ABSTRACT

Title of Thesis: Simplified Protein Purification Using Protein-Polysaccharide Conjugation

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The purification step of protein production and isolation is the most time consuming and complex step in processing. Optimization of the purification process requires rapid, economical, and cost-effective methods to purify proteins at both the small-scale and the large-scale. Instead of using complicated chromatographic techniques and dialysis, a more simplified method is proposed utilizing only a centrifuge. A tyrosine tag with an enterokinase enzymatic cleavage site was added to the C-terminus of green fluorescent protein (GFP). The tyrosine tagged protein was expressed in *E. coli* cells. The cells were harvested and lysed. The lysate containing the tyrosine tagged GFP was covalently coupled to a polysaccharide (chitosan) using tyrosinase enzyme. The GFP in the protein-polysaccharide conjugate was then liberated using the enzyme, enterokinase. This method was effective in conjugating and purifying the protein using a centrifuge with a limited number of processing steps.

SIMPLIFIED PROTEIN PURIFICATION USING PROTEIN-POLYSACCHARIDE CONJUGATION

by

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DEDICATIONS

I would like to dedicate this thesis to my family, my parents, Arnold and Lea Small, my brothers, Scott and Jason Small, and my grandmother, Sophie Waldorf for their love, encouragement, and support in all of my endeavors.

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Chapter 1: Introduction

1.1. Overview

Successful elucidation of genome sequences has increased pressure to mass-produce hypothetical proteins so that new folds are predicted and structural information can be more firmly and rapidly embedded into the fields of cell and developmental biology, medicine, bioengineering, etc. (Machalek, 2001). Rapid and low-cost methods for protein purification are sorely needed, particularly at small scales where automation can accelerate the purification cycle time. Additionally, even at large-scale, low cost techniques that minimize the number of separation stages will increase yield and decrease overall processing time (Ladisch, 2001). Process development time has been cited as a critical bottleneck in the development of new drugs and therapeutics (Boguslavsky, 2002 and Marsh, 2002). Affinity binding techniques based on protein fusions have seen rapid acceptance in process laboratories because of their simplicity and the relative ease with which they can be built into expression vectors. Immobilized metal ion affinity chromatography (IMAC), which exploits the affinity of surface-exposed poly-histidine residues with immobilized transition row metal ions, is widespread. New techniques such as metal-affinity precipitation are promising in that a simple increase in temperature or pH promotes precipitation of the attached proteins (Galaev *et al.*, 1999). In this way,

the need for the expensive and unscaleable chromatography steps is reduced. Despite the one-step purification success of IMAC, the method has several disadvantages associated with large-scale processes, due to costs associated with fouling within the column (Kumar *et al*., 1998).

1.2. Objective and Proposed Process

Our objective for this study was to develop a new method for protein purification using protein-polysaccharide conjugation and enzymatic cleavage to liberate the protein. In order to achieve these objectives the following actions need to be accomplished:

1) Develop a method to attach amino acid residue tags (short sequences, in particular a five poly tyrosine tag) to the carboxylic acid terminus of a desired protein (GFP, green fluorescent protein) with a six poly histidine tag at the amine terminus. Specifically, construct an expression vector wherein the *gfp* gene is flanked by a six histidine codon (N-terminus) and a five tyrosine codon (C-terminus) all downstream of the promoter region.
2) Grow host *E. coli* cells and induce protein synthesis.
3) Purify protein using histidine tags with column chromatography or
4) Directly perform the coupling reaction (tyrosinase-mediated) to covalently couple the protein to a polysaccharide (chitosan).
5) Precipitate the chitosan, wash, and resuspend the protein/polysacchradie complex.

6) Perform the enzymatic cleavage reaction (enterokinase digestion) to liberate the protein from the polysaccharide.

These steps are provided in a schematic as shown in Figure 1. The coupling reaction as depicted is heterogeneous, which is feasible. However, homogeneous coupling enables a one step separation based on centrifugation and this is the principle advantage of this method described here.

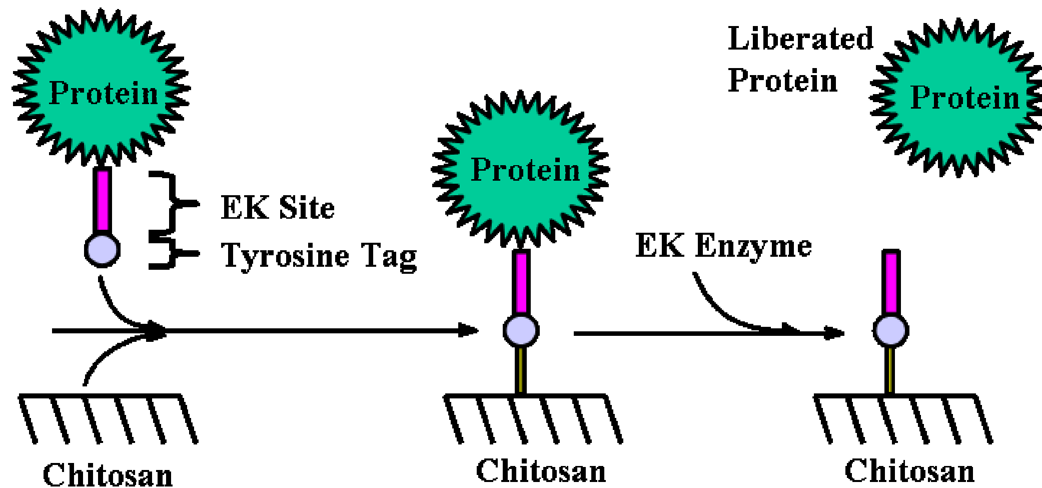

Figure 1: Proposed simple purification scheme.
A tyrosine tag with an enterokinase enzymatic cleavage site was added to the C-terminus of a target protein. The lysate containing the tyrosine tagged protein was coupled to a polysaccharide (chitosan) using tyrosinase enzyme. The target protein in the protein-polysaccharide conjugate was then liberated using enterokinase digestion.

1.3. Green Fluorescent Protein (GFP)

Green Fluroescent Protein (GFP) consists of 238 amino acids with a molecular weight of 27 kDa. The major spectral absorption peak is at 395 nm with a minor peak 470 nm and a single emission peak at 509 nm (Chalfie *et al*., 1994). Three amino acids, Ser65, Tyr66, and Gly67 associate to form the fluorophore of

the molecule (Prasher, 1995). Wild type GFP radiates green light when exposed to blue or ultraviolet (UV) light. GFP was first discovered in the pacific jellyfish, *Aequorea victoria* and was subsequently purified and characterized (Shimomura *et al*., 1962). The gene responsible for GFP was successfully cloned in 1992 (Prasher, *et al*., 1992) and has been successfully expressed in prokaryotic cells such as *E. coli* (Chalfie *et al*., 1994), yeast cells (Sterns, 1995), plant cells (Youvan, 1995), insect cells (Reilander *et al*., 1996), and mammalian cells (Pines, 1995). GFP forms a compact β-can structure (see Figure 2) comprised of 11 strands (Yang, *et al*., 1996). The *gfp* gene used in our experiments (*gfp*UV) is a UV-optimized variant this a 18 times more fluorescent than the wild type (Cody *et al*., 1993).

