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Optimization of expression in *Escherichia coli* BL21 (DE3), IMAC purification, storage, and preliminary functional characterization of recombinant Chitinase C from *Pseudomonas aeruginosa*

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SUMMARY Chitin is a homopolymer that is found in the abdominal lining and exoskeleton of various insects. Given the increasing environmental pollution due to chemical insecticides used in agriculture, there is great interest in the development of bioinsecticides. Proteins that hydrolyze chitin, like chitinases, have potential in this regard. *Pseudomonas aeruginosa* PAO1 Chitinase C (ChiC) is a 55 kDa secreted protein, and while the *chiC gene* has previously been cloned and expressed in *Escherichia coli* BL21 (DE3), the optimal isopropyl β-D-1- thiogalactopyranoside (IPTG) inducer concentration, incubation time, and temperature for soluble recombinant ChiC (rChiC) expression have yet to be elucidated. We used a factorial experiment to optimize the conditions for soluble rChiC expression, purified soluble rChiC using immobilized metal affinity chromatography (IMAC) with Ni-NTA resin, and conducted a preliminary functional characterization of rChiC using a chitin-binding assay. We found that the soluble protein is optimally expressed under 0.1 mM IPTG induction, 24 hr incubation, and at 20°C. The present study used histidine in place of the more commonly used imidazole for competitive washing and elution in IMAC, illustrating the potential of histidine as a competitive agent for protein purification. Finally, we observed that the purified rChiC has a functional chitin-binding domain, demonstrated by a chitin-resin binding assay. Overall, our study demonstrates the preliminary optimization of expression, purification, and functional characterization of rChiC present in the soluble fraction in *E. coli* BL21 (DE3). This study reveals potential for future research aimed at further optimization of soluble rChiC and subsequent functional characterization of the chitinolytic domain of rChiC using *in vitro* and *in vivo* systems.

INTRODUCTION

Chitin is an insoluble homopolymer of 1,4 β-linked N-acetyl-D glucosamine. It is a key structural component of the exoskeleton and abdominal lining of insects, crustacean shells, and the cell wall of numerous fungi and algae (1). Several organisms ranging from bacteria to higher-order plants have shown to produce chitinases, which are extracellular enzymes that catalyze the hydrolysis of chitin into chitodextrins. In bacteria, the expression of chitinolytic enzymes is typically induced upon contact with chitin for use as a carbon or nitrogen source. In the case of plants, chitinolytic enzymes are used to defend against pathogenic insects and fungi (2). Bacteria synthesize and secrete a wide variety of chitinases, including ChiA, ChiB, ChiC, and ChiD. Their ability to synergistically degrade chitin makes them attractive insecticidal and anti-fungal biocontrol agents (3).

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In recent decades, the continued use of synthetic chemical pesticides in agriculture has caused harm to both natural ecosystems and the human population. In particular, pesticides often bioaccumulate in livestock and wildlife that reside near agricultural farms or runoff, resulting in toxic levels of pesticide accumulation in the human food supply (4). With a pressing need for ecologically sustainable alternatives to pesticides and fertilizers, chitinases show promise as a potential biopesticide (5). Interestingly, previous studies have shown that ChiC is found exclusively in highly insecticidal bacteria including *Pseudomonas aeruginosa*, *Pseudomonas protegens* CHA0, and *Serratia marcescens* (1).

The chitinase C (ChiC) protein is a gradually secreted 55 kDa chitinolytic enzyme that is composed of 483 amino acids (a.a) (1). It is involved in several carbohydrate metabolic processes including carbohydrate binding and hydrolysis of O-glycosyl compounds. ChiC consists of three predicted functional domains: a catalytic domain (a.a 27-336), a fibronectin-like type III domain (a.a 345- 430), and a chitin-binding domain (CBD) (a.a 436-482), which work synergistically to cleave random sites of chitin (Fig 1). The CBD is a type 3 chitin-binding domain (InterPro: SM00495) under the carbohydrate-binding module family 5/12 homologous superfamily that possesses carbohydrate binding and O-glycosyl hydrolase activities, allowing ChiC to bind to and aid in the degradation of chitin (InterPro: IPR003610). The hydrolytic domain consists of the glycoside hydrolase family 18 (GH18) domain that houses the active site of the enzyme (amino acids 135-143) (InterPro: IPR001579) which catalyzes the random cleavage of chitin β-1,4 glycosidic bonds (InterPro: IPR001223) into trimeric, dimeric, or monomeric N-acetylglucosamine products. Lastly, the fibronectin-like III domain is involved in cell adhesion and migration, and it facilitates the interaction of ChiC with other proteins (InterPro: IPR003961) (6).

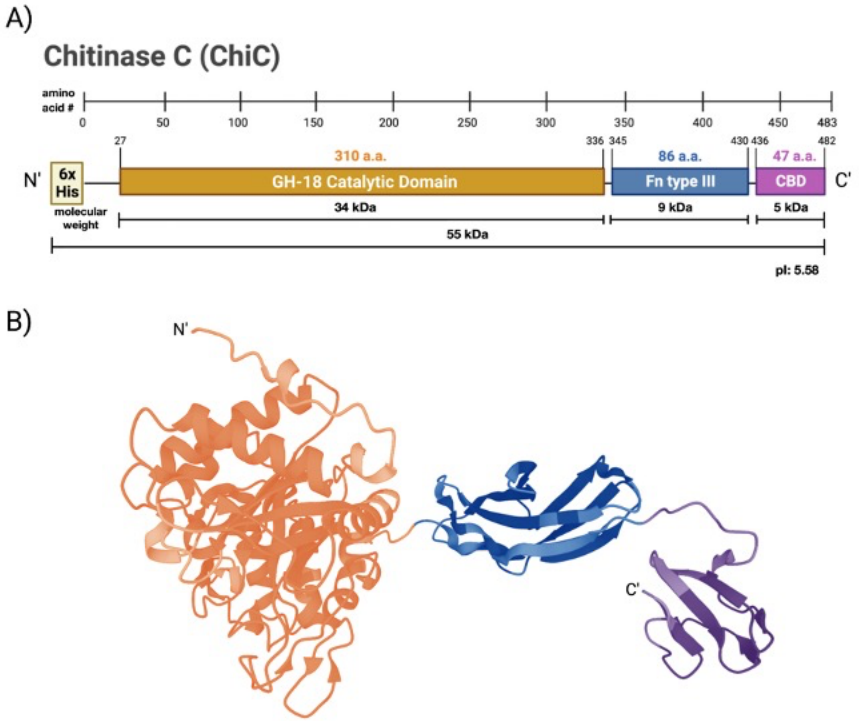

FIG. 1 Domain structures of Chitinase C. (A) Protein domain map of recombinant ChiC cloned from *P. aeruginosa*. The top bar and numbers indicate the positions of amino acid sequences. The protein sequence was analyzed using the Pfam database. The locations of the following domains are as indicated: GH-18 catalytic domain (yellow, Pfam ID: PF00704) fibronectin-like type III domain (blue, Pfam ID: PS50853), and CBD (purple, Pfam ID: PF02839). The molecular weight distribution and isoelectric point of the rChiC used in this study are highlighted in the figure. a.a is amino acids. (B) The three-dimensional, tertiary structure prediction of *P. aeruginosa* rChiC was derived using AlphaFold. The functional ChiC domains are colour-coded with respect to the domains outlined in 1A.

To understand the contributions of ChiC to the entomopathogenicity of these insecticidal strains, the *chiC* gene of *P. aeruginosa* was cloned into an expression vector pET-28 which contains the kanamycin resistance *kanR* gene (7). A 0.8 kDa 6X-polyhistidine (6X-His) tag was added at the N-terminus of the recombinant ChiC (rChiC) protein product, which allows for protein purification using immobilized metal affinity chromatography (IMAC). The constructed plasmid (named pM3CRYY) was transformed into *Escherichia coli* BL21 (DE3) which lacks the *lon* and *ompT* proteases, thus making it an appropriate model organism for recombinant protein expression as it does not degrade foreign protein (8–10). The pET-28 plasmid backbone contains a T7 promoter, which is usually repressed by the lac repressor. However, inducers like IPTG bind to the lac repressor causing it to dissociate, promoting T7

promoter activity and triggering *chiC* expression. The use of IPTG as an inducer allows for precise and adjustable protein production (11, 12).

