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Recombinant Chitinase C produced through cell-free protein synthesis retains chitin-binding capabilities

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SUMMARY Optimizing high-yield production of target proteins is often a time-consuming process for many experiments. Cell-free protein synthesis (CFPS) is a potential solution, as it eliminates preliminary processes, such as cell transformation, while providing high protein concentration. In this study, our target protein for CFPS production was Chitinase C (ChiC) from *Pseudomonas aeruginosa PAO1*. We hypothesized that ChiC would retain its chitin-binding functionality through CFPS synthesis. Our study demonstrated that CFPS can successfully produce recombinant ChiC in Escherichia coli BL21 (DE3) cell extracts that not only fold at the appropriate size, but also possess chitin-binding functionality. Overall, CFPS was revealed as a viable avenue for ChiC synthesis, laying the foundation for future optimization of ChiC production and cell-free production of similar proteins.

INTRODUCTION

Chitin is a polysaccharide polymer consisting of β-linked N-acetyl-D-glucosamine found predominantly in the cell walls of fungi and in the exoskeleton of crustaceans and insects, ranking only behind cellulose as the most abundant polymer on Earth (1). The glycosidic bonds of chitin are degraded by chitinase enzymes, one example being *chiC* in *Pseudomonas aeruginosa* PAO1 that encodes the 53 kDa chitinase ChiC (2). The development and deployment of chitinolytic pesticides by chitinase enzymes has been discussed as a treatment to shelter crops from pests immune to existing methods (3). Although ChiC itself may not be dangerous to work with, *P. aeruginosa* is still a Risk Group 2 organism, so the cloning of *chiC* into the pET-28a plasmid, on a gene regulated by a T7 promoter, and transformation into *Escherichia coli* BL21 (DE3) by Rocha et al. as the pM3CRYY plasmid has been crucial in making working with ChiC more accessible (4). Rocha et al. additionally attached a 1 kDa 6x-His tag to the N-terminus of ChiC for protein extraction as anti-ChiC antibodies have yet to be developed. However, ChiC produced by *E. coli* has only demonstrated binding activity (Lin et al., unpublished data, Mohebat et al., unpublished data, Kim et al., unpublished data, Guo et al., unpublished data) as all assays to measure its chitinase activity have been unsuccessful (Tong et al., unpublished data). Prior yields of ChiC were also low, with optimization of purification being reviewed as a target avenue for further research (Kim et al., unpublished data). While previous studies focused on generating ChiC through cultivating *E. coli* cultures to harvest the pellets in order to extract ChiC from the lysate supernatant, our study focuses on developing a cell-free method to produce ChiC as an alternate approach.

Cell-free protein synthesis (CFPS) utilizes cell extracts rather than live cells to generate proteins (5). Metabolites and template DNA can be fed directly into the cell extracts. Since the cell walls and chromosomal DNA precipitate during centrifugation, the cell extracts do not generate any additional genetic material, allowing for all the energy in the system to be

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directed towards production of the target protein(s). Chaperones can be cloned into the operon of the template DNA, allowing for the production of protein that does not self-fold (6). CFPS formats are flexible, including batch, continuous exchange and continuous flow systems (5). A variety of platforms have been developed for CFPS usage, including *E. coli* (7), *Bacillus sp.* (8), wheat germ (9), *Nicotiana tabacum* (10), insect cells (11), mammalian (HeLa) cells (12) and even archaeal systems (13). The variance in platforms for CFPS highlights the role that cellular environment, à la pH and dielectric constant, plays in protein folding and must be taken into account when choosing a system for CFPS. If the required protein needs to form disulfide bonds reagents can be added to induce a reducing environment. Although yields vary between protocol and target protein, GFP produced from *E. coli* extract through CFPS has an average concentration of 2.3 mg/ml (7). More complex CFPS systems such as the plant *Nicotiana tabacum* platform (10) are capable of producing complex proteins requiring several post-translational modifications, such as monoclonal antibodies and G protein-coupled receptor membrane proteins, to a high yield of 3 mg/ml (14).

