Expression and Purification of WO2 Antibody Antigen-Binding Fragments and Implications for Passive Immunotherapy of Alzheimer's Disease

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Senior Thesis Project

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Abstract

The focus of this project is to produce and study the antigen-binding fragment (Fab) of the antibody WO2. WO2 is a monoclonal anti-amyloid IgM that could give new insights into the study of Alzheimer's disease. Although WO2 does not bind to the precursor proteins of an amyloid fibril, it can bind both Aβ fibrils and amyloid fibrils formed from other proteins. Therefore, elucidating the structure of WO2 could help researchers better understand the structure of the amyloid fibrils as well WO2's potential role in passive immunotherapy and diagnosis of Alzheimer's disease (O'Nuallain and Wetzel 2002).

To study the structure of WO2, x-ray crystallography will be implemented. However, the first challenge was to express the antibody. The yeast expression system *Pichia pastoris* was chosen because it is a well understood eukaryotic system. A eukaryotic system is useful for recombinant expression of eukaryotic proteins such as antibodies, since bacteria often cannot fold and secrete these properly (Lange *et al.* 2001). Fabs expressed in prokaryotes are often deposited within insoluble inclusion bodies, while the yeast system can correctly oxidize and fold a heterodimeric disulfide-linked Fab. After many failed attempts, *Pichia* was transformed with a recombinant plasmid containing the WO2 gene, ultimately leading to successful expression and secretion of WO2. After secretion, the antibody had to be purified. Several different protein purification techniques were utilized, but much of the protein was lost in these steps. Enough was purified, however, for crystallization experiments. We were able to optimize crystallization conditions in order to produce needle-like crystals. These crystals, as well

as the methods developed below, will further the goal of studying the structure of WO2 with x-ray crystallography.

Background

Alzheimer's disease (AD) is a debilitating and tragic illness thought to be caused by the aggregation of a normal peptide, β-Amyloid (Aβ), into an insoluble plaque in the brain. There are other diseases linked to abnormal peptide fibril and plaque formation, and these are collectively referred to as "amyloidoses" (Dobson 2002). Classic symptoms of AD include increasing memory loss, decreasing cognitive function, and personality change (Weiner and Frenkel 2006). AD was first described in 1906 by Dr. Alois Alzheimer, and despite the fact that its pathology has been studied since its discovery, there is not yet a cure (Alzheimer's Association).

There is ample evidence for Aβ as a significant pathogenic agent in AD. The similarities between the neuropathologies of AD and Down's syndrome (Trisomy 21) are confirmed by the location of the gene for APP on chromosome 21 (Walsh and Selkoe 2007). Furthermore, *in vitro* and *in vivo*, various forms (soluble oligomers made of 15-20 monomers, fibrillar, and Aβ-derived diffusible ligands) of Aβ peptide are toxic to neurons (Deshpande *et al.* 2006), and mutations in the gene for amyloid precursor protein (APP) and/or presenilin (a vast assembly of proteins at and within membranes that provides the active site for γ-secretase) can drastically increase the prevalence and rate of development of AD (Walsh and Selkoe 2007).

Upon examination of the plaque deposits left in the brains of AD patients, the secondary and tertiary structures of the peptide Aβ in plaques are different than its

soluble form. This is understandable: modifications and conformational changes to a normal protein could make it more susceptible to aggregation (Dobson 2002). However, the important question is "how and why does this happen?" Knowing the pathological mechanisms of disease can help greatly in developing therapies, and therefore much of the current research on AD therapy is focused on understanding and interrupting these mechanisms.

The first major pathway in AD is the initial formation of the Aβ peptide. Aβ peptide is derived from the aforementioned transmembrane protein APP, which has neither a regular length nor a known function. APP is first cleaved by the aspartyl protease β-secretase (β-amyloid cleaving enzyme-1, BACE-1) directly on the N-terminal region of the peptide sequence that will be Aβ. A large section of APP is removed in this cleavage reaction, but the segment of APP that contains Aβ is still anchored in the membrane and is called C99. Another aspartyl protease, γ-secretase, then cleaves three different sites within the transmembrane segment of C99, an activity which occurs within the presenilin complex. The first section cleaved at the "ε-site" is the C-terminal domain of APP, which is released into the cytoplasm. The second section is only a six residue long sequence cleaved at the "ζ-site". The third cleavage reaction releases the final Aβ peptide extracellularly, and can occur at several different sites collectively called the "γ-site" (Figure 1). The flexibility of this final cleavage reaction is the reason Aβ peptide can be (most commonly) 40 residues long, or two residues shorter or longer (Walsh and Selkoe 2007) Furthermore, Aβ somehow escapes cleavage by α-secretase, a normal enzymatic reaction which cuts the protein on the extracellular side like β-secretase but closer to the membrane. This cutting releases a peptide fragment that is non-toxic; in

other words, α-secretase cleavage of APP prevents the eventual formation of the ~40 residue long pathogenic Aβ (Figure 1).

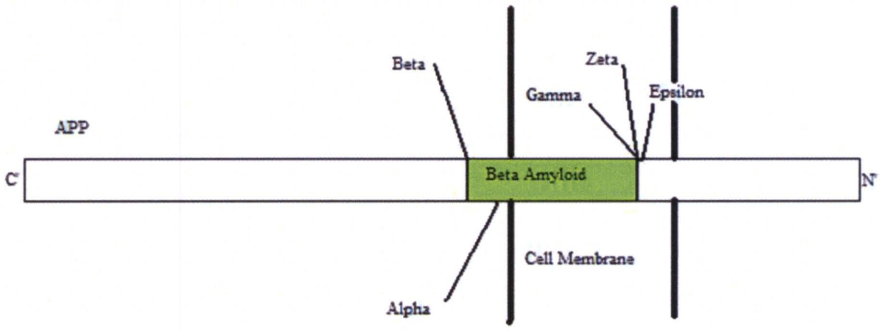

Figure 1: Action of the aspartyl proteases on APP.

The pathway of formation of amyloid plaques, the self-association of Aβ peptide, is the second major process in AD. Proposing a general mechanism of this process is a difficult task because many non-homologous peptide sequences have the ability to form fibrils and plaques, so there appears to be no one universal mechanism of formation. However, structural information about amyloid fibrils can give us clues as to how they assemble. The formation of fibrils is greatly accelerated with nucleation and seeding, similar to the process of crystallization. At some point in this process Aβ peptide folds into a β-sheet and stacks perpendicular to the length of the β-sheet, forming a strand with a structure called a "cross-β" motif. These cross-β strands packing together form the fibrils of amyloid plaques (Tycko 2006).

The formation, overproduction, and buildup of Aβ peptide from APP and the aggregation of this buildup via the mechanisms described is known as the amyloid cascade. Currently, AD research is focused on intervening at points in this cascade, from inhibiting β- and γ-secretases by use of small drug compounds to immunological approaches aimed at preventing or clearing amyloid in the brain. Immunological approaches to AD therapy involve the use of antibody-antigen interactions. Active

immunization involves the direct administration of some form of Aβ peptide, which triggers an immune response that creates anti-Aβ antibodies. Passive immunization involves peripheral administration of anti-Aβ antibodies, and both methods of immunization have been subject to extensive experimentation and have produced promising results (Solomon 2004).

