Chemical Strategies to Enrich Malarial Proteins from Wheat Lysate

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Abstract

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Malaria is a major public health burden and developing the next generation of anti-malarials is vital to control the spread of disease. Protein arrays, which can investigate binding of many proteins to various probes simultaneously, are becoming an important tool in the drug development process. Results from protein arrays can be affected by the sample purity, as high amounts of protein from the translation system can mask positive interactions on the arrays. The results would be improved by separating the malarial protein from the translation system proteins. However, purifying the hundreds of proteins for arrays using traditional affinity fusion tags is extremely time-consuming. To reduce the time required to produce protein samples at the desired purity, enrichment schemes were developed that covalently attach a small molecule to the proteins in the wheat cell-free expression system. After translation, separation matrices with high specificity for the small molecule modification were added to remove the modified wheat proteins, leaving the malarial protein as a higher percentage in the sample (labeled an enriched sample). Three enrichment schemes, based on the affinities between biotin and streptavidin and histidine peptides and Co^{2+} ions, removed more than 675 μg (≥85%) of the wheat protein. This

resulted in samples with equal concentrations of malarial protein and wheat protein (approximately 0.5 mg/mL), down from the initial 20-fold excess of wheat proteins. Enrichment increased specific activity of two expressed enzymes, dihydrofolate reductase-thymidylate synthase and topoisomerase II, by 3.2- to 4.9-fold. Model protein arrays were created by immobilizing enriched protein samples and various controls. The enriched samples produced fluorescence signals 2.9- to 4.1-fold higher than the non-enriched controls. These enrichment schemes will decrease the time required for preparation of hundreds of protein samples to weeks instead of the months or years it would take with traditional affinity tags. Additionally, the increased fluorescence intensities seen on the arrays from enriched samples will provide better identification of binding interactions, especially for proteins with low expression yields or for weak binding interactions.

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Glossary

°C	degrees Celsius
CB	amine-PEG_2-biotin
DHFR-TS	dihydrofolate reductase-thymidylate synthase enzyme
DNA	deoxyribonucleic acid
dUMP	deoxy-uridine monophosphate
GFP	green fluorescent protein
His_{6x}	histidine affinity tag (six histidines)
kDa	kiloDalton
N1	NHS-biotin
N2	NHS-LC-LC-biotin
N3	NHS-PEG_{12}-biotin
NHS	N-hydroxysuccinimide
nm	nanometer
PBS	phosphate buffered saline
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SB	maleimide-PEG_2-biotin
SH	maleimide-His_{6x} peptide
TEMED	*N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine
TB	transcription buffer
TBS	tris buffered saline
TBST	tris buffered saline with 0.2% Tween-20
Topo II	topoisomerase II enzyme
TSB	translation buffer

Acknowledgements

I would like to thank Dr. Pradip Rathod for all of his guidance and support during my graduate career. This project would have never been completed without his input. I would like to thank all of the members of the Rathod lab that I have had the pleasure of working with for creating a pleasant experience during graduate school. There are several who deserve extra acknowledgement because they were fantastic teachers who taught me everything I needed to complete my research. Dr. Devaraja Mudeppa is a master at expressing proteins with the wheat expression system and assaying their function, which he very patiently explained many times over the years. Dr. Jeremy McLean, Dr. Josh Hunter and Dr. Kalyan Krishnamoorthy taught me all of the basic biochemistry techniques I needed and were very willing to help with any problems that I encountered.

Finally, I need to thank all of my friends and family who supported me throughout this process, especially when I was completely frustrated and not enjoyable to be around. I especially appreciate all of the non-science diversions to help keep me balanced.

Dedication

I would like to dedicate my dissertation to my parents, Bruce and Susan Maki, since I would not have pursued this doctorate without their support and emphasis on education, and to my niece, Nicole Kim. I hope that she has as many wonderful opportunities as I have had.

Chapter 1: Introduction

Malaria

Malaria threatens roughly 3.3 billion people across the world and is one of the leading causes of disease across the world and the fifth leading cause of death. The populations most at risk are those living and traveling in Latin America, South and Southeast Asia, and sub-Saharan Africa (Fig. 1). Human malaria is caused by one of five parasite species of the genus *Plasmodium*: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* and is transmitted to human by the bite of the female *Anopheles* mosquito. *P. vivax* and *P. falciparum* are responsible for the majority of malaria cases but most fatalities are caused by *P. falciparum*. In 2008, the World Health Organization reported that there were approximately 250 million disease cases with approximately 800,000 fatalities but that these numbers may be lower than the true numbers since they rely on passive national reporting that may be unreliable.[1-3] The numbers of disease cases and deaths have decreased since the peak in the early 2000s as the methods to control the spread of disease were improved. These improved methods included new drug treatments, like artemisinin based combination therapies, and increased distribution of insecticide treated bed nets by various countries and public health entities.

However, these trends could reverse in the next years due to decreased efficacy of these control methods. Mosquitoes have developed resistance to the insecticides used in indoor sprays and to treat bed nets.[1, 4] Additionally, *P. falciparum* parasites have developed resistance to the majority of the anti-malarials used throughout the decades including the newest artemisinin based combination therapies (Fig. 2A).[5, 6] The drug resistance is widespread, though parasites from Southeast Asia and Africa tend to be resistant to the largest number of drugs (Fig. 2B). To

continue to lessen the clinical burden of the disease and lower the number of deaths, new drug treatments are desperately needed. The drug development process is extensive requiring many different experiments to prove that the antimalarial is potent against the malaria parasite and selectively affects parasites instead of human cells. This process is slow and can take years to complete due to the presence of several bottlenecks, many of which involve generating sufficient reagents for the variety of experiments required.

Protein Supply as Bottleneck

One difficulty in drug development is producing sufficient amounts and purities of proteins necessary for the many types of experiments required.[7, 8] For example, proteins are required for enzyme assays to check inhibitor activity against *Plasmodium* proteins and their mammalian counterparts to look at both potency and selectivity. Crystal structures of protein targets can be used to develop the best scaffolds for tight binding in the active sites. Protein mass spectrometry experiments can help to identify the mechanisms that small molecule compounds use to inhibit enzymes, which can be used to optimize the potency. All of these single-protein experiments need large quantities of expressed malarial protein, which can be challenging for *P. falciparum* enzymes. This difficulty is addressed on an individual protein basis many procedures have been developed to improve yields.

However, interest is shifting to multiple-protein experiments, especially protein arrays. These experiments are appealing because of the lowered reagent consumption, which is beneficial with the low yields of protein expression, and the ease of performing high-throughput experiments. Hundreds or potentially thousands of spots can fit on the microscopy slide used while maintaining very specific protein-probe interactions. The binding of a probe with one

protein spot was detected out of 10,000 other proteins arrayed.[9] These arrays of numerous proteins can be used to simultaneously investigate the immune response of infected patient sera to hundreds of malarial proteins immobilized on a slide.[7, 10, 11] Of more importance to drug development, the arrays can be used to conduct high-throughput versions of pull-down assays that can identify protein-protein interactions that could be potential drug targets.[12] Protein arrays have also been used to identify or quantify interactions between protein and small molecules, including enzymatic activities and binding and inhibition constants, which is of great interest to the Rathod lab.[13, 14] Research from previous students has identified antimalarial compounds that are more potent against parasites than the single enzymes thought to be the target, indicating that the compound might be acting against a different target. Using protein arrays to identify other enzymes as potential targets for these compounds or others identified in whole cell screens can guide derivative design to make a better antimalarial.[15] The challenges for supplying sufficient protein samples differ in these multiple-protein experiments from the single-protein experiments. These multiple protein experiments do not require large quantities of one protein. Rather they need moderate yields of a large number (tens to thousands) of different proteins. It becomes impractical to try to solve expression and purification issues on a single protein level for these array experiments.

Protein Expression

Protein expression is most commonly done with *in vivo* translation using *E. coli*, since it has the highest average translation rates, with average elongation rates greater than ten amino acids per second.[16] However, these *in vivo* systems do not express *Plasmodium* proteins well. The *E. coli* system was only able to express full-length soluble protein for 6.3 percent of the

1000 *P. falciparum* genes tested.[17] Several factors contribute to the inefficient expression: longer gene lengths, biased genome (80% of the *P. falciparum* genome is adenosine and thymine), and lack of the correct folding machinery.[18-20] The longer genes and biased genome cause processing errors and ribosomal stalling, leading to many partial products. The folding machinery in the prokaryotic *E. coli* cannot correctly process the multi-domain proteins from eukaryotic *P. falciparum*, resulting in formation of insoluble product.

From the experience of the Rathod lab, the most efficient system to express soluble malarial proteins is the wheat-germ *in vitro* (cell-free) expression system.[21] In wheat, the essential translation components are stored in a dried form in the embryo, which can be isolated from crushed wheat and combined with various buffers to create the solution (referred to as wheat germ extract or wheat lysates) used for translation. The open nature of the solution-based expression allows for flexibility over the identity and concentration of the reagents used in translation, which can be altered to improve the yields of expression. Wheat is an eukaryotic species, so it also has the proper folding machinery to process the multi-domain proteins, significantly increasing the protein solubility. Using the continuous flow method and optimized buffer conditions, the wheat cell-free system has comparable translation rates and yields to the *in vivo* methods.[22]

Expressing large numbers of full-length and soluble malarial proteins with the wheat-germ system is significantly more successful than with *E. coli*. A 2008 survey showed 93 out of 124 *P. falciparum* membrane proteins were successfully expressed in a wheat cell-free system and a 2010 experiment successfully expressed 89 out of 97 *P. vivax* proteins.[7, 23] Close to thirty proteins, many of which are difficult to express or never were successfully expressed in *E. coli,* have been expressed by colleagues in the Rathod lab and all have shown significant activity

(most data not published).[21] Due to the large numbers of malarial proteins that have been successfully expressed, the wheat-germ system is the ideal choice to express the multiple proteins needed for the experiments necessary for drug development.

Protein Purity

As mentioned previously, protein arrays will be useful proteome-wide applications for malaria research and drug development. Due to the way samples are immobilized on the slides, the results of the experiments can be affected significantly depending on the purity of the protein after translation. The most commonly used array slide chemistries are three-dimensional gel coatings on glass slides, which have the best binding capacities and least protein denaturation. The gels immobilize proteins via non-specific adsorption in pockets in the gel coating, so if a sample contains multiple proteins, the amount of each protein immobilized is proportional to the amount of the protein in the sample.

The fastest and ideal method to prepare protein arrays is to spot translation mixtures directly on to the slide with no purification.[11, 24-26] In these arrays, the lysate proteins were the vast majority of the sample and therefore took up the majority of the non-specific binding capacity. This limited the amount of malarial protein that was able to interact with the probe used. The presence of these non-malarial proteins also increased the non-specific signal, as can be seen in an array detecting interactions between infected patient sera and immobilized *P. vivax* proteins expressed with the wheat system (Fig. 3).[7] The negative controls (translations done with no DNA, signal intensities circled in the upper right corner of the figure) had significant non-specific binding to the patient sera. The signal intensity corresponded to a signal from

greater than 20 ng/μL circumsporozoite protein (CSP), a known *P. vivax* antigen that elicits a strong immune response.

The signal from the negative controls could mask the signal from proteins with expression yields around or below 20 ng/μL. Even using the wheat expression system, there are occasional yields of expressed protein around this 20 ng/μL value. The high signal resulting from the negative controls also affects what signals are classified as positive binding interactions, termed positive hits. A positive hit is defined based on the ratio between sample intensity and negative control, with the cutoff usually set at greater than or equal to two. With a high signal intensity for the negative control, the ratios are lower and positive hits can be mistakenly classified as negative hits. It also is difficult to distinguish between strong and weak interactions with the lower ratios. To improve array experiments, the malaria protein needs to be separated from the wheat proteins. This will both increase the malarial protein concentration in the sample and reduce the non-specific interactions with wheat proteins. The scheme used for this separation should work for tens to hundreds of proteins simultaneously with no optimization required for individual proteins. Optimization of the separation scheme for individual proteins makes the process inefficient for the large-scale expression and purification desired for protein array experiments.

Conventional Approaches to Protein Purification

Proteins are usually separated from the expression system proteins with a combination of salt precipitation, ion exchange chromatography, size-exclusion chromatography or affinity purification utilizing a recombinant peptide sequence (tag) attached to the protein of interest. The first three methods are based on physical properties of the proteins. Salt precipitation affects

the solubility of proteins in the buffers by disrupting interactions between charged residues and the aqueous solvents. Ion exchange chromatography separates proteins based on the overall charge of the protein and how strongly components of the translation mixture interact with the charged column material. Size-exclusion chromatography separates proteins based on the size of the protein and how long the proteins are retained in a porous material. Separations based on charge or size do not provide a good separation for the wheat system since many malarial proteins have a similar overall charge or size to the background wheat proteins. These methods are also less useful when trying to design a generic high-throughput purification system as they have to be altered and optimized for each malarial protein's charge and size.

Affinity purification is the most specific separation method since it is based on binding to a certain peptide sequence. To express a protein with a recombinant affinity tag, an extra gene sequence is added to the plasmid at the N- or C-terminus and the tag is expressed as part of the protein during translation. After expression, a separation matrix is added that will specifically bind to the tag attached to the protein. Since the wheat proteins do not bind to the matrix, they are washed off the column. The protein of interest (in this case a malarial protein) is then eluted off the column using high-salt concentrations or low pH. The use of affinity tags yields samples with greater purity than the other methods. The purity varies depending on the tag and separation matrix, but after optimization the purity is usually around ninety percent.[27]

Despite providing good purity, the presence of an affinity tag can have deleterious effects on expression and function. For instance, larger affinity tags (glutathione S-transferase [GST] monomer adds 26 kDa and maltose binding protein adds 43 kDa) can decrease expression levels by creating a larger metabolic burden, which causes ribosomes to stall partway through translation.[27, 28] Some tags can decrease the translation initiation when placed at the N-

terminus.[29] In some cases, a sequence that would normally increase solubility can be placed in the wrong spot making the protein insoluble.[27] The addition of a fusion tag can affect the overall fold of the protein, which frequently affects the function of the protein. Even small tags, like the Arg_{5x} and His_{6x} (which add 1 kDa each), have an effect on the overall fold and have changed the diffraction patterns of many crystallized proteins.[30] The addition of a His_{6x} tag caused a T-cell receptor to misfold during translation and caused a conformation change in a plant photosystem II subunit, both of which resulted in loss of function.[28, 31] When the proteins fold, the tags can also overlap with various functional sites in the protein, which will also affect the functional activity of the expressed protein.[32-34]

For each protein there is at least one affinity tag that will maintain the correct fold and function but it has to be determined on an individual protein level since a fusion tag. The time required to perform the experiments evaluating different affinity tags for the tens or hundreds of proteins needed for screens and arrays is immense. Altering a plasmid to have a different affinity tag sequence, expressing the new protein, and checking the activity can take months to complete for a single protein. If the tag has no negative impact on the protein, the efficiency of the separation still needs to be tested. For some tags the affinity between tag and the separation matrix can be strongly affected by the placement of the tag.[35] Additionally, the protein fold can be affected by the use of denaturing buffers.[36]

Enrichment via Modification of Wheat Lysates

Using a separation based on affinity is the best approach for a separation scheme since it is specific to the identity of the peptide tag used. However, the need to select the correct tag and optimize separation for each protein makes the approach of attaching the affinity tag to the

malarial protein unappealing for a high-throughput scheme. An alternative approach is to use the affinity pair but attach the tag to the wheat proteins in the translation system. The separation matrix would bind to the translation factors and ribosomes in the translation mixture instead of the expressed protein. The effect of the tag on wheat translation activity, not on the malarial protein activity, will need to be determined. Since one affinity pair should work for expressing a large range of malarial proteins, the amount of effort put into expressing the proteins needed for an array or screen should decrease significantly. Also, since the wheat-germ proteins will be bound to the separation matrix, elution from the separation matrix is unnecessary and the effects of various elution buffers is not important.

Putting tags on the translation machinery has been successfully used before with an *E. coli* translation system where the translation factors and other essential proteins from *E. coli* are expressed with recombinant His_{6x} tags.[37] These translation factors are first expressed and purified using immobilized metal affinity matrices and then used *in vitro* to express other proteins of interest. After expression, the *E. coli* proteins are removed by binding the His_{6x} tag on the translation proteins to metal matrices leaving the protein of interest behind in solution. This *E. coli* system successfully produced dihydrofolate reductase-thymidylate synthase (DHFR) and had an easy purification method for the enzyme. However, this system is prokaryotic and will have similar problems expressing eukaryotic proteins as described previously. Additionally, expressing the necessary translation factors and other proteins from wheat as recombinant proteins with fusion tags is not feasible, however, since the identities of the essential components in this system are unknown.

Since a recombinant affinity tag on wheat is not an option, chemical strategies will need to be utilized to modify the wheat proteins with a small molecule that is part of an affinity pair

(Fig. 4). The small molecule can be attached covalently to the proteins in the wheat germ mixture through reactive amine, thiols or carboxylic acids on the protein surfaces. The small molecule modifier is added to the wheat germ protein mixture and allowed to react for only a short time to minimize loss of translation activity. After translation using the modified wheat-germ protein mixture, the separation matrix is added to remove the modified wheat proteins. The matrix should pull out the majority of the wheat protein while minimizing non-specific binding to the expressed malarial protein. After the separation steps, the resulting solution should be enriched for the target protein, which will be reflected in higher specific activity values for two expressed enzymes, dihydrofolate reductase-thymidylate synthase (DHFR-TS) and topoisomerase II (Topo II).

Schemes that provide good activity enrichment for DHFR-TS and Topo II will be used to produce enriched protein samples for use with protein arrays.[7, 10, 38] These arrays will be used to see how the signals change between non-modified controls or enriched samples spotted onto three-dimensional nitrocellulose coated glass slides. If the ratios between the enriched samples and negative controls are higher than the ratios between non-modified and negative controls, then the enrichment scheme will be considered to have a positive effect on the protein arrays.

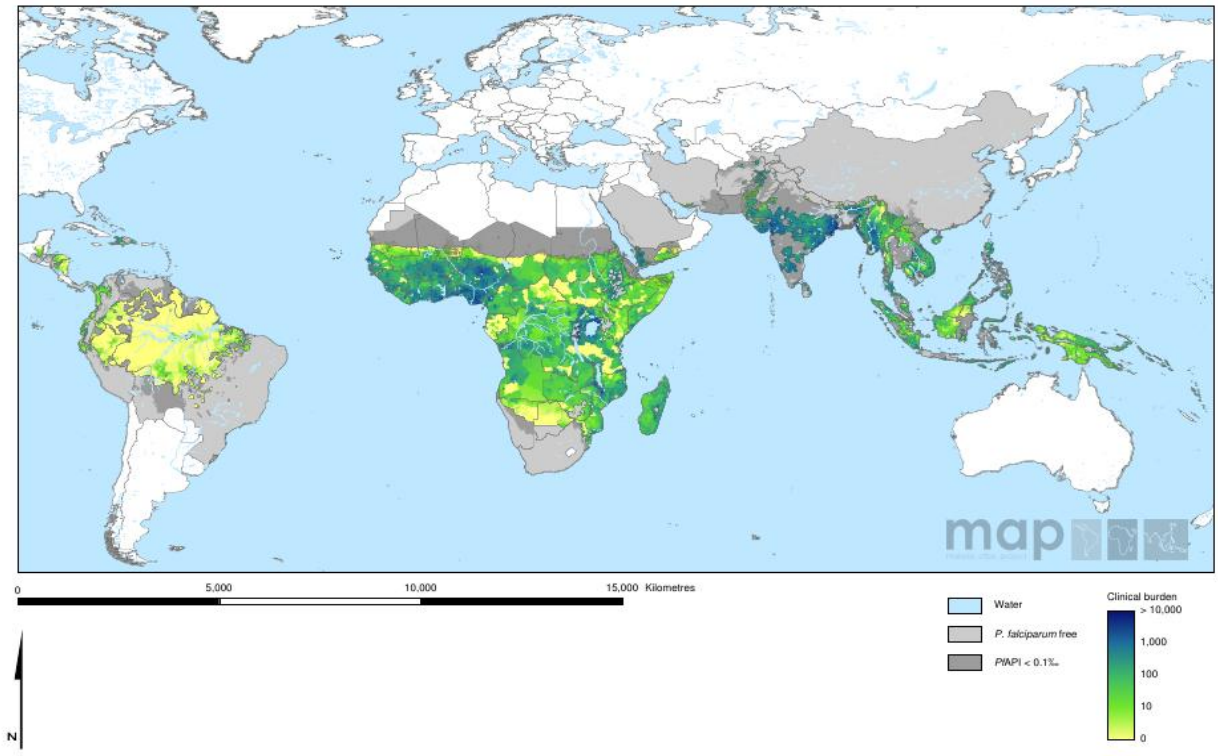
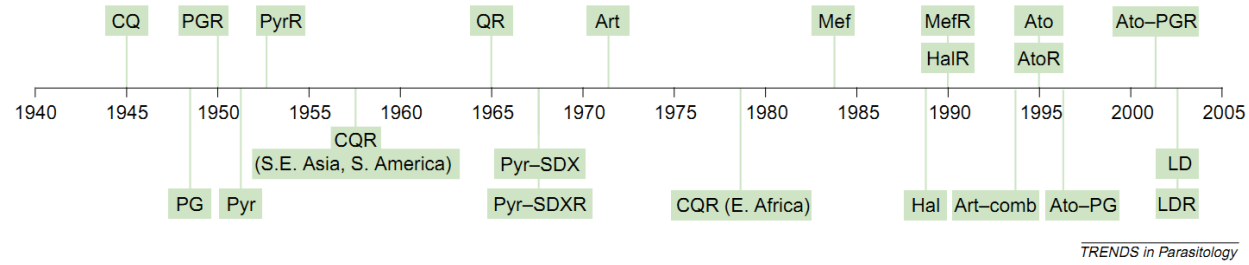


Figure 1: Distribution of *P. falciparum* clinical burden. Reproduced from Malaria Atlas Project.