For the purposes of our study, we chose *E. coli* as our platform for a variety of reasons. *E. coli* is already a popular platform for protein recombination due to its highly characterized systems, high growth rate, and low cost (15, 16). In *E. coli*, the cytosol is a reducing environment while the periplasm is oxidizing (15). Cell lysis generates an overall reducing environment with a pH of 7.5 (17) and an ε of 65 (18). This is especially crucial for the production of ChiC in particular since an oxidizing environment could cause it to aggregate through the formation of disulfide bonds, lowering protein yield from CFPS (2). *E. coli* CFPS systems are also relatively easy to set up, with the preparation of cell extracts taking upwards of three days, followed by the protein synthesis only occurring over a few hours (5).

Guo et al. (unpublished data) were able to demonstrate that recombinant ChiC from pM3CRRY expressed in *E. coli* has chitin-binding capabilities. We hypothesized that ChiC generated from our cell-free system would be equally as capable as the ChiC produced through traditional culturing methods as the conditions of our CFPS system would resemble the *E. coli* system and possess the capability to transcribe and translate recombinant *chiC*. In our study, we found not only that *chiC* from pM3CRYY can be transcribed and translated into ChiC, but that our CFPS-produced ChiC also possessed chitin-binding properties. The resultant findings suggest that ChiC produced by CFPS is functionally identical to protein generated through harvesting *E. coli*.

METHODS AND MATERIALS

Preparation of liquid LB media, agar plates, and kanamycin. Filter-sterilized kanamycin was prepared at 50 mg/mL and stored at -20°C. Gibco liquid Luria-Bertani (LB) broth and LB agar were prepared based on the Lennox formulation, autoclaved at 121°C, 15 psi for 30 minutes, and stored at room temperature and 4°C respectively (19). Kanamycin (50 mg/mL) was added to liquid LB broth and agar.

pM3CRYY plasmid extraction. Isolated colonies of *E. coli* BL21 (DE3) cells containing pM3CRYY plasmids from Rocha et al. were grown overnight in 5 mL LB broth with 1:400 kanamycin at 200 rpm, 37°C. Plasmids were isolated and purified using the EZ-10 Spin Column Plasmid DNA Miniprep kit and manufacturer's protocol (BioBasic) (20). Purified plasmids were quantified using a NanoDrop 2000/2000c Spectrophotometer and stored at -20°C.

Cell-free protein synthesis reaction. Reactions were set up using PURExpress® *In Vitro* Protein Synthesis kit (New England Biolabs®) following recommendations from the Protein Synthesis Reaction using PURExpress (E6800) protocol (21). Reactions were assembled in the following order on ice per 25 µl: 10 µl Solution A (New England Biolabs®), 7.5 µl Solution B (New England Biolabs®), 1 µL Ambion™ RNase Inhibitor, cloned (Invitrogen™), template DNA beginning at 250 ng, and UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™). Incubations were done at 37°C. Reactions were stopped by immediately heating to 90°C in 0.5X BlueJuice™ Gel Loading Buffer (Invitrogen™) for 2 minutes then stored at -20°C.

Sample preparation and SDS-PAGE. Samples were mixed with 2x Laemmli Sample Buffer (Bio-Rad) with 10% β-ME (SIGMA®). Samples were then heated for 5 minutes at 90°C. Electrophoresis was conducted with the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels (Bio-Rad) using Mini-PROTEAN TGX Stain-Free Precast Gels (Bio-Rad). 10 µL of samples were loaded alongside 10 µL Precision Plus Protein™ Unstained Protein Standards Ladder (Bio-Rad) for SDS gels and 10 µL of PageRuler Plus Pre-Stained Protein Ladder (ThermoFisher) for western blots. The inner chamber and outer chambers were loaded with 200 ml and 550 ml TRIS-base/Glycine/sodium-dodecyl-sulfate (SDS) running buffer respectively. The gels were run at 100 V for 100 minutes or at 200 V for 50 minutes with the PowerPac™ Basic Power Supply (Bio-Rad). SDS-PAGE gels were imaged using the ChemiDoc (Bio-Rad).

Chitin-binding assay. Full CFPS reactions (25 µL) were incubated with 150 µL Chitin Resin (New England Biolabs®) in microfuge tubes at room temperature for 30 minutes on a shaker. Samples were centrifuged for 5 minutes at 12,000 rpm and then washed with 150 µL Tris-Buffered Saline (TBS). Washes were repeated 3 times in total. After the last wash, samples were eluted thrice in a 1:1 ratio with 2x Laemmli Sample Buffer (Bio-Rad) with 10% β-ME (SIGMA®). Samples were incubated in the elution buffer for 5 minutes at room temperature on a shaking incubator followed by 5-minute centrifugation at 12,000 rpm for washes and elutions. All supernatants collected after each centrifugation were kept in microfuge tubes labeled accordingly and stored at -20°C.