Proper recombinant protein expression may be challenging due to the production of inclusion bodies, which are insoluble aggregates of recombinant proteins. Consequently, the conditions for soluble recombinant gene expression need to be experimentally determined in *E. coli* BL21 (DE3) since cellular environmental conditions may differ from those seen in the host organism, *P. aeruginosa*. Protein induction may be performed using a wide range of IPTG concentrations (0.005-5 mM), but 1 mM IPTG is most frequently used for expression (13). However, complete induction with 1 mM IPTG can lead to increased metabolic load on the host resulting in incorrect protein folding, aggregation, and the formation of inclusion bodies (14). Similarly, temperature and time of incubation have also been shown to influence recombinant protein expression. Slower rates of protein expression give the newly transcribed protein an opportunity to properly fold, while low incubation temperatures promote weak hydrophobic interactions, resulting in reduced protein aggregation as compared to higher temperatures where these interactions are stronger (10).

Although previous studies have demonstrated the production of rChiC in *E. coli* BL21 (DE3), the optimal conditions to produce soluble rChiC have yet to be elucidated. In this study, we aimed to investigate the optimal conditions for soluble rChiC expression. We hypothesized that lower temperature, lower IPTG inducer concentration, and longer incubation time would provide the optimal set of conditions for soluble rChiC expression. To test this hypothesis, we investigated various combinations of conditions for soluble rChiC expression using a factorial design. Different IPTG induction concentrations (0.1 mM, 0.5 mM, 1.0 mM), incubation times (1 hr, 3 hrs, and 24 hrs), and incubation temperatures (20°C, 30°C, and 37°C) were tested. We then determined the optimal conditions for rChiC purification via IMAC and tested the functionality of the rChiC CBD with an in vitro functional assay using chitin resin beads.

From the tested parameters, we found that the highest proportion of soluble rChiC was expressed at the lowest temperature (20°C), the lowest IPTG induction concentration (0.1 mM), and the longest incubation time (24 hours). Additionally, while previous studies have eluted His-tagged proteins with imidazole during nickel IMAC, our study demonstrates the elution of rChiC using histidine as an alternative competitive agent. This choice was driven by the availability of histidine at the time of experimentation. Furthermore, we determined that storage of rChiC in 1x TBS at 4°C, -20°C, or -80°C without cryoprotectant is adequate for protein preservation. Lastly, the chitin-binding assay we performed on rChiC revealed that it has a functional CBD. This study offers insight into methods that could be applied to the large-scale expression of soluble, purified rChiC that retains chitin-binding activity, potentially aiding in the development of novel bio-insecticides in place of synthetic ones.

METHODS AND MATERIALS

Bacterial Culture. *E. coli* B21 (DE3) were cultured at 37°C in Luria-Bertani (LB) broth or agar plates supplemented with 25 µg/mL kanamycin. For induction of recombinant ChiC (rChiC) expression, LB broth supplemented with 0.1 mM, 0.5 mM, and 1.0 mM IPTG was used. Cultures were grown under constant shaking at 200 rpm at 20°C, 30°C, or 37°C.

Plasmid Isolation and Verification. Overnight culture of pM3CRYY transformed *E. coli* BL21 (DE3) grown in 5 mL of 25 µg/mL-LB kanamycin was used for plasmid extraction following the manufacturer's instructions (Plasmid MiniPrep Kit, BioBasic). The *chiC* insert in the pET28 pM3CRYY plasmid was confirmed via Sanger sequencing (GeneWiz) using the custom primers: 5'-TAT CCG AAT TCA TGA TCA GGA TCG ACT T -3' (forward primer) and 5'-AAT ACA AGC TTT CAG CGC AGC GG -3' (reverse primer), and was aligned against the canonical *P. aeruginosa* chiC gene using NCBI BLAST (9).

Optimization of conditions for rChiC expression using a factorial design. A factorial design for the optimization of rChiC was employed using three levels of IPTG concentration, temperature, and induction time. A culture containing recombinant *E. coli* BL21 was diluted (1:100) with fresh LB-kanamycin broth to a total of 30 mL and grown at 37°C to an OD_{600} of 0.4-0.8. The cells were centrifuged (4,000 rpm, 10 min) and resuspended in fresh 25µg/mL

LB-kanamycin media. As the uninduced negative control, 2 mL of this culture was preserved. Cultures were incubated in 8 separate tubes that varied in three conditions: IPTG concentration (0.1 mM, 1 mM), temperature (20°C, 37°C), and induction time (1hr, 24hr). An additional tube with midpoint conditions (0.5 mM IPTG, 30°C, 3hr) was prepared for a total of 9 experimental samples. Cell growth after complete induction time was measured by OD_{600} and normalized to the OD_{600} of the negative uninduced control (0 mM IPTG, 20°C, 0 hr). Cells were lysed via Bead Beater Homogenizer (MP FastPrep®-24) and separated into soluble (supernatant) and insoluble (pellet) fractions via centrifugation (13,000 rpm, 10 min). 2X Laemmli Sample Buffer with 10% β-mercaptoethanol (BME) (Bio-Rad) was added to the supernatants at a 1:1 ratio and 40 µL of 10% BME was added into pellets. Samples were denatured at 95°C for 5 mins and stored at -20°C before analysis by SDS-PAGE.

Cell Lysis by Bead Beater Homogenization. Induced *E. coli* BL21 (DE3) cultures were split into 1 mL fractions, resuspended in LB broth or 1X TBS, and lysed using the Bead Beater Homogenizer (MP FastPrep®-24) using 0.1g of 0.1 mm glass beads (BioSpec Products, Inc) at 4 m/s for 80 seconds. Cell lysates were collected, centrifuged at 13,000 rpm for 10 min, and separated into soluble (supernatant) and insoluble (pellet) fractions. The fractions were resuspended in 2X Laemmli Sample Buffer with 10% BME (Bio-Rad) in a 1:1 ratio and heated to 95°C for 5 mins. The fractions were stored at -20°C for subsequent analysis by SDS-PAGE.

SDS-PAGE. Cell lysate and protein fractions stored in Laemmli Sample Buffer with 10% BME (Bio-Rad) were thawed on ice, denatured at 95°C for 5 mins, and centrifuged (8,000 rpm, 20 sec). Samples were separated by SDS-PAGE using the Bio-Rad protein electrophoresis chamber system with either 4–20% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad) or gels made from TGX Stain-Free™ FastCast™ Acrylamide Solutions (Bio-Rad). 10 µL of Laemmli buffer-sample solutions alongside 5-10 µL of Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher) or Precision Plus Protein™ Unstained Protein Standards Ladder (Bio-Rad) were loaded into each well. Gels were run at 100 V for 15 min and then 200 V for 20 - 25 min with 1X TRIS-base/Glycine/SDS running buffer. SDS-PAGE gels were imaged using the ChemiDoc Imaging System (Bio-Rad).

Immobilized Metal Affinity Chromatography (IMAC). 6X-His tagged rChiC was purified using 0.6 mL of Ni-NTA resin (ThermoFisher) in a chromatography column. The column was equilibrated with 1X TBS (20 mM Tris/HCl, 200 mM NaCl, pH 7.5) and washed with 1X TBS supplemented with 5 mM histidine and the soluble cell lysates were incubated on the column for 10 min. The protein was eluted with buffers consisting of 1X TBS supplemented with increasing concentrations of histidine (20 mM, 200 mM, 350 mM, 500 mM). Eluted samples were collected in 1 mL aliquots in Eppendorf tubes and placed on ice. Samples were analyzed by SDS-PAGE.

Dialysis and Storage of Soluble rChiC under different conditions. IMAC purified protein was dialyzed in 15mm Spectra-Por dialysis tubing (Spectra/Por® Membrane, MWCO: 12-14,000, ThermoFisher) for 4 hours at 4°C in 1X TBS. The dialyzed proteins were stored in 1 mL aliquots in Eppendorf tubes at 4°C, -20°C, or -80°C, with or without 25% glycerol. Protein concentrations were normalized prior to SDS-PAGE analysis.