Figure 2: RasMol representation of Wild-type (WT-GFP).
(Rasmol v 2.7.2.1, Glaxo Research and Development, Greenford, Middlesex UK). The α-helices are in magenta, the β-sheets are in gold, the fluorophore is in cyan, and the random coils are in white. The β-sheets form an almost perfect cylinder with the fluorophore being located in nearly the geometric center. The random α-helices at the top form a lid for the structure. This conformation is called a β-can.

1.4. Tyrosinase

Tyrosinase is an enzyme that catalyzes the oxidation of phenols to *o*-quinones. Phenol oxidases, like tyrosinase have been found in many natural

processes including melanization, insect sclerotization, etc. In a few cases, tyrosinaste-mediated reactions lead to functional biomaterials. The tyrosinase enzyme found in mussels, barnacles, and other aquatic animals allows them to strongly adhere to underwater surfaces (Waite, 1990). The hardening of insect carapaces is also facilitated by tyrosinase (Sugumaran, 1988). Plant and fungal forms of the tyrosinase enzyme are also responsible for the oxidation of produce, i.e. the browning of fruits, vegetables, and mushrooms (Zawistowski, *et al*., 1991). Vertebrate forms of tyrosinase are stimulated by UV light to cause pigmentation variations within these organisms (Prota 1988). Previous studies have shown that tyrosinase can be used to couple proteins to chitosan with the help of a phenolic chemical linker (Chen, *et al*., 2001). These reactions occur due to the presence of tyrosinase generated *o*-quinones which will react with a strong nucleophile (see Figure 3). However, these processes are still poorly characterized, and the *o*-quinones can participate in numerous reactions producing an array of products that are difficult to categorize (Xu, 1996).

Table 1: Examples of tyrosinase reactions in nature

Example	**Reference**
Ability of marine animals (e.g. mussels, barnacles, etc.) to adhere to underwater surfaces	(Waite 1990)
Insect cuticle sclerotization (the hardening of insect shells)	(Sugumaran 1988)
Browning of fruits, vegetables, and mushrooms	(Prota 1988)
Vertebrate pigmentation and pigmentation reactions with UV light	(Prota 1988)

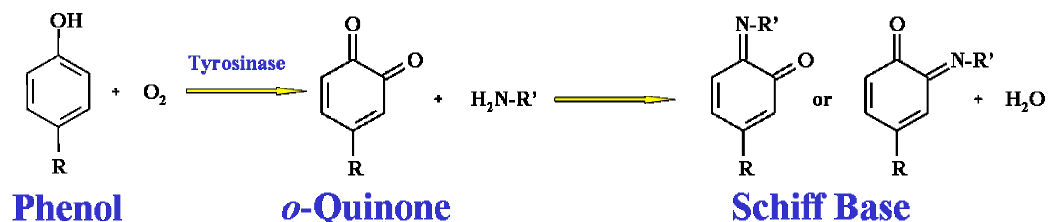

Figure 3: Tyrosinase's reaction scheme.
Tyrosinase catalyzes the oxidation of phenolic compounds. These compounds may then react with nucleophiles to produces Schiff Bases or Michael's Adducts and water in a condensation reaction.

1.5. Chitosan

Chitosan is a biopolymer of chitin. Chitin is the second most abundant polysaccharide found in nature. Chitin is found in a wide variety of biological sources, including the following: insect carapaces, crustacean shells, and fungal cell walls (Goosen, 1997). As such, chitosan is a renewable resource, since it can be generated from the shells of both shrimp and crabs. The presence of a high content of primary amino groups in chitosan allows for two characteristics that make this biopolymer attractive to couple proteins: First, the amino groups are basic with $pK_a \approx 6.3$. Hence, below pH 6.3, protonation occurs and the resulting cationic polyelectrolyte is rendered water-soluble. Second, the amino groups are nucleophilic at a pH above 7. The deprotonation of the amino groups creates an unshared pair of electrons that can participate in a wide variety of chemical reactions, including protein coupling (Kumar, 2000).

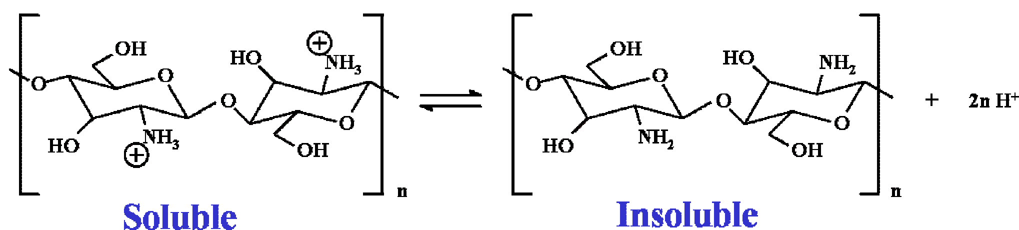

Figure 4: Chitosan.
Protonated and deprotonated equilibria of chitosan subunit. The protonated species will have the following characteristics: polycationic, water-soluble, and unreactive. The deprotonated species will have the following characteristics: a neutral charge, insoluble in water, and nucleophilic and reactive (Kumar, 2000).

1.6. Immobilized Metal Affinity Chromatography (IMAC)

Chromatography is a method to separate molecules based on differences in chemical structure. There are several chromatographic methods:
adsorption chromatography (ADC), liquid-liquid partition chromatography (LLC), ion-exchange chromatography (IEC), gel filtration (molecular sieving) chromatography, affinity chromatography (AFC), hydrophobic chromatography (HC), high pressure liquid chromatography (HPLC). Most researchers are particularly interested in affinity chromatography for the potential for one-step purification of recombinant proteins. This is done to the specific interactions between the target protein with its compliment. Such interactions can include the following: antibody-antigen interactions, ligand-enzyme reactions, and hormone-receptor interactions. One attractive method for affinity for purification of proteins is immobilized metal affinity chromatography (IMAC) (Porath *et al.*, 1975). IMAC columns generally employ the first row transition metals (Zn^{2+}, Ni^{2+}, Cu^{2+}, and Fe^{2+})

chelated by iminodiacetate (IDA) to interact with surface groups of proteins (Porath, *et al*., 1975). Those proteins with a high affinity for the metal ion used in the column are retained on the column while all other proteins are eluted out in the wash (Smith, *et al*., 1990). Three amino acids can theoretically interact with the metal ion in affinity chromatography: histidine, tryptophan, and cysteine (Porath, *et al*., 1975). Both histidine and tryptophan have been shown experimentally to have an affinity for the metal ions (Sulkowski, 1989). Histidine is an uncommon amino acid residue and only accounts for 2.1% of the amino acids in the average globular protein (Klapper, 1977). Therefore, adding histidine to either the carboxyl- or amino-terminus of a protein will cause that protein to be preferentially separated from the bulk proteins in a sample ((Wang, *et al*., 1994), (Lilius, *et al*., 1991), and (Ljungquist, *et al*., 1989)).