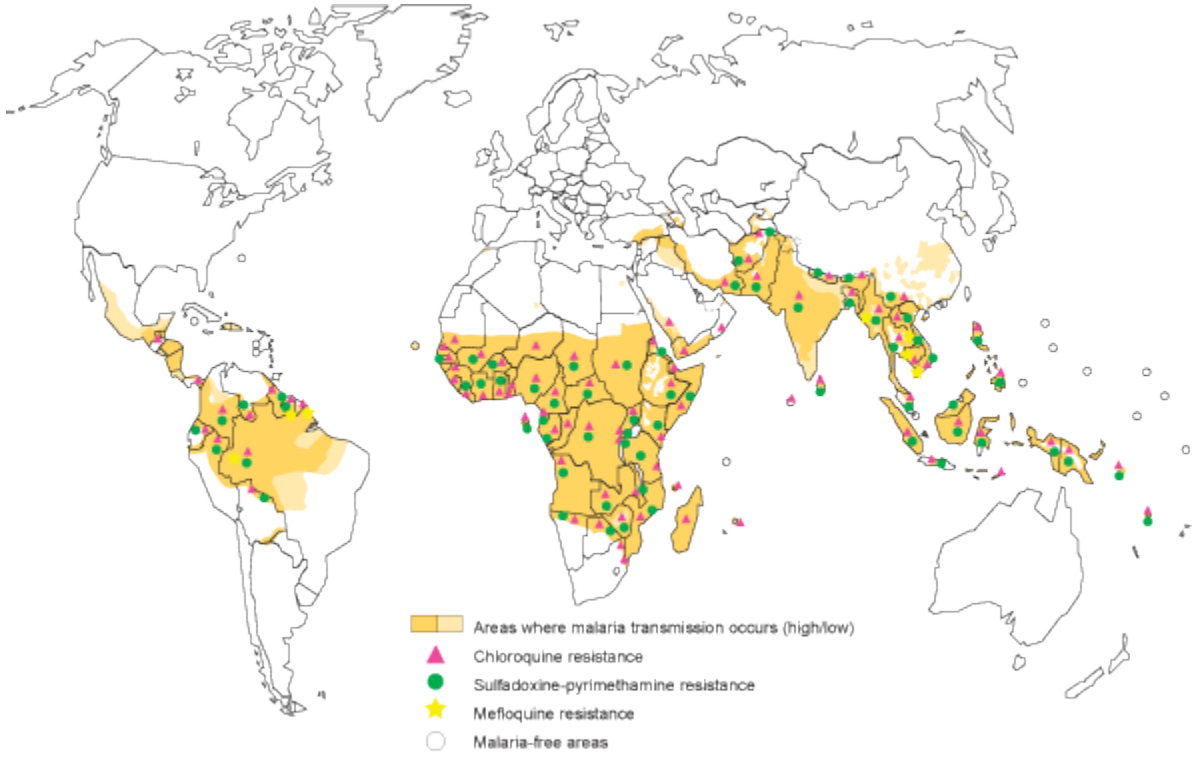


Figure 2: Resistance to multiple anti-malarials. (a, reproduced from reference 4) Approximate dates that each drug was introduced for clinical use and the dates that resistance was first noted. (b, rollbackmalaria.org) Spatial distribution of antimalarial resistance.

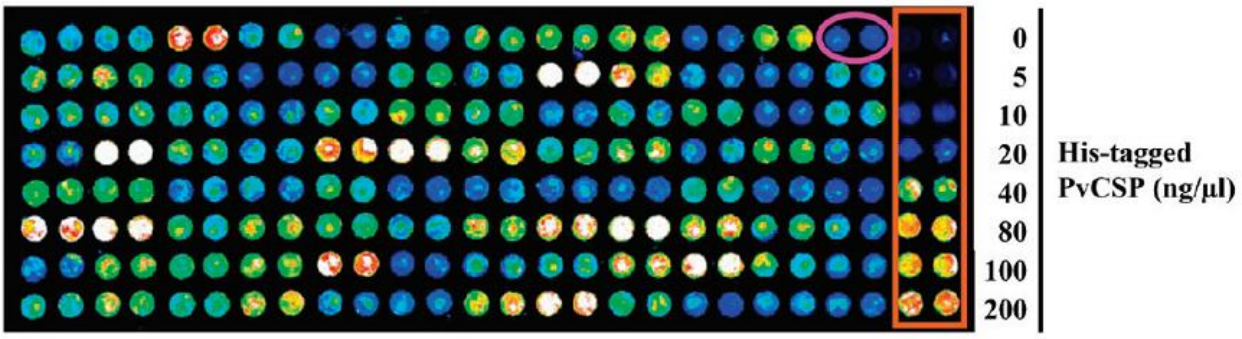


Figure 3: Non-specific interactions between probe and wheat proteins on protein array. Boxed spots are increasing amounts of a purified antigen from *P. vivax* that is known to elicit a strong immune response. Wheat negative controls circled in upper right corner. Reproduced from reference 6.

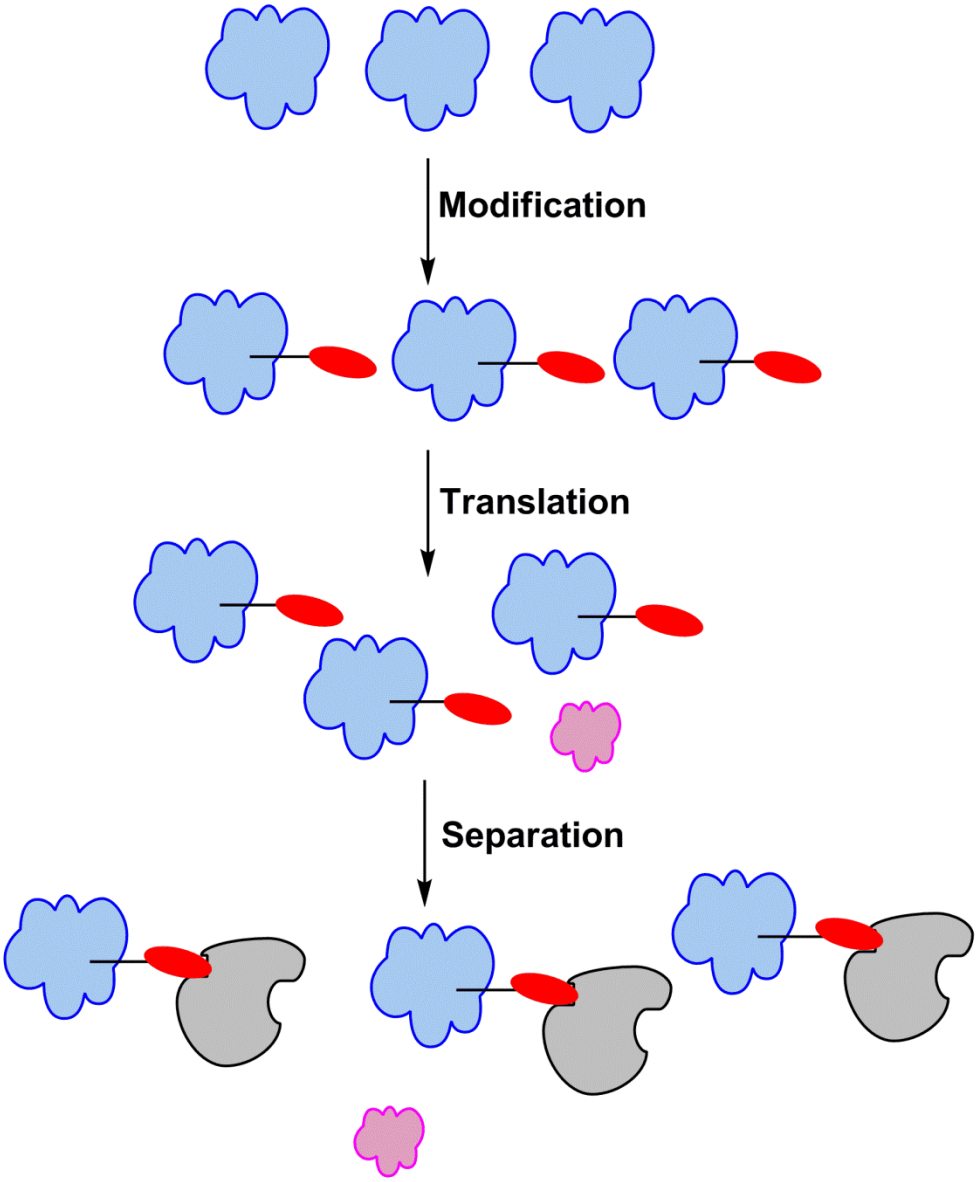


Figure 4: Enrichment scheme. This scheme is designed for high-throughput preparation of malaria proteins for large-scale arrays and screens. The wheat proteins are shown in blue, the separation matrix in grey and the malarial proteins in pink.

Chapter 2: Materials and Methods

Materials

N-hydroxysuccinimide (98%), L-alanine, L-asparagine, L-aspartate, L-arginine, L-cysteine, L-glutamate, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide HCl (EDCI), polyoxyethylene-sorbitan monolaurate (Tween-20), N,N,N',N'-tetramethylethylenediamine (TEMED), monoclonal anti-polyHistidine-alkaline phosphatase antibody from mouse, alkaline phosphatase blue membrane substrate solution, streptavidin-alkaline phosphatase, d-Biotin, Igepal CA630 detergent, tetrahydrofolate (THF), and formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal anti-polyHistidine-alkaline phosphatase antibody came in a 0.05M Tris buffer (pH 8.0) with 1 mM $MgCl_2$, 1% bovine serum albumin (BSA), 50% glycerol and 15 mM sodium azide and was used as is. Streptavidin-alkaline phosphatase was dissolved in PBS at a concentration of 1 mg/mL and stored at -20°C in 5 μL aliquots. EZ-Link NHS-biotin, EZ-Link NHS-LC-LC-biotin, EZ-Link NHS-PEG_{12}-biotin, EZ-Link Maleimide-PEG_2-biotin, EZ-Link Amine-PEG_2-biotin, high-capacity streptavidin agarose resin, HisPur cobalt agarose resin, and Imperial protein stain were purchased from Pierce (Rockford, IL). Dithiothreitol, ammonium persulfate, 30% acrylamide/bis solution (37.5:1, 2.6%C), protein assay reagent, and non-fat dry milk blotting grade blocker were purchased from Bio-Rad (Hercules, CA). BenchMark pre-stained protein ladder (6, 15, 19, 26, 37, 49, 64, 82, 115, 180 kDa markers), SYBR Green I nucleic acid gel stain, methotrexate-fluorescein and Alexa Fluor 488 Goat anti-Rabbit antibody were purchased from Invitrogen (Carlsbad, CA). The methotrexate-fluorescein was dissolved in

equal parts water, methanol and DMSO to a 0.33 M final concentration. The anti-Rabbit antibody was delivered at a 2 mg/mL concentration in 0.1 M sodium phosphate, 0.1 M sodium chloride, 5 mM sodium azide, pH 7.5 and used as is. Plasmid Midi plasmid isolation kits were purchased from Qiagen (Valencia, CA). Recombinant RNAsin ribonuclease inhibitor, rCTP, rATP, rUTP and rGTP were ordered from Promega (Madison, WI). illustra MicroSpin G-25 columns and Ni Sepharose fast flow resin were purchased from GE Healthcare (Pittsburgh, PA). Sodium dodecyl sulphate (SDS), tris(hydroxymethyl) aminomethane (Tris base), Luria broth one gram capsules and glycine were purchased from Research Products International (Mt. Prospect, IL). A maleimide-His-His-His-His-His-His custom peptide was purchased from LifeTein (South Plainfield, NJ). Catenated kinetoplast DNA (kDNA) was purchased from TopoGEN (Port Orange, FL). 2'-deoxyuridine 5'-monophosphate diammonium salt [5-^{3}H] (hot dUMP) was purchased from Moravek (Brea, CA). Opti-fluor scintillation fluid was purchased from Perkin Elmer (Fremont, CA). High mountain organic spring wheat was purchased from Wheatland Milling (Collinston, UT). D-Tube Dialyzer Mini MWCO 12-14 kDa dialysis cups were purchased from EMD Chemicals (Gibbstown, NJ). Immobilon-P PVDF transfer membrane, Microcon centrifugal MWCO 30 kDa centrifugal filter devices and Amicon Ultra 0.5 mL centrifugal filters were purchased from Millipore (Billerica, MA). Vivaspin 15 mL MWCO 10 kDa concentrations were purchased from Sartorius (Goettingen, Germany). Sixty-four pad ONCYTE Nova nitrocellulose film-slides were ordered from Grace Bio-Labs (Bend, OR). A polyclonal antibody raised in rabbits against an epitope in the joining region of *Pf*DHFR-TS and was purchased from Genscript (Piscataway, NJ). The antibody was shipped in lyophilized form. The sample was rehydrated to regenerate the PBS with 0.02% sodium azide buffer and aliquots were stored at -80°C.

Methods

A. Wheat Germ Extract Preparation

Wheat germ extract was prepared according to the procedure described previously.[39] Fifty pounds of organic wheat was ground manually to crack the wheat. The wheat embryos range in size from 710-850 μm. Particles larger than 850 μm were removed first with a sieve collecting the smaller particles. To remove fragments smaller than 710 μm, the fraction that passed through the larger sieve was applied to a smaller sieve (700 μm) and the fraction that does not pass through is collected. Embryos were partially purified from other wheat debris using a solvent floatation method. The fraction collected from the second sieve was added to a cyclohexane/carbon tetrachloride mixture (240:600 vol/vol ratio). The embryos will float in this solvent system and were removed promptly from the solvent and dried in a fume hood. Intact embryos were selected upon visual examination based on color (embryos should be yellow with minimal white matter and no brown or grey particles).

All of the remaining steps of the extract preparation occur at 4°C to maximize extract quality. Purified, intact embryos were wrapped in cheese cloth and washed several times with 4°C sterilized, 18 MΩ resistant water until the water used in the wash runs clear. This was followed by sonication in a solution containing Igepal CA630 to remove ribosome inactivating complexes, like tritin and other endosperm contaminants, which decrease translation yields.[41] After sonication in sterilized water and removal of the detergent, the embryos were dried with paper towels and frozen with liquid nitrogen. The frozen embryos were crushed into a fine powder with a sterilized mortar and pestle. The crushed embryos were ground into the extraction buffer (40 mM HEPES-KOH, pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 2 mM calcium chloride, 4 mM DTT, 0.3 mM of each of the twenty amino acids) until the mixture was

smooth with a light yellow color. Approximately 0.8 mL of extraction buffer was used per gram of intact embryos. Two spins at 30,000xg in a 25° fixed angle rotor centrifuge for 45 minutes separated the extract from the wheat fat which is removed promptly after each spin. Unnecessary and potentially harmful small molecules were removed by passing the extract mixture through G-25 Sephadex spin columns equilibrated with elution buffer (40 mM Hepes-KOH, pH 7.8, 100 mM potassium acetate, 2.5 mM magnesium acetate, 0.2 mM of each of the twenty amino acids, 10 mM dithiothreitol, 0.01% sodium azide). The optical density (OD) of the extract (absorbance at 260 nm) was measured using the NanoDrop 1000 Spectrophotometer. The extract was aliquoted and stored at -80°C until used for translations. Extract is stable for several months when stored at -80°C.

B. Plasmid Isolation

Three μL of an *E. coli* stock solution and 200 μL ampicillin (100 mg/mL) were added to 200 mL of Luria broth medium and the culture was shaken overnight at 37°C. Plasmid was isolated and purified using a Qiagen Midi kit according to the procedure provided by the manufacturer. The bacterial cells were pelleted by spinning at 8000xg for ten minutes. The cells were resuspended in six mL Buffer P1 with RNAse inhibitor by pipetting and inversion and then lysed with six mL of Buffer P2. The mixture was neutralized with six mL of Buffer P3 and incubated on ice for 15 minutes. The cell debris was pelleted by spinning at 12,000xg for 30 minutes. The supernatant was passed over a column, equilibrated with Buffer QBT, which binds to plasmid DNA. The column was washed twice with Buffer QC and then the plasmid DNA was eluted with Buffer QF. Isopropanol was added to the eluant to precipitate the plasmid DNA and the pellet was washed with 70% ethanol and redissolved in sterilized, deionized water. The final concentration

of the plasmid DNA was determined with a NanoDrop 1000 spectrophotometer by measuring the absorbance at 260 nm.

C. mRNA transcription

For 100 μL mRNA transcription, 20 μL transcription buffer (400 mM HEPES-KOH, pH 7.8, 80 mM magnesium acetate, 10 mM spermidine, 50 mM DTT), 12 μL nucleotide tri-phosphates (25 mM each of rATP, rGTP, rCTP, and rUTP), 10 μg plasmid, 2 μL RNAse inhibitor, 3 μL SP6 RNA polymerase and sterilized, deionized water were allowed to react for three hours at 37°C. The turbidity was removed and 7.5 mM ammonium acetate and 100% ethanol were used to precipitate the mRNA, which was pelleted by spinning for 20 minutes at 20,000xg. The mRNA was washed with 70% ethanol and redissolved in 33 μL sterilized water.

D. Protein translation with wheat germ extract using continuous exchange method

Extract (final OD=40), 5 μL creatine kinase (10 mg/mL), 33 μL mRNA, 1 μL RNAse inhibitor and translation buffer (TSB, 30 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM DTT, 0.3 mM of each of the 20 amino acids, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate) were added to a total volume of 100 μL. The translation mixture was placed in a D-Tube Dialyzer Mini dialysis cup which is placed in 5 mL of translation buffer. Translation proceeded for 36-40 hours at 27°C. To improve yields, some of the translation buffer can be replaced by 1M DTT.

E. Modification with one reagent

One protein mixture (one extract equivalent [final OD = 40] and five μL creatine kinase [10 mg/mL]) was brought to room temperature. Biotin solutions were freshly prepared in sterilized, deionized water except for NHS-PEG_{12}-biotin. NHS-PEG_{12}-biotin solutions were prepared by diluting an 1M stock solution (in DMSO) with water. To make the maleimide-His_{6x} peptide solutions, the lyophilized powder was dissolved in sterilized, deionized water to a concentration of 100 mM and stored as aliquots at -20°C. Modifying solutions were prepared by diluting an 100 mM stock solution with water. Three μL of modifying solution was added to the extract and creatine kinase. For modification with amine-PEG_2-biotin, a solution of EDCI in water was added to activate the carboxyl group. After sixty minutes, 12 μL of TSB was added to quench the reaction. Excess modifier was removed by passing the extract and creatine kinase through a G-25 spin column equilibrated with TSB. Desalted extracts were further used in translations or separations.

F. Modification with multiple reagents

i. NHS-biotin and maleimide-PEG_2-biotin

One protein mixture was brought to room temperature. Each biotin reagent was dissolved in sterilized, deionized water immediately prior to use. Three μL of both modifying solutions were added concurrently to the extract and creatine kinase. After an one-hour reaction time, 12 μL of TSB was added to quench the reaction. The protein mixture was passed through a G-25 spin column equilibrated with TSB to remove excess biotin.

ii. Combinations of NHS-biotin, NHS-LC-LC-biotin, and NHS-PEG_{12}-biotin

One protein mixture was freshly thawed. Solutions of NHS-biotin and NHS-LC-LC-biotin were prepared in sterilized, deionized water immediately before modification. The solution of NHS-PEG_{12}-biotin was prepared by diluting an 1M DMSO stock solution with sterilized, deionized water immediately before use. Three µL of the NHS-biotin solution were added to the extract and creatine kinase. After another 30 minutes, three µL NHS-LC-LC-biotin solution and/or three µL NHS-PEG_{12}-biotin solution were added. After 30 minutes (total modification time was 60 minutes), the modification reaction was quenched with 12 µL TSB. Excess biotin was removed by passing the protein mixture through a G-25 spin column equilibrated with TSB.

iii. NHS-biotin and maleimide-His_{6x} peptide or maleimide-PEG_2-biotin and maleimide-His_{6x} peptide

One protein mixture was brought to room temperature. The biotin was dissolved in sterilized, deionized water immediately prior to use. The peptide was diluted from the 100 mM stock solution immediately prior to use. Three µL of each modifying solution were added to the protein mixture. After one hour, the reactions were quenched by the addition of 12 µL of TSB. The excess biotin and peptide were removed by passing the protein mixture through a G-25 spin column equilibrated with TSB.