Chitin Binding Assay. 40 µL of purified rChiC samples were incubated overnight with 160 µL of chitin resin beads (New England Biolabs) at 4°C. Samples were washed with 150 uL wash buffer (20mM Tris-HCl, pH 8.5, 0.5M NaCl) and spun down at 8000 rpm for 10 seconds. Washes were performed 3 times and aliquots of each wash were stored at a 1:1 ratio in 2X Laemmli Sample Buffer with 10% BME(Bio-Rad) for SDS-PAGE analysis. After washing, beads were resuspended in 40 µL 2X Laemmli Sample Buffer with 10% BME (Bio-Rad). All samples were stored at -20°C. The same steps were repeated with 10 µL of 0.5 mg/mL bovine serum albumin (BSA) as a negative control.

RESULTS

***chiC* gene in the pM3CRYY plasmid verified via Sanger sequencing**. We verified the *chiC* gene in the pM3CRYY plasmid via Sanger sequencing (GeneWiz) using forward (5'-TAT CCG AAT TCA TGA TCA GGA TCG ACT T -3') and reverse (5'-AAT ACA AGC TTT CAG CGC AGC GG -3') primers internal to the *rChiC* gene. We analyzed the sequencing results using nucleotide BLAST, revealing a 99% identity with *P. aeruginosa* PAO1 *chiC* gene. The only mismatch was from nucleotide 26 on the Sanger sequencing product, which was assigned N. We then converted the nucleotide sequence to its amino acid sequence using ExPASy Translate. We analyzed this sequence using protein BLAST, revealing 100% identity with *P. aeruginosa* ChiC protein. These results verify that the *chiC* gene present in the pM3CRYY plasmid is the correct gene of interest.

Optimal expression of soluble rChiC observed when *E. coli* BL21 (DE3) is induced at 20°C with 0.1 mM IPTG for 24 hours. To optimize the conditions for soluble rChiC expression in *E. coli* BL21 (DE3) transformed with pM3CRYY, we induced cells under varying temperatures, IPTG concentrations, and incubation times following a factorial design. Subsequently, we normalized samples to the same bacterial concentration (OD_{600}), lysed using a bead beater homogenizer, separated into soluble and insoluble fractions, and resolved using SDS-PAGE prior to visualization. In all conditions, we observed lower amounts of protein in the soluble fraction compared to the insoluble fraction, suggesting that the majority of the expressed rChiC is insoluble (Fig 2A). While the bands seen in the soluble fraction of

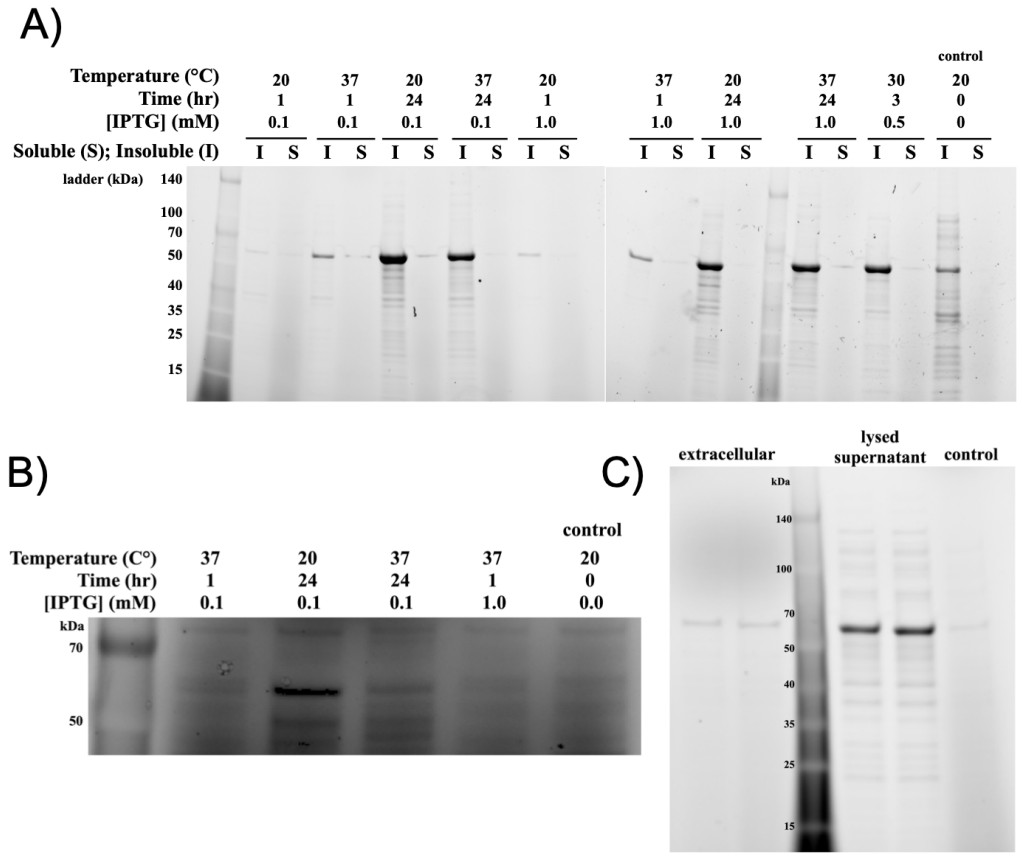

FIG. 2 Factorial design reveals 20°C, 0.1 mM IPTG, and 24 hours of induction as optimal conditions for the expression of soluble rChiC in *E. coli* BL21 (DE3). (A) 9 samples of 1 mL pM3CRYY-transformed *E. coli* BL21 (DE3) were induced to express *rChiC* under different temperatures, IPTG concentrations, and incubation times following a factorial design. Insoluble (I) and soluble (S) fractions of lysed cells were run on SDS-PAGE and visualized under UV illumination at auto-exposure. (B) Soluble fractions in 1A were run on SDS-PAGE and visualized under UV illumination at 45-sec exposure. (C) Protein induction was scaled up (*E. coli* BL21 (DE3) in 30 mL LB-kan, 0.537 OD_{600}) under the most optimal conditions for soluble rChiC expression (20°C, 0.1 mM IPTG, 24 hours). Cells were centrifuged, resuspended in 1X TBS, lysed, and separated into extracellular, soluble intracellular (supernatant) by centrifugation, and insoluble fractions. Extracellular and soluble (supernatant) fractions were loaded in duplicates. Negative control in (A, B, C) is uninduced *E. coli* BL21 (DE3) incubated for 0 hrs at 20°C with 0.0 mM IPTG. The ladder is Spectra ™ Multicolor Broad Range Protein Ladder (ThermoFisher).

all the conditions were faint (Fig 2A), greater amounts of soluble rChiC were visually estimated in conditions induced with the lowest tested IPTG concentration (0.1mM) compared to induction with higher IPTG (1.0 mM) and the uninduced control. Interestingly, the uninduced control showed detectable amounts of rChiC, suggesting leaky expression of the pM3CRYY plasmid.

After identifying the optimal IPTG concentration, we repeated the optimization assay with the soluble fractions of 0.1 mM IPTG-induced samples to assess the impact of different temperatures and times on soluble rChiC expression. From the conditions tested, the most optimal for soluble rChiC expression was 0.1 mM IPTG, 24 hours, and 20°C (Fig 2B). These samples were run alongside a condition that displayed low soluble rChiC expression in Figure 2A (1.0 mM IPTG, 37°C, 1hr) as a point of comparison, verifying that induction under high IPTG concentration, short incubation time, and high-temperature results in noticeably lower amounts of soluble rChiC production.