Some passive immunological approaches to AD therapy are microglial activation, catalytic dissolution, and the peripheral sink hypothesis, all of which describe different ways in which monoclonal antibodies (mAbs) could be utilized to combat Aβ peptide. Microglial activation involves antibodies binding to amyloid plaques and attracting microglia for disposal. Catalytic dissolution uses antibodies in a somewhat different way; in this mechanism, antibodies bind to Aβ peptide in a way that changes its conformation and act as an inhibitor to fibril formation. The peripheral sink hypothesis proposes a much different approach than either of the two discussed. In the peripheral sink hypothesis antibodies do not target Aβ directly in the brain and spinal cord. Instead, anti-Aβ antibodies bind to Aβ in the bloodstream, shifting the equilibrium between Aβ in the central nervous system and Aβ in the bloodstream. By removing Aβ from the bloodstream, Aβ is transported there from the brain to correct the imbalance and subsequently bound by the antibody and removed (Gardberg *et al.* 2007). However, the peripheral sink hypothesis is only applicable for antibodies that bind the linear epitope of Aβ, and data suggests that antibodies WO1 and WO2 do not target this epitope (Rich and Myszka, personal communication from Gardberg). Nonetheless, all three of these methods require antibodies with high affinity for Aβ peptide (Gardberg *et al.* 2007).

Methods and Results

Construction and Propagation of Plasmids

Dr. Anna Gardberg wrote a set of instructions for the creation of a plasmid encoding a chimeric antigen-binding fragment of the antibody WO2 (WO2-Fab), and these were carried out by TopGeneTech. The chimeric Fab fragment consisted of the light chain (L) and the Fd portion of the heavy chain, and it was formed using the human IgG constant domains and the murine variable regions. The cDNAs for the L and Fd portion of WO2 were individually cloned into the pPicZα vector using the BamHI restriction site. Additionally, a thrombin cutting site was added to the light chain gene so that the C-terminal His tag already in the pPicZα vector could be used. Both the L and the Fd genes were then combined into a single vector, pPicZα-WO2 (pza-WO2), by fusing each in frame with the α-factor signal sequence of *Saccharomyces cervisiae*. The linar order on the plasmid is 5'-AOX1 promoter-alpha factor-WO2 light chain (VL-CL)-Thrombin recognition sequence-6xHis tag-stop-BamHI recognition sequence-AOX1 promoter-alpha factor-WO2 heavy chain (VH-CH)-stop-3'. This vector is under the control of the AOX1 promoter. The cloning was confirmed by PCR with L and Fd-specific primers, and subsequently by agarose gel electrophoresis. The plasmid was received in a DH10 *E. coli* stab and transformed into DH5α *E. coli* for propagation. The plasmid was then purified from the DH5α culture using Wizard SV Minipreps (Promega). The methods described above were adapted from Ning *et al.* 2005 and Lange *et al.* 2001, and were carried out by Dr. Anna Gardberg and TopGeneTech.

Transformation using Pichia pastoris

Two cultures of *Pichia pastoris* X-33 were grown overnight in 5-10ml of YPD media with 5ul of 100mg/ml ampicillin (for a final concentration of 100ug/ml). The following day, the optical density of the culture as measured at 600 nm on a visible spectrophotometer (OD_{600}) was taken. From this the amount of yeast culture needed to inoculate two new 100ml overnight cultures could be determined (using a doubling time of 2 hours and $1OD_{600}=5x10^7$ cells/ml) with the fact that the desired OD_{600} would need to be 1-2 the next day. The reasoning is as follows: $8x10^8$ cells are needed for each transformation experiment, so if 1 $OD_{600}=5x10^7$ cells/ml then a 100ml culture at 1OD contains $5x10^9$ cells total. $5x10^9$ cells is enough to do 6.25 transformations at $8x10^8$ cells/transformation. Hence, an OD_{600} = 1-2 should be plenty for five transformations. The following day, the OD_{600} of one of the 100ml cultures reached 1.4, although it took much longer than expected. This OD_{600} translated to $7x10^9$ cells (in 100ml), which was more than enough to do five transformations.

The 100ml culture was divided into two centrifuge tubes and spun down at 1500xG for five minutes. A lithium acetate solution was prepared (100mM Lithium Acetate, 0.6M sorbitol, 10mM Tris-HCl pH 7.5, and within one hour of use add 10mM DTT), and 8ml of this solution were used per number of potential transformations (8.75 potential transformations * 8ml = 70ml). The two cell pellets were each resuspended in 35ml of the lithium acetate solution and allowed to incubate at room temperature for 30 minutes. These cells were then centrifuged as before, and then the cell pellets were resuspended in 1.5ml ice cold sorbitol per number of potential transformations (1.5ml * 8.75 = 13ml total, or 7.5 ml per centrifuge tube.) This protocol is adapted from Wu and

Letchworth 2004, and has a ~100-fold increase in transformation efficiency over protocols not employing the use of lithium acetate.

Prior to transformation by electroporation into yeast, the circular pPicZαA plasmid was linearized (to be taken up effectively and efficiently by the *Pichia* cells.) This was done by restriction digestion, a reaction in which an enzyme called an endonuclease recognizes and cuts a specific nucleotide sequence within the plasmid, causing it to open and become linear. It is important to note that the endonuclease cuts at only one site, and furthermore does not also cut within the cloned gene of interest. Five micrograms of purified pPicZa and pPicZa-WO2 plasmids were digested with BglII and PmeI endonucleases (both purchased as purified enzymes from New England Biolabs (NEB)), respectively, at 37°C for 2 hours. The 40ul total-volume digest reactions were as follows: 1) pPicZa control with BglII: 5.2ul plasmid (2.5ug), 0.5ul BglII (@ 10units/ul), 0.4ul 100X BSA (@ 10mg/ml), 4ul 10X NEB Buffer 3, and 29.9ul molecular biology grade dH_2O; 2) pPicZa control with PmeI: 5.2ul plasmid (2.5ug), 0.5ul PmeI (@ 10units/ul), 0.4ul 100X BSA (@ 10mg/ml), 4ul 10X NEB Buffer 4, and 29.9ul molecular biology grade dH_2O; 3) pPicZa-WO2 with BglII: 3.8ul plasmid (1.25ug), 0.5ul BglII (@ 10units/ul), 0.4ul 100X BSA (@ 10mg/ml), 4ul 10X NEB Buffer 3, and 31.3ul molecular biology grade dH_2O; 4) pPicZa-WO2 with PmeI: 3.8ul 5-fold diluted plasmid (0.25ug total), 2.0ul PmeI (@ 1unit/ul), 0.4ul 100X BSA (@ 10mg/ml), 4ul 10X NEB Buffer 4, and 29.8ul molecular biology grade dH_2O. Reactions 1 and 2 were duplicated, 3 was performed four times, and 4 performed 20 times. Buffers used were those recommended and prepared by NEB (as provided with the enzymes): NEB 10x Buffer 3: 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, NEB 10x Buffer 4:

20 mM Tris-acetate (pH 7.9) , 50 mM potassium acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol. Digests were confirmed using agarose gel electrophoresis using low melting temperature 0.9% agarose and ethidium bromide staining, and a single band at the proper molecular weight in kilobases (the plasmid is 4.96 kilobase pairs in size) was observed. BglII worked better than PmeI in producing the digested plasmid (4.96kb). The digested plasmid was then excised from the low-melt agarose gel and purified using a DNA gel purification kit from Promega. The digested plasmid was eluted from the spin column used in the Promega kit with 10 ul of molecular biology grade distilled water (dH_2O; purchased from Eppendorf). The plasmid was then stored at 4C (if used the same day) or -20C (if used the next day) until needed for yeast transformation by electroporation.

Five experimental transformations were chosen, so five 1.5ml aliquots of the ice-cold sorbitol resuspension were transferred to 1.5ml centrifuge tubes. Each tube of cells was pelleted and resuspended in 1.5ml ice-cold sorbitol. This wash was repeated three times. After the final spin-down, the cell pellets were each resuspended in ice-cold sorbitol to a total volume of 80ul. This was then transferred into 2 mm electroporation cuvettes (Bio-Rad) and placed on ice. The *Pichia* transformations performed involved the following plasmid digests: 1) Positive control: 2ul BglII linearized pPicZa plasmid (354ng/ul), 2) Positive control: 2ul PmeI linearized pPicZa plasmid (335ng/ul), 3) Experimental: 2ul BglII linearized pPicZa-WO2 plasmid (352ng/ul), 4) Experimental: 2ul PmeI linearized pPicZa-WO2 plasmid (49ng/ul), and 5) Experimental: 1ul PmeI linearized pPicZa-WO2 plasmid (49ng/ul) + 1ul ssDNA (2mg/ml). The appropriate digested and gel-purified plasmid DNA was added to each electroporation cuvette and

allowed to incubate on ice for five minutes. Then, each cuvette of cell-plasmid-sorbitol mixture was electroporated using the "Pic" setting on a Bio-Rad MicroPulser Electroporation apparatus. After electroporation, each transformation mixture was transferred to an ice-cold eppendorf tube immediately and 0.5 ml of ice-cold sorbitol was pipetted into the mixture. The cells were allowed a 1 hour recovery at 30°C (with no mixing or shaking). After this, 0.5 ml of YPDS rich growth media was added, and the cells were allowed to recover for another hour (again with no mixing or shaking). After the final recovery, each transformation was plated on YPDS agar + 200 ug/ml final concentration of zeocin antibiotic + 50ug/ml ampicillin. While zeocin is usually lethal to *Pichia,* the pPicZαA plasmid contains a gene whose protein product chemically modifies the zeocin, rendering it ineffective. Therefore, *Pichia* cells which are successfully transformed with pPicZαA-WO2 plasmid are resistant to zeocin and can grow and divide with no complications in its presence. Ampicillin has no detrimental effect on *Pichia*, transformed or not, and is used to prevent bacterial contamination. Twenty ul of each of the positive control transformations were plated, and 2ul, 20ul, 200ul, and the remaining ~700ul of each of the experimental transformations were plated, each on separate YPDS-zeocin plates. The plates were incubated at 30°C, and, after two days, colonies of yeast appeared.

Transformed colonies that had been growing in the presence of 200 ug/ml zeocin + 50ug/ml ampicillin were restreaked onto 1000 ug/ml zeocin + 50 ug/ml ampicillin YPD agar plates to isolate the most resistant colonies. Theoretically, colonies that could withstand 1000ug/ml zeocin would be synthesizing a large number of plasmids to combat the antibiotic. By default, then, these cells would also have the capacity for generating a

large amount of WO2. Restreaked colonies that grew well and did not exhibit contamination were then used to grow overnight cultures for *Pichia* DNA isolation. DNA was isolated from each culture using colony PCR. Several colonies were treated with zymolyase (a proprietary mixture of yeast cell membrane degrading enzymes purchased from Zymo Research) by resuspending a colony in 10 ul of zymolyase solution (1000 units dissolved in 200 ul 0.9M sorbitol and 0.1 M Phosphate pH 7.7) and incubating for 15 min at 37°C. One ul of each of these resuspensions was used for PCR with 12.5 ul of GoTaq Green PCR Master Mix (Promega), 2 ul each of WO2-forward and –reverse primers, and 7.5ul water. A control of 1ul zymolyase solution plus 0.2 ul 50 ng/ul pzaWO2 vector (replacing the colony resuspension) was also used. The PCR program was a follows: 3min @ 94C (initial denaturing), 1min @ 94C (denaturing in cycle), 1min @ 55C (primer-template annealing in cycle), 3min @ 72C (polymerase extension in cycle), and 5min @ 72C (polymerase extension clean-up step. The second, third, and fourth steps were repeated for thirty cycles. The isolated and amplified DNA was then run on an ethidium bromide-stained 1% agarose gel. The colony "3-700A" was determined to have the best AOX amplification as it produced dark insert bands on the gel which matched the control, linearized pza-WO2. "3-700A" (restreaked) was subsequently used for growth and secreted expression of WO2-Fab. This re-streaking was stored at 4C. A colony from the re-streaked "3-700A" was itself re-streaked onto YPD + 50ug/ml ampicillin + 1000ug/ml zeocin agar and incubated at 30C before growth and expression.

The transformation was further confirmed and the methanol utilization phenotype determined using a different colony PCR method not implementing zymolyase or

detergent (although the zymolyase method was successful, it is expensive.) Instead of a zymolyase solution, a colony was resuspended in 10ul of 0.1N NaOH and incubated for 10 minutes at 95°C. One ul of this was used in PCR reactions as a template with the same PCR reagents as listed above, except AOX1-forward and –reverse primers were used instead of WO2. The methanol utilization phenotype (Mut^+ or Mut^S) was verified by a dark 1.2kb insert band on a 1% agarose ethidium bromide stained gel indicating AOX amplification.

Growth and Expression

The transformed *Pichia pastoris* cells from colony "3-700A" (restreaked) were initially grown using the following shake-flask protocol. All cultures were grown at 28-30C, shaking at 250rpm. Two 50ml overnight cultures were grown in BMGY + 50 ug/ml ampilcillin in 250ml flasks. BMGY is a yeast-rich growth media with glycerol as a carbon additive. The next day, each overnight culture was used to inoculate 400-500ml BMGY in 3L flasks. Each of these cultures was grown to and OD_{600} of 10-11. After reaching at least this OD_{600}, the cultures were each divided into four ~200ml aliquots in centrifuge bottles and spun down. In this system, methanol is used as the chemical inducer of expression by activating the AOX1 promoter. Each pellet was resuspended in 500ml BMMY (the same as BMGY, except glycerol replaced by methanol) + 100ug/ml zeocin + 50ug/ml ampicillin, and these four cultures were allowed to grow and express for six days. To ensure continual induction of expression, each day 0.5% methanol was added to each flask.