G. Modification with 2 G-25 desalting steps

Four to eight protein mixtures were freshly thawed. The protein mixtures were passed through a G-25 spin column equilibrated with Hepes buffer, TSB, the TSB variant that had free amines removed (TSB minus NH_2, for experiments with NHS-biotin) or the TSB variant that had free

thiols removed (TSB minus SH, for experiments with maleimide-PEG_2-biotin). Solutions of NHS-biotin or maleimide-PEG_2-biotin were freshly prepared in sterilized, deionized water. Three µL per protein mixture of one of the modifying solutions were added and allowed to react for one hour. After one hour, the reaction was quenched with 12 µL TSB per protein mixture. Excess biotin was removed by passing the protein mixtures through a G-25 spin column equilibrated with Hepes buffer, TSB, TSB minus NH_2 (for experiments with NHS-biotin) or TSB minus SH (for experiments with maleimide-PEG_2-biotin). The solutions were concentrated down to 100-200 µL using Microcon centrifugal molecular weight cutoff 30 kDa centrifugal filter devices.

H. Protein analysis with SDS-PAGE

Protein samples from translations and separations were analyzed by SDS-PAGE, which separates proteins based on the molecular weight. Gels consisting of a 15% resolving gel and a 7% loading gel were cast before use. To cast the resolving gel, two mL polyacrylamide/bis solution, 1.25 mL 1.5M TrisHCl (pH 8.8), 50 µL 10% SDS, 60 µL 10% ammonium persulfate, 5 µL TEMED and 1.835 mL sterilized, deionized water were mixed and the mixture was poured between two glass plates secured with the Bio-Rad Mini Protean casting system. Isopropanol was pipetted onto the top of the resolving gel to remove bubbles and create a level boundary. After the acrylamide solidified, the isopropanol was removed and the loading gel was cast. For the loading gel, 340 µL polyacrylamide/bis solution, 250 µL 1M TrisHCl (pH 6.8), 20 µL 10% SDS, 30 µL 10% ammonium persulfate, three µL TEMED and 1.25 mL sterilized, deionized water were mixed and the mixture was poured on top of the resolving gel. A comb was inserted to form 15 µL wells and the polyacrylamide hardened. Samples were prepared by adding 25 µL

2x SDS loading buffer (3 mL 0.15M Tris (pH 6.8), 1.2 mL 10% SDS, 2 mL 50% glycerol, 0.9 mg bromophenol blue, 0.12 mL β-mercaptoethanol) and sterilized, deionized water to the protein samples to a final volume of 50 μL. The samples were boiled for ten minutes to denature the samples. Ten μL of sample was loaded into the well. Electrophoretic separation was done in running buffer (12 mM Tris, 96 mM glycine, 2 mM SDS) with a constant current of 190 mV. After the separation was finished, the gel was washed for 20 minutes in deionized water and stained for 45 minutes with Coomassie stain (Imperial protein stain). Excess stain was removed by washing the gel with deionized water overnight.

I. Western blot analysis

Protein samples were separated with SDS-PAGE as described above with Coomassie staining. The gel was transferred to a PVDF membrane with a semi-dry method. After the electrophoretic separation was complete, the gel, filter paper and PVDF membrane were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol by volume). Filter paper was placed on the bottom electrode followed by the PVDF membrane, acrylamide gel and finally more filter paper. Excess buffer was removed. The top electrode was secured and transfer proceeded for two hours at a constant 100 mA current. The blot was blocked overnight at 4°C with TBST (50 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 9.3).

The blot was incubated with an antibody specific to each experiment. For biotin-only experiments, the blot was incubated with 2 μL streptavidin-alkaline phosphatase (1 mg/mL) for one hour. Unbound streptavidin was removed by washing the blot with TBST. Streptavidin was visualized by addition of alkaline phosphatase blue membrane substrate.

For His_{6x} peptide or His_{6x} peptide/biotin combination experiments, the blot was incubated initially with a biotinylated anti-His_{6x} epitope antibody (1:1000 dilution). After one hour incubation, the unbound antibody was removed by washing with TBST. Streptavidin-alkaline phosphatase was added (2 µL of a 1 mg/mL solution) and incubated for one hour. Unbound streptavidin was removed by washing the blot with TBST. Streptavidin (attached to biotins on the wheat proteins or the anti-His_{6x} antibody) was visualized by addition of alkaline phosphatase blue membrane substrate.

J. Mass Spectrometry Analysis of Covalently Modified Proteins

Samples of the three test proteins (50 µL, 160 µM) were covalently modified as described in individual chapters. The reaction was quenched with TSB and desalted four times with 100 mM ammonium acetate and Amicon Ultra centrifugal devices. The fourth spin concentrated the sample to 50 µL. Fifty µL acetonitrile and 1 µL 1% formic acid were added to make the appropriate electrospray ionization solution. The samples were sprayed and analyzed with a Bruker Esquire Liquid Chromatograph - Ion Trap Mass Spectrometer in the positive ionization mode. The masses and relative intensities for each protein in the sample were determined using the Bruker Daltonics Data Analysis software. Differences in the protein mass were correlated to the modification reagents and used to determine singly, doubly or multiply modified proteins.

K. Separation with one resin

Three-hundred or four-hundred µL of high-capacity streptavidin agarose resin or one-hundred µL of high-binding immobilized Co^{2+} resin were washed two times with equal volume equivalents of PBS. One translation mixture was added to the resin and incubated with end-

over-end mixing for three hours at room temperature. After three hours, the resin was spun down at 3000xg and unbound proteins were removed. The resin was washed four times with 100 µL PBS and the washes were collected and pooled with the initial unbound fraction. The pooled fractions were concentrated to a final 100 µL volume with Microcon centrifugal MWCO 30 kDa centrifugal filter devices. Bound proteins were eluted by boiling the resin for 45 minutes in 150 µL 2x SDS loading buffer (3 mL 0.15M Tris (pH 6.8), 1.2 mL 10% SDS, 2 mL 50% glycerol, 0.9 mg bromophenol blue, 0.12 mL β-mercaptoethanol). Protein fractions were analyzed qualitatively using SDS-PAGE and the concentrations were quantified using the Bio-Rad Bradford reagent.

L. Separation with two resins

One-hundred µL of high-binding immobilized Co^{2+} resin was washed two times with 100 µL PBS. One translation mixture was added to the washed resin and mixed end-over-end for two hours. After the two hour binding time, the resin was spun down at 3000xg and unbound fraction removed. The resin was washed three times with 100 µL PBS. The wash fractions were collected and combined with the unbound fraction and TSB to a total volume of 500 µL. This total fraction was concentrated down to 100 µL final volume. Three-hundred µL of high-capacity streptavidin resin was washed two times with 300 µL PBS. The unbound and wash concentrate from the first spin was added to the streptavidin resin and mixed end-over-end for two hours. After the binding time, the resin was spun down and unbound fraction removed. The resin was washed three times with 100 µL PBS. The wash fractions were collected and combined with the unbound fraction and TSB to a total volume of 500 µL. This total fraction

was concentrated down to 100 µL final volume. The concentrations of protein were determined by reaction with the Bradford reagent.

M. Measuring protein concentrations

Protein concentrations were determined using the Bio-Rad protein assay (Bradford) reagent following the procedure provided by Bio-Rad. Protein samples were diluted to 800 µL with PBS followed by addition of 200 µL protein assay reagent. Samples were mixed by inversion for five seconds and the protein-Bradford reagent complexes were allowed to form for twenty minutes. Absorbance at 595 nm of each sample was measured with a Beckman DU530 UV-Vis spectrometer. A standard curve generated from measuring the absorbance at 595 nm of solutions with known concentrations of bovine serum albumin (BSA) was used to convert absorbance measurements into protein concentrations. The values reported are the average and standard deviation for triplicate samples.

N. Thymidylate Synthase (TS) enzyme assay

To prepare the methylene tetrahydrofolate (mTHF) cocktail, a 25 mg THF mixture (70% THF, 30% additives) was added to 92.5 mL of TES solution (125 mM TES [pH 7.0], 62.5 mM $MgCl_2$, 2.5 mM EDTA, 187.5 mM β-mercaptoethanol, 16.25 mM formaldehyde). Once the THF was completely solubilized, 0.5 mL aliquots were prepared and stored under argon at -80°C. Sterilized water, protein sample (diluted 1:10 with TSB), 20 µL mTHF cocktail and 5 µL 0.1 mM dUMP solution (0.4 mCi) were added to a final volume of 75 µL and reacted for 40 minutes at room temperature. The enzymatic reaction was quenched with a stop solution (1.5M trichloroacetic acid, 1.33 mM non-radioactive dUMP). As the enzyme reacts, a tritium ion is

cleaved from dUMP and incorporated into tritiated water. Activated charcoal (10% by weight) was added to precipitate unreacted dUMP and other organic compounds from solution. The supernatant was added to five mL scintillation fluid. The amount of tritium incorporated into water was measured as DPM counts with a Beckman LS-600 scintillation counter. DPM counts were converted to activity with units of pmol/µL protein/min by comparison to standards with known dUMP concentrations. Preparation of these standards omitted DHFR-TS protein and the charcoal precipitation step. The enzymatic activities were converted to specific activities using the concentrations determined using the Bradford reagent. The values reported are the average and standard deviation of triplicate samples.

O. Topoisomerase II enzyme assay

To prepare 10x Buffer A, 7.5 mL Tris-HCl (1M, pH 8.0), 4.5 mL NaCl (5M), 1.5 mL $MgCl_2$ (1M), and 1.5 mL sterilized deionized water were mixed and stored as 1 mL aliquots at -20°C. Prior to use in the assay, five µL DTT (1M) were added to the 10x Buffer A. Thirty-eight µL of enzyme mix were needed for each assay reaction and was prepared prior to each assay from 4 µL 10x Buffer A, 2 µL ATP (20 mM), 0.8 µL catenated kDNA (250 ng/µL) and 31.2 µL sterilized, deionized water. Two µL protein solution (diluted with TSB) was added to the freshly prepared enzyme mix. As the enzyme reacts, the kDNA becomes decatenated. After a 30 minute reaction time, the decatenation reaction was stopped by the addition of 5 µL EDTA (0.25M). Catenated kDNA and protein was pelleted by spinning for ten minutes at 13,000xg. Thirty µL of the supernatant was added to one well of a black 96-well plate. Sixty µL SYBR green DNA dye (1:1300 dilution in TAE buffer) was added and mixed with the 30 µL supernatant. Fluorescence (excitation 488 nm, emission 522 nm) was measured using a BioTek Synergy 4 multi-mode plate

reader. Fluorescence intensity was converted to ng decatenated kDNA and activity was reported as ng decatenated kDNA/µg protein using the protein concentrations determined with the Bradford reagent. The values reported are average and standard deviation of triplicate samples.

P. Spotting samples on array slides

One Oncyte Nova 64-pad slide fitted with a 64-well slide module was washed with PBS. Enriched samples were concentrated until they had the same protein concentration as the control samples. Protein samples were diluted with translation buffer with 0.2% Tween 20. Ten µL of the diluted samples were spotted onto each pad manually avoiding contact with the nitrocellulose coating. Proteins in the samples were allowed to bind to the gel coating for a two-hour period at room temperature with slight rocking. After two hours, the unreacted protein was removed and empty pore spots in the nitrocellulose were blocked with a PBS blocking solution (PBS with 0.1% Tween 20, 2% non-fat blotting milk) for four hours at room temperature with gentle rocking. After sufficient blocking, the blocking solution was removed so the slide can be incubated with an appropriate probe.

Q. Probing slides with antibody

Forty µL of a primary antibody solution (anti-*Pf*DHFR-TS diluted 1:600 with the PBS blocking solution) were added to each well on a slide spotted with protein sample. The primary antibody reacted with the epitopes on the proteins on the slide overnight at room temperature with slight rocking. Unreacted antibody was removed by washing the slide four times with the PBS blocking solution. Forty µL of a secondary antibody solution (anti-rabbit IgG conjugated to AlexaFluor 488 diluted 1:500 with the PBS blocking solution) was added to each well and

reacted for 45 minutes at room temperature with slight rocking. Unreacted secondary antibody was removed by washing the slide four times with the PBS blocking solution. Dried slides were scanned with a Typhoon FLA 9000 scanner using a 473 nm laser for excitation.

R. Probing slides with methotrexate

Forty µL of a 15 µM methotrexate solution (methotrexate conjugated to fluorescein diluted with the PBS blocking solution) were added to each well on a slide spotted with protein sample. The small molecule solution was incubated with the slides at room temperature overnight with slight rocking. Unreacted methotrexate was removed by washing the slide for two hours with four 50 µL washes of the PBS blocking solution. After the slide dried sufficiently after the washes, the fluorescein attached to the bound methotrexate was detected by using the 473 nm laser on the Typhoon FLA 9000 scanner.

S. Quantifying the fluorescence intensity ratios

Tiff files (16-bit image, grayscale) for each array image were uploaded into Image J. The background was subtracted by using the sliding paraboloid function with a rolling ball radius of 10 pixels. One pad was outlined by a square and the integrated pixel density was measured inside the square. This was repeated for all 64 pads on the array. The mean pixel intensity was inverted, divided by 10^6, and variant log transformed using the *asinh* function in Excel to get a number between one and twenty. Using the *asinh* function minimized the natural variance between different replicates. The normalized intensities for all blank pads on the slide were averaged to determine the background value. This value was subtracted from the sample measurements to get the background adjusted pixel intensities. The differences in fluorescence

intensities were determined by looking at ratios of pixel intensities of two pads with different sample types (e.g. enriched sample vs. negative control, non-modified control vs. negative control). The ratios for each comparison were determined by calculating the average and standard deviation of all of the individual ratios between sample types on the same slide. The average pixel intensities were converted into concentrations of protein using a calibration curve generated from known amounts of DHFR-TS purified with a N-terminal GST affinity tag.

Chapter 3: Enrichment Schemes

Choice of Affinity Pairs

Two different affinities were the main focus with the enrichment schemes: the affinities between biotin and streptavidin and between metal ions and the imidazole ring in the amino acid histidine. Both of these affinity pairs have been used frequently for protein purification, especially with *in vitro* expression systems.[42-45] These pairs have good affinity, even in the presence of high salt concentrations, but the malarial proteins are likely to be negatively affected if these pairs are used for recombinant affinity purification.

The biotin-streptavidin affinity is a popular purification choice due to the strength of the interaction. With a dissociation constant (K_D) of approximately 10^{-14} M, the interaction between biotin and streptavidin is one of the strongest known non-covalent interactions. The strength of the non-covalent bonding is due to a binding pocket in streptavidin that has a large number of hydrogen bonds, Van der Waals interactions, and hydrophobic interactions. Additionally, a loop from β strands L3 and L4 closes over and traps the bound biotin in the binding pocket.[46] The drawback of using biotin and streptavidin for purification is the difficulty in eluting off the biomolecule of interest. Elution often requires harsh conditions, including 8M guanidinium chloride or boiling in SDS loading buffer, so the proteins with the biotin are frequently denatured or damaged after separation. This is not a problem with the enrichment scheme as elution of wheat proteins is unnecessary.

The high affinity between histidines and metal ions is due to the Lewis base character of the imidazole ring. The lone pair of electrons on the nitrogen in the imidazole side chain is a good Lewis electron pair donor and binds to the electron acceptor metal ions with a micromolar

affinity that is not disrupted by high salt concentrations. The metal ions bind selectively to histidines (out of the twenty amino acids) due to the imidazole ring at physiological pH (pH 7.0). This binding becomes stronger as the length of histidines increases, with six histidines (His_{6x}) being the most common length used in affinity purification. Proteins with a His_{6x} tag can be gently competed off of the metal ions using high concentrations of free imidazole. While the elution conditions are gentler than with biotin, the use of the recombinant affinity tag still creates problems for the protein fold and function as described in the introduction.

The use of the His_{6x} affinity tag for the enrichment scheme has an additional benefit since it should exploit the high histidine character of the wheat proteins (Fig. 5).[47] An antibody that binds to the His_{6x} epitope (demonstrated by binding to a *Mycobacterium tuberculosis* enzyme with a recombinant affinity His_{6x} tag) binds to a majority of the proteins across the entire molecular weight range in the wheat protein (Fig. 5B). The wheat proteins have a natural affinity towards the metal resins, which has created problems when using the recombinant His_{6x} affinity tag for purifying proteins from the wheat system previously After separation with Co^{2+} resin, most of the proteins were in the eluant fraction (third lane of Fig. 5C) with a small, yet significant, portion in the unbound (flowthrough, second lane of Fig. 5C) fraction. Allowing the unmodified wheat proteins to bind to a Co^{2+} resin also provided a two-fold increase in specific activity for two different malarial enzymes (DHFR-TS and Topo II). Covalently adding more histidine sequences to the wheat germ proteins should increase the histidine character of the wheat extracts, especially for proteins that did not bind to the metal ion resins. Using a histidine modification reagent should build on this natural affinity and provide good activity enrichments for the malarial proteins.

For the malarial protein enrichment scheme, the biotin or His_{6x} reagents need to work at neutral pH. Many different biotins and a modified His_{6x} peptide are commercially available for protein labeling and these can be attached to proteins via different functional groups: amines, carboxylic acids, or thiols. In addition, the distance between biotin and reactive functionality can be controlled to target functional groups in different regions of the protein. Biotins with smaller linker lengths will primarily target surface groups on the protein while biotins with longer linker lengths can react with groups that are partially obscured or buried in the protein fold. Various linkers on biotins are available with the length varying from 13.5 Å to 56 Å. Five different biotins and one modified His_{6x} peptide were tested as potential modifiers (Fig. 6). For convenience in figures and captions, each of the different reagents will be assigned an one letter abbreviation: N1 – NHS-biotin, N2 – NHS-LC-LC-biotin, N3 – NHS-PEG_{12}-biotin, SB – maleimide-PEG_2-biotin, CB – amine-PEG_2-biotin, SH – maleimide-His_{6x} peptide. Three of the reagents (N1, N2, N3, all biotins) are amine-reactive. Two of the reagents (SB, SH) are thiol-reactive. The last reagent (CB) is carboxyl-reactive.

The choice of separation matrices (some composition of streptavidin for biotin-modified samples and immobilized Co^{2+} or Ni^{2+} ions for His_{6x}-modified samples for separation) depends on the time required for separation and the binding capacity of the matrix. To ensure a faster separation, using columns to do gravity flow purifications will be avoided due to the extra steps to concentrate the protein solution after separation. Additionally, the cost of separation resins in column format is higher than the cost of bulk agarose resin. The matrices also need to have a high binding capacity since a translation has between 750 to 1000 µg of wheat protein. Magnetic beads and microwells coated with the separation matrix do not have the binding capacity to remove that amount of protein but agarose resins do have sufficient binding capacity. With

streptavidin agarose resins, approximately 100 µL resin can bind to 1000 µg biotinylated protein. Fifty µL Co^{2+} resin can bind to greater than 1000 µg His_{6x} tagged protein.

Methods to Evaluate Enrichment Schemes

Different chemical strategies and modifications will be tested in several ways to determine whether they provide sufficient malarial protein enrichment in the wheat-germ translation mixture. The first experiments will eliminate ineffective or harsh modification reagents and schemes based on three criteria: extent of modification of the wheat protein mixture, the effect on GFP translation, and amount of modified protein removed by the separation matrix. The extent of modification of wheat proteins will be monitored by Western blotting, using antibodies or enzymes that recognize the small molecule selectively over the wheat proteins, and mass spectrometry. Qualitative comparisons can be made between lanes on Western blots indicating that one procedure provided a better modification than another procedure, but no quantitative comparisons can be made. The intensities on Western blots can also verify that the modification is not biased towards a particular molecular weight range Modification will also be detected with mass spectrometry. The three proteins that will be modified are RNAse A, lysozyme, and creatine kinase, which range in size from thirteen to forty-three kDa and differ in the identity of the functional groups on the surface of the protein. The advantage of mass spectrometry is that modification of the three test proteins can be determined semi-quantitatively. The drawback with the mass spectrometry experiments is that it is unknown how representative the three proteins are of the wheat-germ proteins used in the translations.

If a reagent successfully modifies the wheat proteins, the modified translation mixture will be used to express green fluorescent protein (GFP). GFP is a good test protein due to the ease of monitoring protein expression. To monitor GFP expression, the fluorescence signal is checked visually using a UV light source and no further activity assays are performed. Another advantage of GFP is that it is a hardy protein when expressed in the wheat germ expression system and is the positive translation control in the Rathod lab. The translational activity of the wheat can be decreased significantly and GFP will still be expressed. If a modified extract cannot express GFP, it will be considered unable to express more difficult malarial proteins with good enzymatic activity. For modified translation mixtures that express GFP, the amount of protein remaining after separation with the affinity matrix will be quantified using the Bradford reagent. Separations that leave more than fifteen percent of the initial protein mass will not be considered successful.

The schemes will be evaluated by expressing two malarial enzymes and measuring the enzymatic specific activity. The two enzymes chosen, DHFR-TS and Topo II, are both interesting drug targets. DHFR-TS has been an important anti-malarial drug target, while Topo II is an emerging drug target for malaria, though it has been important for other diseases. Besides being proteins of interest for further studies, DHFR-TS and Topo II also provide more challenges for an expression system than GFP. DHFR-TS is a difficult malarial protein to express successfully with good activity in *E. coli* but has been successfully expressed in the wheat-germ translation system.[21] The protein is larger than GFP (80 kDa versus 30 kDa) which puts a larger burden on the translation machinery. The extra difficulty in expressing DFHR-TS will eliminate modification methods that worked well on the robust GFP but are actually too harsh on the wheat system for other malarial proteins.

The expression will be monitored measuring how much the thymidylate synthase (TS) specific activity (nmol $^{3}H_2O$ per hour per mg protein) changes after separation. TS activity is determined using the tritium release assay, which measures how much tritium is cleaved from deoxyuracilmonophosphate (dUMP) by the TS section of the enzyme and then incorporated in water. The amount of tritiated water is measured by a scintillation counter and converted to specific activity using radioactive controls. A separation scheme will enrich the solution for the malarial protein if the specific activity post-separation is larger than the specific activity for a non-modified control pre-separation.