Western blot. Proteins were transferred from a Mini-PROTEAN TGX Stain-Free Precast Gel (Bio-Rad) to a Trans-Blot Turbo Mini 0.2 µm Nitrocellulose Transfer Pack (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad) at 1.3 A and 25 V for 7 minutes. Membranes were blocked for 1 hour with 15 ml 1X Tris-buffered saline with 0.1% Tween®20 (TBS-T) + 1% bovine serum albumin (BSA) (Sigma-Aldrich) at room temperature on a shaker. 9 mL of primary 6x-His Tag Monoclonal Antibody (Invitrogen™) diluted 1:1000 in 1x TBS-T + 1% BSA was used to incubate the membrane overnight on a shaker at 4ºC. The subsequent morning, three 15 ml 1X TBS-T washes were performed for 5 minutes each. Membranes were then incubated in the dark with 15 mL Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP (Invitrogen™) diluted 1:100000 in 1x TBS-T + 1% BSA for 1 hour. The three TBS-T washes were repeated and the membranes were incubated in 7 mL of Clarity Western ECL substrate reagents (BioRad) for 5 minutes. The membranes were imaged using the ChemiDoc (Bio-Rad).

RESULTS

ChiC can be generated with CFPS. To confirm whether ChiC was produced via CFPS, we took aliquots at different time points over the course of the run time. We also included unheated samples to observe whether the heating of samples was necessary to visualize the target proteins. Dihydrofolate reductase (DHFR) provided by the PURExpress® *In Vitro* Protein Synthesis kit (New England Biolabs®) served as a positive control to confirm that template DNA can be transcribed and then translated into protein via CFPS. The gel showed a band around 20 kDA that increased in intensity over a 2-hour CFPS run time in the DHFR sample, which was confirmed as DHFR based on gels provided by PURExpress® (22). The control unheated DHFR samples taken at the 2-hour run time also displayed an intense band of the same size (Figure 1). In the pM3CRYY sample, there was a band around 55 kDa that increased in intensity over a two-hour CFPS run time which was comparable to the purified ChiC band and previous UJEMI findings (Guo et al., unpublished data). The unheated pM3CRYY sample taken at the 2-hour run time does not show a band of 55 kDa (Figure 1). Our results suggested that DHFR and ChiC were produced via CFPS and that heating was a necessity for pM3CRYY sample preparation but not DHFR.

Doubling CFPS run time does not lead to increased ChiC production. Since we confirmed that ChiC was produced via CFPS, we wanted to optimize the protocol to produce a higher yield. Based on the Protein Synthesis Reaction using PURExpress® (E6800) protocol, the recommended run time was 2 hours, however an additional 1 to 2 hours can potentially lead to increased yield (21). Thus, we extended the CFPS run time to 4 hours,

