protein with the cell wall is likely though disulfide bond formation, as the presence of a reducing agent is the critical component in enolase-MelanA release. Since pH 7.6 Tris buffer with 2-mercaptoethanol yields the same amount of fusion protein with the lowest level of contaminating host protein, this was the buffer chosen for all subsequent experiments.

It should be noted that washing cell pellets with a high pH buffer containing 2-mercaptoethanol can lead to perturbation of the plasma membrane (Klis et al. 2007). This work speculated that this perturbation could be responsible for the apparent

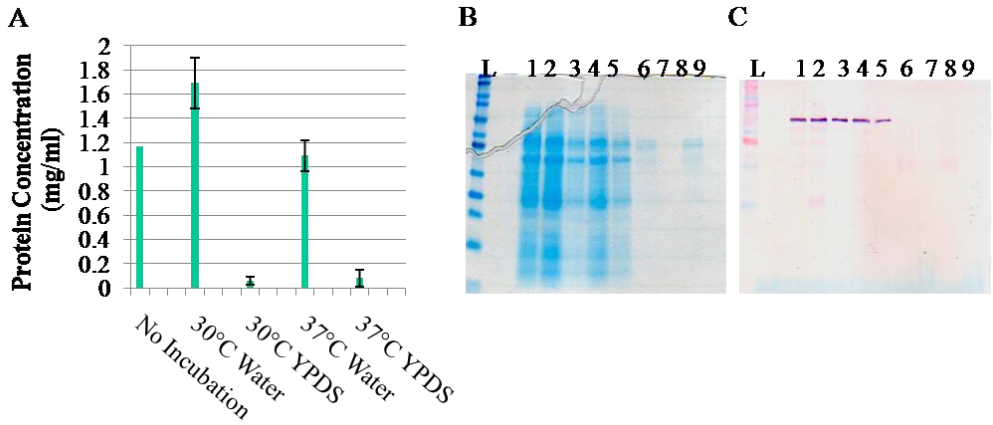

Figure 4-9-One ml samples from a *P. pastoris* EnoMel3 fermentation were harvested and the cells pelleted by centrifugation. The pellets were resuspended in either sterile water or YPDS prewarmed to 30 or 37°C and incubated at the same temperatures for one hour. The cells were then again pelleted and the cell associated protein extracted by incubation with 0.1 M Tris (pH 7.6) with 0.5% 2-mercaptoethanol for 30 minutes. **A**. The protein concentrations for the various treatments were determined by Bradford assay. **B** and **C**- Samples of the extracted protein and the water or YPDS used in the incubation were run on SDS-PAGE (**B**) and analyzed by immunoblotting with anti-MelanA antibodies (**C**). **L**-Molecular weight marker. **1**-No treatment. **2**-30°C water/extraction. **3**-30°C YPDS/extraction. **4**-37°C water/extraction. **5**-37°C YPDS/extraction. **6**-30°C water. **7**-30°C YPDS. **8**-37°C water. **9**-37°C YPDS.

location of traditionally cytoplasmic proteins at the cell surface. Since enolase and other putative unconventionally secreted proteins are highly enriched in the cytoplasm, Klis et al. suggest that even a small amount of cell lysis would lead to a noticeable increase of these proteins at the cell exterior. With enolase-MelanA, however, this explanation seems to be unlikely. Intracellular lysates from these cells exhibit very high levels of fusion protein seen by Western blotting (data not shown). If the pH 9.0

buffers did indeed lead to release of protein from the cytoplasm, it follows that the level of enolase-MelanA fusion in these extractions would increase in relation to the amount released by the pH 7.6 buffers. Enolase-MelanA levels seen in the pH 9.0 wash do not increase, indicating that the increase in total protein is due to the release of ionically bound protein from the cell wall under these conditions.

High cell density fermentation of *P. pastoris* is an effective way to greatly enhance the amount of recombinant protein produced by this organism. For traditionally secreted proteins, fermentation can result in protein concentrations in the gram per liter range. With enolase-MelanA, initial fermentations failed to yield protein in the culture supernatant as was seen in shake flask cultures. Furthermore, washing of the cell pellets failed to release any fusion protein. Elevating the $CaSO_4$ concentration of the media yielded enolase-MelanA in the supernatant at 72 and 96 hours, and throughout fermentation bound to the cell mass. Blotting with anti-enolase polyclonal antibodies indicates that degradation of the fusion protein occurs heavily at 72 and 96 hours.