Topo II is also significantly larger than DHFR-TS (160 kDa versus 80 kDa) which creates another larger metabolic burden and has never been expressed successfully for malaria previously. Topo II activity is quantified using an assay that measures the amount of kDNA decatenated (circular DNA that is not linked in a chain) by the Topo II in a translation mixture. After the decatenation reaction, the catenated kDNA (circular DNA linked in a chain) and proteins are precipitated while the decatenated kDNA remains in the supernatant and reacts with SYBR gold. The amount of fluorescence intensity is converted to specific activity using a fluorescence control. The Topo II specific activity is compared between non-modified controls pre-separation and the modified samples post-separation to see which schemes enriched for Topo II activity.

Schemes that fulfill all five criteria with a minimum amount of time and reagents required for the enrichment will be tested on protein arrays to see what effects enrichment has on the signal intensities.

Results

Enrichment Scheme with a Single Reagent

The six initial enrichment schemes tested were based on modification with one reagent and separation with one matrix. The amine-reactive reagents (N1, N2, N3) were able to modify all three of the test proteins used in mass spectrometry experiments (Table 1). Proteins modified with NHS-biotin were labeled with up to three biotin molecules and at least half of the protein was modified. NHS-LC-LC-biotin added only two biotins and was less active than the NHS-biotin in terms of percentage modified. NHS-PEG_{12}-biotin was the least reactive towards the proteins tested, with small amounts of modification on RNAse A or lysozyme, though it did significantly modify creatine kinase. The modification was not quantitative with any of the amine-reactive biotins.

Neither RNAse A nor lysozyme were modified with the thiol-reactive reagents (SB, SH) or the carboxyl-reactive reagent (CB). The lack of reactivity of the thiol-reactive reagents towards these proteins was due to a lack of free thiols. Reducing disulfide bridges did not help increase the reactivity of maleimide-PEG_2-biotin and maleimide-His_{6x}. Increasing the EDCI concentrations above 200 mM did not help amine-PEG_2-biotin. Creatine kinase was slightly modified with amine-PEG_2-biotin and was quantitatively modified with maleimide-PEG_2-biotin and maleimide-His_{6x}. This complete modification agreed with literature results from other experiments using maleimide groups.[48] The consistent reactivity between the two thiol-reactive reagents indicates that the presence of different groups does not affect the reactivity of the maleimide group. While the thiol reagents were better at completely modifying a protein solution, free thiols were less prevalent on the three proteins tested. Mass spectrometry verified

reactivity of the reagents but does not help with predictions about the extent of modification of wheat proteins due to the lack of information on the wheat protein sequences.

All of the reagents were able to modify the protein mixtures over various concentrations, though amine-PEG_2-biotin required a high concentration (≥100 mM) of EDCI to successfully modify the protein mixture (Figs. 7, 8). This high concentration could cause problems for translations. Increasing the concentration at least four-fold caused an increase in the biotin or His_{6x} peptide detected on Western blots. However, at 50 mM concentrations or higher of the His_{6x} reagent, a significant amount of the protein-His_{6x} peptide conjugates aggregated and precipitated out of solution.

For a successful separation scheme, the modification should not be biased towards any part of the molecular weight range in the protein mixture. With each modifier at the lowest concentrations as compared to higher modification concentrations, fewer proteins at the highest and lowest molecular weight ranges were successfully modified. This likely limits the usefulness of these reagent concentrations in separations. Above 100 mM reagent, no increase was seen in the intensity on the Western blot. Increasing the time from 30 to 60 minutes increased the extent of modification but little change was seen after 60 minutes. This time dependence matches well with previous literature results.[49] Keeping the protein solution at room temperature for extended times can lower translational activity, so the shortest reaction time that had good modification, 60 minutes, will be used.

Good modification conditions (≤100 mM reagent, 60 minute reaction time) were tested to see if modified extracts were able to express GFP. Protein mixtures modified with NHS-biotin, NHS-LC-LC-biotin and NHS-PEG_{12}-biotin were able to express GFP when the reagent concentration was ≤50 mM. Below 50 mM concentrations, the fluorescence signal increased as

the reagent concentration decreased. When the protein mixtures were modified with maleimide-PEG_2-biotin, GFP was expressed even when 100 mM biotin was used for modification, though the fluorescence intensity decreased significantly as the concentration increased above 50 mM. His_{6x}-modified protein mixtures were able to express GFP, as long as the concentration of peptide was 25 mM or less. The fluorescence intensity did not decrease when peptide concentration increased from 12.5 mM to 25 mM, which differed from the concentration dependent GFP expressions using biotin-modified protein mixtures. When concentrations above 25 mM peptide were used for modification, the wheat protein concentration remaining after protein-His_{6x} peptide conjugate precipitation was not sufficient for translation. When the amine-PEG_2-biotin was added by itself, it did not disrupt GFP expression but addition of EDCI at concentrations necessary for modification completely eliminated GFP expression. Since there was no GFP expression with amine-PEG_2-biotin modification, it was not studied in future experiments.

Separations of modified proteins from GFP were done with high capacity streptavidin agarose resin or with high binding capacity immobilized cobalt ion agarose resins. A key factor for a good separation was the desalting step in the modification process. Using a G-25 Sephadex spin column after modification removed excess biotin or peptide that would have bound to the resin instead of the modified protein. Adding this desalting step doubled the amount of modified protein bound to the resins for each modifier.

The amounts of protein remaining were compared between separations of modified samples and non-modified samples to see if the modification provided an improvement (Fig. 9). Modifying the protein mixtures with a single reagent did not provide the desired amount of separation for protein enrichment, as approximately 20% of the protein remained in the best

separation. Using a 50 mM NHS-biotin concentration, the best separation left 19% of the protein in the unbound solution (81% removed). Lowering the modifying concentration increased the amount of protein remaining in solution with the worst separation (12.5 mM NHS-LC-LC-biotin or NHS-PEG_{12}-biotin) leaving 45% of the total protein unbound. However, all samples modified with biotins were an improvement over non-modified controls passed through streptavidin resin. Separations with His_{6x}-modified protein mixtures were not better than non-modified controls passed through Co^{2+} resins, which had a significant amount of protein removed due to the inherent histidine character. Changing the peptide concentration did not provide an improvement.

While a significant amount of protein was removed in each separation, the resins used should bind to more than 1000 µg protein, more than the mass of protein in the translation mixture. The separations were not removing protein amounts near this capacity even when the separation is optimized for various buffers, binding times, etc. This indicates that the percentage of the protein mixture modified was not high enough to enable complete separation. As all of these schemes had at least 20% of the initial protein mass remaining after separation, they failed the criterion for a good separation and were not tested further.

Enrichment Schemes with Multiple Desalting Steps

One problem with the modifications, especially with the thiol-reactive reagents, is the presence of large concentrations of reactive groups in the buffers that react with the reagent before it can react with protein. Along with reacting with amines on the proteins, amine-reactive biotins can bind to N-termini of amino acids or side-chains of lysines in the various buffers. The thiol-reactive reagents can react with side-chains of cysteines or dithiothreitol (DTT). The

concentrations of amines and thiols in the buffers range from 1 to 5 mM, which is only two- to ten-fold less than the modifying concentrations. Removing the reactive buffer components from the protein mixture should prevent the buffers from binding to the modification reagents and increase the protein modification yields. The free amines or thiols can be removed by adding a desalting step using G-25 Sephadex spin columns prior to modification followed by the normal desalting step after modification. An issue with using desalting steps before and after modification is the limited protein recovery after desalting. Doing two desalting steps decreases the protein concentration by approximately 75%, which will significantly decrease the translational activity. Adding concentration steps after the second desalting step could restore the translational activity.

The effect of two desalting steps on modification was tested with an amine-reactive biotin (NHS-biotin) and a thiol-reactive biotin (maleimide-PEG_2-biotin). A Hepes solution, the main component of the translation buffer, was used as the buffer to equilibrate the desalting columns. The extra desalting step prior to addition of biotin reagent improved the modification of the protein mixture as detected by Western blot when maleimide-PEG_2-biotin was used and improved the modification a minimal amount with the amine-reactive biotin (Fig. 10). The increase in modification indicated that the reagents, especially the maleimide-PEG_2-biotin, were being prematurely quenched by components in the buffers. To ensure that this effect was due to removing only the reactive components and not the entire buffer, variants of TSB were prepared that omitted the amino acid solution and spermidine (TSB minus NH_2) for amine-reactive biotins or cysteine and DTT (TSB minus SH) for thiol-reactive biotins (Fig. 10). The large increase in modification was also seen when the variants were used, indicating that the reagent quenching was due to the reactive thiols or amines.

After modification with two desalting steps, neither the NHS-biotin nor maleimide-PEG_2-biotin modified extracts could express GFP, with the loss of protein concentration due to desalting steps being a key factor. Adding a concentration step to return the concentration of the modified mixture to the initial level restored the translational activity for protein mixtures modified with either NHS-biotin or maleimide-PEG_2-biotin. Increasing the concentration of either modifying reagent beyond 25 mM eliminated GFP expression. However, decreasing the concentration to 12.5 mM did not increase the GFP signal detected for either of the biotin modifiers.

Just as with modification, translational activity was also dependent on the identity of the buffers used in the initial desalting steps. When the modification used Hepes buffer for the first desalting steps, the resulting GFP signal was non-existent, likely due to a decrease in the concentration of necessary small molecules. To avoid removing essential small molecules that are not reactive towards the biotin reagents, the TSB variants were tested with translation. Using these variants as the buffer in the first desalting step improved the levels of GFP expression, especially when the buffer in the second desalting step was normal TSB to restore the concentration levels of the buffer components after modification.

Separations were much improved with the addition of the desalting step prior to modification for maleimide-PEG_2-biotin (Fig. 10). Additionally, the buffers chosen for the desalting steps had a large impact on the protein amounts remaining. To maintain a separation that removes approximately 90% of the protein mixture, the Hepes buffer or one of the TSB variants had to be used for the first desalting step (Fig. 10). Using TSB as the buffer for both desalting steps worsened the separations and left over 40% of the protein behind in solution. The separations of NHS-biotin modified protein mixtures were also improved, with 15% of the

protein remaining after separation. As with the thiol-reactive biotin, changing the initial desalting buffer had a large impact on the amount of protein remaining. This was especially true with TSB was used as the initial buffer. After separation of these extracts prepared with TSB as the desalting buffer, approximately 40% of the protein remained.

Varying the NHS-biotin concentrations during procedures with two desalting steps changed the separation results in a similar manner to that seen with the one desalting step procedure. In both cases the separations improved as the concentration increased. When maleimide-PEG_2-biotin was used as the modifier, only a small improvement in the separations was seen when the concentration was increased but all concentrations were better than the initial scheme. Since GFP expression was the same at 12.5 and 25 mM and separations were best at the higher concentration, 25 mM concentrations were chosen as the best option for the two desalting step schemes.

Using a scheme with an extra desalting step did provide activity enrichment over non-modified controls after separation. Using two desalting steps with the thiol-reactive biotin provided an enrichment of 2.2-fold for TS activity. However, using the desalting step before modification with NHS-biotin provided a TS activity enrichment of 3.8-fold compared to the non-modified control. That the TS activity enrichment factor is larger for the NHS-biotin is the reverse of the separation data, where the better separation for maleimide-PEG_2-biotin indicated it would have the better post-separation enrichment. The improvement for the scheme with NHS-biotin modification and the extra desalting step was due primarily to an increased activity pre-separation. This suggested that the extra concentrating step had a very beneficial effect on the translational activity.

The pattern in the enrichment factors for Topo II activity differed from those seen with DHFR-TS specific activity. The scheme involving two desalting steps and modification with the thiol-reactive biotin produced a better enrichment factor (3.2-fold) than modification with amine-reactive biotin (2.5-fold). This pattern matches the results seen from the initial separations done with GFP translation mixtures with the better separations producing the better increases in enrichment.

Enrichment Scheme with Multiple Reagents

Two different types of biotin combinations and one biotin and His_{6x} peptide combination were tested for the enrichment scheme. The first set of combinations used multiple amine-reactive biotins. The rationale behind the combination was that biotins with shorter linkers can react with free amines on the surface and biotins with longer linkers can modify the amines that shorter linkers cannot reach. The other combinations tested involved reagents with different reactivities. NHS and maleimide groups react with two different functional groups, increasing the probability that the protein would have at least one functional group that would be successfully modified if the NHS-biotin/maleimide-PEG_2-biotin or NHS-biotin/maleimide-His_{6x} combinations are used for modification. The presence of reagents with different affinities (biotin plus His_{6x} peptide) should provide better enrichment if both resins are used during separation.

The mass spectrometry results from multiple modifications are essentially the sum of the single modification results, indicating that the reactivities of the reagents were not altered when they were used in combination (Table 1). Both the relative amounts of modified and multiply modified protein were increased in samples modified with all three amine-reactive biotins compared to the single biotin experiments. With the NHS-biotin/maleimide-PEG_2-biotin and the

NHS-biotin/maleimide-His_{6x} peptide combinations, mass spectrometry experiments showed the majority of the creatine kinase sample was multiply modified though some protein was only singly modified with the thiol-reactive reagents. RNAse A and lysozyme were modified only with NHS-biotin and at similar levels to the single biotin experiments. With the NHS-biotin and maleimide-His_{6x} peptide, over 90% of the creatine kinase had two modifiers which can react with either the Co^{2+} resin or streptavidin resin.

The extent of wheat protein modification, detected with Western blots probed with streptavidin, increased when multiple amine-reactive biotins were used as long as the biotins were added sequentially. For sequential addition, NHS-biotin was added initially followed by addition of the second and/or third biotins halfway through the one-hour reaction time (Fig. 11). This increased modification was seen with reagent concentrations of 12.5 mM and 25 mM, though the increase was larger when 25 mM concentrations were used. Simultaneous addition of two or more biotins at the beginning of the one-hour reaction time did not have a major impact as all of the biotins likely reacted with surface groups on the wheat proteins or amino acids in the buffer.

Using two biotins that react with different functional groups produced a slight improvement over the NHS-biotin only modification and a large improvement over modification done with only maleimide-PEG_2-biotin both in intensity and in the molecular weight range of proteins modified (Fig. 11). The combination of NHS-biotin and maleimide-His_{6x} also produced an increase in intensity and across the molecular weight range as detected by Western blot (Fig. 11).

Protein mixtures modified with multiple amine-reactive biotins successfully expressed GFP at both 12.5 and 25 mM modifying concentrations, though the fluorescence intensity from

expressed GFP was very weak at 25 mM reagent. The fluorescence intensity for modifications done with multiple 12.5 mM biotin solutions was as strong as the intensity for a single biotin modification. Attempts to express GFP with protein mixtures modified with low (12.5 mM or less) concentrations of NHS-biotin and maleimide-PEG_2-biotin were unsuccessful. However, protein mixtures modified with the combination of NHS-biotin and maleimide-His_{6x} peptide were able to express GFP. The signal was slightly brighter when 12.5 mM instead of 25 mM concentrations of modifying reagents were used and compared well to the levels of GFP expression when only a single reagent was used (either peptide or NHS-biotin). Mass spectrometry results show that the reactivities of both NHS and maleimide combinations were similar, indicating that the same number of small molecules would be added to the wheat proteins and the same number of functional groups would be modified with both combinations. The stark differences in the effects on GFP translations are likely due to the identity of the modifier (i.e. too many biotins) instead of changes to the side chains of the proteins. The NHS-biotin plus maleimide-PEG_2-biotin combination was not tested further.

The separations were much improved with translation mixtures modified with multiple reagents (Fig. 12). Using 25 mM NHS-biotin and NHS-LC-LC-biotin as the modifying reagents, the separation using streptavidin resin had 11% of the translation mixture remaining after separation. Using the 25 mM concentrations of all three biotins caused a further decrease in the amount of protein remaining after separation to 7%. The combination of 25 mM NHS-biotin and NHS-PEG_{12}-biotin did not provide the same amount of separation (14% of the protein mixture was in the unbound fraction) but was still better than the separations with 25 mM of any single biotin separation. Decreasing the concentrations to 12.5 mM worsened the separations slightly (14% for NHS-biotin and NHS-LC-LC-biotin, 15% for NHS-biotin and NHS-PEG_{12}-biotin, 12%

for all three biotins) but these combinations were still better than single biotin modifications. Considering separation and GFP expression, using combinations of 12.5 mM amine-reactive biotins are promising separation schemes to evaluate with other enzymes.

To optimize separations for the presence of both NHS-biotin and maleimide-His_{6x} modifiers, both separation matrices were used to remove the modified proteins. The separations using the two resins were much improved compared to single resin and single modification experiments (Fig. 12). The best separation happened with the 25 mM concentration for both reagents, independent of the order of resins used during separation. After separation with both resins, this scheme had less than ten percent of the protein remaining in solution, compared to 20-30% with one modification, one resin separations. Schemes using the modifiers and resins from both affinity pairs removed the most wheat protein of the combinations.

Enrichment schemes that rely on multiple amine-reactive biotins provided a large activity increase post-separation even though they had a negative impact on TS activity pre-separation (Fig. 13). Modification schemes adding 12.5 mM concentrations of NHS-biotin and NHS-LC-LC-biotin sequentially showed a 2.9-fold improvement in activity enrichment and the sequential addition of 12.5 mM concentrations of three biotins provided a 3.2-fold enrichment. The sequential addition of NHS-biotin and NHS-PEG_{12}-biotin (12.5 mM) was the best modification for this scheme providing an enrichment of 4.2-fold after separation with streptavidin resin. Increasing the concentrations of the amine-reactive biotins to 25 mM did not provide enrichment factors comparable to lower concentration modification schemes for all three combinations. In fact, using 25 mM concentrations with all three biotins actually provided no improvement in specific activity over the non-modified control. Schemes using both NHS-biotin and maleimide-His_{6x} peptide and both Co^{2+} and streptavidin resins resulted in a 3.4-fold enrichment over the

non-modified activity pre-separation and 2-fold enrichment over the non-modified activity post-separation. There was not much difference in enrichment between 12.5 mM (3.1-fold) and 25 mM (3.4-fold) concentrations, with the results from modification with 25 mM being slightly better.

The modification combination of 12.5 mM NHS-biotin and 12.5 mM NHS-PEG_{12}-biotin was the best amine-reactive combination for Topo II activity enrichment (3.6-fold improvement) though the combination of 12.5 mM of all three amine-reactive biotins was also good at enriching for Topo II activity (3.3-fold improvement). Using the combination of biotin and His_{6x} peptide with two separation resins provided Topo II enrichment over the non-modified control. Increasing the concentration from 12.5 mM to 25 mM of both modifiers in the combination resulted in a significant increase in enrichment from 2.6-fold to 4.9-fold over the non-modified pre-separation activity. For the multiple resin separations, the order of resins used was important for activity enrichment (Fig. 14). If the separation started with the streptavidin resin, the enrichment is less than 2-fold for both enzymes. Using the immobilized cobalt resin first followed by the streptavidin resin resulted in the high enrichment.

Discussion

This is the first example of protein expression using covalently modified wheat germ extract. Of the three reactive groups targeted, two could be successfully modified without eliminating translational activity but modifying carboxyl groups eliminated all translational activity. The loss of activity occurred upon addition of EDCI, the reagent necessary to activate the carboxyl group in order to facilitate amide bond formation, even without the addition of amine-PEG_2-biotin. EDCI can cause protein cross-linking, which can have a negative effect on

activity. While some wheat protein may have been covalently cross-linked due to the presence of EDCI, it was negligible and not apparent on Coomassie-stained SDS-PAGE gels. The loss of activity is likely due to other complications from covalent modification of the carboxylic acids. Converting a carboxylic acid to an amide results in a net increase in overall positive charge of the wheat mixture. This amide bond can form in the absence of the biotin reagent due to the presence of free amines in the buffer, which explains why the activity is affected even without the amine-PEG_2-biotin reagent. Potential byproducts from the EDCI activation could also have negatively affected the translational activity.

Covalently modifying amines did not completely eliminate translation activity as long as the modifier concentration was less than 100 mM. The loss of activity was concentration dependent, as samples modified with the lowest concentrations retained the most activity. Two potential reasons for activity loss are the change in the net charge and the loss of reactive groups. As with carboxylic acids, the formation of an amide bond out of an amine group results in a change in the net charge of the wheat mixture. However, the net charge decreases since the amine groups from lysines and the N-terminus are positively charged and the newly formed amide bond is neutral at the 7.0 pH of the translation buffer. Additionally, when an amine group is converted to an amide functionality, it loses its reactivity. If an amine essential for translation is modified, the activity would decrease.

The final reactive side chain that was targeted was the thiol on the amino acid cysteine. Previous work with the wheat germ system established the fact that the presence of dithiothreitol (DTT), a reducing agent, was essential to maintain good activity.[23, 41, 50] Since DTT was essential, it suggested that the presence of free thiols was also essential to maintaining translation activity. However, the thiols were covalently modified to form a thioether bond with the

maleimide group that caused minimal effects on translation activity. The activity loss was relatively minimal, even at modifier concentrations of 100 mM, so the loss of free thiols did not have as significant an impact on translational activity as the previous experiments suggested. The data from the maleimide modification experiments indicate that the activity may be affected more by the presence of disulfide bridges instead of the absence of free thiols.