Seth Albright's (of the Jain Lab at the University of Tennessee) 5L fermentor protocol was also tried. A single colony was inoculated into 100ml of YPD + 50ug/ml ampicillin and allowed to grow at 28-30C and 250rpm for 32-48 hours (until OD>10). This culture was then used to inoculate 2.5L of BMGY in a 5L fermentation apparatus. This culture was grown for 24 hours at 28C, pH 6.0 (using a 30%NH_4OH feed for pH control), dissolved oxygen (DO) 30%, agitation 200-1000, and air flow 5L/min. After this 24 hour period, all the initial glycerol was exhausted as seen by the increase in DO. The culture was then fed 70ml of 50% glycerol plus 12ml/L PTM1 metals solution (acquired from Seth Albright; from Invitrogen's Pichia Fermentation guidelines, composed of 6.0g cupric sulfate-$5H_2O$, 0.08g sodium iodide, 3.0g manganese sulfate, 0.2g sodium molybdate, 0.02g boric acid, 0.5g cobalt chloride, 20.0g zinc chloride, 65.0g ferrous sulfate-$7H_2O$, 0.2g biotin, 5ml concentrated sulfuric acid, all dissolved in 1L H_2O, filter sterilized, and stored at room temperature) at 6.9% pump action. This glycerol feed was tested using a DO spike test, which is performed by stopping the feed and timing a 15% increase in DO. If the time is less than a minute, the feed is actively being used.

After the glycerol-fed batch phase, the methanol adaptation was started. Anhydrous methanol plus 12ml/L PTM1 metals solution was fed to the culture at 1.6% pump action until the DO began to increase. The pump action was then increased to 2.3% when a rapid DO spike occurred, and this feed continued for 14-16 hours. When the DO again increased and remained high and/or a rapid spike could be observed, the feed rate was increased to 2.7% pump action. 50% antifoam/water solution was used throughout the fermentation to decrease foaming. This rate was maintained for the remainder of the fermentation, which was about two days. At the end of the fermentation the OD_{600} was

>300, and the culture was removed from the flask with sterile tubing and a peristaltic pump.

Purification

In either the shake-flask or the fermentor protocols, at the end of the expression period each culture was centrifuged and the supernatant was vacuum filtered. The supernatants were combined and any protein secreted into the supernatant (hopefully including the recombinant WO2-Fab) by the yeast was precipitated with 65% ammonium sulfate. A small amount (~200ul) of supernatant untreated with ammonium sulfate was saved and microdialyzed overnight for an SDS-PAGE gel sample; this was to ensure that the target protein (WO2-Fab) was present before continuing purification steps (Figure 2). *Note: In every gel figure, the standard used is Bio-Rad Prestained Low Molecular Weight Standards. The bands are, in descending order, Phosphorylase B 106,904Da, BSA 93,636Da, Ovalbumin 52,264Da, Carbonic Anhydrase 37,226Da, Soybean Trypsin Inhibitor 28,224Da, and Lysozyme 18,833Da. The WO2-Fab is composed of the light chain linked by disulfide bond to the Fd portion of the heavy chain of the antibody. Both the light chain and the Fd portion weigh 25-30kDa. So, the unreduced Fab migrates to around 50kDa on an SDS-PAGE gel, and the reduced fragments migrate and blend together at around 30kDa. Beta-mercaptoethanol (BME) was used as a reducing agent for all reduced gel samples. On the following gels using prestained standard, the unreduced Fab (Light chain plus Fd) migrates near the third standard band (ovalbumin) and the reduced Fab (Light chain and Fd separated) migrates in between the fourth and the fifth standards (carbonic anhydrase and soybean trypsin inhibitor, respectively.)*

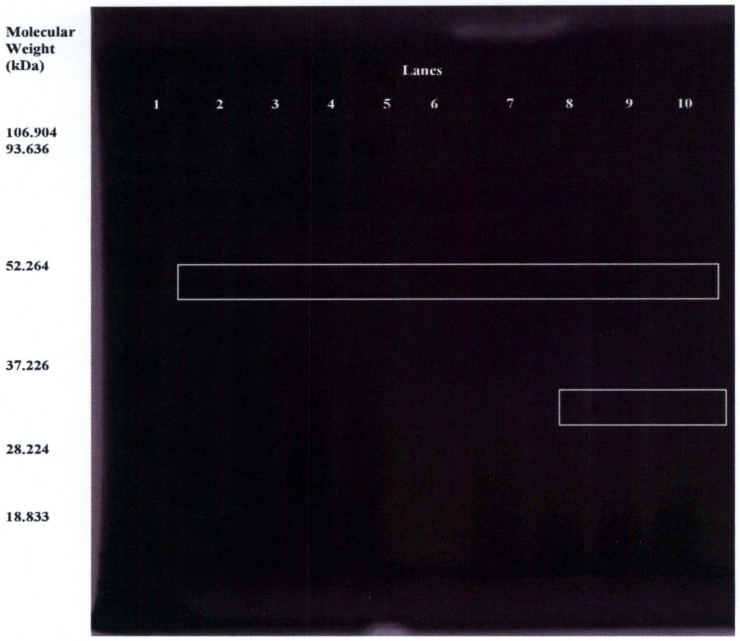

Figure 2: 12% SDS-PAGE Gel of Microdialyzed Supernatants taken from the Fermentation. Lanes: 1) Prestained Standard, 2) Beginning of fermentation methanol adaptation, 3) 6 hours of methanol adaptation, 4) 1 day of methanol adaptation, 5) 2 days of methanol adaptation, 6) vacant, 7-10) Same as lanes 2-5, but with BME-reduced samples. The unreduced Fab is visible in all samples at ~50kDa, and the reduced Fab is visible in the last two lanes at ~30kDa. All following gel figures show one or both of these bands.

After 2+ hours of ammonium sufate precipitation, the supernatant was centrifuged in aliquots to separate the ammonium sulfate pellet. The ammonium sulfate pellets were then divided into smaller aliquots (0.5-3g) and the mass of each small pellet was recorded. The ammonium sulfate pellets were stored at 4C in a wash solution of 65% ammonium sulfate, 0.1% sodium azide, and 1mM EDTA. Overall, about 2g of ammonium sulfate pellet per liter of culture resulted from the fermentor (~10g pellet/5L), while 1.5-2.5 g ammonium sulfate pellet per liter of culture resulted from the shake flask protocol (~3-5g pellet/2L).