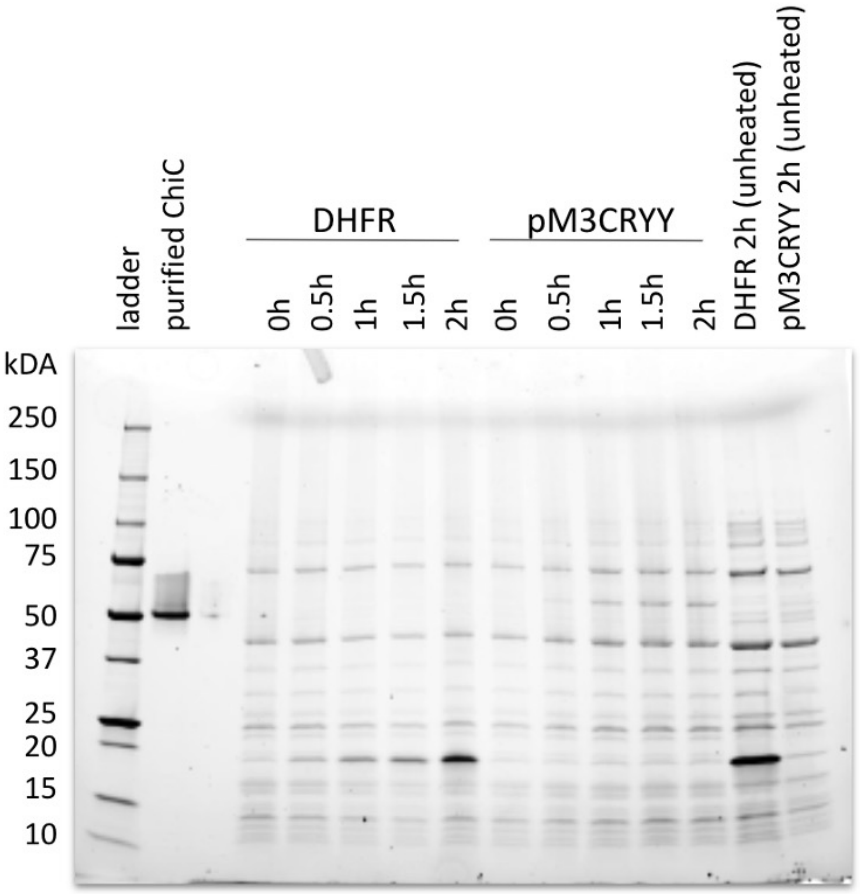

FIG. 1 Increased production of DHFR and ChiC over 2 hours with CFPS. SDS-PAGE gel showing total proteins synthesized from DFHR and pM3CRYY plasmids at different time points via CFPS. Concentration of DHFR plasmid was 20 ng/µL. Concentration of pM3CRYY plasmid was 10 ng/µL. Ladder used was Precision Plus Protein Standard (Bio-rad, 10 µL).

comparing its production to the 2-hour time point. We saw no noticeable intensity difference at the target 55 kDa band between the 2 and 4-hour lanes (Figure 2A**)**. This showed that doubling the reaction time in CFPS did not improve ChiC yield.

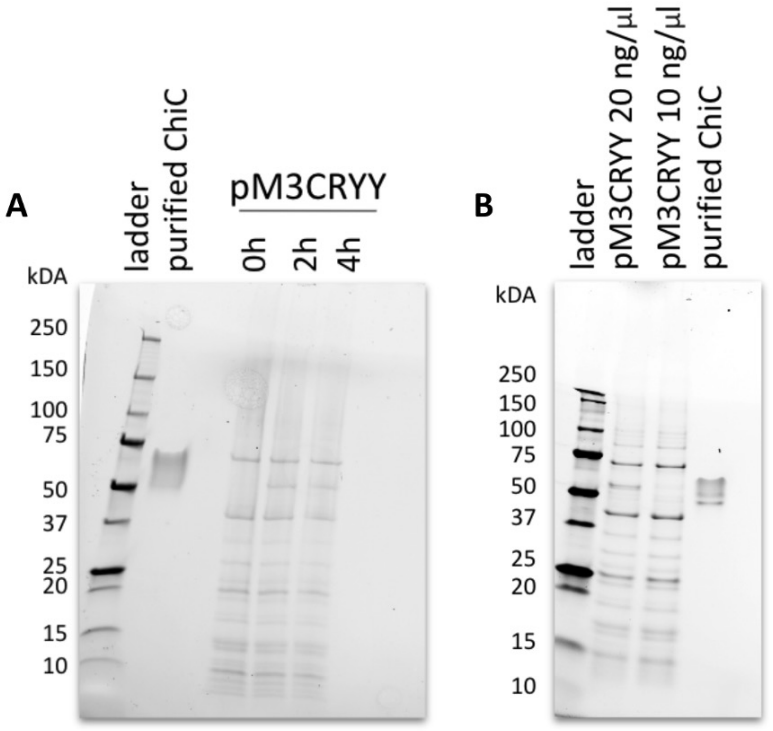

FIG. 2 Doubling pM3CRYY plasmid concentration yielded higher ChiC levels, but increasing run time did not. (A) SDS-PAGE gel showing total proteins synthesized from pM3CRYY plasmid at different time points via CFPS reaction. Plasmid concentration used was 20 ng/µL. Ladder used was Precision Plus Protein Standard (Bio-rad, 10 µL). **(B)** SDS-PAGE gel showing total proteins synthesized from pM3CRYY plasmids at different concentrations via CFPS reaction. Total reaction time was 2 hours. Ladder used was Precision Plus Protein Standard (Bio-rad, 10 µL).

