ABSTRACT

Title of thesis: TYROSINE-BASED “ACTIVATABLE PRO-TAG”: ENZYME-CATALYZED PROTEIN CAPTURE AND RELEASE

Angela T. Lewandowski, Master of Science, 2004

Thesis directed by: Professor William E. Bentley
Department of Chemical Engineering

Fusion tags are widely used in the recovery and purification of recombinant proteins. We have investigated the capture and release of two fusion proteins from cell extracts using the aminopolysaccharide chitosan. We have fused to green fluorescent protein (GFP) and to S-ribosylhomocysteinase (LuxS) a “pro-tag” consisting of five tyrosine residues that are “activated” by tyrosinase-catalyzed conversion into reactive o-quinones. The o-quinones react with the amino groups of chitosan, resulting in the covalent conjugation of the fusion protein to chitosan. The fusion protein is captured from solution by precipitation of the protein-chitosan conjugate due to the decrease in solubility of chitosan at higher pH. Additionally, chitosan is used to “pre-precipitate” cell extract contaminants such as nucleic acids and phospholipids, and thus, crudely purify the fusion protein remaining in solution. Finally, we released the fusion protein from chitosan back into solution using the chitosan-hydrolyzing enzyme chitosanase as an alternative to protease cleavage.
TYROSINE-BASED “ACTIVATABLE PRO-TAG”: ENZYME-CATALYZED PROTEIN CAPTURE AND RELEASE

by

Angela T. Lewandowski

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Advisory Committee:

Professor William E. Bentley, Chair
Professor John Fisher
Professor Maria Klapa
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Abbreviations: LYS, clarified lysate; PPT, “pre-precipitation” supernatant; C, conjugation reaction supernatant; W₁ and W₂, ₁ˢᵗ and ₂ⁿᵈ wash of conjugate, respectively; CE, chitosanase reaction mixture; CE-IM, IMAC elution of chitosanase reaction mixture. 
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INTRODUCTION

The selective capture of recombinant proteins has been greatly facilitated by the fusion of affinity tags to termini of target proteins. Traditionally, these tags have been employed to recover and purify target proteins, while more recent applications have exploited fusion tags for the assembly of target proteins onto patterned surfaces. Additionally, there are many examples (e.g. production of therapeutic proteins) where protein capture is followed by removal of the affinity tag to release the target protein. Typically, this is accomplished by insertion of a protease cleavage site between the affinity tag and the target protein.

Most affinity fusion tags exhibit strong non-covalent interactions with their respective ligands. Examples of commonly used affinity tags include cellulose-binding domain (Greenwood et al. 1989; Ong et al. 1991), maltose-binding domain (di Guan et al. 1988), chitin-binding domain (Chong et al. 1997), biotin (Smith et al. 1998; Tsao et al. 1996), poly-histidine (Smith et al. 1988), and glutathione S-transferase (Smith and Johnson 1988). An exception is the work of Chilkoti et al. 1994, who chemically “activated” a genetically engineered protein by reduction of disulfide bonds, allowing for its covalent conjugation to a thermally responsive polymer.

In this work, we have investigated an “activatable pro-tag” for its ability to capture a fusion protein by covalent conjugation to the aminopolysaccharide chitosan. The “pro-tag” consists of a pentatyrosine moiety located at the C-terminus of the two proteins being investigated, green fluorescent protein (GFP) and S-ribosylhomocysteinase (LuxS). Scheme 1 shows that the tyrosine residues of the “pro-tag” are activated by
tyrosinase-catalyzed oxidation and converted to reactive \(\alpha\)-quinones (Chen et al. 2001; Chen et al. 2003; Kumar et al. 1999; Wu et al. 2002a; Wu et al. 2002b; Yi et al. 2004; Yi et al. 2003). The electrophilic \(\alpha\)-quinones of the “pro-tag” can then undergo non-enzymatic reactions with the nucleophilic amino groups of chitosan to yield a protein-chitosan conjugate. Chitosan’s primary amines confer both nucleophilic and pH-responsive properties (Chen et al. 2001; Chen et al. 2003; Kumar et al. 1999; Wu et al. 2002a; Wu et al. 2002b; Yi et al. 2004; Yi et al. 2003). At low pH, the amino groups are protonated, so that chitosan is a water-soluble cationic polyelectrolyte. As the pH is increased above chitosan’s pKa (~ 6.3), the amines become deprotonated, and chitosan becomes neutral in charge and insoluble. The precipitation of chitosan at higher pH allows separation of the chitosan-protein conjugates from solution. Thus, chitosan confers to the protein-chitosan conjugates pH-dependent solubility that can be exploited for recovery and purification of the fusion proteins.

\[ \text{Chitosan} \quad [\text{GlcN}]_{x} - [\text{GlcNAc}]_{y} \]

**Scheme 1.** Schematic representation of tyrosinase-catalyzed conjugation of protein to chitosan.
To further facilitate recovery and purification of the fusion proteins from cell lysates, we utilize chitosan in a “pre-precipitation” step that is aimed to clear lysate of nucleic acids and phospholipids (Scheme 2A). That is, chitosan’s positive net charge allows us to exploit chitosan as a capturer of negatively charged species. Chitosan has already been used to coagulate lipids and proteins in the treatment of wastes from various process industries (Hwang and Damodaran 1995; Jun et al. 1994). Additionally, Agerkvist et al. 1990, have shown that chitosan could be used as a cationic polyelectrolyte to flocculate *E. coli* cell debris, proteins, and nucleic acids. In this study, the recombinant protein in the cell lysate is not “pre-precipitated” by chitosan, but is retained in the clarified supernatant. In a second step, chitosan is added to the clarified supernatant, and enzymatic activation of the “pro-tag” (via tyrosinase addition) results in conjugation of the fusion protein to chitosan. Thus, after the second centrifugation, the conjugated protein is captured in the pellet.
Scheme 2. Schematic representation of fusion protein capture and release. (A) Capture of fusion protein via chitosan “pre-precipitation” of cell extract components and tyrosinase-catalyzed conjugation of fusion protein to chitosan. (B) Enzymatic release of proteins via enterokinase (EK) or chitosanase.
We demonstrate the release of the recombinant proteins from chitosan through the action of two enzymes, enterokinase and chitosanase (Scheme 2B). Enterokinase (EK) recognizes the specific amino acid sequence D-D-D-D-K in the tethering linker, and cleaves just after the lysine residue. We have inserted two EK cleavage sites into GFP’s sequence for the release of wild type GFP from chitosan. EK is commonly used for the selective cleavage of the target protein from the affinity tag, however, its effectiveness varies widely (Gaillard et al. 1996; Martinez et al. 1995; Rabbani et al. 1997; Reddi et al. 2002). As an alternative to EK, we also use chitosanase, which catalyzes the hydrolysis of chitosan. Chitosanase was used in the release of both GFP and LuxS. This approach is expected to yield the fusion protein decorated with residual sugar residues.