The Hi-Trap (AP Biotech) system uses a scaleable column configuration for IMAC, allowing six poly-histidine tagged proteins to be purified. Figure 5 shows the basic methodology to run the IMAC system. Initially, the column needs to be equilibrated with the starting buffer elution. The sample is then passed through the column, effectively loading the sample containing the tagged protein of interest to the column. The six poly-histidine tag will then complex the target protein to the Ni^{2+} immobilized ion, thus binding the target protein to the support matrix. The column is then washed with starting buffer elution to remove protein impurities. Finally, either a constant or step-wise buffer elution salt gradient is passed through the column to remove the target protein from the column. After all the target protein

is removed, the column may be equilibrated with the starting buffer solution for reuse.

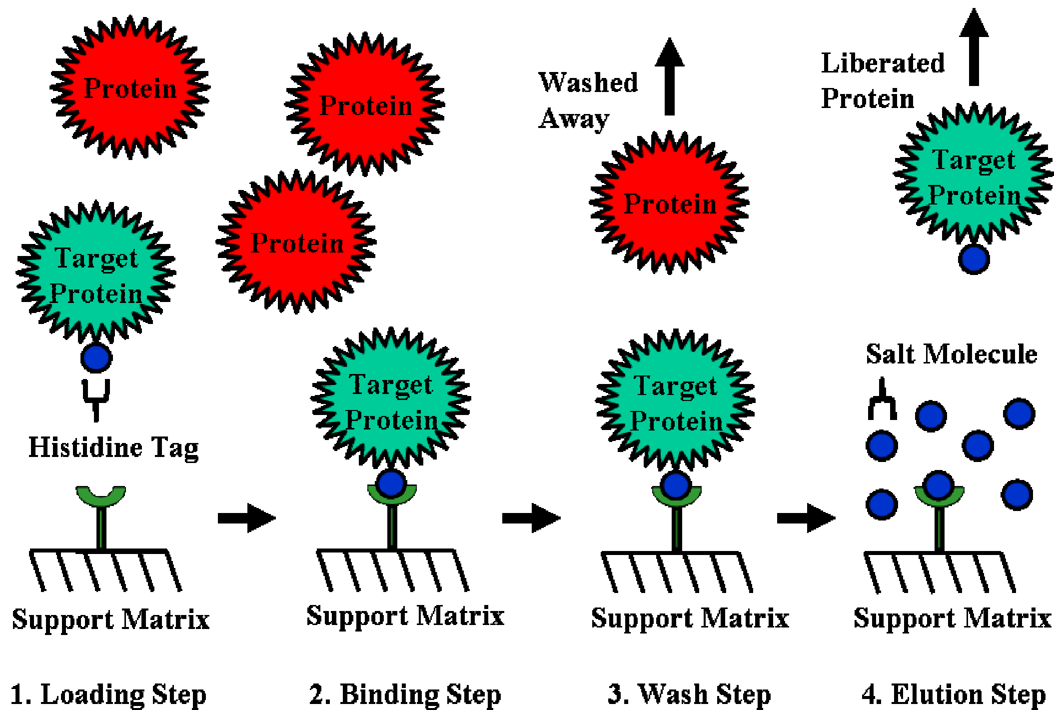

Figure 5: Hi-Trap system for His tagged protein purification.
The Hi-Trap system uses a Ni^{2+} immobilized ion on an agarose highly cross-linked support matrix to chelate target proteins. This purification generally takes four steps as follows: a loading step, a binding step, a wash step, and an elution step.

A typical column chromatography system consists of the following: a sample reservoir, a gradient elution hopper, an external pump, a series of columns, and the waster reservoir (See Figure 6). IMAC systems have obvious advantages to other chromatographic systems in that they are one-step purification systems and run under moderately low pressure. However, there are numerous disadvantages as follows: (1) the heavy metal chelating agents are not environmentally friendly and are difficult to dispose of; (2) although the system may be reused several times, the

repeated pressure drops and compression/expansion steps placed on the support matrix often result in the loss of mechanical rigidity and generally lead to gel compaction over time; (3) once the column has been compressed the flow dynamics are irreparably damaged, and the column support matrix needs to be replaced; (4) globular proteins or proteins with inclusion bodies may have a tendency to precipitate in the column; (5) reproducibility of column results is not always consistent, i.e. the column never really runs the same way twice; (6) finally, although the system is modular, it is not linearly scaled. Thus, simply multiplying column units by a scale-up factor will often not yield consistent results.

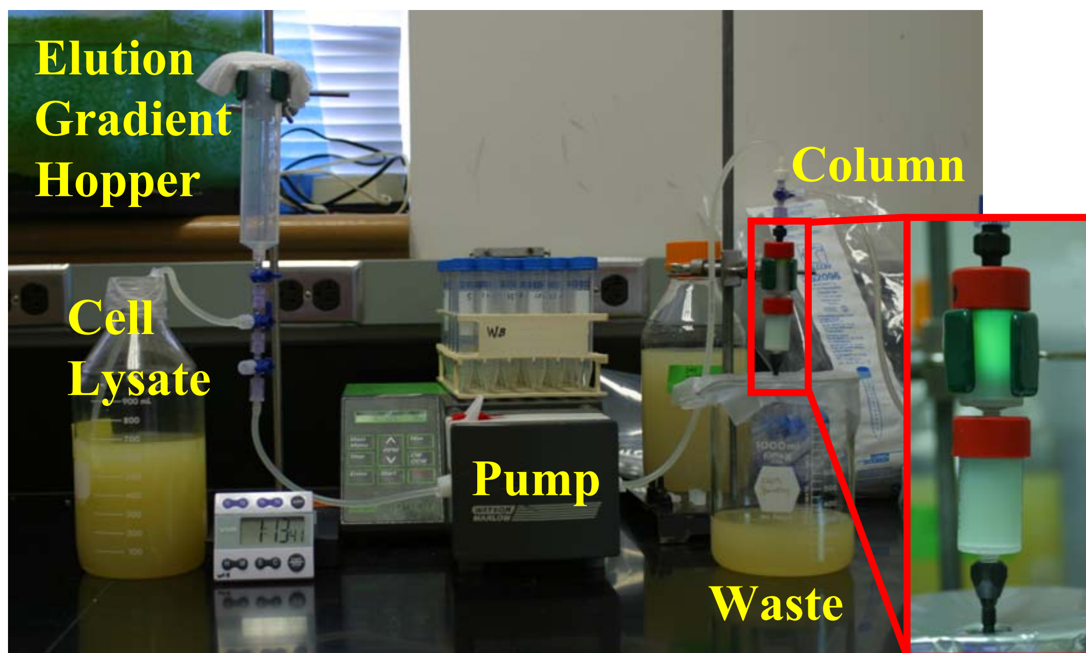

Figure 6: Column chromatography system.
A typical column chromatography system is shown consisting of the following: a sample reservoir, a gradient elution hopper, an external pump, a series of columns, and the waster reservoir. A six poly-histidine tag allows purification of the fusion protein using an IMAC column loaded with nickel ion.