Doubling pM3CRYY concentration increased ChiC production via CFPS. Per the recommendations of the Protein Synthesis Reaction using PURExpress® (E6800) protocol, another factor that can lead to increased protein production was by increasing the plasmid concentration (21). We noticed that doubling the DHFR plasmid concentration led to a darker band (Figure 1), thus we hypothesized that doubling the pM3CRYY plasmid would also increase production (Figure 2B). We saw a noticeable band at ~55 kDa in the lane that had double the plasmid concentration (20 ng/µL), which was more pronounced than the original plasmid concentration we used (10 ng/µL). From this, we determined that increasing plasmid concentration led to increased ChiC yield.

ChiC produced via CFPS possesses an intact 6x-His tag and retains chitin-binding capabilities. To determine if the pM3CRYY produced by CFPS folded into a functional conformation, we performed a chitin-binding assay to determine if our recombinant ChiC retained chitin-binding capabilities. Elution lanes in an SDS-PAGE gel showed bands just above 50 kDa; no other bands at that molecular weight were observed in the washes (Figures S1, 3). The elution bands for the ChiC lanes are also observed to be in the range of the previously isolated ChiC (unpublished data). DHFR protein bands appeared in the washes as expected since it is unable to bind to chitin. However, it was also detected in the elutions. While intensities of the other protein bands diminished as the washes progressed, they increased during successive elutions as well. Since the protein bands corresponding to the 55 kDa molecular weight of ChiC are present in only the elution lanes and not the wash lanes, it can be inferred that ChiC was bound to the resin during the washes. This was corroborated by a western blot, using anti-6x His mouse and anti-mouse HRP rabbit antibodies to stain (Figure 4). Again, ChiC elutions bands were the only visible bands for their molecular weight, indicating that they successfully bound to the chitin resin during the assay. No bands with a molecular weight corresponding to DHFR were observed. Signal from other proteins in the cell extract was also detectable as bands in both the wash and elution lanes.

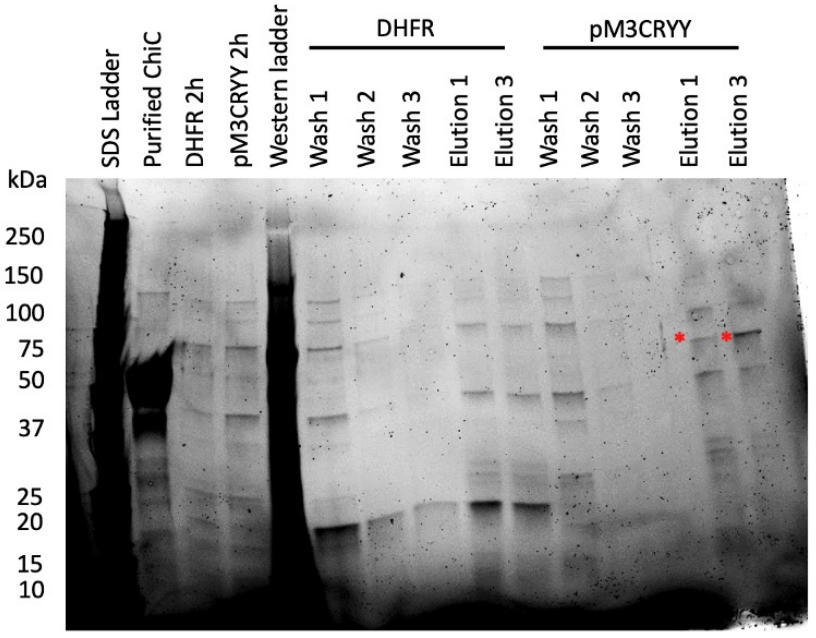

FIG. 3 ChiC is present in elutions of a chitin-binding assay and absent in the washes. SDS-PAGE gel showing chitin-binding assay washes and elutions with products from DHFR and pM3CRYY samples. SDS ladder used was Precision Plus Protein Standard (Bio-rad, 10 µL). Total CFPS run time was 2 hours with lanes DHFR 2h and pM3CRYY 2h as references. Concentration of plasmids was 20 ng/µL. Western ladder used was PageRuler Plus Pre-Stained Protein Ladder (ThermoFisher, 10 µL). ChiC bands are indicated by red asterisks.