This research into an enrichment scheme demonstrated that lowering the protein concentration of the translation mixture below a certain point eliminated translation activity. Activity was not dependent on the total mass of protein in the translation. Using a sufficient volume of the low concentration mixture to ensure that the translation mixture had the correct mass of protein did not restore activity. Adding a concentration step to restore the initial concentration of the extract did restore activity.

The various enrichment schemes were evaluated looking at five criteria: extent of modification, amount of protein removed during separation, GFP expression, and expression and enrichment of DHFR-TS and Topo II. Five schemes satisfied all five criteria and provided activity enrichment: modification with NHS-biotin after an initial desalting step, modification with maleimide-PEG_2-biotin after an initial desalting step, modification with NHS-biotin and NHS-PEG_{12}-biotin combination, modification with NHS-biotin, NHS-LC-LC-biotin and NHS-PEG_{12}-biotin combination, and modification with NHS-biotin and maleimide-His_{6x} peptide. The first four schemes used streptavidin resin as the only separation matrix. The last scheme used both streptavidin resin and Co^{2+} resin as the separation matrices.

While all of the schemes provided similar levels of enrichment, two of them are not as attractive for the high-throughput approach desired to express the numbers of proteins envisioned for protein arrays. The schemes that use an extra desalting step prior to addition of the

modification reagent required more time and reagents than the other steps. In the other three schemes, the modification steps were completed within one and a half hours. The time required for modification almost doubled when the initial desalting step plus the final concentration step were added. There is a limit on the number of translations, approximately twenty, that can be started using the continuous exchange expression method without introducing errors from human fatigue or causing loss of activity. The ideal protein arrays experiments need more than twenty proteins, so they require multiple rounds of translation initiation. Since modification needs to be done immediately prior to translation initiation to preserve activity, these schemes require multiple rounds of modification. When trying to express more than twenty proteins, the time differences between the modification procedures become larger and the schemes using the extra desalting step become more impractical.

The extra desalting step and concentration step in these enrichment procedures also resulted in the loss of at least one-third of the wheat extract. Preparing the extract from wheat is a long, intensive process, making the extract a very expensive reagent. To create enough extract required for three months of translations requires full-time effort of three people for at least a week. Losing one-third of the extract during the modification process is not acceptable based on the time required to make the extract. If advances are made in the wheat expression system that increase the efficiency of extract preparation and the efficiency of translation initiation, then these two schemes with the initial desalting step might become more attractive for high-throughput enrichment.

While all enrichment schemes provided an increase in enzymatic specific activity, the maximum increase was five-fold. The four- to five-fold increases are envisioned as a good start to an enrichment scheme but further improvements to at least ten-fold enrichment are ideal. The

limitations to the tested enrichment schemes lie in both the modification and separation steps. Complete modification is hampered by the uneven distribution of functional groups on the protein surfaces. Some proteins may have three amines on the protein surface while other proteins have one amine. The three amines can react with and use up the modifying reagent before it reaches the other proteins, leaving some protein unmodified. While increasing the modifying concentration usually addresses this issue for most reactions, an increased concentration corresponded with a decrease in translation activity as groups essential for translation were altered covalently. Finding another reactivity that is more evenly distributed on the proteins could address some of the issues of good modification while maintaining good activity.

For each functional group targeted for modification there are proteins in the wheat extract that do not have a group accessible by the reagent. Unbound fractions from separations were subjected to additional modification and separation steps to test this theory. After additional rounds of modification and separation, there was always a fraction of protein remaining that would not show a signal on the Western blot and would not bind to the resin. This fraction was largest when the modification targeted the thiol group. Modification via a different reaction, perhaps using non-specific binding, in combination with the modification of other functional groups might effectively target these proteins.

The separation is limited by the accessibility of the modifying reagents to the resins used. If the modifier adds to a more interior part of the protein structure, the part of the modifier that binds to the resin might be obscured. Additionally, throughout the translation, the protein fold might change due to denaturation or due to the use of the protein in the translation reaction. If the protein fold changes, a modifier that was once on the surface of the protein may now be

obscured by the protein fold. Or a modifier might be cleaved off of the proteins during translation. Any of these situations would result in decreased binding of the wheat proteins to the resins used. A way to address some of these potential problems is to find a smaller separation matrix than the agarose resins with a similar binding capacity and a similar ease of removal from solution. Both of the separation matrices used for the enrichment schemes were modified agarose resins, which are very large. The size of the resin would limit the size of the spaces in proteins accessible to the Co^{2+} ions or streptavidin.

Conclusions

Various modifications and separations were tested to develop an enrichment scheme to remove wheat proteins and increase the concentration of malarial protein in the translation mixture. Different functional groups were successfully modified by covalent addition of biotin or His_{6x} peptide. Depending on which functional group was targeted, the wheat extract could be covalently modified and retain translation activity. Several schemes produced good separation (at least 85% of the wheat protein removed) and good activity enrichment (2-fold or higher). Three of these schemes provided at least three-fold enrichment in DHFR-TS and Topo II activity using the minimum amount of time for modification and separation, making them the most amenable to high-throughput procedures. Importantly, the amount of time required for protein sample preparation decreased significantly. Purifying the number of proteins of interest on protein arrays with different affinity tags would take months to years to complete, depending on how many different affinity tags need to be tested with each protein of interest. These new enrichment schemes would prepare the enriched samples for arrays in a few weeks or less.

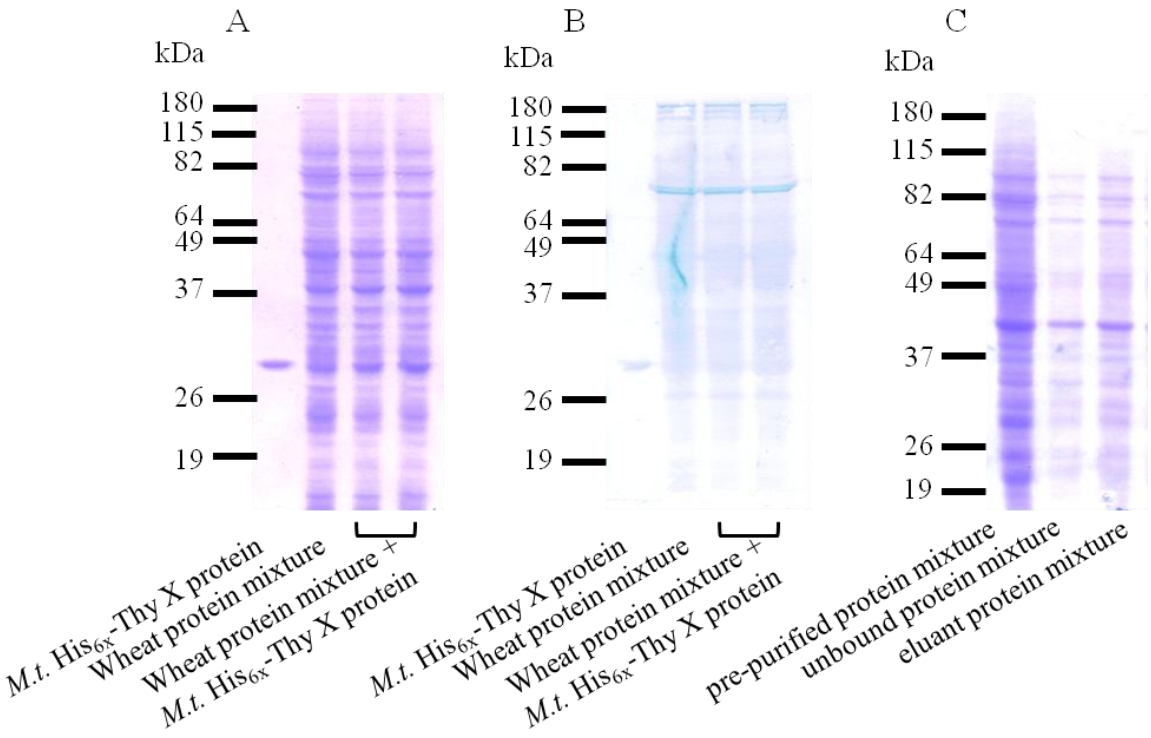


Figure 5: High histidine character of the wheat-germ extract. (a) Coomassie stained SDS-PAGE used as a loading control for the Western Blot. (b) Western blot probed by an antibody that binds to the His_{6x} epitope. (c) Coomassie stained SDS-PAGE analyzing samples from separation of wheat extract done with Co^{2+} resin.

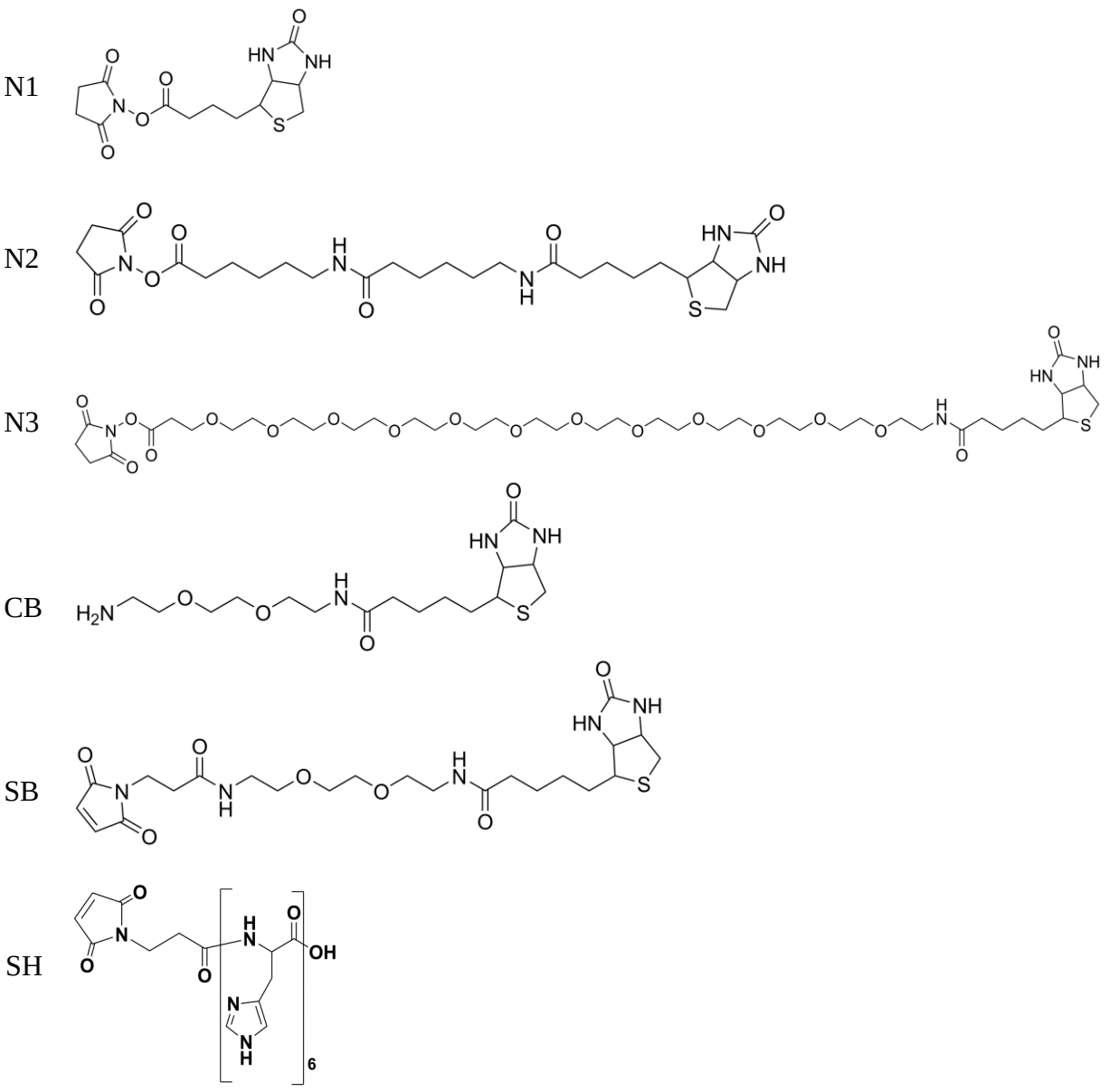


Figure 6: Various reagents used for modification and their one-letter abbreviations. N1 – NHS-biotin, N2 – NHS-LC-LC-biotin, N3 – NHS-PEG_{12}-biotin, CB – amine-PEG_2-biotin, SB – maleimide-PEG_2-biotin, SH – maleimide-His_{6x}.

Table 1: Mass spectrometry data for modified test proteins.

Modifier	Protein	Mass	Modified Masses	% Modified
NHS-biotin	RNAse A	13681	13908 (s), 14134 (d), 14360 (t)	48
	Lysozyme	14305	14531(s), 14759(d), 14982(t), 15209(q)	61
	Creatine Kinase	42986	43210 (s), 43437 (d), 43663 (t)	95
NHS-LC-LC-biotin	RNAse A	13681	14133 (s), 14582 (d)	38
	Lysozyme	14305	14757 (s), 15210 (d), 15663 (t)	91
	Creatine Kinase	42986	43436 (s), 43890 (d)	66
NHS-PEG_{12}-biotin	RNAse A	13681	14508 (s)	11
	Lysozyme	14305	15128 (s)	5
	Creatine Kinase	42986	43813 (s), 44640 (d), 45467 (t)	58
Maleimide-PEG_2-biotin	RNAse A	13681	-	0
	Lysozyme	14305	-	0
	Creatine Kinase	42986	43509 (s)	100
Amine-PEG_2-biotin	RNAse A	13681	-	0
	Lysozyme	14305	-	0
	Creatine Kinase	42986	43342 (s)	33
Maleimide-His_{6x} peptide	RNAse A	13681	-	0
	Lysozyme	14305	-	0
	Creatine Kinase	42986	43977 (s)	100
Multiple amine-reactive biotin	RNAse A	13681	13909 (s), 14135 (s/d), 14361 (m), 14508 (s), 14587 (m), 14734 (m), 14813 (m), 14961 (m), 15040 (m)	90
	Lysozyme	14305	14531 (s), 14757 (s/d), 14984 (m), 15210 (m), 15436 (m), 15664 (m)	77
	Creatine Kinase	42986	43211 (s), 43442 (s/d), 43442 (m), 43886 (m)	95
NHS-biotin + Maleimide-PEG_2-biotin	RNAse A	13681	13907 (s), 14134 (d), 14361 (t)	51
	Lysozyme	14305	14532(s), 14757(d), 14984(t), 15209(q)	91
	Creatine Kinase	42986	43512 (s), 43743 (d), 43964 (t)	100
Maleimide-His_{6x} + Maleimide-PEG_2biotin	RNAse A	13681	-	0
	Lysozyme	14305	-	0
	Creatine Kinase	42986	-	100
Maleimide-His_{6x} + NHS-biotin	RNAse A	13681	13908 (s), 14134 (d), 14360 (t)	51
	Lysozyme	14305	14531 (s), 14759 (d), 14982 (t), 15209 (t)	85
	Creatine Kinase	42986	43978 (s), 44203 (d)	100

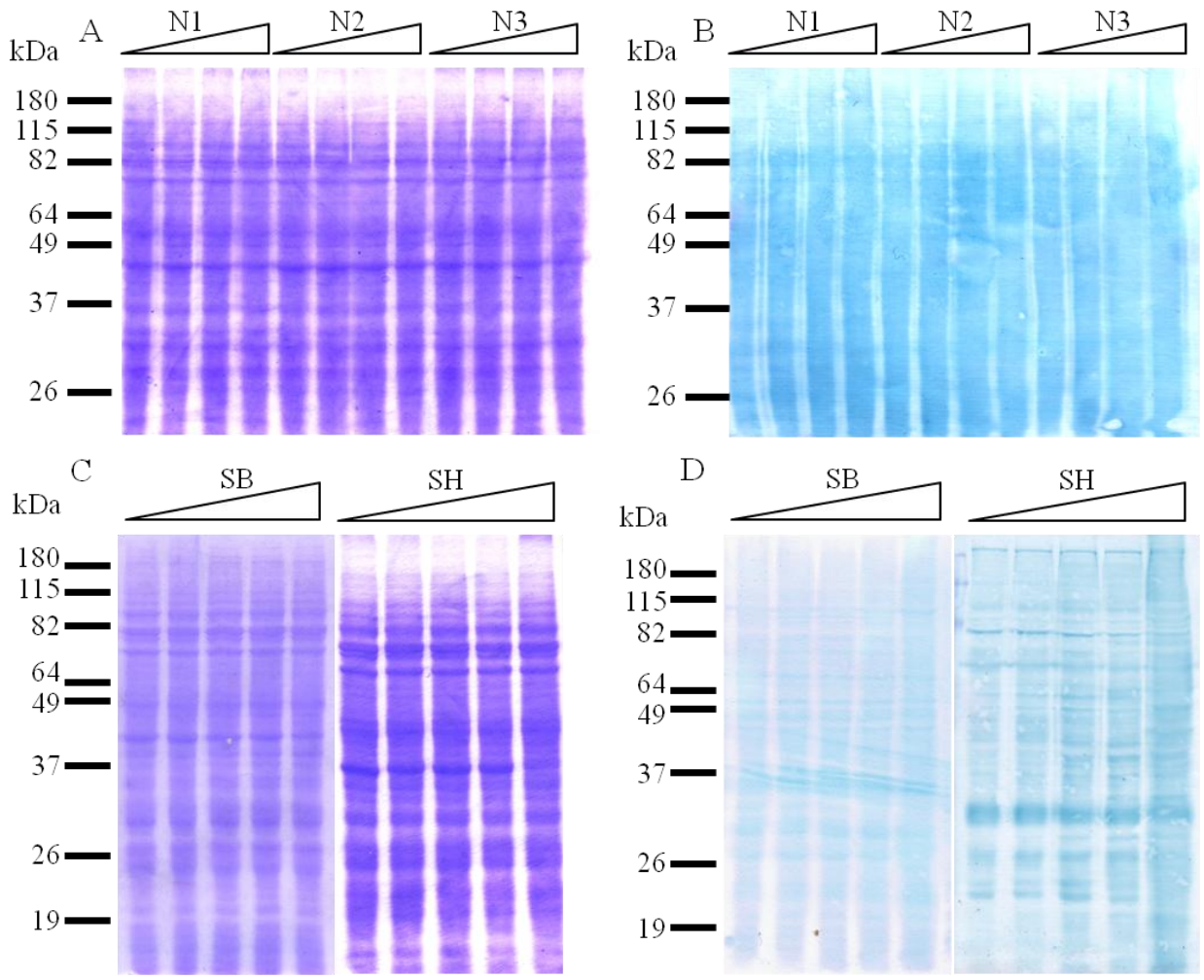


Figure 7: Modification with amine-reactive biotins and thiol-reactive reagents. (a, c) Coomassie-stained gels used as loading controls for Western blots. (b) Western blot with increasing amine-reactive biotin concentrations. (d) Western blot with increasing thiol-reactive biotin or thiol-reactive peptide concentrations.

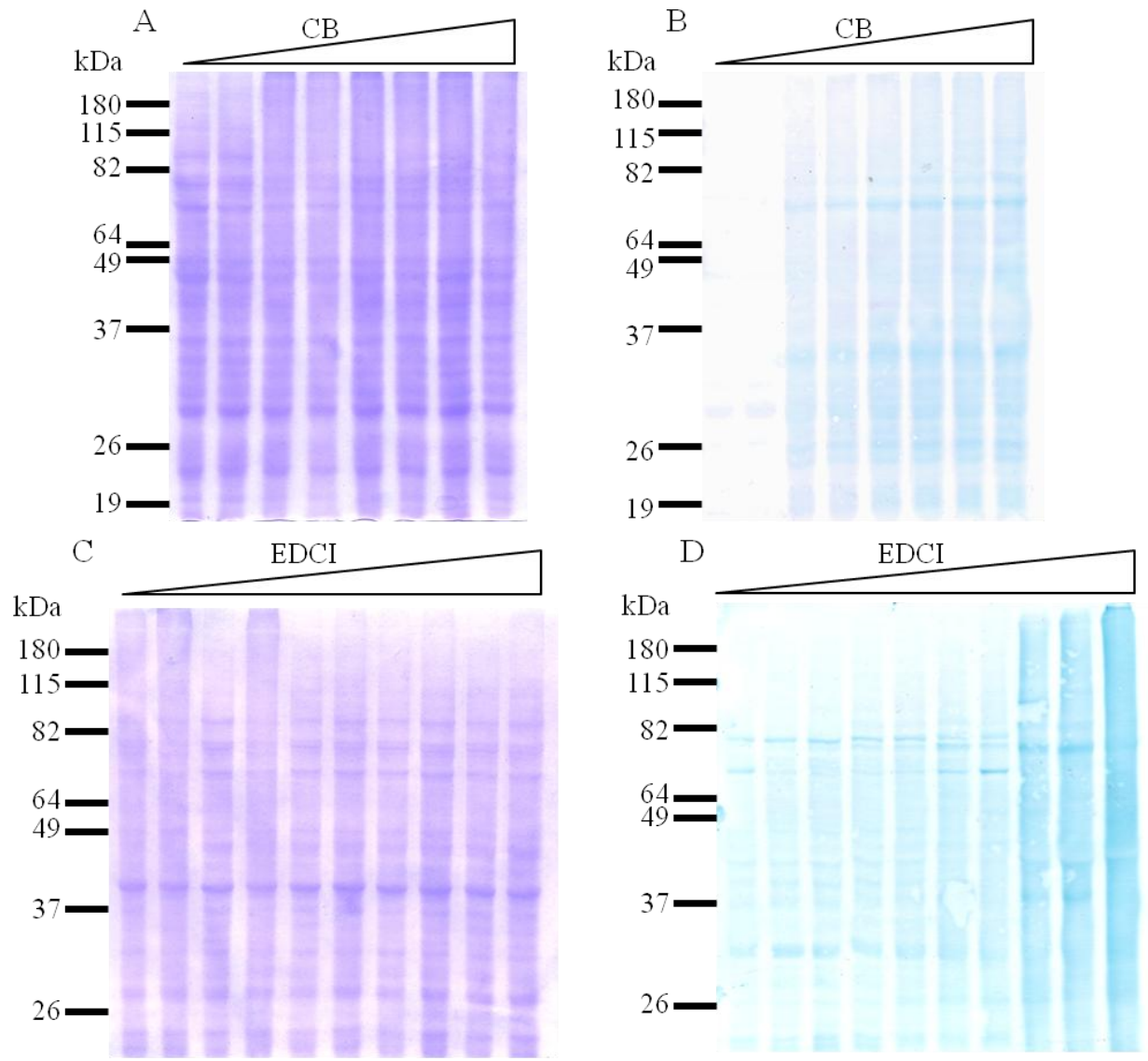


Figure 8: Modification with carboxyl-reactive amine-PEG_2-biotin. (a, c) Coomassie-stained gels used as loading controls for Western blots. (b) Western blot with increasing biotin concentration. (d) Western blot with increasing EDCI concentration.