To purify the WO2-Fabs from the ammonium sulfate pellets, ~1-3g, or roughly two individual pellets, was used. Each pellet was resuspended in 20mM MES (pH 6.0) to a total volume of 2.5ml and desalted on a GE Healthcare PD-10 G25 desalting column according to the packaged instructions. The desalted pellet resuspensions were then subjected to further purification in one of two ways.

The first method used to treat the desalted pellet suspensions was cation exchange purification on a BioCad perfusion HPLC system (Perceptive Biosystems). HPLC. All resuspended and desalted pellets were combined and brought to a 10ml load volume. They were purified using a Poros HS column, with 20mM MES pH 6.0 used as running buffer ("buffer A") and 20mM MES pH 6.0 + 100-250mM NaCl used as elution buffer ("buffer B"). The appropriate fractions were then analyzed using SDS-PAGE, and the best (most pure and most highly-concentrated) fractions according to the gel were pooled (Figures 3, 4).

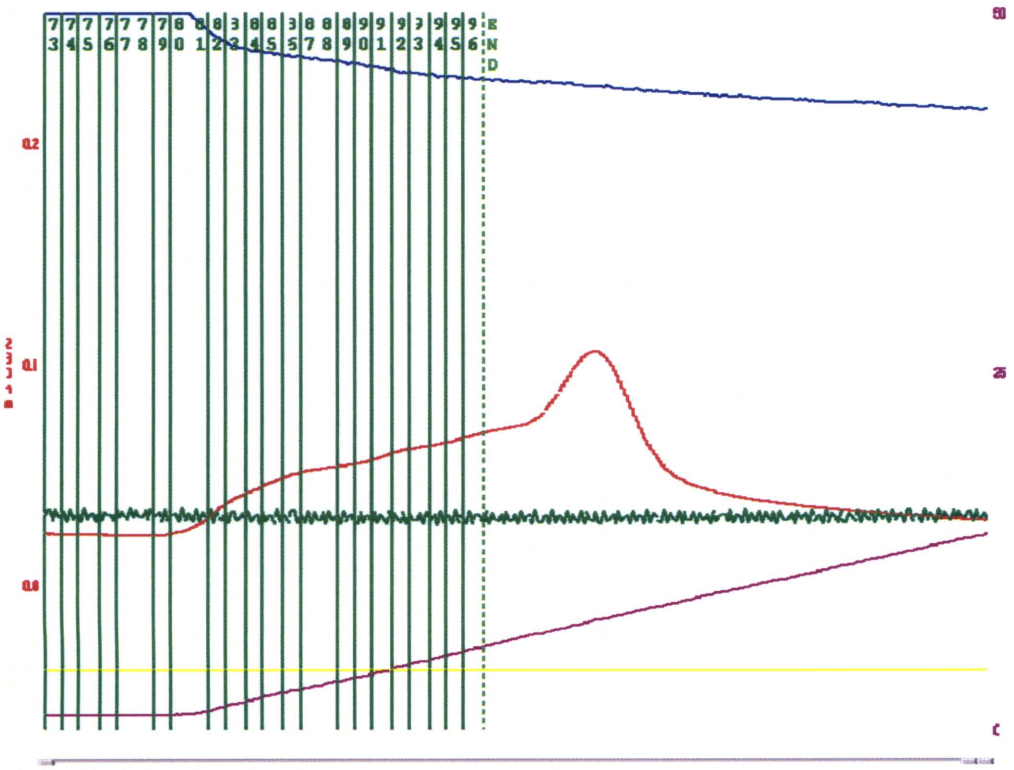

Figure 3: Chromatogram from Poros HS Cation Exchange Purification from 6-25-07. The HPLC program was not correct here and stopped collecting fractions after 96. However, this problem was identified immediately and the peak was captured in a beaker. See corresponding SDS-PAGE gel below.

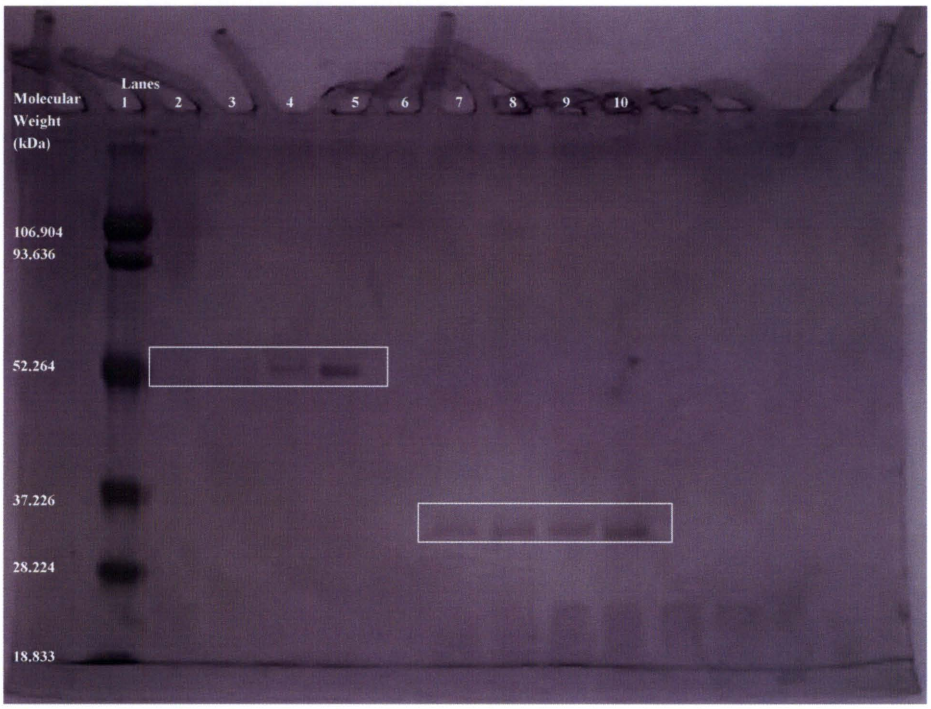

Figure 4: 12% SDS-PAGE Gel, 6-25-07 Poros HS Cation Exchange of WO2-Fabs (Figure 3). Shoulder 1 is fractions 82-84, Shoulder 2 is fractions 85-87, Shoulder 3 is fractions 91-93, Peak was collected in a small beaker. Lanes: 1) Prestained Standard, 2)Shoulder 1, 3)Shoulder 2, 4)Shoulder 3, 5)Peak, 6)Blank, 7)Shoulder 1 BME Reduced, 8)Shoulder 2 BME Reduced, 9) Shoulder 3 BME Reduced, 10)Peak BME Reduced. The unreduced bands (2-5) show unreduced WO2-Fab (~50kDa). The reduced bands (7-10) show reduced WO2-Fab (~30kDa). Both of these bands are described in the note on pages 14-15.