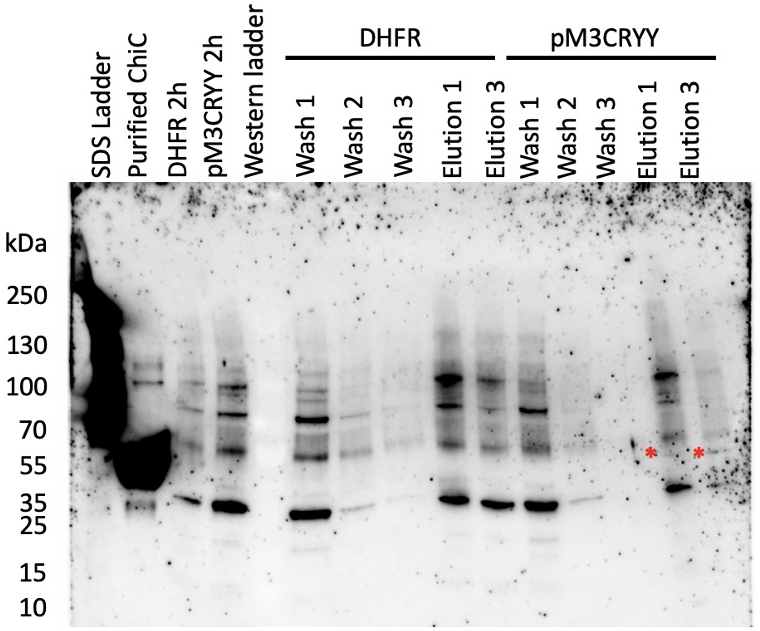

FIG. 4 ChiC exhibits chitin-binding ability. Western blot showing chitin-binding assay washes and elutions with products from DHFR and pM3CRYY samples. Primary antibody was anti-6x His mouse (1:1000) and secondary antibody was anti-mouse HRP rabbit (1:100000). SDS ladder used was Precision Plus Protein Standard (Bio-rad, 10 µL). Total CFPS run time was 2 hours with lanes DHFR 2h and pM3CRYY 2h as references. Concentration of plasmids was 20 ng/µL. Western ladder used was PageRuler Plus Pre-Stained Protein Ladder (ThermoFisher, 10 µL). ChiC bands are indicated by red asterisks. Note that this is only the chemiluminescent blot and as such, the western ladder is not visualized, but it is present on the full blot (S2).

DISCUSSION

Being able to produce large volumes of protein in an efficient manner reduces complications in downstream assays that require high protein amounts. In this study, we showed that ChiC production increased over a 2-hour period by CFPS with an optimized plasmid concentration. The ChiC produced from CFPS also demonstrated chitin-binding capability, indicating that the protein synthesized retains functional properties.

Codon usage bias can be a limiting factor for ChiC production. Although we were able to increase ChiC production by doubling our template plasmid concentration, the resulting level of intensity, thus protein production, was lower than DHFR (Figures 1, 2B). We speculate that a part of the observed difference in protein production between DHFR and ChiC is due to differences in codon usage between *E. coli* and *P. aeruginosa*, as there are codons that are common to *P. aeruginosa* are rare in *E. coli*, causing bottlenecks during translation. One notable example of this is the CCC codon that codes for Pro, appearing four times in chiC (2). In *P. aeruginosa PAO1*, it has a frequency of 13.0 per thousand codons while in *E. coli* it only has a frequency of 2.4 per thousand (23). While *E. coli* has a mean GC content of around 50%, *P. aeruginosa* skews higher with a mean GC content of 67% (2). This difference in codon usage can be accommodated for, with the ExpOptimizer web server from NovoPro Bioscience (https://www.novoprolabs.com/tools/codon-optimization) by reducing GC content of *chiC* from 67.36% to 56.18%. While this reduction is not quite down to 50%, codon optimized *chiC* is still more partial to *E. coli* than *P. aeruginosa* and should result in a measurable increase in protein production.