Additionally, we compare the recoveries of the two fusion proteins in the capture and release from chitosan. The first protein GFP exists as a monomer in solution, and wild type has a molecular weight of ~ 27 kD ((His)6-EK-GFP-EK-(Tyr)5 ~ 32 kD). It is a stable protein, only denatured under harsh conditions (i.e. pH 2, pH 13, or 6 M guanidine-HCl) (Ward and Bokman 1982). Its structure is a \( \beta \)-barrel 42 Å long and 24 Å wide around a central helix containing the chromophore (Ormo et al. 1996). GFP absorbs light at 395 nm, and emits green light at 509 nm (Cody et al. 1993).

The second protein LuxS exists as a dimer in solution, and the wild type monomer has a molecular weight of ~ 19 kD ((His)6-LuxS-(Tyr)5 ~ 25 kD). The monomer has approximate dimensions 25 Å x 35 Å x 45 Å (Ruzheinikov et al. 2001). Each monomer is mainly composed of a four-stranded antiparallel \( \beta \)-sheet and three \( \alpha \)-helices (Ruzheinikov et al. 2001). In the unique dimer structure, the \( \beta \)-sheets face each other at the dimer interface to form a \( \beta \)-barrel, which is flanked by the \( \alpha \)-helices (Ruzheinikov et
The two identical active sites are found in deep pockets formed at the dimer interface, each containing a Fe$^{2+}$ ion, which is required for enzyme activity (Zhu et al. 2003). As shown in Scheme 3 (adapted from Xavier and Bassler 2003), LuxS catalyzes the formation of homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) from S-ribosylhomocysteine (SRH) (Miller and Duerre 1968). SRH is formed from S-adenosylhomocysteine (SAH) by the nucleosidase Pfs (Della Ragione et al. 1985). DPD then goes on to become autoinducer-2 (AI-2), a small signaling molecule found in many bacterial species (Chen et al. 2002). LuxS is considerably less stable than GFP, unable to withstand high temperatures and extremes of pH.

There were two major goals of this study. The first was to recover and partially purify both fusion proteins, (His)$_6$-LuxS-(Tyr)$_5$ and (His)$_6$-EK-GFP-EK-(Tyr)$_5$, without chromatography. This will be done by a “pre-precipitation” of contaminants with chitosan followed by the tyrosinase-catalyzed capture of the two fusion proteins to chitosan through their “pro-tags.” The second goal was to release the two fusion proteins from chitosan into solution using an alternative to protease cleavage.
MATERIALS AND METHODS

**Materials.** Chitosan (minimum 85% deacetylated chitin; molecular weight 300,000 g/mol) from crab shells, isopropyl β-D-thiogalactopyranoside (IPTG), phosphate buffered saline (PBS) (0.5 mM MgCl₂, 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.5), imidazole, nickel sulfate, β-mercaptoethanol, bromophenol blue, mouse monoclonal anti-polyhistidine, goat anti-mouse IgG conjugated to alkaline phosphatase, tyrosinase from mushroom, enterokinase (enteropeptidase) from porcine intestine, and chitosanase from *Streptomyces griseus* were purchased from Sigma (St. Louis, MO). Tyrosinase, enterokinase, and chitosanase were reported by the manufacturer to have activities of 3620, 192, and 102.3 Units/mg solid, respectively. Luria-Bertani (LB) medium was purchased from Becton Dickinson (Cockeysville, MD). Ampicillin sodium salt, glycerol, sodium citrate, citric acid, sodium borohydride, sodium phosphate (monobasic), sodium phosphate (dibasic), zinc acetate, trichloroacetic acid, Tris base, acrylamide, Bis-acrylamide, methanol, MgCl₂·6H₂O, and sodium chloride were purchased from Fisher Chemical (Fair Lawn, NJ). Hydrochloric acid and sodium hydroxide were purchased from J. T. Baker (Phillipsburg, NJ). Sodium dodecyl sulfate (SDS), glycine, non-fat dry milk, and Tween 20 were purchased from BioRad (Hercules, CA). Mouse monoclonal anti-GFP was purchased from Clontech (Palo Alto, CA).

**Plasmid construction and bacterial strains.** The p6His-LuxS-5Tyr plasmid was constructed from pTrcHisC (Invitrogen). After inserting the pentatyrosine tag, the resulting plasmid was subsequently transformed into *E. coli pfs* null-mutant strain NC13.
(genotype RK4353Δpfs(8-226)::Kan′) (Hashimoto, unpublished). The p6His-EK-GFP-EK-5Tyr plasmid was constructed from pTrcHisB (Invitrogen), which contains one enterokinase recognition site. The pentatyrosine tag and the second enterokinase recognition site were inserted, and the resulting plasmid was subsequently transformed into E. coli strain BL21 (Small, unpublished). Note that the hexahistidine tag on each protein enables purification using immobilized metal affinity chromatography (IMAC) when needed.