To scale up the production of soluble rChiC for subsequent IMAC purification, we induced 30 mL *E. coli* BL21 (DE3) culture using the most optimal condition (0.1 mM IPTG, 20°C, 24hr) determined from our factorial experiment (Fig 2B, 2C). Darker bands at ~55 kDa appeared in the lysed supernatant (soluble intracellular) fraction compared to the extracellular fraction (Fig 2C). These results suggest that a larger portion of soluble rChiC remained inside the cell while a smaller portion was either actively secreted or released upon cell death (i.e. lysis) during incubation. The soluble intracellular rChiC produced from the scale-up was subsequently purified using IMAC.

Soluble rChiC optimally eluted with 350mM histidine in IMAC purification. To purify the 6X-His tagged rChiC from the scale-up intracellular soluble fraction, we conducted IMAC purification using Ni-NTA resin as the binding agent. Varying concentrations of histidine elution buffers (1X TBS supplemented with 20 mM, 200 mM, 350 mM, or 500 mM histidine) were used as the agent for competitive elution in a gradient. The SDS-PAGE results of IMAC purified samples exhibited a single ~55 kDa band, with the darkest bands belonging to samples eluted with 350 mM histidine (Fig 3). This is in contrast with the unpurified, crude (flowthrough), and wash buffer samples which had many bands of different molecular weights, but still had their darkest band at ~55 kDa. Our results demonstrate that histidine is an appropriate competitive agent for the elution of His-tagged proteins, with 350 mM being the optimal histidine concentration for eluting 6X-His-tagged rChiC. The purified protein was then dialyzed using Spectra-Por dialysis tubing to remove histidine to prevent it from interfering with downstream experiments.

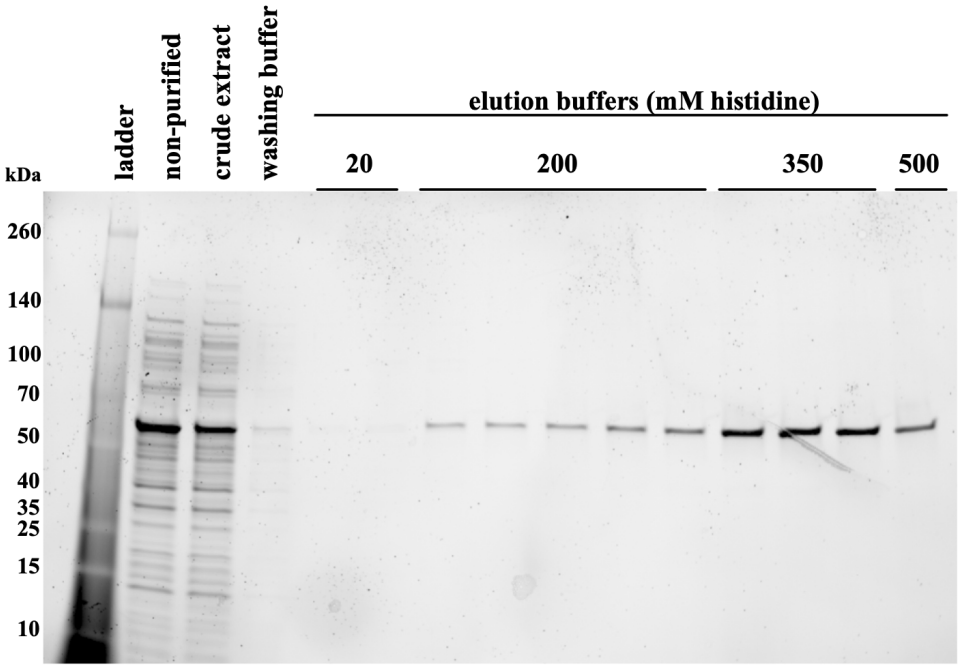

FIG. 3 350 mM Histidine is the most optimal condition for purification of 6X-His tagged rChiC via IMAC. pM3CRYY transformed *E. coli* BL21 (DE3) was induced with 0.1 mM IPTG at 20°C for 24 hours. The soluble (intracellular) fraction from 1C was purified for rChiC via IMAC with 0.6 mL Ni-NTA resin. rChiC was eluted using 20 mM, 200 mM, 350 mM, and 500 mM histidine in 1X TBS (pH 7.5). The negative control is the unpurified soluble cell lysate. The crude extract is the soluble fraction extracted after 10 min incubation in the Ni-NTA resin. The washing buffer contains 5 mM histidine. All samples were run on SDS-PAGE and visualized under UV illumination at auto-exposure. Ladder is Spectra ™ Multicolor Broad Range Protein Ladder (ThermoFisher).

Purified rChiC can be stored at 4°C, -20°C, and -80°C without glycerol cryoprotectant. To determine the effects of different storage conditions on purified, dialyzed rChiC, we stored them under varying temperatures (4°C, -20°C, and -80°C) with or without 25% glycerol. We normalized small fractions from each storage condition for the protein solution dilution factor and stored them in 2X Laemmli Sample buffer at different time points (Fig 4). One week after storage, we observed that all samples without glycerol developed insoluble, white needle-like crystals at the bottom of the storage tubes, indicative of protein precipitation (Supplemental Fig S1). Samples that were stored in 25% glycerol did not develop protein precipitates, suggesting that glycerol promoted rChiC solubilization. However, these samples had very low amounts of soluble proteins at all time points under all temperature conditions (Fig 4). This implies that while glycerol solubilizes rChiC, it did not aid in the preservation of our samples. Protein degradation was not observed over time in samples stored without glycerol, suggesting that the protein is stable for short-term storage under these conditions (Fig 4).