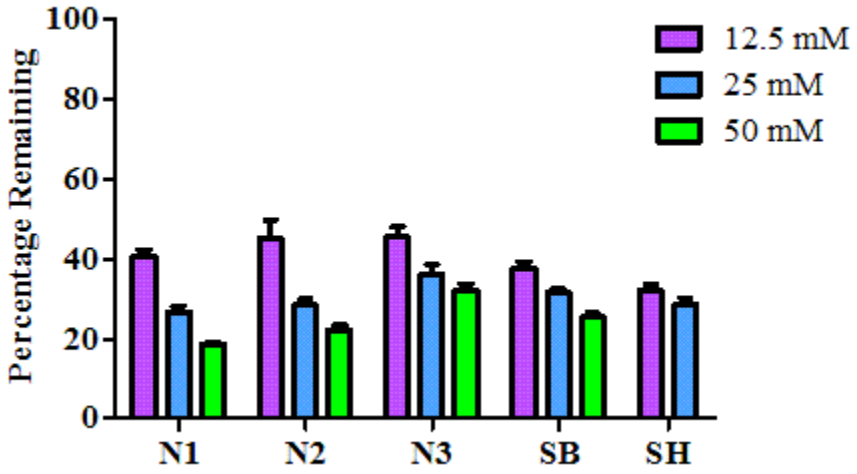


Figure 9: Separation of protein mixtures modified with a single reagent. The numbers in the legend reflect the concentration used for modification. The 50 mM concentration of maleimide-His_{6x} peptide (H) was not tested since it eliminated GFP translation.

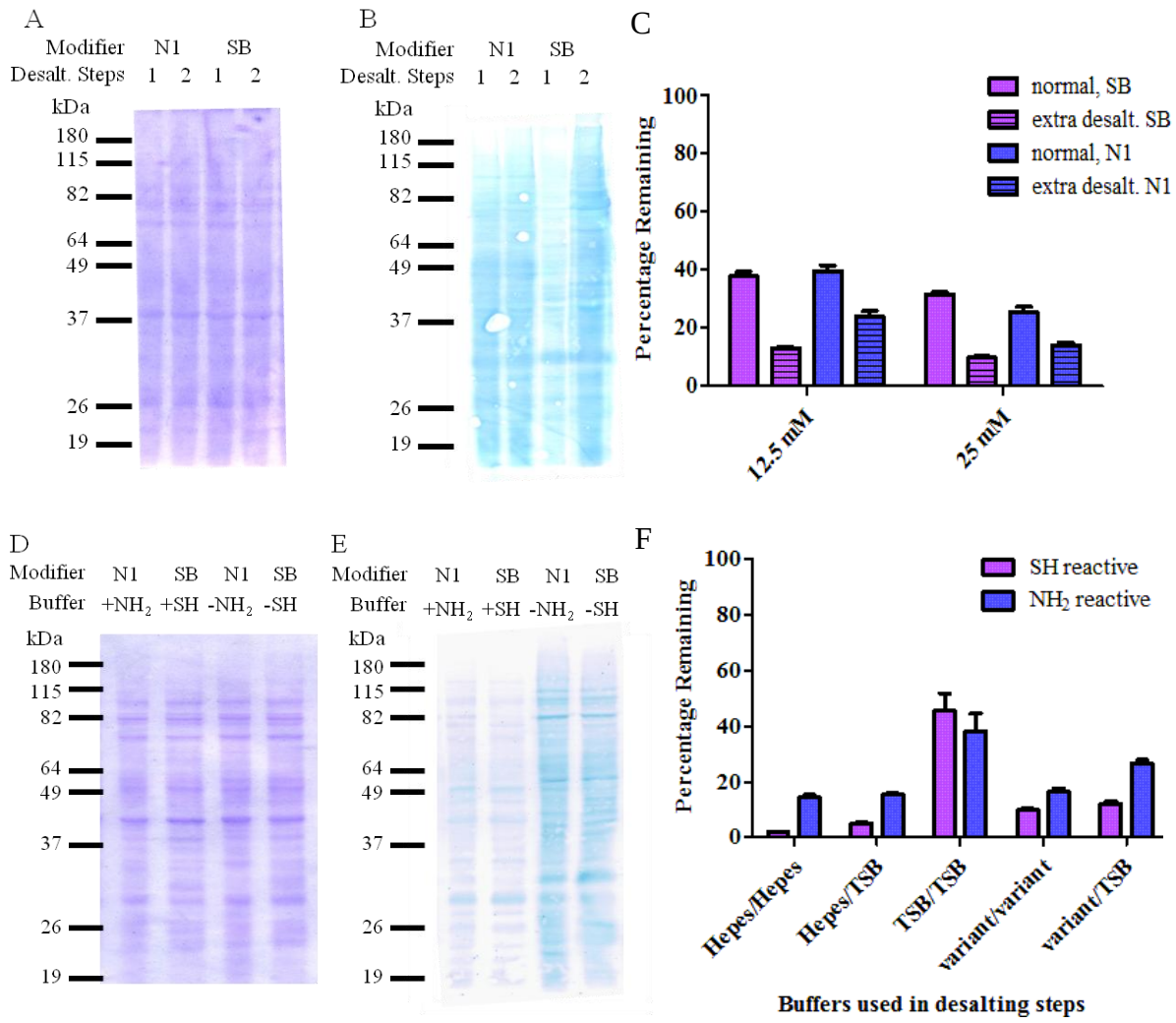


Figure 10: Modification and separation of protein mixtures with two desalting steps. (a, d) Coomassie-stained gels used for loading controls. (b) Western blot looking at samples modified with one or two desalting (desalt.) steps. (c) Separation data for various concentrations used in modification. (d) Western blot looking at modification of samples prepared with TSB or TSB variants ($-NH_2$ or –SH) used to equilibrate G-25 columns of initial desalting step. (f) Separation data for modification schemes using various buffers in the initial or second desalting steps. $+NH_2$ or +SH = TSB, $-NH_2$ = TSB minus amino acids and spermidine, -SH = TSB minus cysteine and DTT.

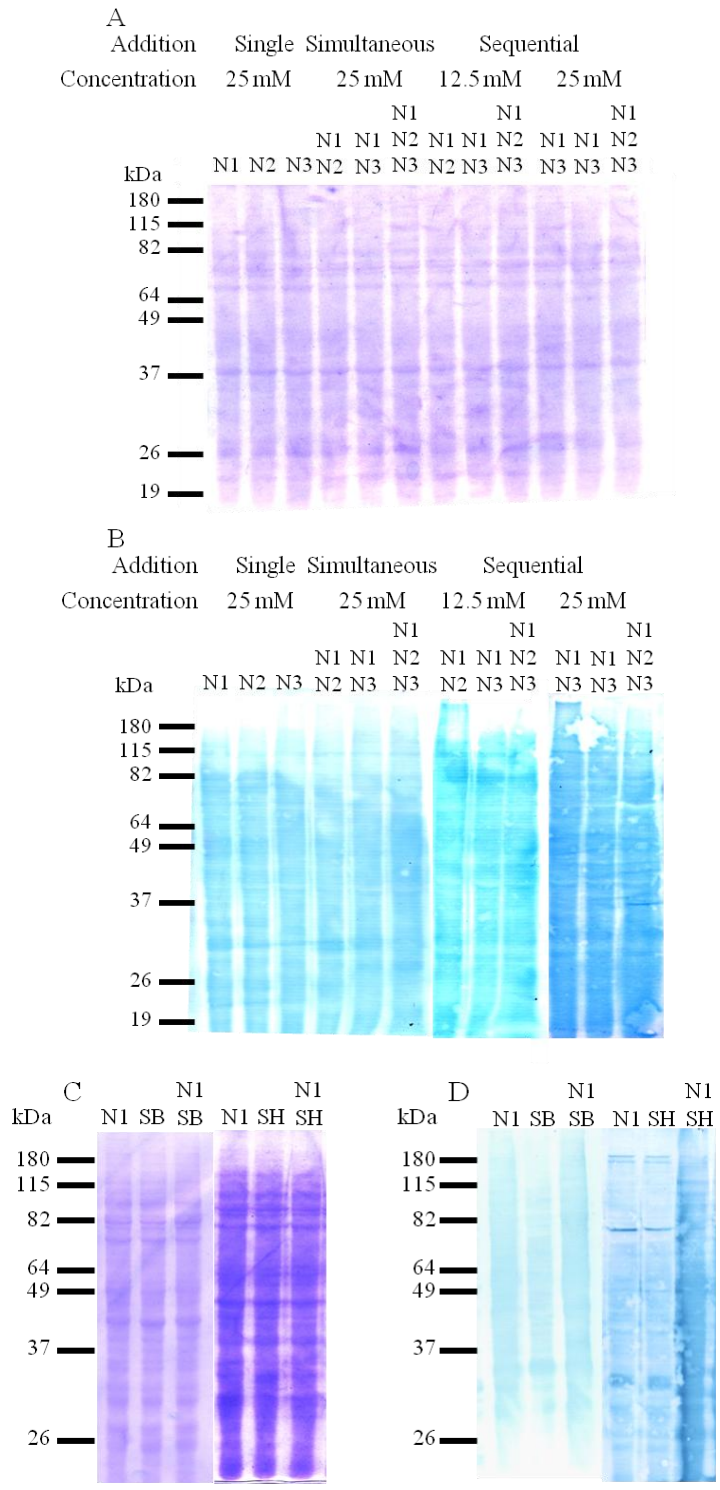


Figure 11: Modification with multiple reagents. (a, c) Coomassie-stained gels used as loading controls. (b) Western blots looking at multiple amine-reactive biotin combinations. (d) Western blots looking at combinations of reagents with different reactivities.

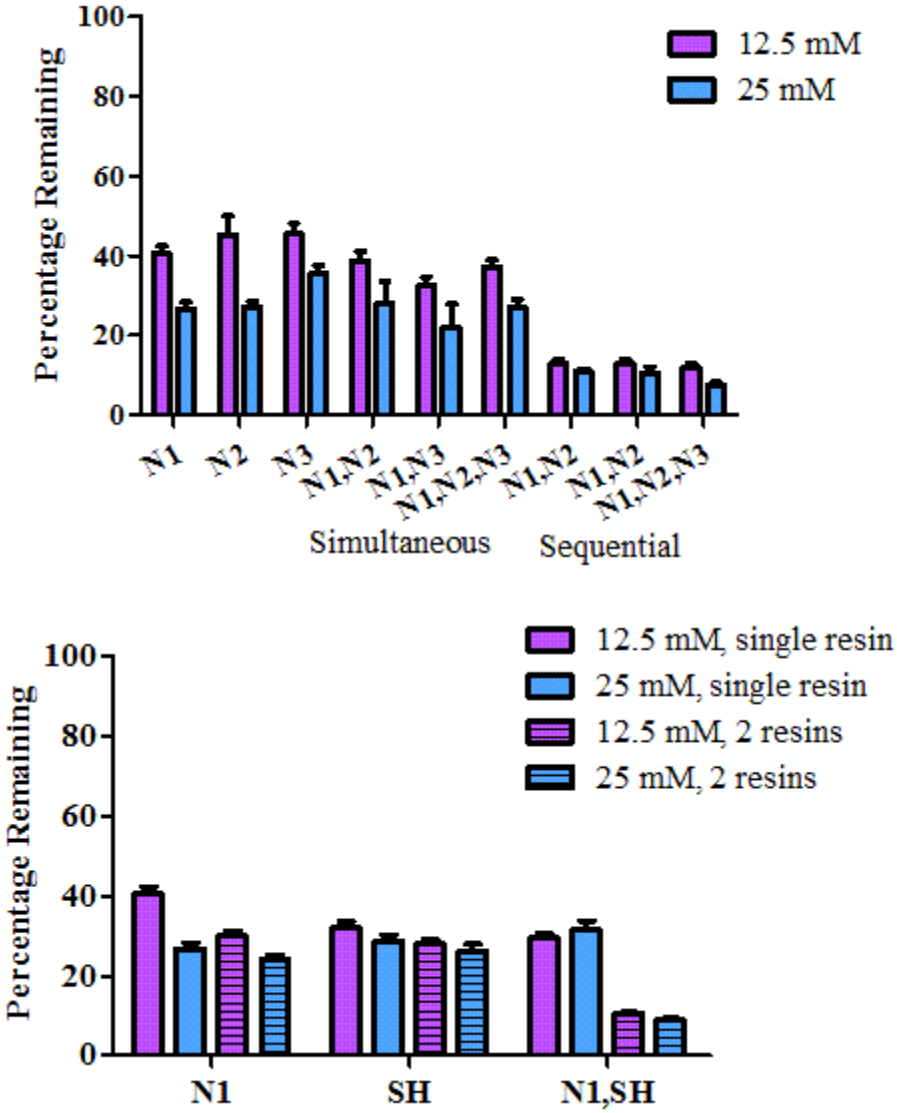


Figure 12: Separation of protein mixtures modified with multiple reagents. In the case of the N1,SH modification, the single resin used was the Co^{2+} resin. The numbers in the legend represent the concentration of the modifying reagent. Simult – simultaneous addition of multiple reagents, Sequen – sequential addition of multiple reagents.

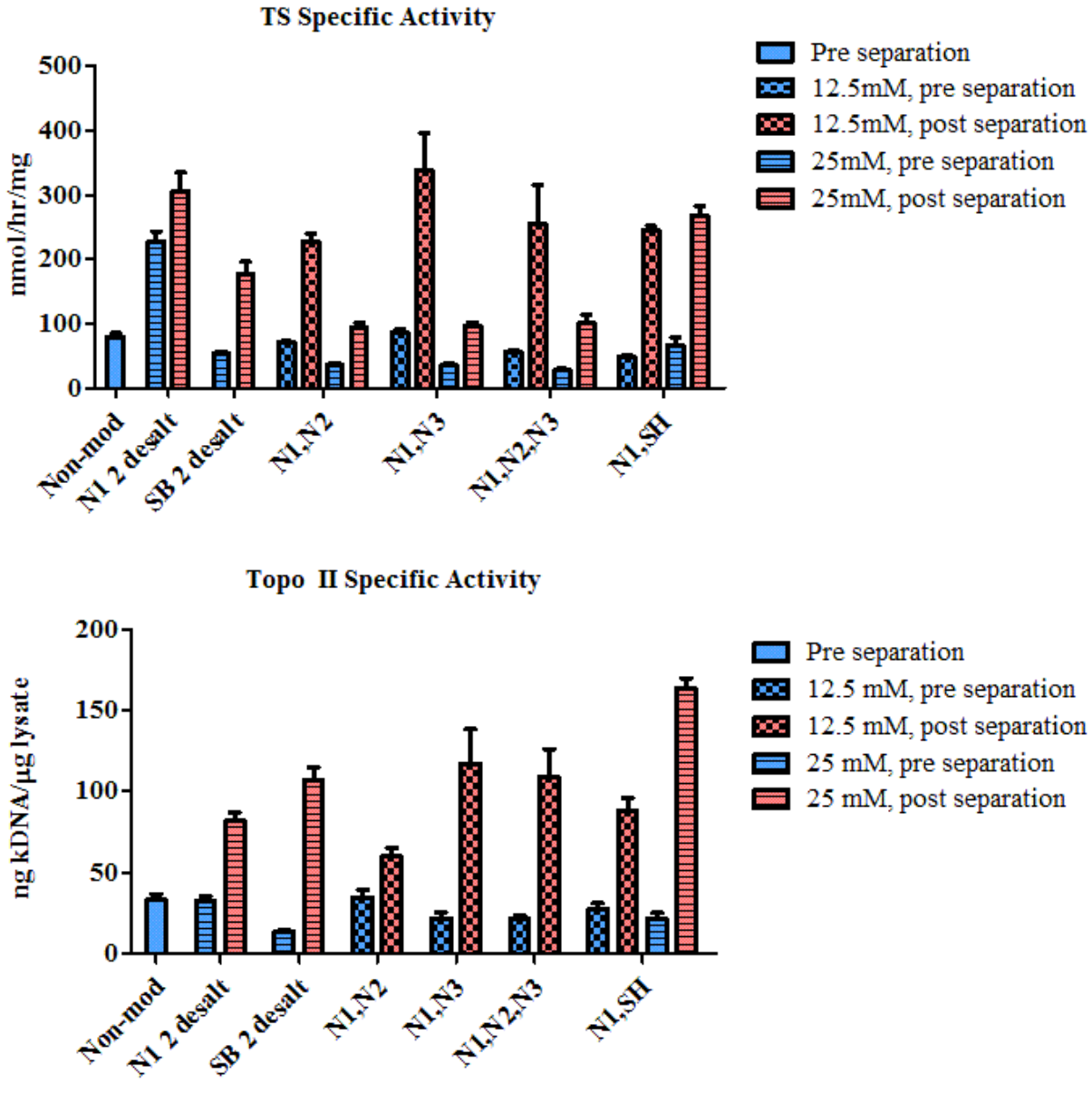


Figure 13: Enzymatic specific activity for non-modified and enriched samples. The schemes using 25 mM concentrations of multiple amine-reactive biotins was not tested with Topo II activity because the enrichment factors were low for TS specific activity.

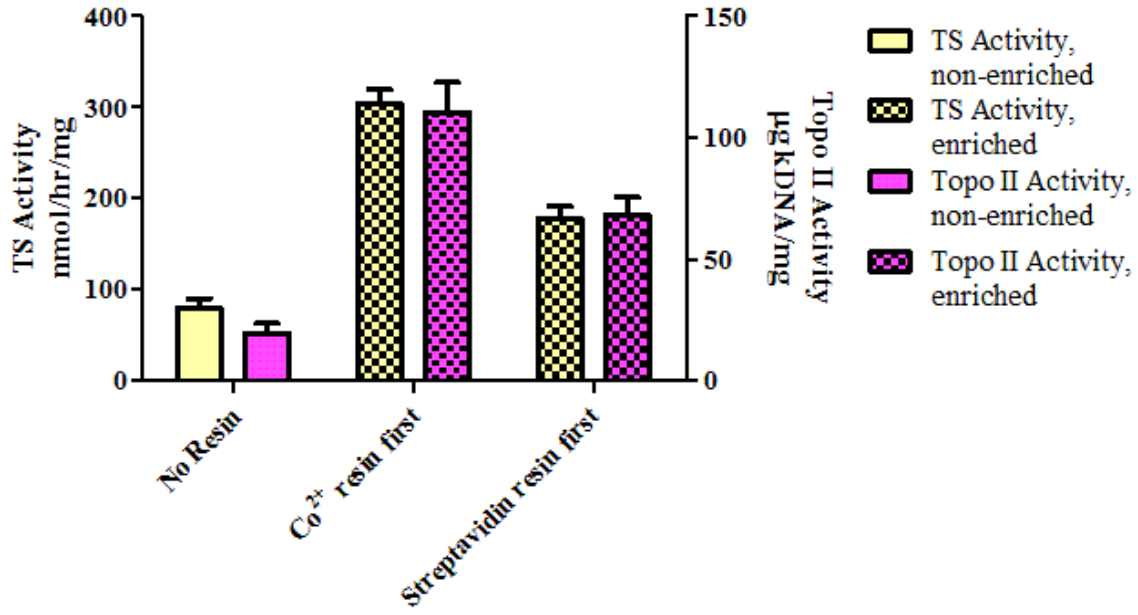


Figure 14: Changes in enzymatic specific activities based on the order of separation resins. The enriched samples were prepared using 25 mM concentrations of the N1 and SH reagents. Using the Co^{2+} resin first provided better enrichment factors than using the streptavidin resin first.

Chapter 4: Protein Arrays

Major Challenge for Array Production

The largest difficulty in creating protein arrays is expressing the numbers of proteins desired for these arrays. While protein arrays tend to be very low density, having between three to sixty different proteins spotted on the slide, there are experiments that have spotted thousands of proteins.[51, 52] Expression of even relatively small numbers of proteins, especially of malaria proteins, is very challenging and becomes very time-consuming if purified proteins are desired. Recent array experiments have spotted translation mixtures from cell-free expressions directly onto arrays with little or no purification, which minimizes the time required for protein expression and array production.[7, 11, 53] Since malarial proteins are expressed more successfully with cell-free systems, this method of constructing arrays is becoming a predominant method for constructing malarial protein arrays.