**Cell culture.** Primary E. coli inocula consisting of 2 mL LB medium, 50 µg/mL ampicillin, and one loop E. coli freezer stock were grown overnight (12 - 14 h) in 20 mL culture tubes at 30°C and 250 rpm in a shaker-incubator (New Brunswick Scientific). Overnight cultures were used to inoculate 500 mL Erlenmeyer flasks containing 100 mL LB medium and 50 µg/mL ampicillin. The 100 mL cultures were grown at 37°C and 250 rpm to OD$_{600nm}$ of 0.5 – 0.6, and then added to 3.2 L baffled Erlenmeyer flasks containing 1.5 L LB medium and 50 µg/mL ampicillin. The 1.5 L cultures were grown at 37°C and 300 rpm to OD$_{600nm}$ of 0.5 – 0.6, when they were induced with 1 mM IPTG, and grown for an additional 5 h. Additionally, zinc acetate was added at induction at a final concentration of 100 µM to cells making (His)$_6$-LuxS-(Tyr)$_5$ to improve stability of the LuxS protein (Zhu et al. 2003). The cells were then centrifuged 20 minutes at 4,800 g into pellets (Dupont Sorvall), and the cell pellet was stored at -20°C.

**Sonication.** The cell pellet containing (His)$_6$-LuxS-(Tyr)$_5$ was resuspended in 50 mM sodium phosphate pH 5.8. The cell pellet containing (His)$_6$-EK-GFP-EK-(Tyr)$_5$ was
resuspended PBS. The resuspended cells were placed in an ice-water bath and lysed by 10 min of sonication (Fisher Scientific Sonic Dismembrator). Cell lysate was centrifuged for 20 minutes at 10,000 g to remove insoluble cellular debris (Eppendorf). The supernatant was decanted, and the pellet was again resuspended in buffer to repeat the cycle. This cycle was performed a total of three times.

**Chitosan preparation.** Chitosan solutions were prepared by dissolving chitosan flakes in de-ionized water, with dilute hydrochloric acid added dropwise until reaching pH 2. After dissolving completely, 1 M NaOH was added dropwise until the pH 5.8 was reached. It was then filtered and stored at 4°C.

**Addition of chitosan to clarified cell lysate.** Clarified cell lysate was “pre-precipitated” by mixing 4:1 with 0.2 – 0.6 % (w/w) chitosan-water solution pH 5.8. The “pre-precipitated” (PPT) solution was centrifuged 15 minutes at 10,000 g to remove insoluble material and impurities. A 1.2 – 1.6 % (w/w) chitosan-water solution, pH 5.8 was added to PPT supernatants in a 1:1 ratio. The PPT supernatant-chitosan mixture was reacted overnight (14 – 16 h) in a shaker-incubator at 30°C and 250 rpm with 140 Units/mL tyrosinase. The reaction mixtures were then brought to pH ~ 7 by the dropwise addition of 1 M sodium hydroxide. Additionally, the GFP-chitosan conjugates to be used in enterokinase reactions were reduced using sodium borohydride at a concentration of 0.06 mg/mL. The protein-chitosan conjugates were then centrifuged for 15 minutes at 10,000 g into pellets, and the supernatants were decanted and saved for analysis.
Enterokinase reactions. GFP-chitosan conjugates were washed twice with PBS. The conjugates were then diluted 1:4 with PBS, and vortexed to resuspend the pellet. This solution was reacted ~ 2 days with 5 Units/mL enterokinase in a shaker-incubator at 37°C and 250 rpm. The reaction mixture was then centrifuged for 15 minutes at 10,000 g, and the supernatant decanted and saved.

Chitosanase reactions. Protein-chitosan conjugates were washed twice with 20 mM sodium phosphate pH 6.4. The conjugates were then diluted 1:3 with the phosphate buffer and vortexed to resuspend the pellet. This solution was reacted ~ 3 days with 1 Unit/mL chitosanase in a shaker-incubator at 30°C and 250 rpm. The reaction mixture was then purified via IMAC.

IMAC purification. The 5 mL HisTrap chelating column (Amersham Biosciences) was charged with Ni$^{2+}$ ions using 0.1 M NiSO$_4$. The column was thoroughly washed with de-ionized water, and then equilibrated with 3 column volumes (CVs) of 20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4. The sample was loaded onto the column. The column was washed with 3 CVs of the previous buffer, and the protein was eluted using 3 CVs of 20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4. All steps were performed at 2 mL/min (1 cm/min linear velocity). The eluted sample was dialyzed overnight (16 hr) at 4°C into PBS. Purified protein concentration was determined with an UV/vis spectrophotometer (DU 640, Beckman, Fullerton, CA) using UV light at 280nm wavelength. The dialyzed protein was stored at 4°C.
UV/visible light readings and fluorescence intensity measurements. A Beckman DU 640 Spectrophotometer using UV/visible light at 260, 280, and 595 nm was used to measure the content of DNA/RNA, total protein, and cell debris, respectively. A Perkin Elmer LS55 Luminescence Spectrometer was used with an excitation wavelength of 395 nm, an emission wavelength of 509 nm, a slit width of 10 nm, and an emission cut off of 430 nm.

Addition of chitosan to purified fusion protein. (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and (His)$_6$-LuxS-(Tyr)$_5$ were previously purified via IMAC and dialyzed into PBS as described above. A 1.2 % (w/w) chitosan-water solution pH 5.8 was added in a 1:1 ratio. This protein-chitosan mixture was reacted overnight (14 – 16 h) in a shaker-incubator at 30$^\circ$C and 250 rpm with and without 140 Units/mL tyrosinase. The reaction mixtures were then brought to pH ~ 7 with 1 M sodium hydroxide, reduced with 0.06 mg/mL sodium borohydride, and centrifuged at 10,000 g for 15 minutes. The supernatant was decanted and saved for analysis.