Figure 7 shows the results of a typical IMAC purification of His_6GFP. The protein does not elute off the column at one elution point (or column eluate volume), but over a series of elution points. In order to remove the salt gradient that is used to elute the target protein, the sample must undergo dialysis. Dialysis uses porous membrane tubing that is permeable to small molecules but retains large macromolecules (proteins) (ref). The dialysis solution consists of a buffer solution that has the same concentration as the elution buffer used in the gradient elutions (See Figure 8). This solution must be repeatedly changed to allow the salt concentration to decrease.

Figure 7: Imidazole elution gradients.
Pictured above are a series of imidazole elution gradients. The following elutions of imidazole in 1X PBS were passed through the column successively and are shown here: 20 mM, 40 mM, 60 mM, 100 mM, 300 mM, and 500 mM. Note that the His_6GFP began eluting at 100 mM of imidazole in 1X PBS and is completely eluted off the column at 300 mM of imidazole in 1X PBS.

Figure 8: Dialysis.
The final step in purifying the protein is dialysis. The elutions containing the protein are placed in porous tubing that allows diffusion of the gradient salt out of the tubing when placed in a large volume buffer solution. Pictured above are the His_6GFP protein 100 and 300 mM imidazole in 1X PBS elutions combined and dialyzed in 1X PBS to remove the imidazole salt.

Chapter 2: Materials and Methods

2.1. Plasmid Constructs

The *gfp* gene in pTG (a *gfp* gene in the pTRCHisB plasmid, Invitrogen) was digested with *Sac*I and *Hin*dIII. Annealing two complementary primers together that would fit in the space between the digested plasmid space created a site for enterokinase cleavage and a pentatyrosyl tag. The annealed primers were as follows: forward primer 5'- CTACAAATATTATTATTATTATTAAGGTACCA and reverse primer 5'- AGCTTGGTACCTTAATAATAATAATAATATTTGTAGAGCT. Annealing was performed at 75°C and then slowly cooled to 25°C in a 25°C water bath. The insert was ligated into the pTG-digested plasmid and resulting plasmids were transformed into *E. coli* DH5α. Upon confirmation of the insert (using *Nhe*I and *Kpn*I), the plasmid was transformed into *E. coli* BL21 (see Figure 9).

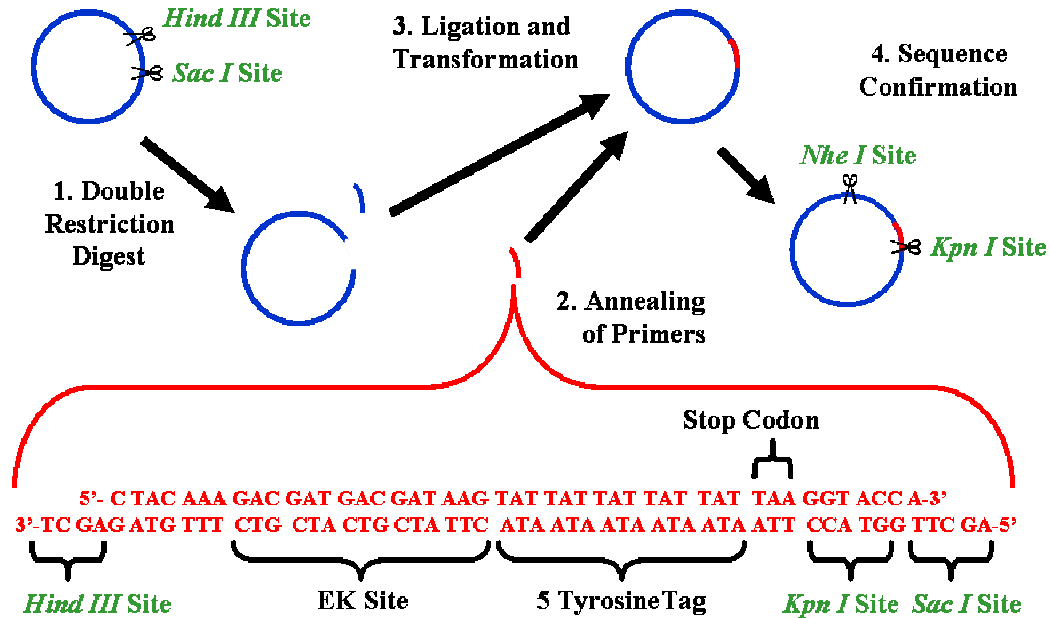

Figure 9: Insertion of five poly-tyrosine tag

1. A small section of the His_6GFP plasmid was removed using DNA restriction enzymes (*Hind III* and *Sac I*).
2. Two complimentary single stranded DNA primers that contain the *Hind III* site, the enterokinase (EK) protein restriction site, the five poly-tyrosine tag, a *Kpn I* site, and the *Sac I* site are annealed together.
3. The annealed primers are inserted into the His_6GFP plasmid and transformed in *E. coli*, thus creating the His_6GFPEKTyr_5 plasmid construct.
4. The sequence was later confirmed by isolating the His_6GFPEKTyr_5 plasmid from the *E. coli*. A double DNA restriction digest was performed with *Nhe I* and *Kpn I* to confirm the insertion of the EK site and five poly-tyrosine tag. Additionally, DNA sequencing was performed as a redundant verification.

2.2. Fermentations

Luria Bertani (LB) medium containing 5 g/L$^-$yeast extract, 10 g/L bacto tryptone, and 10 g/L NaCl (Becton Dickenson) was used as the growth media for all cultures. Primary *E. coli* inoculums consisting of 100 mL of LB medium, 100 µL

ampicillin (100 μg/mL, Sigma) and 1 mL frozen *E. coli,* were grown overnight (~12 h) at 37°C and 250 rpm. These overnight cultures were used to inoculate 2 L LB medium and 2 mL of ampicillin (100 μg/mL, Sigma) and grown to log phase (OD_{600} = 0.6 to 1.0). At log phase, the cultures were induced with 2 mL of 1 M isopropylthiogalactoside (IPTG). Cultures were then grown for an additional 5 h and harvested by centrifugation into pellets (30 min at 12,000 x g, Sorval).

2.3. Chitosan Conjugation

Pellets were resuspended in 100 mL of 1X phosphate buffered saline (PBS) pH 7.4. Aliquots of cell suspension (~3 mL) were lysed using sonication (4 min in an ice bath, Fisher Sonic Dismembrator 550). The cell lysate was centrifuged (10 min at 15,000 x g, Eppendorf 5810 R) to remove insoluble cellular debris. Cell lysate supernatants were then preprecipitated with 2 mL of 0.64% chitosan solution (0.64 g chitosan (Sigma), dissolved in 99.36 g water, 2 M HCl used to pH adjust to 2-3, then raised to pH 6.0 with 1 M NaOH). Preprecipitated material was centrifuged to remove insoluble material and impurities (10 min at 15,000 x g, Eppendorf 5810 R). Supernatants were then poured off and 5 mL of 1% chitosan solution (1 g chitosan (Sigma), dissolved in 99 g water, 2 M HCl used to pH adjust to pH 2 to 3, then raised to pH 6.0 with 1 M NaOH). The supernatant was reacted overnight in an incubator shaker (~12 h) at 30°C and 250 rpm with 200 μL of tyrosinase solution (1 mg/mL, pH 7, Sigma). The protein-polysaccharide (GFP-

chitosan) conjugates were then centrifuged into pellets (10 min at 15,000 x g, Eppendorf 5810 R).