There are a number of factors in *P. pastoris* fermentation that can lead to high levels of degradation. As growth of *P. pastoris* on methanol has been shown to be very stressful, with up to 20% cell death being seen shortly after induction (Hohenblum et al. 2003), increased protease activity in the culture supernatant is the likely cause of enolase-MelanA degradation. Compounding the increased amount of proteases, studies of proteolytic activity in *P. pastoris* supernatants have shown that at pH 6.0 where enolase-MelanA secretion is maximized in shake flasks, protease activity is very high (Shi et al. 2003). Higher salt concentrations in *P. pastoris* fermentations have been shown to result in a lipid like substance in the culture supernatant that can interfere with protein purification (Brady et al. 2001). Brady et al. speculated that this

was due to cell lysis. The amount of cell lysis late in fermentation and the resulting increase is proteolytic activity could be quickly degrading any fusion protein located in the culture supernatant. The lower yield of extracted protein from cell pellets incubated in YPDS for one hour is interesting, especially since there is no appreciable difference in the amount of enolase-MelanA fusion. YPDS incubation for one hr specifically results in lower levels of contaminating host protein in the final extraction. It is possible that incubation in YPDS allows the cells to continue to grow and remodel their cell walls such that there is less protein available for extraction, however more work needs to be done to study this phenomenon.

Ultimately, the yields achieved in this work are very low, and releasing protein from the cell mass results in the concurrent release of a number of *P. pastoris* host proteins. As the primary goal of protein secretion is to increase initial purity prior to downstream purification, these results suggest that enolase driven secretion is not effective for industrial scale protein production. Furthermore, cleavage of enolase and the target protein must occur. The inclusion of specific protease sites between the two proteins could be an effective means to achieve this, but this type of processing is expensive and difficult to implement at an industrial scale. Future work elucidating the precise mechanism of enolase export might provide insight that could increase the yield of secreted fusion protein. For example, it has been shown that certain mutations of *S. cerevisae* can lead to the increased export of enolase to the culture supernatant (Kim and Park 2004), something that might also be possible with *P. pastoris*. Simple methods to separate enolase from the protein of interest also must be developed. However, in certain cases where intracellular expression and traditional secretion proves ineffective, unconventional secretion could provide an additional method to achieve protein for further studies.

BIBLIOGRAPHY

Agaphonov, M. O., Romanova, N. V., Trushkina, P. M., Smirnov, V. N., and Ter-Avanesyan, M. D. (2002). Aggregation and retention of human urokinase type plasminogen activator in the yeast endoplasmic reticulum. BMC Mol Biol *3*, 15.

Agaphonov, M. O., Sokolov, S. S., Romanova, N. V., Sohn, J., Kim, S., Kalebina, T. S., Choi, E., and Ter-Avanesyan, M. D. (2005). Mutation of the protein-O-mannosyltransferase enhances secretion of the human urokinase-type plasminogen activator in Hansenula polymorpha. Yeast *22*, 1037-47.

Andrei, C., Dazzi, C., Lotti, L., Torrisi, M. R., Chimini, G., and Rubartelli, A. (1999). The Secretory Route of the Leaderless Protein Interleukin 1beta Involves Exocytosis of Endolysosome-related Vesicles. Mol. Biol. Cell *10*, 1463-1475.

Brady, C. P., Shimp, R. L., Miles, A. P., Whitmore, M., and Stowers, A. W. (2001). High-level production and purification of P30P2MSP1(19), an important vaccine antigen for malaria, expressed in the methylotropic yeast Pichia pastoris. Protein Expr. Purif *23*, 468-475.

Chaffin, W. L. (2008). Candida albicans Cell Wall Proteins. Microbiol. Mol. Biol. Rev. *72*, 495-544.

Damasceno, L. M., Pla, I., Chang, H., Cohen, L., Ritter, G., Old, L. J., and Batt, C. A. (2004). An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in Pichia pastoris. Protein Expr Purif *37*,

18-26.

Delgado, M. L., Gil, M. L., and Gozalbo, D. (2003). Starvation and temperature upshift cause an increase in the enzymatically active cell wall-associated glyceraldehyde-3-phosphate dehydrogenase protein in yeast. FEMS Yeast Research *4*, 297-303.

Edwards, S., Braley, R., and Chaffin, W. (1999). Enolase is present in the cell wall of Saccharomyces cerevisiae. Fems Microbiology Letters *177*, 211-216.