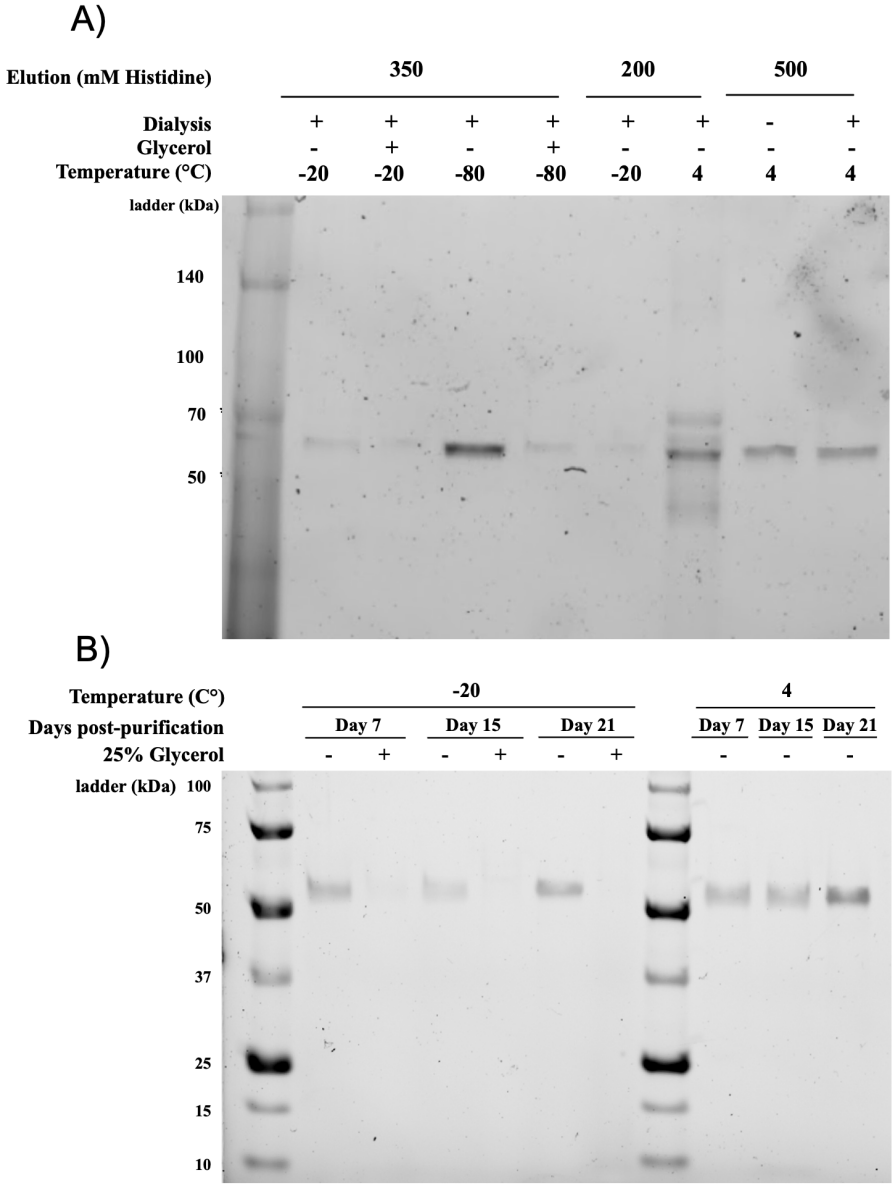

FIG. 4 Variable amounts of rChiC preserved in storage conditions differing in time, presence of glycerol, and temperature. The purified, dialyzed rChiC was stored in 1X TBS (pH 7.5) at 4°C, -20°C, or -80°C with or without 25% glycerol. Aliquots of the stored samples were ran on a SDS-PAGE (A) after 9 days and (B) at three different time points (Day 7, Day 15, Day 21). Samples were normalized for solvent volume, run on SDS-PAGE, and visualized under UV illumination at auto-exposure. The different storage conditions and time points of the collection are shown in the figure. Histidine concentrations of the sample when eluted from the IMAC are shown in figure (A). Ladder is Spectra ™ Multicolor Broad Range Protein Ladder (ThermoFisher) (A) and Precision Plus Protein™ Unstained Protein Standards (Bio-Rad) (B).

While the protein seems to be equally stable at 4°C and -20°C, it appears to be most stable under -80°C (not shown in figure) as the highest amount of protein was present under this condition across all time points. In addition, we did not observe any degradation trends at any of the storage temperatures over 21 days (Fig 4B, not shown for -80°C). Based on the SDS-PAGE results and visual inspection of stored samples, we could not identify an ideal storage

condition for long-term protein preservation. However, our results suggest that rChiC can be stored short-term at 4°C, -20°C, and -80°C without glycerol (Fig 4, S1).

Purified rChiC binds to chitin beads. To test for a functional CBD, we incubated rChiC with 40 µL chitin resin beads overnight. Input lanes demonstrate the size and quantity of protein incubated, serving as a relative comparison for the washes and SDS-elution fractions. The beads were then washed three times with 1X TBS, centrifuged, and treated with 2X Laemmli Sample buffer to denature and elute any protein bound to the beads. The washes and final elution fractions were run on SDS-PAGE. We observed a ~55 kDa band in the elution (SDS-denatured) fraction, which was not observed in the washes (Fig 5A). This demonstrates that the purified rChiC binds to the chitin resin, suggesting it contains a properly folded and functional CBD.

To confirm the binding specificity of the chitin resin, we used 0.5 mg/mL BSA as a negative control (Fig 5B). Following incubation of BSA in the chitin resin, protein bands were observed in the washes of chitin beads but were absent in the elution (SDS-denatured) fraction. This suggests that the binding to the chitin resin is specific for rChiC. While protein samples stored at 4°C and -80°C in the absence of glycerol had a functional CBD as shown by the ~55 kDa band in their elution fractions, no protein was observed in the elution fraction of beads incubated with rChiC stored in glycerol (Fig 5A). However, rChiC stored in glycerol also showed no protein in the input control lane (Fig 5A), suggesting that no protein was incubated with the beads. Thus, no conclusions can be made regarding the functionality of proteins stored in glycerol at -20°C.

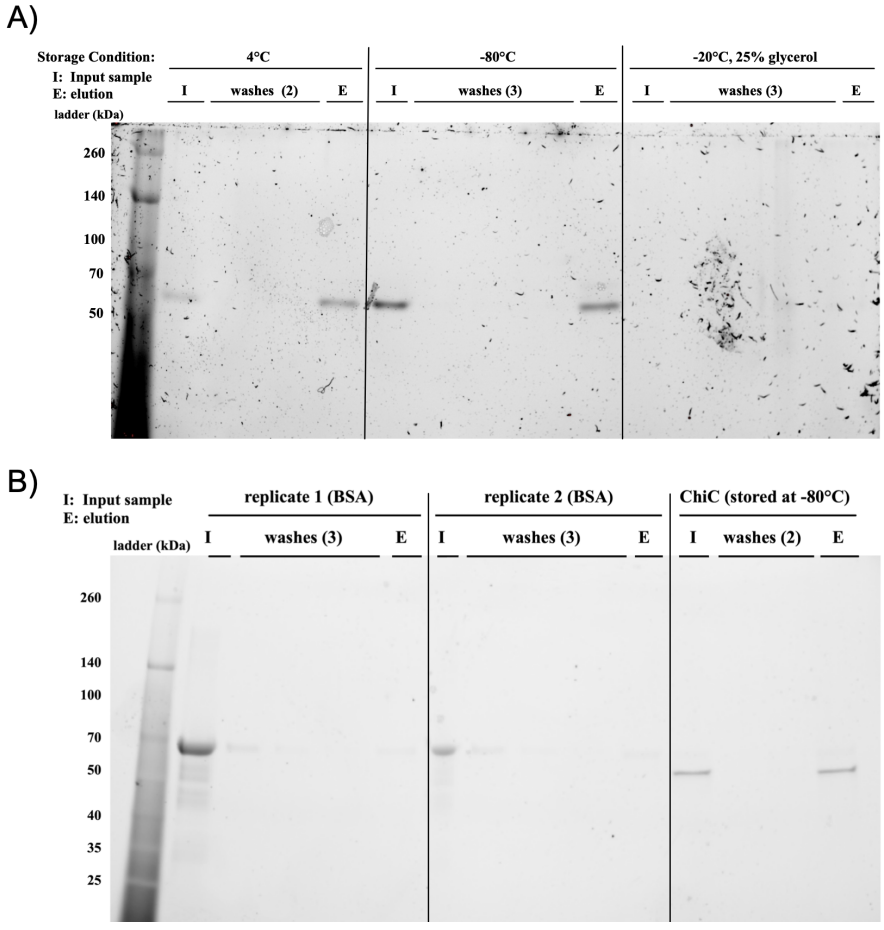

FIG. 5 Chitin-binding domain of rChiC retains function. (A) Purified rChiC from different storage conditions were incubated with 40 µL chitin resin beads overnight, washed with 1X TBS, and eluted in 2X SDS Laemmli Sample buffer with 10% BME. Input, wash, and elution samples were run on SDS-PAGE and visualized with UV illumination under auto-exposure. The different storage conditions and elution fractions are highlighted in the figure. (B) The negative control is 0.5 mg/mL BSA. Purified rChiC stored in -80°C with no glycerol was incubated with 40 µL chitin resin beads overnight, washed with 1X TBS, and eluted in 2X SDS Laemmli Sample buffer with 10% BME. The ladder is Spectra ™ Protein Ladder with 10 µL used in (A) and 5 µL in (B).

DISCUSSION

In this study, we optimized the expression of soluble rChiC in pM3CRYY transformed *E. coli* BL21 (DE3), purified the protein via IMAC, and performed a preliminary functional characterization of its chitin-binding domain. We found that soluble rChiC was optimally expressed under the lowest tested IPTG concentration of 0.1 mM, lowest tested incubation temperature of 20°C, and longest tested incubation time of 24 hours. Subsequently, we determined that the purified rChiC, which was optimally eluted from an IMAC column using 350 mM histidine, had a functional chitin-binding domain as determined by the chitin bead binding assay. In addition, our study suggests that rChiC can be kept in 1X TBS at 4°C, -20°C, and -80°C without glycerol cryoprotectant for short-term storage.