2.4. Protein Liberation

GFP-chitosan conjugates were vortexed in 5 mL of 1X PBS solution. Enterokinase enzyme (200 μL, 0.909 mg/mL, Sigma) was added to the GFP-chitosan conjugate suspensions. The reaction mixtures were reacted overnight (~12 h) in an incubator shaker at 37°C and 250 rpm. The reaction mixtures were then centrifuged into pellets (10 min at 15,000 x g, Eppendorf 5810 R).

2.5. Protein Assay

Samples were heated to 100°C for 2 min, and vortexed. Proteins were separated by SDS polyacrylamide gel electrophoresis using 12.5% acrylamide gels and blotted onto supported nitrocellulose membranes (BioRad) using a mini-trans blot cell (BioRad) and Bjerrum and Schafer-Nielsen transfer buffer (48 m*M* Tris, 29 m*M* glycine, 20% methanol) for 18 min at 10V and another 18 min at 20V. Anti-GFP antibody (BioRad) was diluted 1:2000 in 0.5% Tween-20 (v/v), Tris-buffered saline with 1% (w/v) non-fat dry milk and used to probe for recombinant proteins., Tris-buffered saline with 1% (w/v) non-fat dry milk and used to probe for recombinant proteins. The membranes were then transferred to a 1:4000 diluted anti-rabbit antibody conjugated with alkaline phosphatase (Kirkegaard & Perry

Laboratories) solution. Membranes were washed and developed colorometrically with 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (Sigma). Finally, the membranes were scanned, and the images were analyzed. GFP concentration of the supernatant was measured using a Perkin-Elmer LS55 Luminescence Spectrometer at excitation and emission wavelengths of 395 and 509 nm, respectively. A GFP standard concentration curve was generated using purified GFP 1 μg/μL (Clontech) and 0.01 μg/mL scale dilutions (see Figure 10).

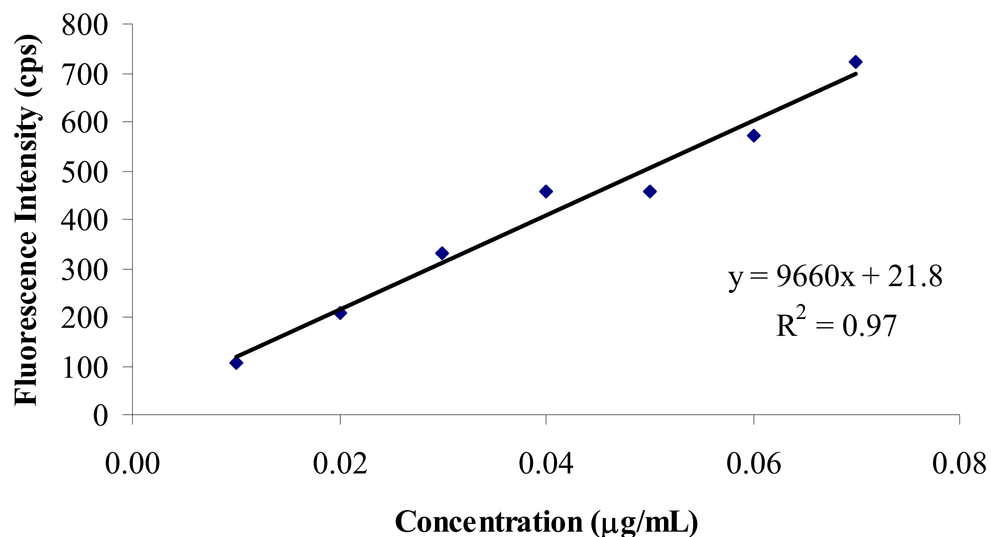

Figure 10: GFP standard curve.
A standard GFP curve generated with and 0.01 μg/mL serial scale dilutions. This linear curve is the best way to determine GFP concentration based on fluorescent intensity counts per second (cps).

Chapter 3: Results and Discussion

3.1. Specificity of Tyrosinase Reaction

Previous studies have shown the tyrosinase coupling reaction is not very specific, i.e. any tyrosine residue that is sufficiently exposed and available will couple to the chitosan. There are four tyrosine residues on the GFP molecule that are thought to be on the outside exterior of the protein (see Figure 11). By placing a pentatyrosine tag on the C-terminus, this should increase the chances of the GFP coupling through the tag rather than other surface exposed tyrosine residues.