Engling, A., Backhaus, R., Stegmayer, C., Zehe, C., Seelenmeyer, C., Kehlenbach, A., Schwappach, B., Wegehingel, S., and Nickel, W. (2002). Biosynthetic FGF-2 is targeted to non-lipid raft microdomains following translocation to the extracellular surface of CHO cells. J Cell Sci *115*, 3619-3631.

Hohenblum, H., Borth, N., and Mattanovich, D. (2003). Assessing viability and cell-associated product of recombinant protein producing Pichia pastoris with flow cytometry. Journal of Biotechnology *102*, 281.

Jong, A. Y., Chen, S. H. M., Stins, M. F., Kim, K. S., Tuan, T., and Huang, S. (2003). Binding of Candida albicans enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. J Med Microbiol *52*, 615-622.

Kim, K., and Park, H. (2004). Enhanced secretion of cell wall bound enolase into culture medium by the soo1-1 mutation of Saccharomyces cerevisiae. Journal

of Microbiology *42*, 248-252.

Klis, F. M., Jong, M. D., Brul, S., and Groot, P. W. J. D. (2007). Extraction of cell surface-associated proteins from living yeast cells. Yeast *24*, 253-258.

Lin-Cereghino, J., Wong, W. W., Xiong, S., Giang, W., Luong, L. T., Vu, J., Johnson, S. D., and Lin-Cereghino, G. P. (2005). Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris. Biotechniques. *38*, 44–48.

López-Villar, E., Monteoliva, L., Larsen, M. R., Sachon, E., Shabaz, M., Pardo, M., Pla, J., Gil, C., Roepstorff, P., and Nombela, C. (2006). Genetic and proteomic evidences support the localization of yeast enolase in the cell surface. Proteomics *6 Suppl 1*, S107-18.

McGrath, J. P., and Varshavsky, A. (1989). The yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. Nature *340*, 400-4.

Nickel, W., and Seedorf, M. (2008). Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. Annu Rev Cell Dev Biol *24*, 287-308.

Nombela, C., Gil, C., and Chaffin, W. (2006). Non-conventional protein secretion in yeast. Trends in Microbiology *14*, 15-21.

Sagt, C. M. J., Müller, W. H., van der Heide, L., Boonstra, J., Verkleij, A. J., and Verrips, C. T. (2002). Impaired cutinase secretion in Saccharomyces cerevisiae

induces irregular endoplasmic reticulum (ER) membrane proliferation, oxidative stress, and ER-associated degradation. Appl Environ Microbiol *68*, 2155-60.

Sambrook, J. (2001). Molecular Cloning: A Laboratory Manual 3rd ed. (Cold Spring Harbor Laboratory Press).

Savina, A., Fader, C. M., Damiani, M. T., and Colombo, M. I. (2005). Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. Traffic *6*, 131-43.

Shi, X., Karkut, T., Chamankhah, M., Alting-Mees, M., Hemmingsen, S. M., and Hegedus, D. (2003). Optimal conditions for the expression of a single-chain antibody (scFv) gene in Pichia pastoris. Protein Expr. Purif *28*, 321-330.

Chapter 5-Conclusions and Perspectives

Pichia pastoris is proving to be a capable alternative to bacterial and mammalian expression platforms. This work has described the use of *P. pastoris* to produce a clinically useful protein, NY-ESO-1. Using purification methods originally designed for *Escherichia coli*, protein expressed in an intracellular insoluble fraction was able to be collected and solubilized, however the protein exhibited the loss of the C-terminal 6X his tag. The loss of the his tag was shown to be due to the presence of a Kex2 protease site directly upstream of the feature, and the removal of this cleavage signal resulted in the maintenance of the his tag on the final protein. The secretory pathway capacity was enhanced by cooverexpressing the *Saccharomyces cerevisae* SNARE protein Sso1, which led to the improvement of secretion of the single chain antibody fragment against A33. Cooverexpression of the *S. cerevisae* SNARE protein Snc2, however, did not lead to an enhancement in yields, highlighting the importance of SNARE protein sequence homology in driving successful fusion events. Finally, an attempt was made to secrete another clinically interesting protein, MelanA, through an unconventional secretory route. In shake flasks it appears that the fusion protein does escape to the extracellular space, in particular becoming bound to the cell walls. In fermentor culture, however, fusion protein is only detected in the supernatant at very late time points, possibly as a result of cell lysis. In addition to the work described in this manuscript, there are numerous other exciting and innovative research projects that aim to expand the capabilities of this interesting organism.