Further optimization is required for ChiC extraction in low volumes. In our chitin-binding assay, we observed the presence of other proteins in the DHFR and pM3CRYY elutions (Figures S1, 3). This finding was unexpected, as we hypothesized only ChiC should be present in the pM3CRYY elutions and non-specific binding proteins should be removed during the preceding wash processes. Technical issues could be the basis of this cause. Since the chitin resin remained a slurry even after centrifugation, it was difficult to completely decant the supernatant after the washes without disrupting the settled resin, which may help explain the presence of non-specific proteins. To mitigate this, we propose using a chitin-binding column protocol optimized for lower volumes which would avoid the issue of excess supernatant remaining within the chitin resin (24). This method should allow for complete flow-through due to gravity thus reducing the risk of contamination.

Non-specific proteins persist in the elution fractions and western blot. In both our SDS-Page gels (Figure S1, 3) and western blot (Figure 4), we observed the presence of non-specific proteins in all of our elution lanes. Because we also used the chitin-binding assay as a method

to purify ChiC, the large presence of contaminants in our elutions resulted in the observation of a significant amount of non-specific binding during the western blot. Additionally, there are no ChiC-specific primary antibodies available that would allow us to observe ChiC-specific binding on the western blot for troubleshooting purposes. While we were still able to definitively resolve ChiC in the elution lanes, the signal of our western blot was dominated by these persistent non-specific protein-antibody interactions.

Despite the contamination observed in our final elutions, the ChiC band (~ 55 kDa) intensity increased in elution 3 compared to elution 1, while non-specific proteins decreased in intensity (Figure 3). This allows us to believe that ChiC produced via CFPS has chitin-binding properties, and optimizing the protocol will yield robust results.

Apart from a chitin-binding column protocol, New England Biolabs® also provides an extraction protocol that is recommended with the CFPS kit (E6800, New England Biolabs®) we used in this study. The procedure involves spin-concentrators that can concentrate low protein volumes and collect non-specific proteins (the flow-through) at the bottom of the concentrator (25). This approach would also prevent contamination during washes while being able to work with low volumes, such as CFPS, efficiently. This would also allow our western blot to better resolve ChiC in chitin-binding assays where the presence of non-specific proteins would be minimized.

Limitations CFPS reaction volumes were low due to the expensive nature of the NEB PURExpress® *In Vitro* Protein Synthesis kit, only allowing us to produce a maximum of 250 µL of soluble protein. In addition to the aforementioned codon bias, limited reagents reduced the total amount of ChiC we were able to generate, impacting our difficulty to distinctly detect it and impairing our ability to do downstream analyses that required intensive amounts of protein as we were working from a limited supply. One such instance of this was in the chitin-binding assay, where the 25 µL yields of each CFPS reaction caused us to perform the assay using chitin resin beads in a microfuge tube as opposed to using a chitin column. This is most likely the reason why cell extract protein bands were observed in the elution fractions of the chitin-binding assay.

Conclusions In this study, we explicitly showed that CFPS can produce ChiC from pM3CRYY in *E. coli* cell extracts. The synthesized protein can be extracted through optimized chitin-binding assays and shows chitin-binding functionality. The optimization of these methodologies will open up avenues for downstream applications, such as assessing chitinase activity in ChiC and in exploring its role as an industrial product.

Future Directions Our discoveries demonstrate that *P. aeruginosa* PAO1 *chiC* on the *chiC*-expression vector pM3CRYY can be expressed via CFPS in *E. coli* extract. While we were able to confirm that the recombinant ChiC possesses chitin-binding properties, future studies could work to prove that ChiC demonstrates full activity by gauging its ability to degrade chitin through a chitinase assay. Codon optimization can also be addressed since it is a known issue and would require ligating a codon-optimized *chiC* into a pET-28a expression vector to create a new plasmid optimized for ChiC production *E. coli*. This would be an especially important accomplishment since a codon-optimized recombinant ChiC would benefit both traditional and CFPS production.

Moving forwards beyond proof of concept, future studies can be used to look at scaling CFPS to potentially test its market viability. Creating a homemade brew to handcrafted mixture of CFPS reagents would allow for the most flexibility in tailoring the reaction for ChiC production. Directly manufacturing the solution of CFPS reagents would circumvent codon optimization issues by allowing for tRNA and amino acid optimization (5). A long-term goal would be to work towards constructing a cost-benefit analysis of ChiC production by CFPS and production by culturing *E. coli*. Use of a bicinchoninic acid assay would determine the concentration of the produced ChiC to further assess its efficacy. This would be the most crucial step in determining if CFPS would be a viable choice to produce recombinant ChiC in quantities sufficient for pesticide production or any other use.

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