A factorial experiment testing three condition parameters showed that samples induced with 0.1 mM IPTG, the lowest tested concentration, generally showed a greater relative amount of soluble rChiC than at higher concentrations of IPTG (Fig 2A). This is consistent with our hypothesis that lower IPTG concentrations minimize host metabolic load, thereby promoting soluble recombinant protein expression. Longer induction time (24 hours) and lower temperature (20°C) also improved soluble rChiC expression, though this only became apparent after closer inspection of the soluble fractions induced with 0.1 mM IPTG (Fig 2B). The effect of time is consistent with previous studies which have reported optimal expression of recombinant proteins at ~72 hours of protein induction (15). While these induction times will depend on the nature of the host and protein, better soluble recombinant protein expression is generally observed at longer rather than shorter induction periods possibly due to longer induction times promoting proper protein folding (11, 16). Additionally, our results displayed optimal incubation temperature at 20°C from the temperatures tested, aligning with our hypothesis that lower temperatures improve soluble rChiC expression potentially due to reduced inclusion body formation due to fewer hydrophobic interactions. Previous studies have reported maximal soluble recombinant protein production at temperatures between 15-20°C in *E. coli*, with some studies reporting that temperatures as low as 4°C improve recombinant protein solubility (16–18). Two points of contention within the results of our factorial experiment include the presence of a ~55 kDa band in the uninduced control and consistently greater proportions of insoluble rChiC relative to soluble rChiC (Fig 2A). The ~55 kDa band in the uninduced control is likely due to leaky expression caused by analogues in the yeast extract of LB media, similar to IPTG, which can bind the *lac* repressor and enable the expression of genes with the T7 promoter. This renders our uninduced sample a less suitable negative control but does not necessarily affect our objective of investigating and characterizing soluble rChiC. Likewise, although the majority of expressed rChiC was insoluble, the detection of rChiC within the soluble fractions was sufficient evidence for the presence of soluble rChiC. Thus, we proceeded with scaling up soluble rChiC expression under 0.1 mM IPTG, 20°C, and 24-hour induction. In our study, we reported the effects of three temperatures (20, 30, 37°C) and induction times (1, 3, 24 hours) within a small range, so future studies may choose to further optimize soluble rChiC expression by exploring lower temperatures and longer induction times.

Throughout our experiment, we lysed cells by pelleting cultures, resuspending in either LB broth or 1X TBS, and subjecting them to bead beater homogenization. Mechanical lysis by bead beating was chosen to minimize the number of compounds and reagents that may interfere with downstream experiments while maximizing protein extraction. Heat lysis was not used as it may denature proteins resulting in aggregation, precipitation, and overall loss of properly folded soluble protein. Interestingly, when bead beating, there was less foaming and more protein collected when cells were lysed in TBS versus LB. It is possible that the comparatively low viscosity of TBS contributed to improved lysis by bead-beating and thus, the collection of a greater amount of intracellular soluble rChiC. Nevertheless, bead beating may be insufficient for complete cell lysis, so to maximize intracellular protein extraction, future studies should consider resuspending cells in a Triton X-100 lysis buffer, which is less dense than LB broth, prior to bead beater homogenization.

While verifying the expression of soluble rChiC in the scale-up, we noticed a small proportion of ~55 kDa protein in the extracellular fraction (Fig 2C), which may suggest that *E. coli* BL21 (DE3) has a secretion mechanism for rChiC. Rocha et al. previously hypothesized that rChiC would be secreted in *E. coli*, but their experiment was unable to show

any rChiC secretion (9). One possible explanation is that secretion is time-dependent, thus their 2-hour induction, compared to our 24-hour induction, may have been too short to detect any secretion. Alternatively, it is possible that some cells reached the death phase during overnight incubation and released their intracellular contents into the media. To determine if the protein is being actively secreted in *E. coli* BL21 (DE3), future projects can perform a pulse-chase experiment, which is a common method for monitoring the transport and secretion of proteins (19). This experiment involves exposing cells to radioactive amino acids and examining the trajectory of the radiolabeled protein of interest using real-time autoradiography. If the result of this experiment shows that rChiC is actively secreted, the Tat and Sec pathways could be investigated since they are commonly found in *E. coli* and have been shown to be involved in the secretion of recombinant proteins (20). If rChiC is found to not be secreted, future works can investigate ways to engineer a secretion mechanism in *E. coli* BL21 (DE3). Since previous studies have confirmed that the ChiC protein is actively secreted from *P. aeruginosa* PAO1 over time, it would be interesting to elucidate the corresponding genes comprising the ChiC secretion system using a forward genetics screen. Subsequently, the identified genes could be cloned into *E. coli* BL21 (DE3) to facilitate the active secretion of soluble rChiC.

Previous studies have conducted a comparable genetic screen in *S. marcescens,* whose ChiC protein is homologous to the one found in *P. aeruginosa.* It was found that mutants with a defective *chiW* gene, which encodes a member of the holin family of proteases, were unable to secrete ChiC which remained trapped in the periplasm. Further investigation by Hamilton et al. revealed that in addition to ChiW, an L-alanyl-D-glutamate endopeptidase ChiX is also required for ChiC secretion (21). It is currently hypothesized that ChiW and ChiX work together to facilitate chitinase secretion, where ChiW acts as a specific inner-membrane holin transporter for the ChiX endopeptidase. The ChiX then clears a path through the peptidoglycan layer for the chitinolytic enzymes to navigate to the cell exterior. Despite the identification of this novel potential secretion pathway, how the ChiC protein crosses the inner cell membrane is still unknown. Additionally, whether this secretory system is conserved in *P. aeruginosa* is yet to be explored.

From our factorial experiment and scale-up, we observed rChiC expressed as an intracellular soluble, insoluble, or potentially secreted protein. Thus, we considered three potential pathways for further investigation and purification. One option was to conduct native protein purification of the soluble intracellular rChiC from our scale-up. Another approach was to focus on insoluble protein purification and refolding using a protein denaturant such as urea. Lastly, the potentially secreted soluble protein could have been collected and purified via native protein purification. We proceeded with the first option, as soluble rChiC was likely already in its native conformation and thus most likely to be correctly folded and retain functionality (22). Although the insoluble fractions contained the largest quantity of rChiC, denaturing and refolding insoluble protein aggregates can be an inconvenient process requiring a variety of reagents. In addition, we observed a greater amount of soluble rChiC in the intracellular soluble cell fraction compared to the extracellular fraction. Together with the uncertainty surrounding a possible rChiC secretion mechanism in *E. coli* BL21 (DE3), we proceeded with the native purification of intracellular soluble protein.

Intracellular soluble rChiC from the scale-up was purified using immobilized metal affinity chromatography (IMAC) with Ni-NTA (nickel) resin beads. The 6X-His tag on the N-terminal of rChiC preferentially binds to the nickel beads while all other proteins elute out of the column when washed with a buffer. In IMAC, imidazole is normally used as a competitive agent for displacing the bound protein of interest from the Ni-NTA resin. In place of imidazole, we used histidine, an amino acid that contains an imidazole ring as its organic R-group and is thus structurally and chemically similar to imidazole. We hypothesized that histidine could replace imidazole as a competitive agent for the elution of bead-bound rChiC. Furthermore, since different proteins exhibit different binding affinities to the Ni-NTA resin, the concentration of the competitive agent for protein elution must be optimized. We used a gradient of histidine concentrations to elute the rChiC and found that optimal elution occurred using 350 mM histidine in 1X TBS (Fig 3). This is consistent with a previous study that demonstrated His tagged proteins eluting under an imidazole concentration of at least 200 mM or greater (23). However, very high concentrations of imidazole (~500 mM) have been

shown to decrease the yield of eluted protein. Thus, high protein quality and yield are expected when His tagged proteins are eluted with histidine concentrations above 200 mM and below 500 mM (24). This is consistent with the thickest band of purified rChiC protein appearing in the 350 mM histidine elution (Fig 3). Our study sheds light on the use of histidine as an alternative to imidazole for the purification of His-tagged proteins in IMAC using Ni-NTA resin. Our work also demonstrates that rChiC can be purified and eluted from an IMAC column in its native conformation without using a denaturing buffer, which is often needed to expose N or C-terminal His tags for the purification of other recombinant proteins.