The second method used to treat the desalted pellet suspensions was immobilized metal affinity chromatography (IMAC), and this method worked better as a first step (after ammonium sulfate cut) than the cation exchange. IMAC takes advantage of the C-terminal His tag in the WO2-Fab construct by using metals like nickel or cobalt to coordinate with the string of histidines. Additionally, if the cation exchange was chosen initially but did not adequately purify the sample, IMAC was then utilized. Ni-NTA resin (Qiagen) was used with 50mM dibasic phosphate buffer pH 8 + 300mM NaCl, and varying imidazole concentrations for binding, wash, and elution (5, 10, and 250mM, respectively.) Imidazole is used for elution because it competes with the His tag for metal coordination. Each pellet solution was first brought to 100mM phosphate buffer +

300mM NaCl. 5ml of Ni-NTA resin slurry was collected and centrifuged in a new 50ml tube. The storage buffer was removed, and the resin was washed with 20ml of binding buffer twice. The pellet solution sample was then added and incubated at 4C while rocking gently for one hour. After this incubation period, the resin-protein complex was transferred to a Bio-Rad Econocolumn of appropriate size, attached to a corresponding flow adapter, washed on an HPLC with 10 column volumes of wash buffer. The protein was finally eluted on a gradient (1→100% wash→elution buffer) over 60ml, and collected in 1ml aliquots. According to the chromatogram, the best aliquots were chosen and analyzed using SDS-PAGE. According to the gel, the best fractions were collected and combined. It was subsequently dialysed into 20mM MES pH 6.0 and further purified via cation exchange chromatography as previously described (Figures 5, 6, 7).

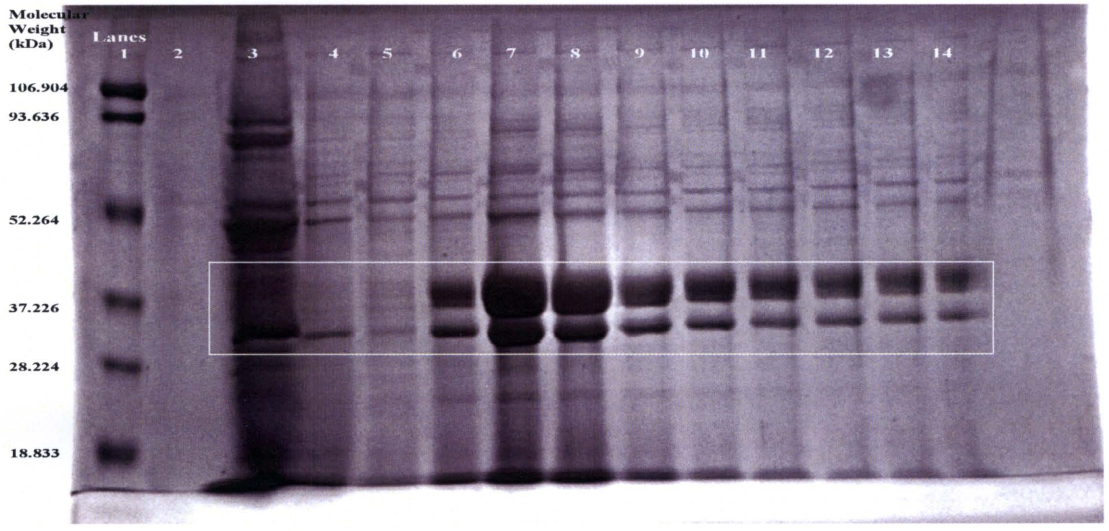

Figure 5: 12% SDS-PAGE gel from 7-25-07, BME reduced samples. From a Ni-NTA IMAC, 1ml fractions collected by hand. Lanes: 1) Prestained Standard, 2) Vacant 3) IMAC binding flowthrough, 4) IMAC wash flowthrough, Lanes 5-14) Fractions 1-10, respectively. Note double band only seen after fermentation. Because this gel looked so dirty, these fractions (1-10) were combined, dialysed into 20mM MES pH6.0, and run on a Poros HS cation exchange. That run produced a small peak which was subsequently lost in a spill accident. The flowthrough from this IMAC, combined with the flowthrough from the subsequent cation exchange were then combined and run on another cation exchange as seen below in figures 6 and 7. Note the unidentified heavier double band above the reduced WO2-Fabs at 30kDa (as previously described in note on p.14-15.)

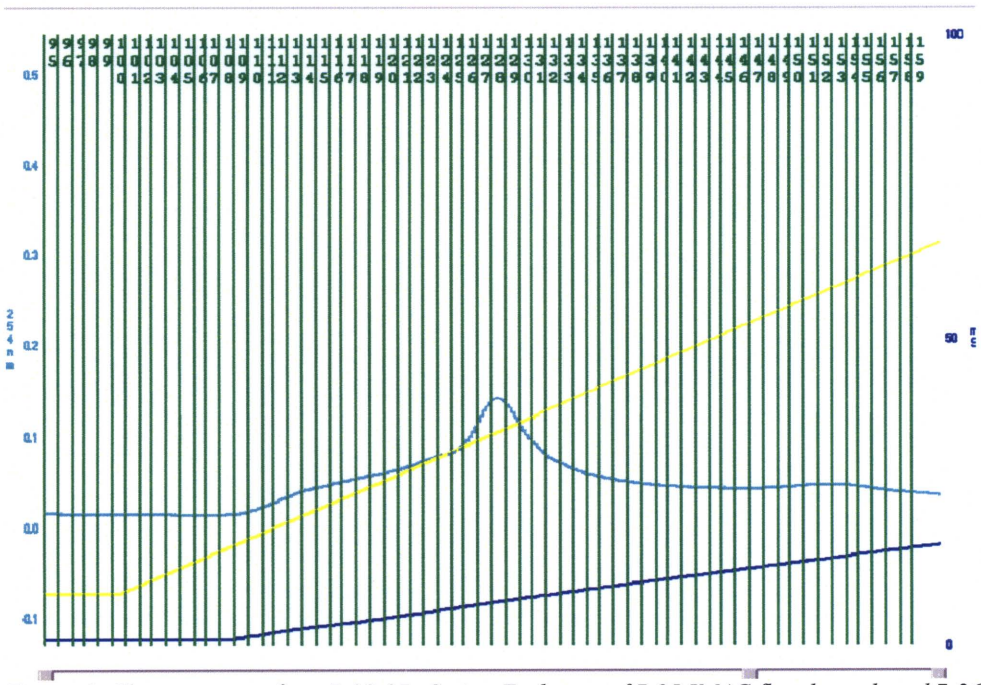

Figure 6: Chromatogram from 7-30-07: Cation Exchange of 7-25 IMAC flowthrough and 7-26 Cation Exchange Flowthrough (from 7-25 IMAC.) The 7-26 Cation Exchange was subject to an accident and all material was lost. As this chromatogram shows, however, some protein was salvaged from the flowthroughs. See corresponding gel (Figure 7).