SDS-PAGE and Western blot analysis. Sample buffer 2x concentration (0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue) was mixed with protein samples in a 1:1 ratio, and these mixtures were then heated at 95 $^\circ$C for 5 minutes. Proteins were separated by SDS polyacrylamide gel electrophoresis using 12.5% acrylamide gels at 180 V for ~ 1 hour using the BioRad Mini Protean 3 system, and blotted onto BioRad nitrocellulose membranes using a BioRad Trans-Blot semi-dry transfer cell and Bjerrum-Schafer-
Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 20% (v/v) methanol, 0.0375% (w/v) SDS) for 30 minutes at 15 V. Unbound membrane sites were blocked using 5% (w/v) non-fat dry milk in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl. The membrane was first incubated at room temperature for 2 hours in mouse monoclonal anti-GFP at 1:500 dilution or for 1 hour in mouse monoclonal anti-polyhistidine at 1:4,000 dilution. The membrane was then incubated at room temperature for 1 hour in goat anti-mouse IgG conjugated to alkaline phosphatase at 1:4,000 dilution. Both antibodies were diluted in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% (w/v) non-fat dry milk, 0.05% (v/v) Tween 20. Membranes were developed colorimetrically using Roche NBT/BCIP stock diluted 1:50 in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M MgCl₂.

**Isoelectric focusing gel electrophoresis.** (His)₆-LuxS-(Tyr)₅ and (His)₆-EK-GFP-EK-(Tyr)₅ were previously purified via IMAC and dialyzed into PBS as described above. They were mixed 1:1 with Novex IEF Sample Buffer pH 3 – 7 2x concentration (Invitrogen) and then loaded onto a Novex IEF pre-cast gel pH 3 – 7 (Invitrogen) using Novex cathode and anode running buffers (Invitrogen). The gel was run at 100 V for 1 hour, and then 200 V for 2 hours. It was then fixed in 12% (w/v) trichloroacetic acid for 30 minutes before staining.

**In vitro AI-2 synthesis and AI-2 activity assay.** (His)₆-LuxS-(Tyr)₅ were previously purified via IMAC, dialyzed into PBS, and conjugated to chitosan using tyrosinase as described above. The LuxS-chitosan conjugate was centrifuged to form a pellet, and the pellet was washed with PBS. A solution containing the LuxS substrate
SRH was previously made in vitro by the addition of 1 mM SAH in 50 mM Tris-HCl pH 7.8 to IMAC-purified (His)$_6$-Pfs-(Tyr)$_5$ conjugated to chitosan via tyrosinase (Yi et al., unpublished). The presence of SRH was verified by RP-HPLC analysis (Yi et al., unpublished). The SRH solution was added to the LuxS-chitosan conjugate, and the reaction was carried out at 37°C and 250 rpm for 4 hours. After the reaction, the solution was extracted with chloroform, and AI-2 activity was recovered in the aqueous phase. AI-2 activity was measured using the cell-based assay described in Surette and Bassler 1998. In this assay, *Vibrio harveyi* AI-2 reporter strain BB170 was grown 16 hours at 30°C and 250 rpm in autoinducer bioassay (AB) medium (medium recipe according to Greenberg et al. 1979), and then diluted 1:5,000 with fresh AB medium. Then, 180 µL of the diluted BB170 cell suspension was mixed with 20 µL of the sample to be analyzed. Fresh AB medium was used as a negative control, and 12 hour conditioned medium from *E. coli* wild type strain W3110 grown in LB medium with 0.8% supplemental glucose was used as a positive control. Bioluminescence results were normalized by dividing by that of the negative control and by the mg of (His)$_6$-LuxS-(Tyr)$_5$. 
RESULTS AND DISCUSSION

Conjugation of purified fusion protein to chitosan via tyrosinase.

In initial studies, we examined tyrosinase-catalyzed conjugation of purified (His)_6-EK-GFP-EK-(Tyr)_5 and (His)_6-LuxS-(Tyr)_5 to chitosan. The experimental design is shown schematically in Fig. 1A. The two fusion proteins were purified via IMAC, incubated with chitosan and tyrosinase for 12 hours, brought to pH ~ 7, and centrifuged to form chitosan pellet. Additionally, to assess the relative amounts of covalently-coupled conjugate versus physically entrapped protein, the IMAC-purified fusion proteins were incubated with chitosan but without tyrosinase for 12 hours, brought to pH ~ 7, and centrifuged to form chitosan pellet. For all experiments, the decanted supernatants were analyzed by Western blots. By comparing band intensities of the supernatants to those of calibration samples, we calculated the amounts remaining in the supernatants, which were then compared with the amounts at the start of the experiments to calculate the amounts precipitated in the chitosan pellets.
Figure 1. Tyrosinase-catalyzed conjugation of purified fusion protein to chitosan. (A) Experimental set-up. (B) Photograph taken under UV light of GFP-chitosan pellets. (C) Western blot analysis of supernatants decanted from centrifugation of GFP-chitosan incubation ("sup no rxn") and GFP-chitosan-tyrosinase incubation ("sup rxn"). At left is a known amount of IMAC-purified GFP fusion. (D) Western blot analysis of supernatants decanted from centrifugation of LuxS-chitosan incubation ("sup no rxn") and LuxS-chitosan-tyrosinase incubation ("sup rxn"). At left is a known amount of IMAC-purified LuxS fusion.
The (His)$_6$-EK-GFP-EK-(Tyr)$_5$ reaction pellet and also the pellet formed without tyrosinase are both shown in Fig. 1B under UV light to illustrate GFP’s fluorescence. It is apparent from Fig. 1B that a considerable amount of GFP fluorescence was precipitated with chitosan. Additionally, it is shown that significantly more GFP fluorescence precipitated with chitosan during tyrosinase-catalyzed conjugation. We calculated from the Western blot in Fig. 1C that 0.80 mg (His)$_6$-EK-GFP-EK-(Tyr)$_5$ was bound to each mg of chitosan in the experiment with tyrosinase, and 0.031 ± 0.043 mg (His)$_6$-EK-GFP-EK-(Tyr)$_5$ was bound to each mg chitosan in the experiment without tyrosinase. It is interesting to note that the supernatant from the tyrosinase reaction in Fig. 1C appears as a double band on the Western blot. In additional experiments (not shown) we incubated (His)$_6$-EK-GFP-EK-(Tyr)$_5$ with tyrosinase (and not chitosan) and also observed a double band. Similarly, we calculated from the Western blot in Fig. 1D that 1.3 mg (His)$_6$-LuxS-(Tyr)$_5$ was bound to each mg of chitosan in the experiment with tyrosinase, and 0.25 ± 0.10 mg (His)$_6$-LuxS-(Tyr)$_5$ was bound to each mg chitosan in the experiment without tyrosinase. We assume that the amount of fusion protein bound to chitosan in the experiment with tyrosinase represents the total amount of fusion protein bound to chitosan, both covalently and non-covalently, and the amount of fusion protein bound to chitosan in the experiment without tyrosinase represents the amount of fusion protein bound to chitosan non-covalently, presumably by electrostatic interactions. The difference between these values represents the amount of fusion protein bound covalently, or 0.77 mg (His)$_6$-EK-GFP-EK-(Tyr)$_5$ per mg chitosan and 1.25 mg (His)$_6$-LuxS-(Tyr)$_5$ per mg chitosan. More (His)$_6$-LuxS-(Tyr)$_5$ than (His)$_6$-EK-GFP-EK-(Tyr)$_5$ was covalently conjugated to chitosan. Additionally, more (His)$_6$-LuxS-(Tyr)$_5$ than
(His)$_6$-EK-GFP-EK-(Tyr)$_5$ was bound non-covalently to chitosan. Results may be explained by differences in non-covalent interactions between chitosan and the two fusion proteins and by differences in protein 3-dimensional size and shape.