One well developed project with great potential is the humanization of the *P. pastoris* glycosylation pathway. Proper glycosylation of recombinant proteins is important to ensure proper protein activity and stability (Helenius and Aebi 2001), but also to limit the potential for adverse immune responses caused by non-native carbohydrate structures. The importance of proper glycosylation is one of the primary reasons

mammalian cell culture systems are so heavily used, despite their limitations. The first step in achieving humanized glycosylation was to remove the ability to hypermannosylate glycoproteins. A strain of *P. pastoris* that is fully humanized has been developed that required the removal of four genes with the subsequent introduction of 14 heterologous genes (Hamilton et al. 2006). A recombinant erythropoietin was generated in these strains with activity similar to that produced in mammalian cell culture systems. Also in this system, different glycoforms of IgG have been produced that vary in their ability to mediate immune function. This system has the potential to supplant mammalian cells as the workhorse for therapeutic protein production.

Another active area of research is the cooverexpression of chaperones in *P. pastoris*. Work in our lab has shown the effectiveness of overexpressing BiP in addition to A33 scFv to enhance yields of secreted protein three fold (Damasceno et al. 2007). Inan et al improved the secretory yields of *Necator americanus* secretory protein (Na-ASP1), which contains 20 cysteine residues, by the cooverexpression of protein disulfide isomerase (Inan et al. 2006). Zhang et al. demonstrated that secretory yields of granulocyte colony stimulating factor could be improved not only by the co-overexpression of BiP or PDI, but also by the co-overexpression of the cytosolic chaperones Ssa1p and YDJ1p from *S. cerevisae* (Zhang et al.). In this case, the overexpression of cytosolic chaperones is thought to stabilize the unfolded intermediate protein prior to its import into the ER. A library of *P. pastoris* clones harboring various combinations of ER and cytosolic chaperones could be constructed to quickly screen for their effectiveness at improving secretory yields of each new target protein.

The AOX1 promoter is by far the most commonly used promoter for heterologous protein expression in *P. pastoris*. Hartner et al. recently fully characterized a library of AOX1 promoter variants with activities that range between 6% and 160% of wild type (Hartner et al. 2008). They were also able to generate AOX1 promoter fragments that were tightly repressed by glycerol, but were strongly activated either by starvation conditions or other carbon sources. This is in contrast with the wild type promoter, where the presence of methanol is necessary for strong induction. This library of promoters will provide researchers a toolbox to fine tune the strength of induction to maximize protein yields, while also providing a means for inducing other accessory proteins, such as chaperones, at low levels.

The use of *P. pastoris* for high level recombinant protein production will continue to improve in popularity. The advantages it offers over other well established production systems are many. The construction of stable expressing strains is straightforward, the growth is rapid and occurs to very high cell densities, and most importantly it can successfully modify and secrete many heterologous proteins. With the recent public release of the *P. pastoris* genome, there now exists even greater potential to rationally design strains optimized for expression of a wide array of proteins. *P. pastoris* is poised to make significant contributions to recombinant protein expression in the future.

BIBLIOGRAPHY

Damasceno, L. M., Anderson, K. A., Ritter, G., Cregg, J. M., Old, L. J., and Batt, C. A. (2007). Cooverexpression of chaperones for enhanced secretion of a single-chain antibody fragment in Pichia pastoris. Appl Microbiol Biotechnol *74*, 381-9.

Hamilton, S. R., Davidson, R. C., Sethuraman, N., Nett, J. H., Jiang, Y., Rios, S., Bobrowicz, P., Stadheim, T. A., Li, H., Choi, B., et al. (2006). Humanization of yeast to produce complex terminally sialylated glycoproteins. Science *313*, 1441-3.

Hartner, F. S., Ruth, C., Langenegger, D., Johnson, S. N., Hyka, P., Lin-Cereghino, G. P., Lin-Cereghino, J., Kovar, K., Cregg, J. M., and Glieder, A. (2008). Promoter library designed for fine-tuned gene expression in Pichia pastoris. Nucl. Acids Res., gkn369.

Helenius, A., and Aebi, M. (2001). Intracellular functions of N-linked glycans. Science *291*, 2364-9.

Inan, M., Aryasomayajula, D., Sinha, J., and Meagher, M. M. (2006). Enhancement of protein secretion in Pichia pastoris by overexpression of protein disulfide isomerase. Biotechnol. Bioeng *93*, 771-778.

Zhang, W., Zhao, H., Xue, C., Xiong, X., Yao, X., Li, X., Chen, H., and Liu, Z. Enhanced secretion of proteins in P. pastoris following overexpression of S. cerevisiae chaperone proteins. Biotechnol Prog *22*, 1090-5.