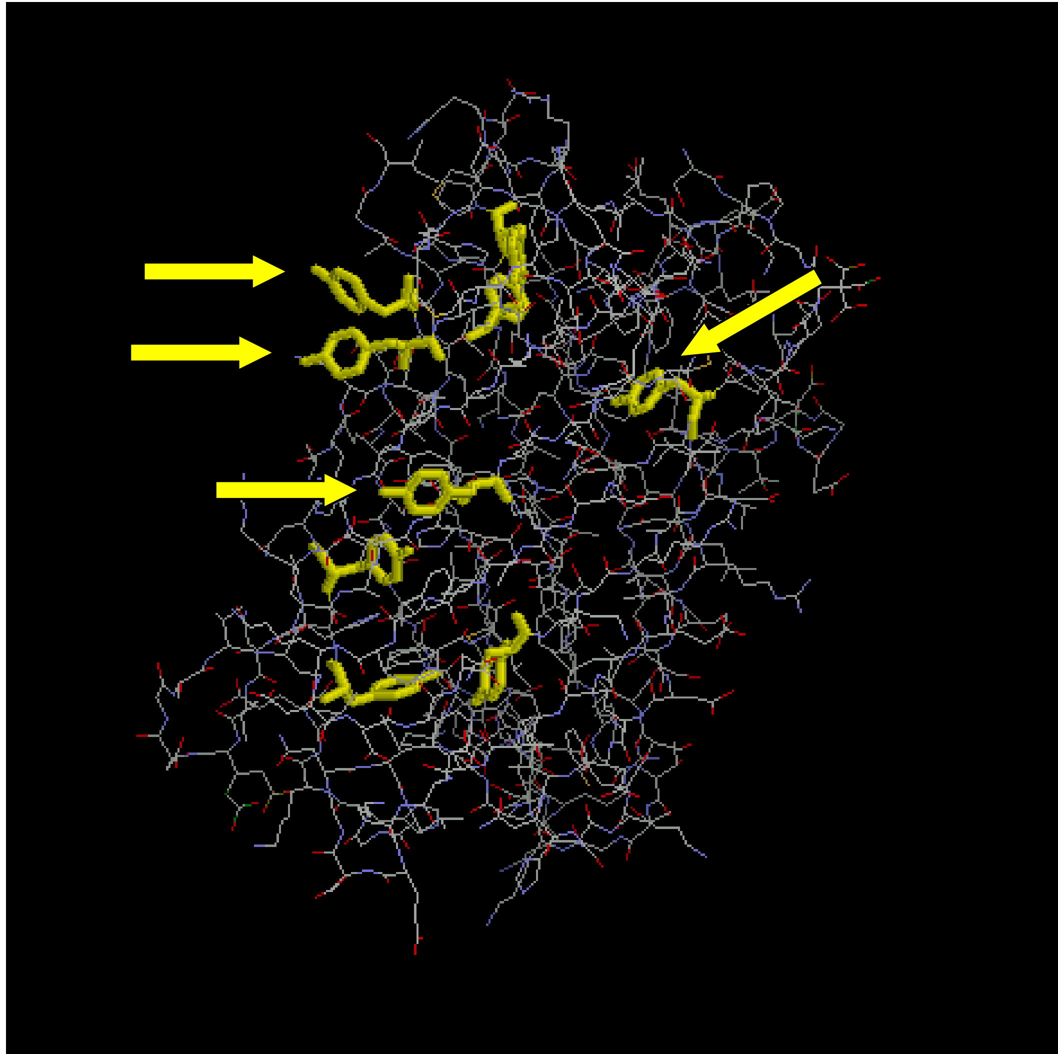

Figure 11: RasMol wirefream representation of Wild-type (WT-GFP).
(Rasmol v 2.6, Glaxo Research and Development, Greenford, Middlesex UK). This is wireframe representation of the GFP molecule. Tyrosine residues are yellow stick representations. The yellow arrows point to the four tyrosine residues that are suspected to be on the outside exposed surface of the molecule.

3.2. Cell Lysate Preprecipitation

A *preprecipitation* of the cell lysates containing the six poly-histidine N-terminus tagged GFP (His_6GFP) and five poly-tyrosine C-terminus tagged GFP ($His_6GFPEKTyr_5$) was done with a 0.64% chitosan solution. This step enables quick precipitation of extraneous cell debris and strongly negatively charged macromolecules and is based on the coagulating properties of chitosan already exploited in the wastewater recovery unites of process industries (Huang *et al*., 2000). Before and after the preprecipitation step the following readings were taken: A_{260}, A_{280}, and OD_{595}. A_{260} is empirically associated with DNA concentration. A_{280} is empirically associated with protein concentration. OD_{595} is empirically associated with the presence of insoluble cell debris. These results are summarized in Table 2. The decrease in the A_{260}, A_{280}, and OD_{595} measurements after the preprecipitation, suggests that DNA, protein impurities, and insoluble cellular debris in the cell lysate were removed as expected in the preprecipitation. Fluorescence intensity readings were also taken and converted into GFP concentrations using a standard curve and adjusted for dilution. These results are also summarized in Table 3. However, the preprecipitation also removes some of the desired GFP protein, approximately 23% of the GFP in the His_6GFP protein and approximately 33% of the GFP in the $His_6GFPEKTyr_5$ protein.

Table 2: Absorbance spectra (A_{260}, A_{280}, and OD_{595}) measurements before and after preprecipitation with 0.64% chitosan solution.

		His_6GFP	**$His_6GFPEKTyr_5$**
Stock Solution	A_{260}	76.3	54.8
After Preprecipitation	A_{260}	21.6	9.82
Stock Solution	A_{280}	43.0	30.9
After Preprecipitation	A_{280}	12.8	6.68
Stock Solution	OD_{595}	0.198	0.273
After Preprecipitation	OD_{595}	0.145	0.269

3.3. GFP-Chitosan Conjugation and Washing

The tyrosinase reaction covalently grafted the His_6GFP and $His_6GFPEKTyr_5$ proteins to the chitosan. A larger volume and higher fluorescence intensity pellet was created from the $His_6GFPEKTyr_5$ protein than the His_6GFP protein (see Figure 12). The fluorescence intensity of the GFP-chitosan conjugate in the $His_6GFPEKTyr_5$ protein pellet is much brighter than the GFP-chitosan conjugate in the His_6GFP protein pellet. The fluorescence intensity of the His_6GFP protein supernatant is much brighter than the for the fluorescence intensity of the $His_6GFPEKTyr_5$ protein supernatant. Thus, the His_6GFP protein supernatant has a higher concentration of the GFP than the $His_6GFPEKTyr_5$ protein supernatant. The measurements of the fluorescence intensity of the supernatants quantify this difference (see Table 3). These attributes all suggest that the $His_6GFPEKTyr_5$ protein was more selectively coupled to the chitosan than the His_6GFP protein.

After covalent grafting of the His_6GFP and $His_6GFPEKTyr_5$ proteins to the chitosan, a washing step was performed using 1X PBS buffer at pH 7.4 to remove protein that is not covalently bound to the chitosan (see Figure 13). Fluorescence intensity measurements were taken and converted into GFP concentrations, again using a standard curve. These results are summarized in Table 4. The relatively small concentrations of GFP in the supernatants suggest that one wash was sufficient to remove non-covalently bound GFP that may still reside in the chitosan pellet. A second wash was not necessary because of the low amount of fluorescence detected in the wash supernatant. As noted 20.1% of the available GFP_{uv} in the His_6GFP protein was bound to the chitosan and 28.7% of available GFP_{uv} in the $His_6GFPEKTyr_5$ protein was bound to the chitosan. These values indicate a strong preference for the pentatyrosine tag, but also indicate significant binding through natural tyrosine residues.

Table 3: Florescence Intensity (FI) measurements and corresponding GFP concentrations (μg/mL) for His_6GFP and $His_6GFPEKTyr_5$ proteins before and after preprecipitation with 0.64% chitosan solution, after pelleting, and after washing.

	His_6GFP		**$His_6GFPEKTyr_5$**	
	FI	**μg/mL**	**FI**	**μg/mL**
Stock Solution	389	3.77	83.6	0.602
After Preprecipitation	306	2.91	64.7	0.407
Supernatant After Pelleting	829	0.0832	227.5	0.0209
Supernatant After Washing	221	0.0202	48.5	0.0023

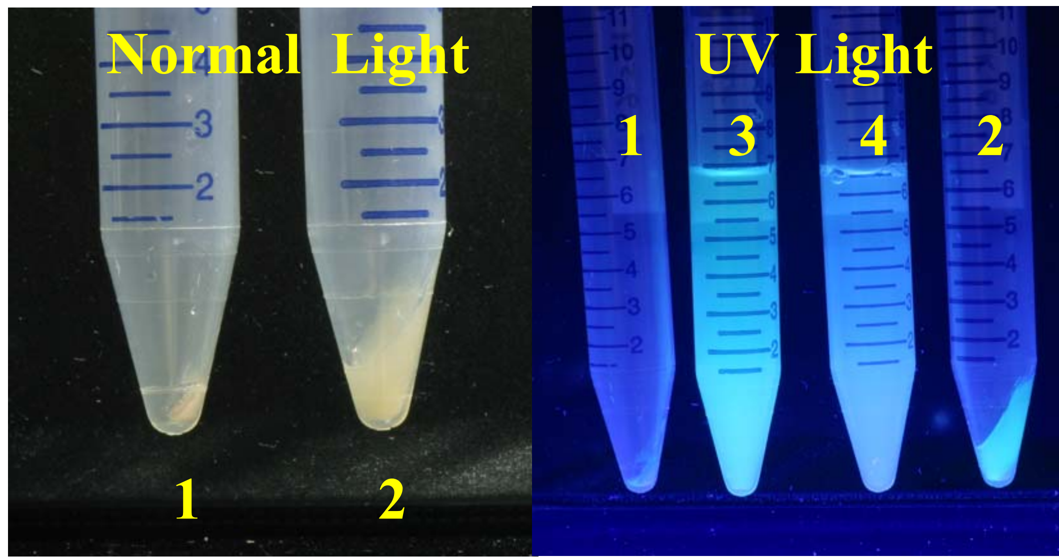

Figure 12: Tyrosinase reaction.
The five poly-tyrosine C-terminus tag allows the GFP to be selectively and covalently coupled to the chitosan resulting in more precipitation of insoluble material (comparing #1 and #2 pellet volumes and fluorescence intensity).