**Activity of purified fusion protein after tyrosinase-catalyzed conjugation to chitosan.**

We examined the activity of (His)$_6$-LuxS-(Tyr)$_5$ and (His)$_6$-EK-GFP-EK-(Tyr)$_5$ after tyrosinase-catalyzed conjugation to chitosan. Activity of the GFP-chitosan conjugate was determined by measuring fluorescence intensity (FI) after resuspension in PBS (395 nm excitation wavelength, 509 nm emission wavelength). We compared FI of the resuspended conjugate to that of soluble unconjugated (His)$_6$-EK-GFP-EK-(Tyr)$_5$ by normalizing results based on mg (His)$_6$-EK-GFP-EK-(Tyr)$_5$ in each sample. The GFP-chitosan conjugate remained active. The FI of the conjugate was $\frac{1}{2}$ that of soluble (His)$_6$-EK-GFP-EK-(Tyr)$_5$, suggesting that some GFP activity was lost during the conjugation reaction.

Activity of the LuxS-chitosan conjugate was determined by *in vitro* synthesis of the LuxS product AI-2. As shown in Scheme 3, AI-2 is the product of several steps of an enzymatic pathway. First, we synthesized SRH, the LuxS substrate. A solution containing SRH was previously made *in vitro* by the addition of 1 mM SAH in 50 mM Tris-HCl pH 7.8 to IMAC-purified (His)$_6$-Pfs-(Tyr)$_5$ conjugated to chitosan via tyrosinase (Yi et al., unpublished). The presence of SRH was verified by RP-HPLC analysis (Yi et al., unpublished). The SRH solution was added to both the (His)$_6$-LuxS-(Tyr)$_5$-chitosan conjugate and also to soluble unconjugated (His)$_6$-LuxS-(Tyr)$_5$, and the
reaction was carried out at 37°C and 250 rpm for 4 hours. After the reaction, the solution was then extracted with chloroform, and AI-2 activity was recovered in the aqueous phase. AI-2 activity was measured using the cell-based assay described in Surette and Bassler 1998. AI-2 activity of the conjugate (denoted “conjugate”) and soluble unconjugated (His)_6-LuxS-(Tyr)_5 (denoted “soluble”) are shown in Fig. 2. AI-2 activity was normalized by dividing by the activity of the negative control and also by dividing by the mg (His)_6-LuxS-(Tyr)_5 in each sample. The LuxS-chitosan conjugate remained active. Activity of the conjugate was \( \frac{1}{5} \) that of soluble (His)_6-LuxS-(Tyr)_5, suggesting that some LuxS activity was lost during the conjugation reaction. This may be due to a decrease in stability of the LuxS due to the conditions of the conjugation reaction (1 hour 30°C incubation with tyrosinase). This may also be due to burying of (His)_6-LuxS-(Tyr)_5 within the chitosan pellet interior, thus decreasing accessibility of its active site. Indeed, we do not know the amount of (His)_6-LuxS-(Tyr)_5 attached to the surface of the chitosan pellet (i.e. exposed to the substrate). Optimization of the conjugation reaction conditions is needed to maximize activity of the (His)_6-LuxS-(Tyr)_5-chitosan conjugate.
Chitosan “pre-precipitation” of cell extract components.

Having established that the “activatable pro-tag” facilitates removal of purified fusion protein from solution by simple precipitation with chitosan, we turned upstream to examine the initial separation of fusion protein from cell extracts. That is, to remove nucleic acids and phospholipids in the cell lysates and recover \((\text{His})_6\text{-LuxS-(Tyr)}_5\) and \((\text{His})_6\text{-EK-GFP-EK-(Tyr)}_5\), we performed an initial “pre-precipitation” (PPT) step as illustrated in Scheme 2A. In this step, chitosan solution pH 5.8 (without tyrosinase) was added to the clarified cell lysates to coagulate negatively charged species. All clarified lysates were at the same concentration of wet cell weight per volume resuspension buffer. Upon addition of chitosan, precipitation was immediately observed at all pH conditions. The lysate-chitosan mixtures were centrifuged to remove the precipitated chitosan. The clarified lysates and the PPT supernatants were analyzed by UV light at 260nm and 280nm and by visible light at 595nm. We have used UV light at 260 nm and 280 nm to
measure nucleic acid content and total protein content, respectively, and visible light at 595 nm to measure cell debris. The clarified lysates and the PPT supernatants were also analyzed by Western blots to determine recovery of (His)$_6$-LuxS-(Tyr)$_5$ and (His)$_6$-EK-GFP-EK-(Tyr)$_5$.