However, the signals on the arrays resulting from non-specific interactions with lysate proteins (*E. coli* or wheat plasmid-negative controls) were significant with fluorescence signals from the negative controls corresponding to greater than twenty ng/μL concentrations of purified antigenic protein (Fig 1).[7, 11] As described in the introduction, this high background can have a big impact on the results of the experiments because of the way an interaction is defined as a positive hit. A hit is based on the ratio of the spot signal to the signal from the negative control, with a ratio of 2 or 3 being the cutoff for a positive interaction. Having a high value for the negative control lowers the ratios, making it harder to identify hits. This high signal results from the presence of antibodies against the expression lysates (*E. coli* or wheat) and non-specific interactions. The antibodies against the expression lysates occurs when using patient samples

and this immune response can be minimized if the patient samples are pre-incubated with lysates to remove those human antibodies.

The other factor that contributes, with all probe types, to the signal from the negative control is non-specific interactions. Decreasing the amount of wheat protein from the translation system, through enrichment, will decrease the amount of non-specific signal and will help improve the ratios of signal intensities used to identify binding interactions. Enrichment will also improve by increasing the amount of the target protein on the spot allowing for more specific interactions to occur between probe and immobilized protein. Improved ratios allow for better discriminations of actual hits from non-specific interactions and discrimination of strong and weak interactions. It also allows for interactions to be identified with proteins that have low levels of expression that would preclude a signal detectable above wheat protein (negative control).

Evaluating Effects of Enrichment on Arrays

The enrichment schemes discussed in chapters three and four increase the overall percentage of expressed malarial protein and remove the majority of the wheat proteins, so they should have a beneficial effect on the signal ratios from arrays. An enrichment scheme will be considered to have a positive impact on the arrays if the ratio between enriched sample and negative control is greater than the ratio of non-modified control to negative control. The ratios will be measured for two binding partners to DHFR-TS: an antibody, directed against an epitope in the joining region of *P. falciparum* DHFR-TS, and methotrexate, a small molecule inhibitor of the DHFR enzyme in many species. Interactions between the antibody and the wheat proteins are expected to be minimal non-specific interactions. It is expected that there will be some

specific binding between methotrexate and the wheat protein only samples, as the wheat-germ extract has its own DHFR protein. Binding of both probes will be quantified using the Typhoon FLA 9000 gel imager. A secondary antibody conjugated to an AlexaFluor fluorophore will bind to the anti-DHFR-TS antibody and will be used for detection. The methotrexate used for DHFR binding is conjugated to fluorescein and will be directly detected by the scanner (Fig. 15). Previous experiments show that the fluorophore does not eliminate binding to DHFR.

Results

Signal Ratios to Wheat Negative Controls

Various samples, including enriched DHFR-TS, non-modified DHFR-TS controls and wheat protein only controls, were diluted with translation buffer (TSB) with 0.1% Tween 20 to ensure that all samples had similar protein concentrations before manually spotting onto sections (called pads) of a 3D nitrocellulose coated microscopy slide. All samples needed similar concentrations to ensure similar adsorption to the coating. The arrays were created with manual spotting instead of using an automated printer due to the increased ease and speed of the manual printing process. Manual spotting also reduced the cost and required technical knowledge of the printing process. However, the manual spotting process created spot dimensions (2.5 mm by 2.5 mm with a 4.5 mm distance between the center of the spots) that are an order of magnitude larger than spots generated with automated printers (circular spots with 180-200 µm diameter, 250-400 µm center to center distance) and consumed larger volumes of protein samples for immobilization.

Samples immobilized on the nitrocellulose coating were able to bind to the two different molecules: an antibody and a small molecule inhibitor of DHFR, methotrexate (Fig. 16). Since

DHFR binding to the small molecule is dependent on the protein having the correct fold, [54, 55] the fact that the immobilized proteins bind successfully to this small molecule indicates that the extent of denaturation upon immobilization is minimal. The epitope in *Pf*-DHFR-TS recognized by the antibody is not a conformational epitope, so binding of the antibody to protein does not provide information about the protein fold. However, antibody binding proved that the gel coating used to immobilize the protein still provides sufficient access for large protein molecules to find the binding region. The slides will be able to probe antibody-antigen or other protein-protein interactions. There was non-negligible binding between both probes and the wheat protein only samples, with higher methotrexate binding to the wheat than antibody binding to the wheat. The binding to methotrexate is the result of the wheat extract having its own copies of DHFR. The non-specific binding to the antibody could result from wheat proteins with epitopes similar to the one detected with the antibody, but is likely non-specific interactions.

The effect of the enrichment schemes was determined by measuring the ratios of enriched signal intensity to negative control and comparing to the ratio of non-modified to negative control. When the anti-*Pf*DHFR-TS antibody was used as the binding probe, the non-modified DHFR-TS control samples had a fluorescence signal 2.9 times larger than the signal from the negative controls (Fig. 17). This ratio, representing a positive binding interaction, which is similar to the ratios seen for positive hits in previous arrays.[7, 10, 11] Using enrichment schemes to remove wheat proteins improved the ratios of signal intensities, with all schemes producing samples that had ratios greater than five. The enriched sample resulting from modification with 12.5 mM NHS-biotin and 12.5 mM NHS-PEG_{12}-biotin added sequentially (labeled N1,N3 in figures) had a fluorescence signal 8.4 times larger than the negative control, which was the largest increase for the three enrichment schemes tested with the antibody as the probe. Samples

that were enriched using modification with 12.5 mM NHS-biotin, 12.5 mM NHS-LC-LC-biotin and 12.5 mM NHS-PEG_{12}-biotin added sequentially (labeled N1,N2,N3 in figures) had a fluorescence signal 5.8 times higher than the negative control. The third enrichment scheme tested, using modification with 25 mM NHS-biotin and 25 mM maleimide-His_{6x} peptide (labeled N1,SH in figures) generated samples that had fluorescence intensities 6.1 times greater than the negative control. The increased intensities were not due to the presence of the modifiers (Fig. 16).

The enrichment schemes also provided better ratios when the binding probe used was the fluorescently labeled methotrexate. Non-modified DHFR-TS control samples had a signal 2.1 times greater than the negative controls. The samples prepared with the N1,N3 enrichment scheme bound to more methotrexate than the non-enriched, with a ratio between sample and negative control of 8.1. The N1,N2,N3 enrichment scheme produced samples with fluorescence signals 5.9 times larger than the negative control. The N1,SH enrichment scheme provided a slightly better signal increase than the N1,N3 scheme, with enriched samples having fluorescence 8.5 times higher than negative controls. The increased methotrexate binding for all enriched samples was not due to the presence of the modifiers (Fig. 16).

Concentration Dependence of Probe Binding

The binding between both probes and immobilized protein was also concentration-dependent (Fig. 18). As the concentration of immobilized protein increased, the fluorescence intensity increased in a linear fashion. This linear increase occurred for all five immobilized sample types, including negative controls and non-enriched samples, but the increase was larger for the enriched samples than with the negative controls or non-enriched controls.

Since probe binding was concentration dependent, the arrays could also be used to quantify the amount of DHFR-TS in the different sample types. Quantification is generally difficult to do with samples expressed with the continuous flow methods used here without the use of affinity tags. Quantification of the protein concentrations was done using a calibration curve generated by immobilizing known amounts of a *Pf*DHFR-TS protein purified using a GST affinity tag and probing the arrays with the anti-*Pf*DHFR-TS antibody (Fig. 19). The calibration curve had very good linearity (R^2 value of 0.9944). The lowest protein concentration detected was 10^{-7} M and the arrays had a detection range over four orders of magnitude (10^{-7} to 10^{-4} M). This calibration curve was used to convert the normalized fluorescence intensities measured for each sample to DHFR-TS protein concentrations (Table 2). The sample with the most DHFR-TS protein was the NP enriched sample. The NLP enriched sample had slightly more protein than the NH enriched sample. All of the enriched samples had at least three times more protein than the non-enriched controls. The amounts measure were eight times higher than the wheat negative controls. The quantification of the wheat protein is likely inaccurate since the antibody should have mostly non-specific interactions with the wheat.

Specificity of Binding Interactions

The binding between probes and immobilized proteins was also specific. Both binding probes showed good discrimination between samples with expressed DHFR-TS proteins and samples with expressed Topo II proteins immobilized in adjacent spots on the array (antibody response shown in Fig. 20). The fluorescence signal resulting from probe binding to the samples with Topo II was equivalent to that seen with the wheat proteins. The antibody used as a binding probe was specific for the *P. falciparum* DHFR-TS protein as compared to *P. vivax* DHFR-TS

when the two proteins were spotted in adjacent pads of the nitrocellulose coating (Fig. 21). The signals from samples expressing *P. vivax* proteins were similar to wheat proteins. Since methotrexate binds to the DHFR enzyme of many species,[54, 55] methotrexate was not expected to show specificity between the *Pf* and *Pv* enzymes and was not tested

Discussion

The enrichment schemes provided an improvement in the signal intensities on the protein arrays. The ratios of the fluorescence intensities increased from three with the non-modified samples to six or more with the enriched samples. These ratios were determined for well-known binding interactions using a protein, *Pf*DHFR-TS, that is expressed well in the wheat system. The antibody used was designed to bind to *Pf*-DHFR-TS with good strength and specificity. Methotrexate is a known binder to the DHFR region with good strength and is often used in experiments probing localization of DHFR, including *Pf*DHFR-TS. The protein and probes chosen are well-behaved and represent ideal interactions on an array. For most interactions, the ratios detected will not be as high as those measured for DHFR-TS. However, the use of the enrichment schemes should allow for these non-ideal interactions to be detected better than with previous experiments.

Other proteins that are not expressed in the same yields, like GTP cyclohydrolase or GTP-AMP phosphotransferase, are of much interest in protein arrays but detecting positive interactions with these proteins would have been difficult previously. The average yield for these proteins is below the 20 ng/μL concentration that the wheat negative controls display. Without enrichment, any positive binding interactions would be below the background level and thus misclassified as negative results. With the enrichment scheme to increase the concentration

and intensities three- to five-fold, interactions should then be detected above the negative control. Enrichment should also positively impact probes with weaker or non-specific binding. If a probe binds with less specificity, the non-specific interactions would increase relative to the specific. Removing protein involved in non-specific interactions would limit this increase. For a probe that binds more weakly to its protein target than the antibody or methotrexate did to DHFR-TS, the interaction could be disrupted more frequently during the washing steps. This would leave a fraction of the initially bound probe remaining for detection. Since enrichment increases the amount of target protein immobilized, the total amount of bound probe corresponding to this weakly bound fraction increases, making the interaction easier to detect.

Currently, only fluorescently labeled probes are chosen for protein arrays but the initial probes had radioactive labels. Incorporating radioactive isotopes into the probe is more appealing than attaching a fluorophore, since the different isotopes do not have any effect on function or binding, though the presence of a fluorophore might. However, the phosphorimaging detection method to measure radioactive probe binding does not have the appropriate spatial resolution. The background from the phosphorimager obscures the boundaries of the spots, making it impossible to discriminate protein-probe interactions from background. The instrument background for many different fluorescence scanners is substantially lower than for phosphorimaging, allowing for easy identification of different spots on the arrays.

Even with the improvement in signals from the enriched samples, the protein arrays still have many limitations that can be addressed to improve arrays further. The limits of detection of purified DHFR-TS are 10^{-7} M and the detection range spans four orders of magnitude. The detection limits and detection range are likely negatively impacted by the size of the protein spots and the instrument used to measure the fluorescence signal. With manual spotting on the

64 pad array slide, the spot size is limited by the size of the pad (2.5 mm by 2.5 mm). Protein samples diffuse over the entire spot with diffusion stopping at the boundary of the nitrocellulose pad and glass slide. Using contact printers to spot the proteins should produce smaller spot sizes. Spots created with contact printers typically have diameters of around 200 µm. The smaller spots typically have less diffusion and retain a higher protein concentration which should be detected better with the Typhoon gel scanner. Arrays with smaller spot sizes also consume less sample, decreasing the cost of reagents used on the array, and improve the signal seen on arrays.[56, 57]

The typical instruments for detecting fluorescently labeled probes on microarrays are specifically designed to scan one microscope slide with good detection limits and range. Until recently, though, these scanners were limited to detecting Cy3 and Cy5 fluorophores or fluorophores with spectral overlap to Cy3 or Cy5. However, many commonly used fluorophores, like fluorescein and Alexa Fluor 488 used in these studies, do not have spectral overlap with Cy3 or Cy5. The Typhoon scanner can detect a range of fluorophores with excitation and emission across the entire visible spectrum but it is designed to scan larger areas than a microscope slide. While the scanner has a proper resolution for the arrays used (the spatial resolution should be at least one-tenth of the spot size), it is unknown whether the photomultiplier tubes have the capabilities to detect low concentrations of fluorophores at the most stringent spatial resolution. Recently, companies have started to offer array scanners equipped with an increased spectral range for the fluorophores that can be detected at the same sensitivity as the previous array scanners. Switching to one of these array scanners could improve the results of the array.

Finally, protein arrays will be limited by how rapidly proteins can be expressed and enriched to the desired purity and concentrations. The conventional protein purification methods were replaced with schemes that were able to enrich at least twenty translation mixtures within five hours (the enrichment of more than twenty samples was never done simultaneously). However, the enrichment schemes were limited by how many different translations can be initiated simultaneously as discussed in chapter three. Without further developments in the speed of protein translation, constructing protein arrays will still be time-consuming if more than ten or twenty different proteins are immobilized.

Conclusions

Using the enriched samples as the source for proteins immobilized on the arrays provided increased fluorescence signals compared to the non-enriched samples. This resulted in better ratios to the negative controls than seen in the previous array experiments. These better signal ratios should allow for better identification of positive binding interactions, especially for proteins that have very low expression yields in the wheat system. The arrays display a concentration dependent response between various probes and immobilized proteins, making this method a feasible option for experiments determining binding kinetics between proteins and small molecules. The response is linear over four orders of magnitude and includes the range of concentrations that match the yields of protein expressed using the wheat germ cell-free system. Interactions on the array are specific, with no binding to Topo II or *Pv*DHFR-TS detected over the wheat negative controls.

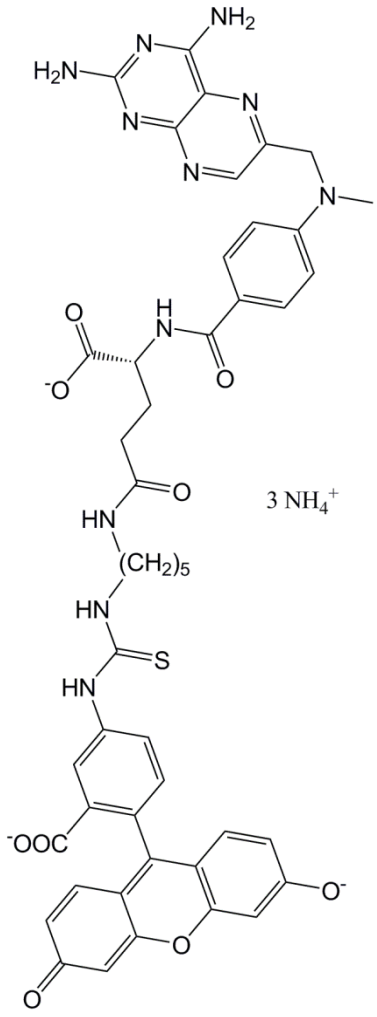


Figure 15: Structure of fluorescent methotrexate.

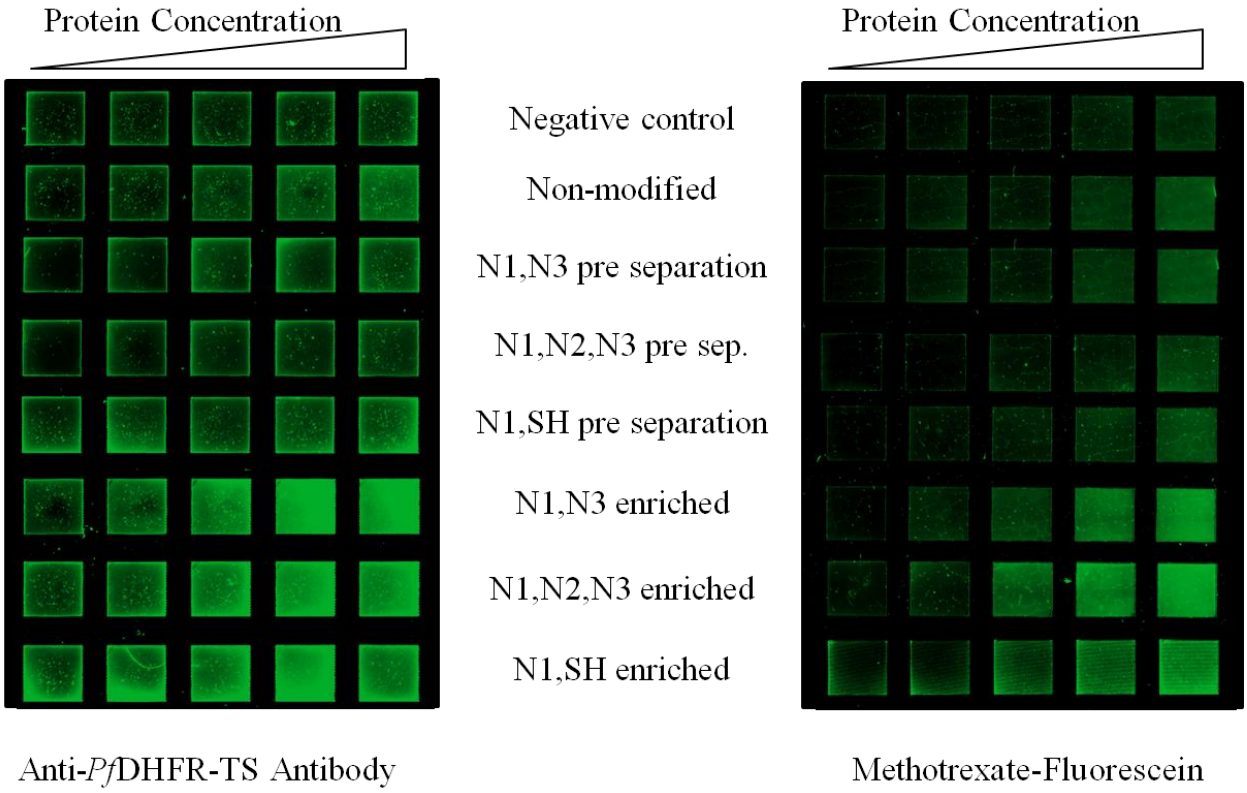


Figure 16: Example protein array images. N1,N3 = DHFR-TS sample modified using scheme with 12.5 mM NHS-biotin and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,N2,N3 = DHFR-TS sample modified using scheme with 12.5 mM NHS-biotin, 12.5 mM NHS-LC-LC-biotin, and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,SH = DHFR-TS sample modified using scheme with 25 mM NHS-biotin and 25 mM Mal-His_{6x} peptide. Enriched = samples after separation with streptavidin or Co^{2+} followed by streptavidin resins.

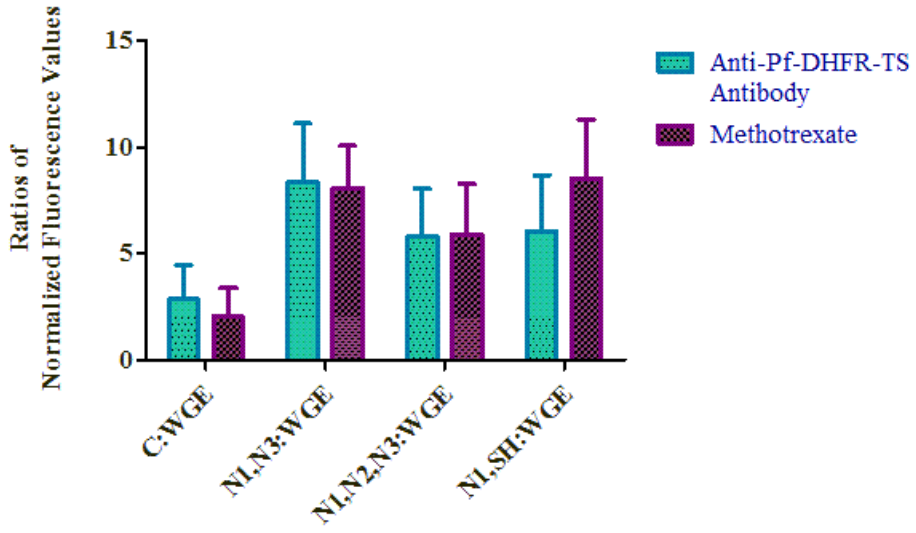


Figure 17: Fluorescence intensity signal ratios. WGE = wheat germ extract, the negative control; C = non-modified control with expressed *Pf* DHFR-TS; N1,N3 = DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,N2,N3 = DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin, 12.5 mM NHS-LC-LC-biotin, and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,SH = DHFR-TS sample enriched using scheme with 25 mM NHS-biotin and 25 mM Mal-His_{6x} peptide and both resins as separation matrix.