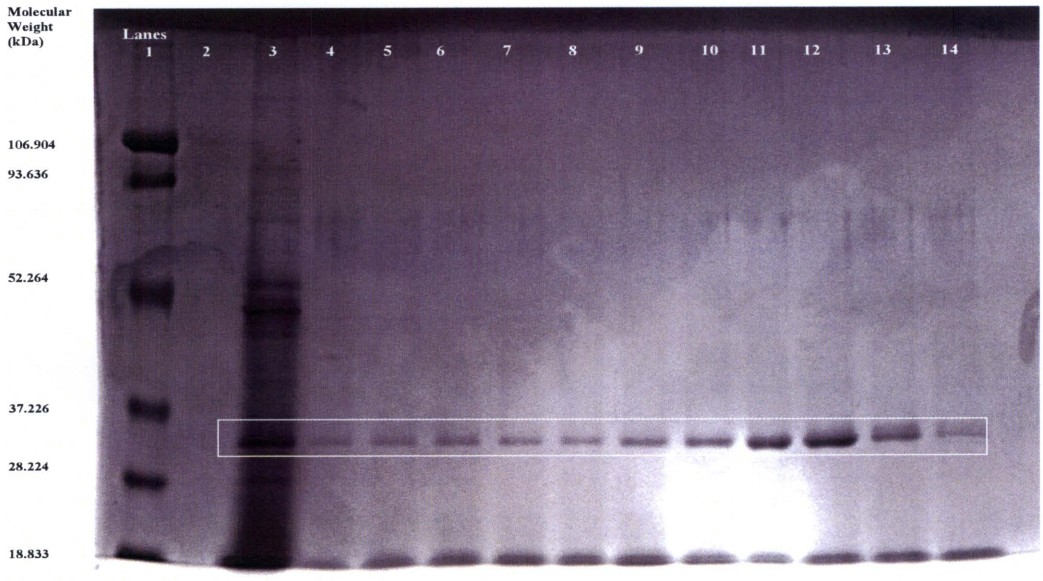

Figure 7: 12% SDS-PAGE gel of 7-30-07 Cation Exchange (Chromatogram Figure 6). All samples were reduced with BME. Lanes: 1)Prestained Standard, 2) Vacant, 3) Flowthrough, Lanes 4-14) Odd numbered fractions 113-133 (i.e. 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133). Note that after the Cation Exchange, the heavier unidentified band is gone. The bands at ~30kDa show reduced WO2-Fab as previously described in note on p.14-15.

After purifying the sample via IMAC and/or cation exchange chromatography, the sample was concentrated on a Millipore Centriplus YM-30 centrifuge filter to an appropriate concentration (as measured by Bradford assay) for Thrombin cleavage of the C-terminal His tag (typically >1mg/ml.) A thrombin cleavage protocol was developed based on the instructions and information in Novagen's Thrombin Kit. Novagen's 10X Thrombin cutting buffer (200mM Tris-HCl pH 8.4, 1.5M NaCl, 25mM $CaCl_2$) was prepared, and dilutions of thrombin were made with 50% glycerol. After testing various concentrations of Thrombin (stock from Haematologic Technologies, Inc., ~3800 NIH U/mg, 13.6 mg/ml) and digestion times, it was determined that a four-hour digestion with a 1:500 thrombin dilution worked the best with our protein. The tests were scaled to 50ug protein per 50ul total digestion volume, and we scaled this up to 100X (i.e. 1mg protein per 1ml digestion volume) for cleaving the purified protein. Whether IMAC and cation exchange or only cation exchange was used as described above, the thrombin cut was thoroughly dialyzed into 20mM MES pH 6.0 (to remove the salt from the IMAC solutions and/or the thrombin cutting buffer) in preparation for the final purification step, size-exclusion chromatography.

The sample was finally purified on the BioLogic HR HPLC using a Superdex 200 Column with 20mM MES pH 6.0 + 250mM NaCl. This method was not ideal, each time causing 50-90% loss of protein. The protein that was left was pooled according to the chromatogram (Figure 8) and concentrated using a Millipore YM-30 centriplus and/or a Millipore YM-30 Centricon to 5-10mg/ml (measured by Bradford assay.) The purified WO2-Fab was stored at 4C until crystallization trials.

To summarize, the purification process started with ~1-3 grams of ammonium sulfate pellet (at ~1.5-2.5g ammonium sulfate pellet per liter of *Pichia* culture). After the IMAC/Cation Exchange steps the ammonium sulfate pellet yielded approximately 1-2mg of WO2-Fabs. This was used for the thrombin cut, and subsequently purified by gel filtration on the Superdex 200 column. After this final purification step, usually less than a milligram of protein remained for an overall yield of a few hundred micrograms of protein produced per liter of *Pichia* culture. The following gel filtration chromatogram (Figure 8) shows a purification with one of the best yields, almost 2mg of WO2-Fabs per liter of *Pichia* culture. This purification did not implement IMAC at all. However, when the same protocol was repeated, much lower yields were observed—that is, these good results could never be replicated. This is why IMAC was tried. Overall, the most thorough and replicable purification process used IMAC, then cation exchange, then gel filtration. Although it did not produce the highest yields, enough WO2-Fabs were purified to conduct crystallization experiments.

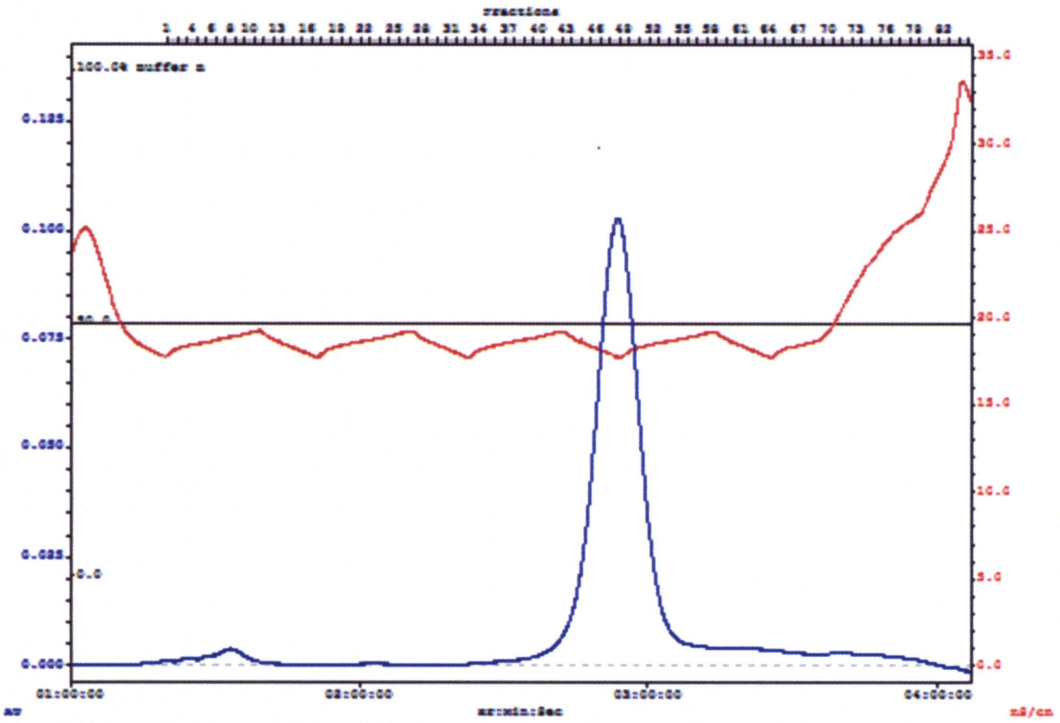

Figure 8: Final Purification Step: Size-Exclusion Chromatography on GE Superdex 200; WO2, from 5-24-07. This sample had approximately 2mg of WO2-Fabs as measured by Bradford assay.