The pre-precipitation of (His)$_6$-EK-GFP-EK-(Tyr)$_5$ clarified lysate was performed at pH 7.0. Shown in Fig. 3A, absorbance measurements of the PPT supernatant at all three wavelengths were low compared to absorbance measurements of the clarified lysate. There was $25 \pm 2.7\%$ remaining at 260 nm, $29 \pm 2.5\%$ remaining at 280 nm, and $62 \pm 5.7\%$ remaining at 595 nm. Results indicate that nucleic acids, cell debris, and total protein were precipitated by chitosan. The amount of (His)$_6$-EK-GFP-EK-(Tyr)$_5$ remaining after pre-precipitation was determined by comparing the Western blot band intensities of the clarified lysate and the PPT supernatant to that of a known amount of IMAC-purified (His)$_6$-EK-GFP-EK-(Tyr)$_5$, and is depicted in Fig. 3B. Recovery of (His)$_6$-EK-GFP-EK-(Tyr)$_5$ was $94 \pm 6.7\%$, and only $0.0027 \pm 0.0046$ mg (His)$_6$-EK-GFP-EK-(Tyr)$_5$ was lost per mg of chitosan. The amount of (His)$_6$-EK-GFP-EK-(Tyr)$_5$ lost during the PPT step differs from the amount lost in the experiments without tyrosinase described previously ($0.031 \pm 0.043$ mg lost per mg chitosan). We assume this is due to differences in experimental conditions (i.e. clarified lysate (His)$_6$-EK-GFP-EK-(Tyr)$_5$ vs. IMAC-purified (His)$_6$-EK-GFP-EK-(Tyr)$_5$).
Figure 3. “Pre-precipitation” (PPT) of cell extract components with chitosan. (A) % remaining in PPT supernatant: PPT with GFP fusion. (B) % GFP fusion remaining in PPT supernatant and mg GFP fusion lost per mg chitosan during PPT. (C) % LuxS fusion remaining in PPT supernatant and mg LuxS fusion lost per mg chitosan during PPT at pH 7.0, pH 6.4, pH 6.1, and pH 5.5. (D) % remaining in PPT supernatant: PPT with LuxS fusion. “UV260” and “UV280” denote UV light absorption at 260 nm and 280 nm, respectively. “Vis595” denotes visible light absorption at 595 nm.
In the pre-precipitation of (His)$_6$-LuxS-(Tyr)$_5$, several different pH conditions were examined to determine optimal recovery. The cell pellet was initially resuspended in four different buffers: PBS pH 7.5, 50 mM sodium phosphate pH 6.2, 50 mM sodium phosphate pH 5.8, and 50 mM sodium citrate-citric acid pH 5.2, resulting in four different lysates at pH 7.0, pH 6.4, pH 6.1, and pH 5.5, respectively. After addition of chitosan solution pH 5.8 and centrifugation, the four PPT supernatants remained at pH 7.0, pH 6.4, pH 6.1, and pH 5.5, respectively. The amount of (His)$_6$-LuxS-(Tyr)$_5$ remaining after pre-precipitation was determined by comparing the Western blot band intensities of the clarified lysate and the PPT supernatant at each pH condition to that of a known amount of IMAC-purified (His)$_6$-LuxS-(Tyr)$_5$, and is depicted in Fig. 3C. Recovery at pH 7.0 was 23 ± 0.81 %, with 0.50 ± 0.0053 mg (His)$_6$-LuxS-(Tyr)$_5$ lost per mg chitosan. Recovery at pH 6.4 was 39 ± 3.5 %, with 0.20 ± 0.012 mg (His)$_6$-LuxS-(Tyr)$_5$ lost per mg chitosan. Recovery at pH 6.1 was 42 ± 13 %, with 0.086 ± 0.0012 mg (His)$_6$-LuxS-(Tyr)$_5$ lost per mg chitosan. Recovery at pH 5.5 was 45 ± 1.6 %, with 0.070 ± 0.0021 mg (His)$_6$-LuxS-(Tyr)$_5$ lost per mg chitosan. More LuxS precipitates with chitosan as the pH of the PPT step increases. This may be explained by electrostatic interactions between LuxS and chitosan. As stated previously, chitosan has a pKa of 6.3, and is theoretically more positively charged at pH 5.5 than at pH 7.0. To determine the theoretical overall charge of (His)$_6$-LuxS-(Tyr)$_5$ at different pH conditions, we performed isoelectric focusing gel electrophoresis on IMAC-purified (His)$_6$-LuxS-(Tyr)$_5$ and found it has a pI of 6.0. (His)$_6$-LuxS-(Tyr)$_5$ theoretically has overall positive charge at pH 5.5 and increasing overall negative charge as pH increases above 6.0. Thus, we expect more LuxS to precipitate with chitosan as pH is increased. Although PPT pH 5.5 resulted in
higher recovery of LuxS, we chose pH 6.1 for all future PPT experiments because we were concerned that LuxS is less stable at lower pH. Indeed, we have evidence that LuxS decreases in stability as pH decreases below neutrality based on mg (His)$_6$-LuxS-(Tyr)$_5$ recovered in clarified lysate at different pH conditions.