Aliquots of IMAC purified rChiC were stored in 1X TBS with or without 25% glycerol under varying temperatures (4°C, -20°C, -80°C). After one week of storage, 4°C samples stored without glycerol contained translucent needle-like crystals (Supplemental Fig S1B). Likewise, rChiC stored at -20°C and -80°C displayed white, snow-like protein precipitates. In the presence of glycerol, we did not observe any visible signs of protein precipitation, suggesting that glycerol helps solubilize the rChiC. However, SDS-PAGE analysis revealed that samples stored in glycerol retained less rChiC compared to those stored without it (Fig 4), suggesting that glycerol may have a negative effect on the preservation of rChiC. Our observations contrast previous studies, where the formation of a protein-glycerol interface prevents protein aggregation by disrupting the hydration shell surrounding potential hydrophobic regions of the protein (25). Future investigations can focus on understanding glycerol-rChiC interactions, and how it contributes to protein instability. Determining optimal conditions for rChiC storage to prevent protein precipitation and crystallization is crucial as protein aggregates are non-functional and difficult to recover. In terms of storage temperature, SDS-PAGE analysis showed that rChiC is stable when kept at 4°C, -20°C, or -80°C without glycerol for short-term storage. Specifically, samples stored at -80°C retained the greatest amount of rChiC 7 days after IMAC purification, while those stored at 4°C and -20°C demonstrated roughly equal levels of protein retention (Fig 4A). By 21 days of storage, there were no apparent degradation patterns in any of the storage conditions. Future studies can explore whether these conditions are appropriate for long-term storage.

Using our stored rChiC samples, we conducted a chitin-binding assay to test the functionality of the chitin-binding domain (CBD). In this assay, we incubated rChiC samples overnight with chitin resin beads, which are normally used to purify recombinant proteins bearing a CBD tag. After washing, we eluted bead-bound proteins using 2X Laemmli Sample buffer, which contains SDS that denatures proteins and releases them from the beads. Our assay showed that rChiC from samples stored at 4°C and -80°C without glycerol retained function in their CBD. This also verifies that storage with glycerol is not necessary for preserving rChiC CBD functionality, at least for short-term storage. Interestingly, the input sample stored at -20°C with glycerol did not contain any ~55 kDa band, so no conclusion could be drawn on whether the presence of glycerol affects CBD functionality. While our results confirm the chitin-binding capability of the rChiC CBD, we cannot fully extrapolate these results to the functionality of the hydrolytic domain and fibronectin-like III domain. Future studies can focus on investigating the chitinolytic domain of rChiC using a colloidal chitin plate assay or an *in vivo* insecticidal assay with *Drosophila melanogaster*. Likewise, future projects can explore the potential functions of the fibronectin-like III domain, namely determining what proteins it binds to using co-immunoprecipitation.

Similar studies that expressed rChiC from other organisms in *E. coli* have also observed retention of CBD functionality. One such study subcloned *Streptomyces griseus* rChiC, expressed it in *E. coli* BL21 (DE3), and found that rChiC exhibited chitin-binding activity on both soluble and insoluble chitin (25). Interestingly, when the CBD was removed from the recombinant plasmid, the delta-ChiC exhibited a 50% and 90% decrease in chitinolytic and antifungal activities respectively (26). Furthermore, a study done by Hashimoto et al. demonstrated that deletion of rCBD from *Bacillus circulans* ChiA1 was not strictly required to hydrolyze β-chitin microcrystals but greatly enhances degradation efficacy (27). Since our study was able to obtain purified rChiC with a functional CBD, the results of Itoh et al. and Hashimoto et al. propound that a properly folded, functional CBD contributes to the functionality of the chitinolytic domain; this suggests that the functional CBD from the purified rChiC in our study may aid in the proper folding and presence of a functional chitinolytic domain.

Likewise, Souse et al. expressed and purified His-tagged recombinant *Chromobacterium violaceum* chitinase (CvChi47) in *E. coli* with an experimental design analogous to ours using Nickel Affinity Chromatography (28). The study verified CBD functionality using a chitin matrix whereby purified protein was incubated with the matrix and any unbound protein was washed off. This was similar to the chitin-binding assay conducted in our study. The purified CBD-binding protein also exhibited significant chitinolytic activity, but only under a specific temperature and pH range, with optimal activity observed at 60°C and pH = 5 (97.2% chitin degradation) (28). Future investigations could assess the chitinolytic activity of the rChiC obtained in our study using the temperature and pH conditions determined by Sousa et al. and optimize using a factorial design.

Overall, the optimal expression, purification, and storage of ChiC could aid in the mass production of rChiC for use as a bioinsecticide once its chitinolytic activity is confirmed. Previous studies have demonstrated the insecticidal activity of rChiC from *S. marcescens* against the larvae of *Malacosoma neustria* (lackey moth) and *Helicoverpa armigera* (cotton bollworm). These insects are potent pests for various agricultural crops including cotton, tomatoes, maize, and citrus (29). The insecticidal assays using rChiC revealed 66% and 50% insecticidal activities against the *M. neustria* and *H. armigera* larvae respectively. Similarly, ChiC from *Pseudomonas sp.* enhanced the insecticidal activity of SpltNPV, a baculovirus, by 1.78-fold after 192 hours of ingestion. This suggests that the *Pseudomonas* ChiC, in addition to exhibiting insecticidal activity on its own, could also potentially increase the pathogenicity of baculoviruses (30).

Conclusions In conclusion, soluble rChiC (from *P. aeruginosa* PAO1) was expressed in *E. coli* BL21 (DE3) under 0.1 mM IPTG, 20°C, and 24 hours. Soluble rChiC can be purified via IMAC using 350 mM histidine as the competitive agent and can be stored in 1x TBS at 4°C, -20°C, or -80°C without cryoprotectants. Lastly, soluble rChiC retained chitin-binding functionality, suggesting its proper folding and potential chitinolytic activity. Further testing is required to confirm its chitinolytic activity and assess its functional significance.

Future Directions Besides the future directions outlined in our discussion, short-term future investigations could examine the biophysical characteristics of the purified rChiC using circular dichroism (CD) spectroscopy. This would help elucidate the percent composition of alpha helices and beta sheets, aiding in the secondary structure characterization of the protein. Additionally, we can probe the tertiary structure of rChiC using limited proteolysis, which involves nonspecific digestion of peptides using proteolytic enzymes such as trypsin. SDS-PAGE of digested fragments can be compared to the tertiary structure of ChiC predicted by AlphaFold (Fig 1). The pursuit of structural characterization will form the cornerstone for more accurate functional characterization, enabling a deeper exploration of its mechanisms and broader biological implications.

Future investigations could also explore the use of certain chemical additives to enhance soluble recombinant protein expression. Specifically, adding osmotic stress-inducing chemicals is known to increase the synthesis of osmolytes in *E. coli* cells, which act as ‘chemical chaperones’ by promoting the refolding of unfolded recombinant proteins (31). Similarly, the addition of ethanol to the growth media can improve the solubility of recombinant proteins as ethanol elicits a heat-shock response in the *E. coli* cells (16, 32). This response induces the production of chaperones and co-factors that facilitate the proper folding of recombinant proteins. Such additives could be investigated for enhanced expression of soluble rChiC. Likewise, future investigations could focus on a two-step incubation system where the induced culture is heat shocked at a high temperature to induce the synthesis of chaperones, followed by incubation at ~20°C. This method has been shown to improve the expression of several soluble recombinant proteins whilst mitigating the production of inclusion bodies (31).

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CONTRIBUTIONS

Laboratory Work. Apsara took the lead in plasmid verification, bioinformatic analysis, and storage. Haein took the lead in scale-up production and IMAC. Leo and Davey took the lead in the chitin-binding assay. The team collaborated on the factorial experiment.

Manuscript. Apsara worked on the abstract, introduction, methods, figure captions, results, discussion, and edited all sections of the manuscript. Haein worked on the methods, figures, figure captions, results, discussion, and edited all sections of the manuscript. Leo worked on the introduction, methods, results, and discussion, and edited all sections of the manuscript. Davey worked on the figure captions, methods, discussion, references, and edited all sections of the manuscript.