Crystallization

The WO2-Fab protein was first screened for crystallization with the Quiagen PEGS Suite and the Hampton Index screens using a Phoenix Nanodispensing Robot (Art Robbins Instruments). The conditions that yielded crystals in these screens predominantly contained ammonium salts, were buffered with Tris or Bis-Tris, and used varied PEGs as a precipitant. Further optimization was carried out using a range of concentrations of various ammonium salts, a range of pHs of Tris, Bis-Tris, and Citrate, and appropriate concentrations (15-25%) of PEGs of polymer lengths of 3350, 6000, 8000, 10,000, 20,000 and PEG-MME5000. Many more optimizations were carried out, and the most productive conditions usually involved an ~0.1-0.3M ammonium salt, ~0.1M citrate

pH5.0, and either 25% w/v PEG3350 or PEG-MME5000. Needle crystals 400-700 x 20um were most commonly observed (Figure 9.) The most promising crystals grew in 0.3M ammonium sulfate, 0.1M citric acid pH 5.0, and 25% PEG-MME 5000 via microseeding from a similar condition. Because X-ray diffraction experiments are usually performed at liquid nitrogen temperatures (-180C), cryogenic buffer conditions for each crystal condition had to be prepared and tested. The successful conditions were used to freeze the most promising crystals in liquid nitrogen to store for later x-ray diffraction experiments.

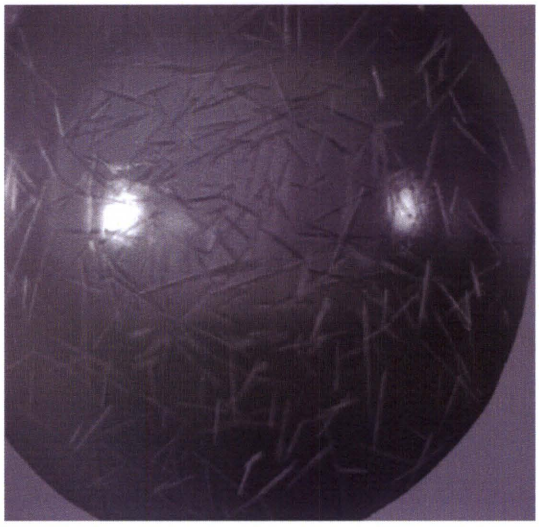

Figure 9: WO2-Fab Crystals, plate 141-D1. 0.2M Ammonium Sulfate, 0.1M Citric Acid pH 5.0, 25% PEG-MME 5000. These crystal needles are ~400x50um. There were crystals that were longer in other wells, but they were bundles of many very thin crystals. These were more separate, individually larger, and less aggregated.

Discussion

The methods developed and crystals produced in this project will further advance the structure-based study of WO2 and other mAbs. However, there are certainly some aspects of the project that need refinement, most notably the expression and purification of the protein. The project began by attempting transformation with the methods outlined in the Invitrogen *Pichia pastoris* manual, but we were unable to succeed using these. Furthermore, *Pichia* is known to have low transformation efficiency (Wu and Letchworth 2004). To overcome these problems, we adopted parts of the protocol reported by Wu

and Letchworth 2004, and this worked well. Although the transformation of *Pichia pastoris* was, in itself, a hard-earned success, the shake-flask preps typically only produced a milligram or less of protein per liter of culture. This was what drove us to consider fermentation instead of a shake-flask protocol, but the fermentation produced similar yields. Additionally, the fermentation produced not only WO2 but also what might have been an unidentified form of WO2, as seen in an extra band on SDS-PAGE gels (Figure 5.) However, only one fermentation was attempted, and the methods certainly need to be further studied, altered, and refined for better expression.

The major area of the project that requires refinement is the purification of the WO2-Fabs. Low levels of protein eluted in the cation exchange steps (peak absorbances at 280nm were frequently below 0.2), but the size-exclusion chromatography steps saw the most drastic reductions in material. Throughout the entire process, ~1-3g of ammonium sulfate cut pellet would yield a few hundred micrograms of protein at most. Overall, the loss was primarily in the HPLC steps, and this, of course seriously limited the number of crystallizations that could be performed.

Aside from the need for improvements to the current protocol for expressing and purifying WO2 using *Pichia pastoris*, the results of this project will be very useful for further study. Using *Pichia pastoris* for expression of mAbs is advantageous over other eukaryotic systems because of the fact that *Pichia* is well-studied, can be used for plasmid-based recombinant gene expression, can link the mAb heterodimer and secrete it in the correctly folded state, has relatively simple growth and expression, and provides timely confirmation of results. Our successful transformation protocol for *Pichia* should be of help to anyone wanting to use *Pichia* for expressing mAbs. Furthermore, both the

successes and letdowns throughout this project provide valuable information for those working with or planning to do similar work with *Pichia.* Lastly, even with the problems of low expression and low purification yield, we did manage to purify and produce crystals of WO2-Fabs, the original goal.

The crystals produced will serve as a beginning step in x-ray crystallographic studies of WO2. Furthermore, the methods developed for producing WO2 can be refined and used for further production of WO2 and other mAb crystals. I hope that the inheritors of this project can learn from what we have done thus far and go on to accomplish much more.

Conclusion

Research on Alzheimer's Disease is exceedingly important to a society with a rapidly increasing elderly population. As the abilities of medicine advance, many more people will be living into old age than in previous eras, and in the next few decades this will be especially true of the baby-boomer generation. Diseases primarily affecting the aged population have profound social and economic consequences, and developing therapies for such diseases is one of our biggest challenges. Projects like the one described here continue to address that challenge and further our understanding of Alzheimer's Disease and potential treatments.

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Media Recipies (from Invitrogen's *Pichia pastoris* manual)

Yeast Extract Peptone Dextrose Medium (YPD)

1% yeast extract
2% peptone
±2% agar (for plates only)

After Autoclaving
2% dextrose (glucose)

Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS)

1% yeast extract
2% peptone
1M sorbitol
±2% agar (for plates only)

After autoclaving
2% dextrose (glucose)

Buffered Glycerol- or Methanol- complex Medium (BMGY/BMMY)

1% yeast extract
2% peptone
100 mM potassium phosphate, pH 6.0
1% glycerol or methanol, respectively
in nanopure or endotoxin free water

After autoclaving
1.34% yeast nitrogen base
4 x 10-5% biotin

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