1. His_6GFP pellet
2. His_6GFPEKTyr$_5$ pellet
3. His_6GFP supernatant after reaction
4. His_6GFPEKTyr$_5$ supernatant after reaction

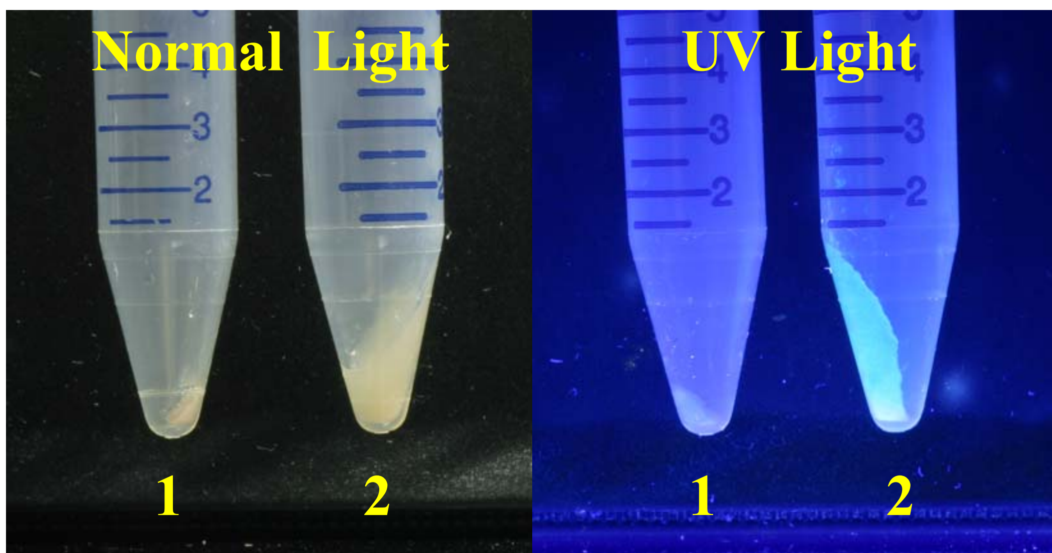

Figure 13: 1X PBS washing.
The pellets were washed with 1X PBS and centrifuged to wash away non-specifically bound impurities.

1. His_6GFP pellet (pellet volume still small)
2. His_6GFPEKTyr_5 (pellet volume still large)

3.4. Enterokinase Enzymatic Cleavage

GFP-chitosan pellets were then reacted heterogeneously with enterokinase enzyme in 1X PBS buffer solution to liberate GFP through the appropriate enterokinase cleavage site. Additionally, GFP-chitosan pellets were diluted with 1X PBS to see the effect protein leaching from the GFP-chitosan conjugate (See Figure 14). Fluorescence intensity measurements were taken of the supernatants and converted into GFP concentrations using a standard curve. These results are summarized in Table 4. Western Blots were done on the supernatants and are shown in Figure 15. The 9.5 fold increase in concentration between the supernatants for the

$His_6GFPEKTyr_5$ pellets treated with enterokinase enzyme compared to the pellets that were diluted suggests that the GFP was cleaved and liberated from the GFP-chitosan conjugate. The HisGFP proteins have virtually the same concentration when diluted or treated with enterokinase enzyme, because there is no enterokinase cleavage site for the enterokinase to react and remove the GFP from the GFP-chitosan conjugate.

Table 4: Florescent Intensity (FI) measurements and corresponding GFP concentrations (μg/mL) for His_6GFP and $His_6GFPEKTyr_5$ proteins after dilution with 1 X PBS and enterokinase addition.

	His_6GFP		$His_6GFPEKTyr_5$	
	FI	**μg/mL**	**FI**	**μg/mL**
Supernatant after Dilution with 1 X PBS	54.8	0.0030	29.7	0.0004
Supernatant after Enterokinase Addition	50.4	0.0025	62.2	0.0038

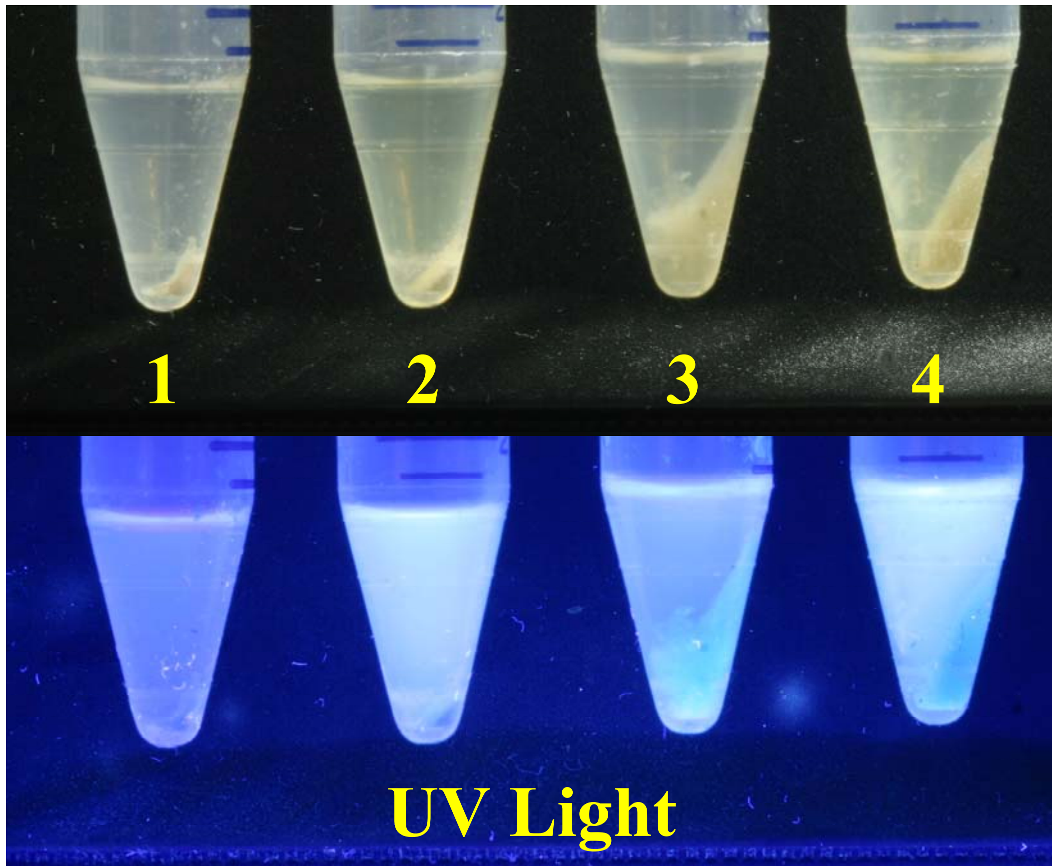

Figure 14: Enterokinase (EK) Digest
Control pellets (#1 and #3) were diluted with 1X PBS. Experimental pellets (#2 and #4) were reacted with a solution of EK enzyme to liberate GFP. Fluorescence intensity was diminished in the #4 pellet, indicating that His_6GFP had been liberated.