REFERENCES

1. **Folders J, Algra J, Roelofs MS, van Loon LC, Tommassen J, Bitter W.** 2001. Characterization of *Pseudomonas aeruginosa* Chitinase, a Gradually Secreted Protein. J Bacteriol **183**:7044–7052.
2. **Fujii T, Miyashita K.** 1993. Multiple domain structure in a chitinase gene (chiC) of *Streptomyces lividans*. Microbiology **139**:677–686.
3. **Singh G, Bhalla A, Bhatti J, Chandel S, Rajput A, Abdullah A, Andrabi S, Kaur P.** 2013. Potential of Chitinases as a Biopesticide against Agriculturally Harmful Fungi and Insects. Research and Reviews: Journal of Microbiology and Biotechnology **3**:27–32.
4. **van der Werf HMG.** 1996. Assessing the impact of pesticides on the environment. Agriculture, Ecosystems & Environment **60:**81–96.
5. **Fenibo EO, Ijoma GN, Matambo T.** 2022. Biopesticides in Sustainable Agriculture: Current Status and Future Prospects, p. 1–53. *In* Mandal, SD, Ramkumar, G, Karthi, S, Jin, F (eds.), New and Future Development in Biopesticide Research: Biotechnological Exploration. Springer Nature, Singapore.
6. **Bork P, Doolittle RF.** 1992. Proposed acquisition of an animal protein domain by bacteria. Proc Natl Acad Sci U S A **89**:8990–8994.
7. **Chan N, Pannu S, Xiong L, Yeung D.** 2022. Construction of inducible expression vector pDNLS-22n containing *P. aeruginosa* PAO1 ChiC. Undergraduate Journal of Experimental Microbiology and Immunology **27.**
8. **Jeong H, Kim HJ, Lee SJ.** 2015. Complete Genome Sequence of *Escherichia coli* Strain BL21. Genome Announcements **3**:e00134-15.
9. **Rocha M, Yap M, Yoon M**. 2022. Expression of *Pseudomonas aeruginosa* PAO1 Chitinase C in *Escherichia coli* BL21 (DE3). Undergraduate Journal of Experimental Microbiology and Immunology **27**.
10. **Rosano GL, Ceccarelli EA.** 2014. Recombinant protein expression in *Escherichia coli*: advances and challenges. Frontiers in Microbiology **5.**
11. **Reznikoff WS, Abelson JN.** 1980. The lac Promoter. 0. Cold Spring Harbor Monograph Archive **7:**221–243.
12. **Mühlmann M, Forsten E, Noack S, Büchs J.** 2017. Optimizing recombinant protein expression via automated induction profiling in microtiter plates at different temperatures. Microb Cell Fact **16:**220.
13. **Donovan RS, Robinson CW, Glick BR.** 2000. Optimizing the expression of a monoclonal antibody fragment under the transcriptional control of the *Escherichia coli* lac promoter. Can J Microbiol **46:**532–541.
14. **Dvorak P, Chrast L, Nikel PI, Fedr R, Soucek K, Sedlackova M, Chaloupkova R, de Lorenzo V, Prokop Z, Damborsky J.** 2015. Exacerbation of substrate toxicity by IPTG in *Escherichia coli* BL21(DE3) carrying a synthetic metabolic pathway. Microbial Cell Factories **14:**201.
15. **Tampubolon PM, Pambudi S, Erlyandi BSP, Sitepu FA, Lestari R.** 2020. Incubation time optimization for the expression of recombinant CHIKV envelope protein E2 in the yeast *Saccharomyces cerevisiae* using SDS-PAGE. AIP Conference Proceedings **2242:**050011.
16. **Bhatwa A, Wang W, Hassan YI, Abraham N, Li X-Z, Zhou T.** 2021. Challenges Associated With the Formation of Recombinant Protein Inclusion Bodies in *Escherichia coli* and Strategies to Address Them for Industrial Applications. Frontiers in Bioengineering and Biotechnology **9**.
17. **Hassan YI, Zhu H, Zhu Y, Zhou T.** 2016. Beyond Ribosomal Binding: The Increased Polarity and Aberrant Molecular Interactions of 3-epi-deoxynivalenol. Toxins (Basel) **8:**261.

18. **San-Miguel T, Pérez-Bermúdez P, Gavidia I.** 2013. Production of soluble eukaryotic recombinant proteins in *E. coli* is favoured in early log-phase cultures induced at low temperature. Springerplus **2:**89.
19. **Hou T, Rinderknecht CH, Hadjinicolaou AV, Busch R, Mellins E.** 2013. Pulse-chase analysis for studies of MHC class II biosynthesis, maturation, and peptide loading. Methods Mol Biol **960:**411–432.
20. **Kleiner-Grote GRM, Risse JM, Friehs K.** 2018. Secretion of recombinant proteins from *E. coli*. Eng Life Sci **18:**532–550.
21. **Hamilton JJ, Marlow VL, Owen RA, Costa M de AA, Guo M, Buchanan G, Chandra G, Trost M, Coulthurst SJ, Palmer T, Stanley-Wall NR, Sargent F.** 2014. A holin and an endopeptidase are essential for chitinolytic protein secretion in *Serratia marcescens*. J Cell Biol **207**:615–626.
22. **Singh SM, Panda AK.** 2005. Solubilization and refolding of bacterial inclusion body proteins. J Biosci Bioeng **99:**303–310.
23. His-tagged Proteins–Production and Purification - CA. https://www.thermofisher.com/ca/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/his-tagged-proteins-production-purification.html. Retrieved 9 December 2022.
24. Optimizing Purification of Histidine-Tagged Proteins. https://www.sigmaaldrich.com/CA/en/technical-documents/technical-article/protein-biology/protein-purification/optimizing-purification-of-histidine-tagged-proteins. Retrieved 9 December 2022.
25. **Vagenende V, Yap MGS, Trout BL.** 2009. Mechanisms of Protein Stabilization and Prevention of Protein Aggregation by Glycerol. Biochemistry **48:**11084–11096.
26. **Itoh Y, Kawase T, Nikaidou N, Fukada H, Mitsutomi M, Watanabe T, Itoh Y**. 2002. Functional Analysis of the Chitin-binding Domain of a Family 19 Chitinase from *Streptomyces griseus* HUT6037: Substrate-binding Affinity and cis-Dominant Increase of Antifungal Function. Bioscience, Biotechnology, and Biochemistry **66:**1084–1092.
27. **Hashimoto M, Ikegami T, Seino S, Ohuchi N, Fukada H, Sugiyama J, Shirakawa M, Watanabe T.** 2000. Expression and Characterization of the Chitin-Binding Domain of Chitinase A1 from *Bacillus circulans* WL-12. Journal of Bacteriology **182:**3045–3054.
28. **Sousa AJS, Silva CFB, Sousa JS, Monteiro JE, Freire JEC, Sousa BL, Lobo MDP, Monteiro-Moreira ACO, Grangeiro TB.** 2019. A thermostable chitinase from the antagonistic *Chromobacterium violaceum* that inhibits the development of phytopathogenic fungi. Enzyme and Microbial Technology **126:**50–61.
29. **Danişmazoğlu M, Demir I, Sezen K, Muratoğlu H, Nalcacioglu R.** 2015. Cloning and expression of chitinase A, B, and C (chiA, chiB, chiC) genes from *Serratia marcescens* originating from *Helicoverpa armigera* and determining their activities. TURKISH JOURNAL OF BIOLOGY **39:**78–87.
30. **Zhong W, Ding S, Guo H.** 2015. The chitinase C gene PsChiC from *Pseudomonas* sp. and its synergistic effects on larvicidal activity. Genet Mol Biol **38:**366–372.
31. **Oganesyan N, Ankoudinova I, Kim S-H, Kim R.** 2007. Effect of osmotic stress and heat shock in recombinant protein overexpression and crystallization. Protein Expression and Purification 52:280–285.
32. **Kusano K, Waterman MR, Sakaguchi M, Omura T, Kagawa N.** 1999. Protein synthesis inhibitors and ethanol selectively enhance heterologous expression of P450s and related proteins in *Escherichia coli*. Arch Biochem Biophys **367:**129–136.