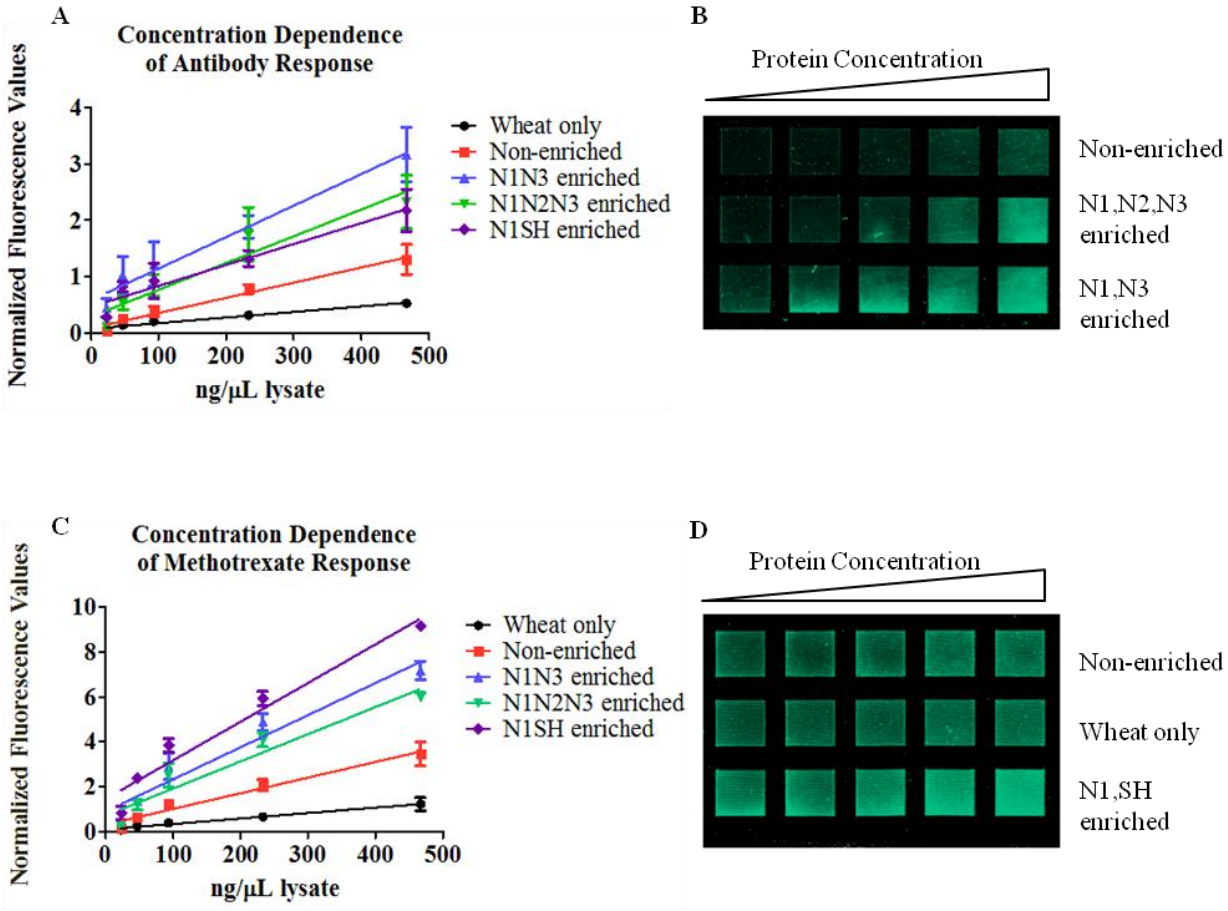
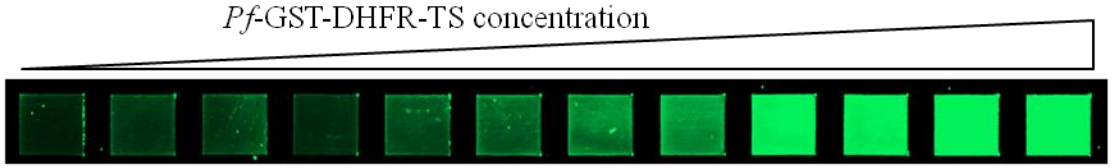


Figure 18: Concentration dependent response of binding probes to immobilized *Pf*-DHFR-TS. (a, b) Anti-*Pf*-DHFR-TS response to increasing protein concentrations. (c, d) Methotrexate response to increasing protein concentrations. N1,N3 = DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,N2,N3 = DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin, 12.5 mM NHS-LC-LC-biotin, and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,SH = DHFR-TS sample enriched using scheme with 25 mM NHS-biotin and 25 mM Mal-His_{6x} peptide and both resins as separation matrix.

A



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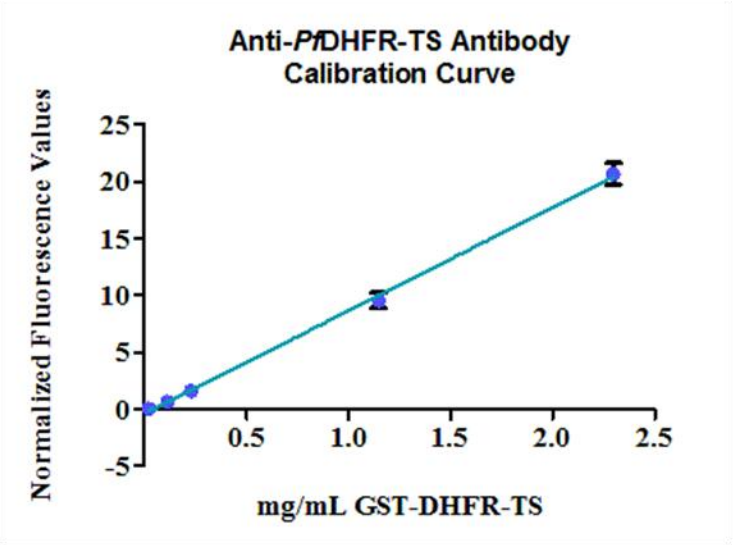

Figure 19: Calibration curve created from anti-*Pf*-DHFR-TS antibody binding to *Pf*DHFR-TS purified with a GST tag. The intensities measured from the array image (a) were converted to normalized fluorescence values as described in the experimental section and used to generate a linear regression line (b). There was a good linearity to the data, with a R^2 value of 0.9944.

Table 2: *Pf*-DHFR-TS concentrations determined with protein arrays. N1,N3 = DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,N2,N3 = DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin, 12.5 mM NHS-LC-LC-biotin, and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,SH = DHFR-TS sample enriched using scheme with 25 mM NHS-biotin and 25 mM Mal-His_{6x} peptide and both resins as separation matrix.

Sample	*Pf*-DHFR-TS concentration (µg/mL), antibody
Wheat only	47.7 ± 2.8
Non-enriched	128.0 ± 44.4
N1,N3 enriched	572.7 ± 43.9
N1,N2,N3 enriched	409.0 ± 24.6
N1,SH enriched	378.8 ± 37.5

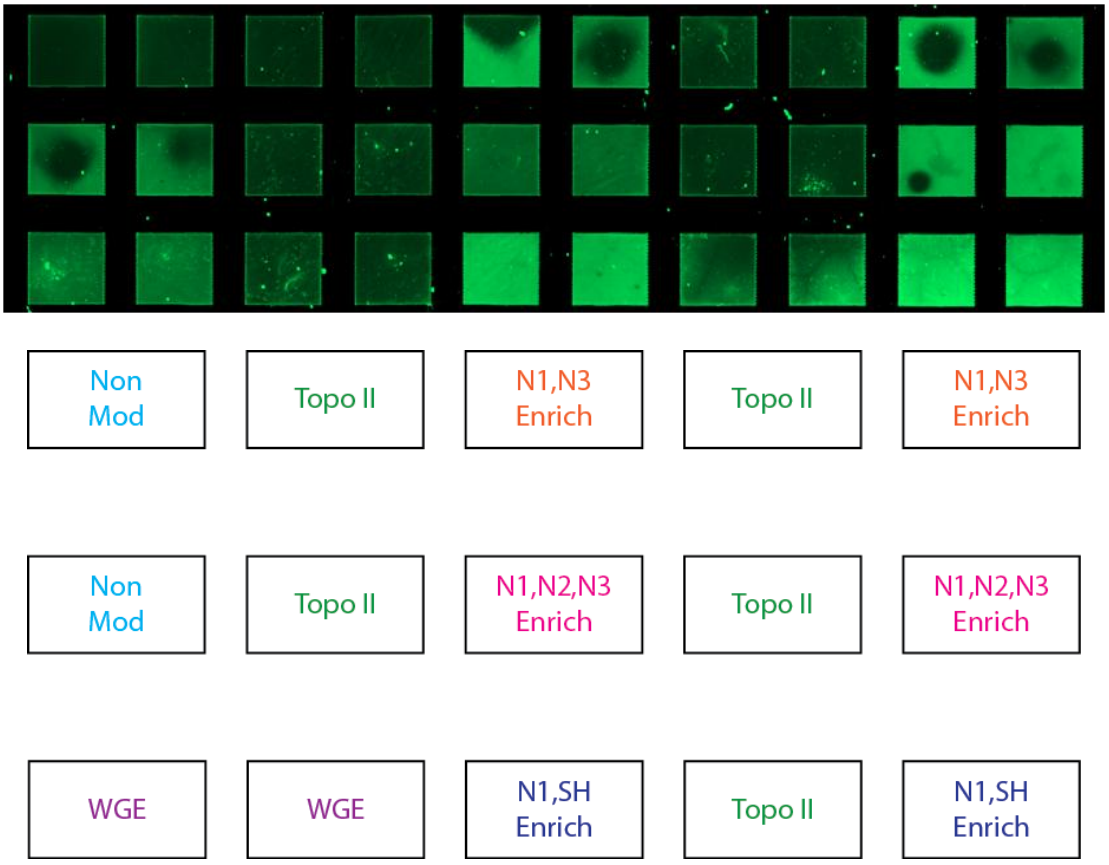

Figure 20: Specificity of binding between probe and two different enzymes. The two enzymes are DHFR-TS and Topo II. In the bottom panel, each box represents two pads on the array image. WGE = wheat negative controls, Non mod = non-modified DHFR-TS samples, Topo II = non-modified Topo II samples, N1,N3 Enrich = DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,N2,N3 Enrich. = DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin, 12.5 mM NHS-LC-LC-biotin, and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,SH Enrich. = DHFR-TS sample enriched using scheme with 25 mM NHS-biotin and 25 mM Mal-His_{6x} peptide and both resins as separation matrix.

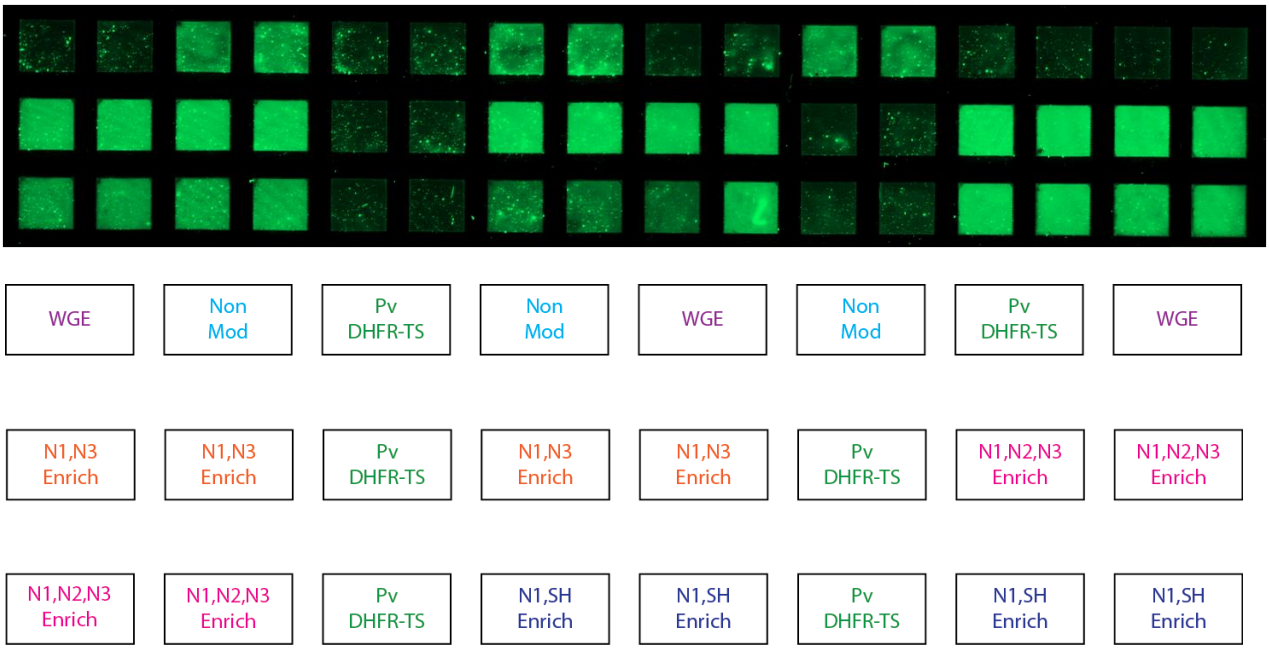

Figure 21: Specificity of binding between probe and enzyme from two *Plasmodium* species. In the bottom panel, each box represents two pads on the array image. WGE = wheat negative controls, Non mod = non-modified *Pf*DHFR-TS samples, N1,N3 Enrich = *Pf*DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,N2,N3 Enrich. = *Pf*DHFR-TS sample enriched using scheme with 12.5 mM NHS-biotin, 12.5 mM NHS-LC-LC-biotin, and 12.5 mM NHS-PEG_{12}-biotin added sequentially; N1,SH Enrich. = *Pf*DHFR-TS sample enriched using scheme with 25 mM NHS-biotin and 25 mM Mal-His_{6x} peptide and both resins as separation matrix, Pv DHFR-TS = non-modified *Pv*DHFR-TS samples.

Chapter 5: Project Conclusions

Malaria, caused by various species of the genus *Plasmodium*, is a devastating disease that causes approximately 300 million disease cases and one million deaths per year. While there is a significant amount of money and research devoted to finding the next generation of drugs or vaccines, the research is limited by the difficulties in expressing and purifying the quantities of proteins necessary for the various experiments. As the proteome becomes a larger focus and experiments transition from single- to multiple-protein experiments, the numbers of proteins of that can be studied are increasing. The increasing use of the wheat-germ system to express *Plasmodium falciparum* and *Plasmodium vivax* proteins addresses one of the issues that limits the production of protein samples. However, the presence of the wheat proteins after translation can affect the results of protein experiments. This is most apparent in protein arrays since wheat proteins increase the non-specific background interactions that can obscure positive binding interactions. To ensure better results on the arrays, the wheat proteins and malarial proteins should be separated via some method before use. The conventional purification method involves the use of recombinant affinity fusion tags, which are peptide sequences attached to the protein of interest. This peptide binds to a separation matrix and will be removed (along with the protein of interest) from the other wheat proteins. The presence of different tags can have deleterious effects on the fold and function of the expressed protein but the effects cannot be predicted. Instead, these effects need to be determined experimentally on an individual protein basis, which becomes time-consuming when dealing with the tens to hundreds of proteins studied on a protein array.

An alternate approach is to attach the affinity tag to the proteins in the wheat expression system. This still utilizes the high, specific affinity of various pairs developed for recombinant affinity tags but does not modify the protein of interest. The open nature of the *in vitro* system allows for addition of a small molecule to covalently modify the wheat proteins prior to translation. After translation, the modified wheat proteins are removed using a separation matrix that binds with high affinity to the small-molecule. The separation leaves the expressed malarial protein at a higher concentration in solution. This process has been termed protein enrichment instead of protein purification.

Potential enrichment schemes were tested against five criteria: successful modification of wheat proteins, separation and removal of wheat proteins, and expression of three proteins. The three proteins expressed with modified wheat lysates were green fluorescent protein (GFP), dihydrofolate reductase-thymidylate synthase (DHFR-TS) and topoisomerase II (Topo II) and each was tested to ensure it retained activity. GFP was used as a positive translation control and the other two proteins are interesting drug targets in *Plasmodium*. The specific activity of DHFR-TS and Topo II were measured post-separation and if the scheme successfully enriched the solution, the activity increased compared to the non-modified control. The potential schemes used reagents based on the high affinity between biotin and streptavidin and between metal ions and histidine amino acids.

Schemes using one modifying reagent, either a biotin or His_{6x} peptide, were able to modify the majority of the protein but did not remove enough of the wheat protein (the separation steps had to remove approximately 85% of the initial wheat protein). An issue with these single modifier schemes was that the reagents were reacting with components in the buffer instead of the proteins. To address this, the wheat lysates were passed through desalting columns

prior to modification to remove the reactive buffer components. Using the initial desalting step followed by a concentration step after modification improved the extent of modification of lysates and increased the amount of protein removed during the separation steps, while maintaining good translational activity. This procedure provided good increases in specific activity but the extra steps make it less ideal for producing the numbers of samples needed for protein arrays.

The best enrichment schemes relied on combinations of reagents for modification. Using multiple amine-reactive biotins or the combination of NHS-biotin and maleimide-His_{6x} peptide produced better results for modification and separation than single reagent schemes. These modified lysates were able to express all three proteins and increased the specific activities for DHFR-TS and Topo II at least three-fold.

DHFR-TS samples enriched using the multiple reagent enrichment schemes provided better signals on the protein arrays. The ratios between the non-modified samples and negative controls were between two and three for both the anti-*Pf*DHFR-TS antibody and the fluorescently labeled methotrexate. However, the ratios measured between enriched samples and the negative controls were between six and nine for all schemes with both probes. Enrichment increased the ratios for positive interactions between three- and five-fold, matching the results seen for increases in specific activity.

While protein enrichment, based on modification and removal of wheat proteins from the translation lysates, produced an increase in both enzymatic specific activity and fluorescence signals on protein arrays, the increases never surpassed five-fold. Several factors were discussed that could be limiting the increases measured, including uneven distribution of reactive groups on the protein. Altering the enrichment schemes and protein array production to address these

factors should help improve the enrichment results even further than the three- to five-fold enrichment already demonstrated.

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Curriculum Vitae

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Education:

Ph.D (Chemistry) University of Washington 2012
Advisor: Pradipsinh K. Rathod

B.A. (Chemistry) Lewis and Clark College 2006

Research Experience:

Ph.D in Chemistry, University of Washington 2006-present
Advisor: Professor Pradipsinh K. Rathod, Department of Chemistry
Thesis Title: Chemical Strategies to Enrich Malarial Enzymes from Wheat Lysates
Research Summary:
Developed protein enrichment scheme to increase concentration of expressed malarial protein in wheat-germ expression lysate. Wheat proteins were covalently modified with a small-molecule reagent. A separation matrix bound and removed the wheat proteins specifically. Enriched samples provided better signal intensities when used on protein arrays.

Undergraduate Research Assistant, Lewis and Clark College 2004-2006
Advisor: Professor Nikolaus Loening
Research Summary:
Developed probe to measure temperature of sample in NMR spectrometer. Probes with thulium in the molecule have temperature dependent chemical shifts but affect the spin relaxation rates and peak widths of the sample protons. Encapsulating the temperature probe or sample in a hydrophilic dendrimer mitigated the effects of the thulium probe without affecting the temperature dependence.

Skills and Techniques:

- Protein expression and purification
- Immunoblotting
- Covalent modification of proteins
- Protein immobilization
- *In vitro* enzymatic assays
- HPLC
- Mass spectrometry (ESI)
- NMR (^{1}H, ^{13}C, NOESY)

Peer-Reviewed Publications:

Narayanasamy, K., Chery, L., Basu, A., Duraisingh, M.T., Escalante, A., Fowble, J., Guler, J.L., Herricks, T., Kumar, A., Majumder, P., Maki, J., Mascarenhas, A., Rodrigues, J., Roy, B., Sen, S., Shastri, J., Smith, J., Valecha, N., White, J., Rathod, P.K. (2012) Malaria Evolution in South Asia: Knowledge for control and elimination. Acta Tropica. 121 (3): 256-266.

Maki, J.N. and N.M. Loening. (2007) Measuring Molecular Motion: Using NMR Spectroscopy to Understand Translational Diffusion. In *Modern Nuclear Magnetic Resonance in Undergraduate Education*. Eds. David Rovnyak, Robert A. Stockland, and Steven P. Lee; American Chemical Society Books: Washington, DC, July 2007.

Teaching Experience:

Teaching Assistant, University of Washington 2006-2012
Department of Chemistry
General Chemistry, Fall 2006, Spring 2007
Organic Chemistry, Winter 2007, Fall 2007, Winter 2008, Spring 2008, Fall 2008, Winter 2009, Summer 2009, Winter 2010, Spring 2010, Winter 2012

Teaching Assistant, Lewis and Clark College 2004-2006
Department of Chemistry
General Chemistry Honors Lab, Spring 2004, Spring 2005
Organic Chemistry, Spring 2005
Organic Chemistry Lab, Fall 2005, Spring 2006

Affiliations and Professional Memberships:

Phi Beta Kappa	2006 – present
American Chemical Society	2010 – present
AAAS	2010 – present

Awards:

Howard J. Ringold Graduate Student Fellowship	2006 – 2007
Graduated *magna cum laude*, Lewis and Clark College	2006
Graduated with departmental honors, Lewis and Clark College	2006