1. His_6GFP pellet with 1X PBS dilution
2. $His_6GFPEKTyr_5$ pellet with EK digestion
3. His_6GFP pellet with 1X PBS dilution
4. $His_6GFPEKTyr_5$ pellet with EK digestion

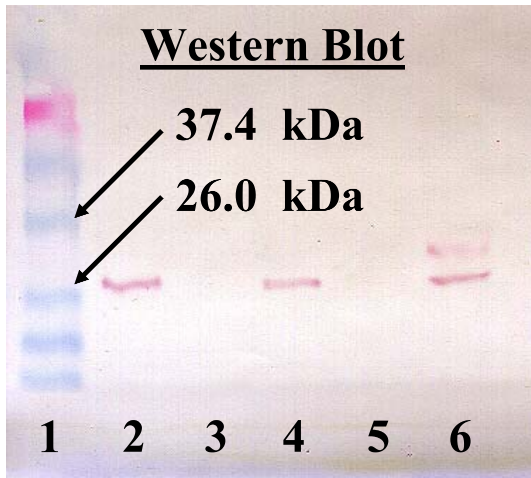

Figure 15: Western blot of supernatants after EK digest reaction
The lanes for the Western blot are as follows:

1. Molecular Weight standard
2. GFP standard, approximately 27 kDa
3. His_6GFP pellet supernatant after 1X PBS dilution
4. His_6GFP pellet supernatant after EK digestion
5. His_6GFPEKTyr_5 pellet supernatant after 1X PBS dilution
6. His_6GFPEKTyr_5 pellet supernatant after EK digestion

Notice that the His_6GFP was liberated in this sample contained in lane 6 and corresponds to the GFP standard in lane 2. The bands in lane 6 are the darkest in the development. Although lane 4 shows that some His_6GFP was released during the digest, there is hardly any bound to the chitosan pellet as seen in previous figures.

Chapter 4: Conclusions and Future Directions

4.1. Conclusions

This project has demonstrated the following: tyrosinase catalyzed the covalent coupling of the protein (GFP) to the polysaccharide (chitosan), resulting in a protein-polysaccharide conjugate. The conjugate was more insoluble then naitive chitosan when it is reacted through the tyrosine tag. This conclusion was supported visually by the large volume of the pellet and the green fluorescence of the pellet when observed in UV light. Additionally, the absorbency spectra and fluorescence readings suggested that the pellet contained green fluorescent protein. The washing step was effective in removing non-specifically bound protein to the chitosan without dissolving the pellet. The protein can be removed and purified in one step by cleavage with a site-specific enzyme (enterokinase cleavage). Again, this was supported visually by the disappearance of the green fluorescence in the pellet. Absorbency spectra and fluorescence readings also support this since a notable increase was seen in the supernatants after enterokinase digestion. Appearance of the matching bands on the Western blot gave concrete evidence that this GFP was liberated and is present in the supernatants. In summary, a target protein was successfully coupled to a polysaccharide, washed, and the liberated using a

digestive enzyme. This method was effective in conjugating and purifying the protein using a centrifuge with a limited number of processing steps.

4.2. Future Directions

This project successfully demonstrates that the concept is feasible. However, there may be more efficient methods to accomplish the purification. For example, enterokinase was used as the cleavage enzyme. Enterokinase is at best 50% efficient at digesting. Other digestive enzymes may be more efficient in cleaving the protein. The incorporation of inteins, small protein constituents that are self-cleaving at pH point may further enhance the decoupling of the protein from the polysaccharide (Wood *et al*., 1999). This method would be highly desirable due the high efficiency of intein cleavage and the robustness associated with pH control.

The chitosan-GFP conjugate was obtained through a homogeneous reaction in this project. Alternatively, this reaction may be done heterogeneously with tyrosinase adsorbed on the surface of a chitosan gel. Preliminary tests of this method have proved effective in binding purified forms of the His_6GFP and $His_6GFPEKTyr_5$ proteins to chitosan gels (see Figure 16). The chitosan gel could also potentially be digested by chitosanase enzyme to release the protein.

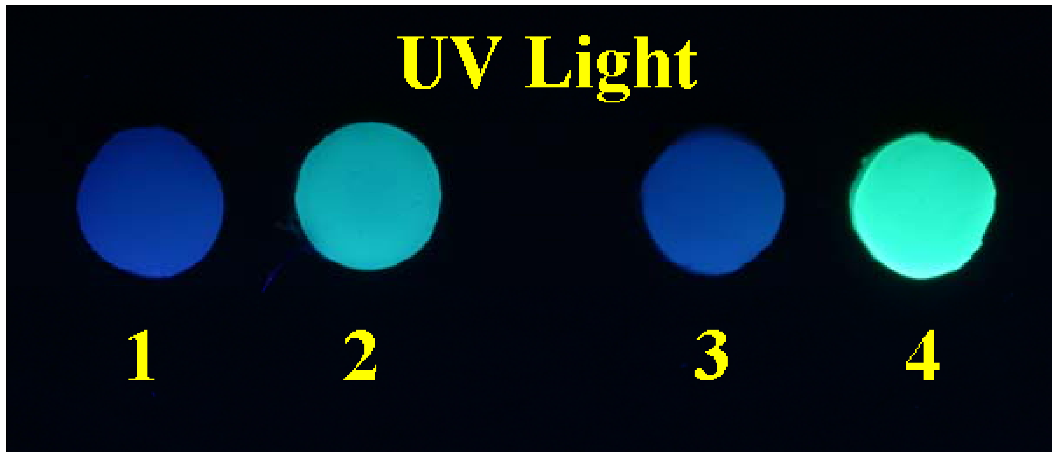

Figure 16: Heterogeneously reacted GFP on chitosan films.
Pictures above are four chitosan films with the following combinations of tyrosinase enzyme and recombinant GFP proteins as follows:

1. His_6GFP without tyrosinase on a chitosan film
2. His_6GFP with tyrosinase on a chitosan film
3. His_6GFPEKTyr_5 without tyrosinase on a chitosan film
4. His_6GFPEKTyr_5 with tyrosinase on a chitosan film

Notice that film #4 is much brighter than film #3, indicating that the tyrosine tag greatly increases the amount of protein that can be bound to the chitosan film surface.

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