The difference in recovery of (His)$_6$-LuxS-(Tyr)$_5$ and (His)$_6$-EK-GFP-EK-(Tyr)$_5$ during pre-precipitation was substantial (42% and 94%, respectively). We first believed that results could be explained by differences in electrostatic interactions between the fusion proteins and chitosan. To determine the theoretical overall charge of (His)$_6$-EK-GFP-EK-(Tyr)$_5$ at PPT pH 7.0, we performed isoelectric focusing gel electrophoresis on IMAC-purified (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and found it has a pI of 5.3. (His)$_6$-EK-GFP-EK-(Tyr)$_5$ theoretically has greater overall negative charge at pH 7.0 than (His)$_6$-LuxS-(Tyr)$_5$ at pH 7.0, yet more (His)$_6$-EK-GFP-EK-(Tyr)$_5$ than (His)$_6$-LuxS-(Tyr)$_5$ precipitated with chitosan at pH 7.0. Thus, the differences in recoveries of the two fusion proteins cannot be explained by electrostatic interactions alone. Instead, the lower yield of LuxS from the pre-precipitation step may be because it is less stable than GFP. As stated previously, GFP is known to be very stable even under harsh conditions (Ward and Bokman 1982), while LuxS has been shown to have low stability (Zhu et al. 2003). The low stability of LuxS may cause it to denature and precipitate, thus decreasing the soluble recovery in the PPT supernatant. Additionally, as stated previously, differences in 3-dimensional size and shape of the two fusion proteins may also affect chitosan binding.

Shown in Fig. 3D, absorbance measurements of the (His)$_6$-LuxS-(Tyr)$_5$ PPT supernatant at pH 6.1 at all three wavelengths were low compared to absorbance measurements of the (His)$_6$-LuxS-(Tyr)$_5$ clarified lysate at pH 6.1. There was $20 \pm 0.040$
% remaining at 260 nm, 22 ± 0.20 % remaining at 280 nm, and 4.1 ± 1.3 % remaining at 595 nm. Results indicate that nucleic acids, cell debris, and total protein were precipitated by chitosan. Results from PPT experiments with both (His)$_6$-LuxS-(Tyr)$_5$ and (His)$_6$-EK-GFP-EK-(Tyr)$_5$ are supported by the work of Agerkvist et al. 1990, who demonstrated that flocculation of \textit{E. coli} cell extracts with chitosan removed 98% of cell debris, 50% of proteins, and 85% of nucleic acids, while 60% of a protein of interest (in their case, β-galactosidase) was retained in the supernatant.

**Capture of fusion protein by conjugation to chitosan.**

To capture (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and (His)$_6$-LuxS-(Tyr)$_5$ from the PPT supernatant, we conjugated (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and (His)$_6$-LuxS-(Tyr)$_5$ to chitosan using tyrosinase, thus completing the entire procedure illustrated in Scheme 2A. PPT supernatant was incubated with chitosan and tyrosinase overnight, and the reaction mixture was then centrifuged. The reaction supernatant was decanted, and the pellet was washed twice with buffer. The reaction supernatant and subsequent washes were analyzed by SDS-PAGE and Western blot. Our results indicated that 75 ± 9.5 % of (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and 54 ± 17% of (His)$_6$-LuxS-(Tyr)$_5$ in the clarified lysates was captured by conjugation to chitosan. SDS-PAGE analyses (not shown) revealed that there was a significant amount of unprecipitated native protein in the reaction supernatant and the first buffer wash of the pellet, but that the second buffer wash was essentially protein free. Due to the large amount of lysate proteins in the reaction supernatant and subsequent washes, we surmise that a minimal amount of such proteins were captured by
chitosan. These results indicate that (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and (His)$_6$-LuxS-(Tyr)$_5$ were successfully conjugated to chitosan directly from clarified lysates.

**Enterokinase release of GFP.**

We then explored the release of GFP into solution from chitosan using the protease enzyme enterokinase (EK, Scheme 2B). Unlike the initial experiments that used IMAC-purified GFP, these experiments were aimed at releasing GFP that was first captured from the PPT supernatant by tyrosinase-catalyzed conjugation to chitosan. The conjugate was centrifuged to form a pellet, and the pellet was washed with PBS (pH 7.5). The pellet was then resuspended in PBS and incubated with EK. Shown schematically in Fig. 4A, EK clips the protein sequence at the two specific EK recognition sites. After EK cleavage, the pentatyrosine tag should remain attached to the chitosan chain and appear in the pellet. The hexahistidine tag should separate from wild type GFP, and both should be released into solution.
**Figure 4.** Enterokinase (EK) release of GFP. (A) Schematic representation of EK reaction with GFP-chitosan conjugate. (B) Western blot analysis of each experimental step leading to EK release of GFP. “LYS” denotes clarified lysate. “PPT” denotes “pre-precipitation” supernatant. “C” denotes conjugation reaction supernatant. “W1” and “W2” denote 1st and 2nd wash of conjugate, respectively. “EK” denotes EK reaction supernatant. Shown above Western blot is a bar graph of relative band intensities corrected for dilution of samples.
Shown in Fig. 4B is the Western blot analysis of the EK reaction supernatant (denoted “EK”), the initial clarified lysate (denoted “LYS”), the PPT supernatant (denoted “PPT”), the conjugation reaction supernatant (denoted “C”), and the conjugate buffer washes (denoted “W1” and W2”). Additionally, a known amount of (His)_6-EK-GFP-EK-(Tyr)_5 was also analyzed (not shown). The EK reaction supernatant does indeed contain soluble GFP. Thus, EK can cleave GFP from the GFP-chitosan conjugate, and release the GFP into solution. However, as indicated by the bar chart, the enzyme was not effective. By comparing Western blot band intensities, we calculated that less than 10 % of the GFP conjugated to chitosan was recovered in solution via EK. In comparison, when the reaction was performed over the same time period with soluble unconjugated (His)_6-EK-GFP-EK-(Tyr)_5, greater than 70 % was released from its N- and C-terminal tags (not shown). A possible explanation for the ineffectiveness of EK when used with the conjugate is that the EK was not able to access its cleavage site (Gaillard et al. 1996). This could be due to a change in folding of the (His)_6-EK-GFP-EK-(Tyr)_5 when bound to the chitosan or due to blockage by the chitosan chains.

It is important to note that the release of GFP into solution demonstrates that at least some (His)_6-EK-GFP-EK-(Tyr)_5 was initially conjugated to chitosan through the “pro-tag,” rather than through its naturally occurring tyrosine residues. That is, the “pro-tag” can be activated by tyrosinase to allow for the covalent conjugation of the fusion protein to chitosan.
Chitosanase release of fusion protein.

To release (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and (His)$_6$-LuxS-(Tyr)$_5$ into solution by an alternative method, we used the chitosan-hydrolyzing enzyme chitosanase. For these experiments, (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and (His)$_6$-LuxS-(Tyr)$_5$ were captured from PPT supernatants by tyrosinase-catalyzed conjugation to chitosan. The conjugates were each centrifuged to form a pellet, and each pellet was washed with 20 mM sodium phosphate pH 6.4. Each pellet was then resuspended in the phosphate buffer and incubated with chitosanase. Additionally, the chitosanase reaction solution was purified via IMAC for analysis.

To anticipate results from chitosanase release, it is necessary to consider some of the characteristics of this enzyme-polysaccharide system. Chitosan is a copolymer of glucosamine and N-acetylglucosamine, and chitosanase-catalyzed hydrolysis results in a mixture of monomeric and oligomeric products (Wu et al. 2002a). Because chitosanase was used after fusion protein capture by tyrosinase-catalyzed conjugation to chitosan, the released proteins should retain both hexahistidine and pentatyrosine tags, and be decorated by sugars of varying size. Additionally, we anticipate the reaction mixture will also contain oligomers of hydrolyzed chitosan not conjugated to (His)$_6$-EK-GFP-EK-(Tyr)$_5$ or (His)$_6$-LuxS-(Tyr)$_5$.

We observed a color change during the chitosanase reaction. Initially, the chitosan pellet appeared white and opaque. By the end of the chitosanase reaction, the chitosan pellet had disappeared, and the resulting solution was opaque brown/orange and contained some small precipitates. In addition, we observed a dramatic reduction in the
viscosity of the solution, which allowed us to directly load the reaction mixture onto SDS-PAGE and onto IMAC columns.

Western blot analyses of (His)_{6}-EK-GFP-EK-(Tyr)_{5} and (His)_{6}-LuxS-(Tyr)_{5} chitosanase reaction mixtures are shown in Fig. 5A and Fig. 5B, respectively. Also shown are Western blot analyses of the initial clarified lysate (denoted “LYS”), the PPT supernatant (denoted “PPT”), the conjugation reaction supernatant (denoted “C”), and the conjugate buffer washes (denoted “W1” and “W2”; not shown for (His)_{6}-LuxS-(Tyr)_{5}). Additionally, known amounts of (His)_{6}-EK-GFP-EK-(Tyr)_{5} and (His)_{6}-LuxS-(Tyr)_{5} were also analyzed (not shown). There was a significant amount of released (His)_{6}-EK-GFP-EK-(Tyr)_{5} in the corresponding chitosanase reaction solution (denoted “CE” in Fig. 5A). However, there was virtually no released (His)_{6}-LuxS-(Tyr)_{5} observed in its chitosanase reaction mixture (denoted “CE” in Fig. 5B). We believe this is due to blockage of the anti-poly-histidine binding sites by the hydrolyzed chitosan. Anti-GFP antibody was used to analyze the GFP samples in Fig. 5A, and anti-poly-histidine antibody was used to analyze the LuxS samples in Fig. 5B. When the same GFP samples were analyzed using anti-poly-histidine antibody, we similarly observed no released GFP in the chitosanase reaction mixture (not shown). Additionally, when the chitosanase reaction mixtures were purified via IMAC, the eluted protein samples were shown to contain significant quantities of fusion proteins (denoted “CE-IM” in Fig. 5A and Fig. 5B). Therefore, we believe the hydrolyzed chitosan present in the chitosanase reaction samples prevents the anti-poly-histidine antibody from accessing its binding site on each fusion protein. By comparing Western blot band intensities, we calculated that the IMAC-purified chitosanase reaction mixtures in Fig. 5A and Fig. 5B contained greater than 90 % of the
amount captured by conjugation to chitosan. Thus, the chitosanase enzyme was effective at hydrolyzing the chitosan, and the majority of (His)$_6$-EK-GFP-EK-(Tyr)$_5$ and (His)$_6$-LuxS-(Tyr)$_5$ captured could be released through the chitosanase reaction. This compares favorably with enterokinase release of GFP.

![Figure 5](image_url)

**Figure 5.** Chitosanase (CE) release of fusion proteins. (A) CE release of GFP fusion. LYS at 25x dilution; PPT at 25x dilution; C, W1, W2 at 1x dilution; CE at 20x dilution; CE-IM at 20x dilution. (B) CE release of LuxS fusion. All at 1x dilution. “LYS” denotes clarified lysate. “PPT” denotes “pre-precipitation” supernatant. “C” denotes conjugation reaction supernatant. “W1” and “W2” denote 1st and 2nd wash of conjugate, respectively. “CE” denotes CE reaction mixture. “CE-IM” denotes IMAC elution of CE reaction mixture. Shown above Western blot is a bar graph of relative band intensities corrected for dilution of samples.
CONCLUSIONS

We report a facile method for the capture and release of recombinant protein from cell extract. Protein capture from solution is based on a “pro-tag” that is enzymatically activated to conjugate the protein to the aminopolysaccharide chitosan. Specifically, the “pro-tag” consists of five tyrosine residues that are activated by tyrosinase-catalyzed conversion into reactive o-quinones, which subsequently react with chitosan. Because the “pro-tag” is “inactive” in the absence of tyrosinase, it is possible to use chitosan in initial protein recovery steps to coagulate cell debris from the cell extracts. Additionally, we show that the recombinant proteins retain activity after being conjugated to chitosan. Recombinant protein release from the chitosan and into solution is achieved by an alternative to protease cleavage using the chitosan-hydrolyzing enzyme chitosanase.
REFERENCES


