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Refolding of Recombinant Chitinase C of *Pseudomonas aeruginosa* purified from insoluble fractions using IMAC purification

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SUMMARY Chitinase C (ChiC) from *Pseudomonas aeruginosa* can potentially be developed into a bioinsecticide agent, as it can break down chitin, a crucial component of most insect exoskeletons. However, due to the pathogenic potential of *P. aeruginosa,* developing ChiC for use in agricultural industries is not desirable. To express recombinant ChiC (rChiC), previous studies transformed *Escherichia coli* BL21 with pM3CRYY, a pET-28α plasmid containing the *chiC* gene. Although they observed the expression of rChiC in *E. coli,* they did not go further to purify rChiC. The aim of this study was to obtain purified and functional rChiC. We investigated the yield of rChiC expression under different sets of conditions in *E. coli* BL21 transformed with pM3CRYY, followed by purification and refolding of rChiC. The conditions that led to the highest yield of expressed rChiC were found to be 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) concentration with a 2 hrs induction period at 37 °C. At these conditions, the majority of the expressed rChiC was insoluble. The rChiC was purified from the insoluble fraction of the cell lysates using immobilized metal (Ni^{2+}) affinity chromatography under denaturing conditions. Subsequently, we refolded the enzyme through dialysis, which yielded 11.7 g of rChiC per 1 L of *E. coli* culture. The unique pattern of cleavage in limited proteolysis demonstrated that the purified rChiC was refolded into a uniform tertiary structure in dialysis. The successful purification of the refolded rChiC using a chitin-binding column suggested that the enzyme binds to the chitin beads. This suggests that the binding domain of the refolded rChiC is functional. The overall results indicate functional rChiC can be extracted from insoluble fraction of cell lysates with a high yield in a time and cost-efficient manner, which paves the way for future biotechnological applications of ChiC in agricultural industries.

INTRODUCTION

Approximately 45% of annual food production is wasted due to pests, such as insects (1). Many chemical pesticides have been developed; however, they can have negative effects for the environment and human health (2). Pesticides with fewer negative impacts are being researched. Chitin, an insoluble polymer of β-1,4-N-acetyl-d-glucosamine and a major component of the insect exoskeleton (3), can be degraded by the bacterially derived chitinase C, making this enzyme a potential pesticide (4). There are a variety of phylogenetically diverse bacteria, including *Vibrio, Pseudomonas,* and *Flavobacterium* that degrade chitin through the secretion of chitinases (5,6). *Pseudomonas aeruginosa* was shown to have insecticidal capabilities, primarily due to having chitinase C (ChiC) enzyme (7). ChiC of *P. aeruginosa* is a 483-amino acid protein containing a chitin-binding domain and a chitinolytic

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domain, which is responsible for cleaving internal glycosidic bonds in chitin to form oligosaccharides (5).

ChiC from *P. aeruginosa* is a 55-kDa protein (5, 8). It contains three domains, a glycoside hydrolase family 18 (GH18) catalytic domain, a carbohydrate-binding module (CBM) family 5/12, and a fibronectin type III (FN3) domain. The CBM 5/12 (InterPro ID: IPR003610) consists of a 3-stranded β-sheet, which has six aromatic groups that might be significant for binding to chitin (InterPro ID: IPR003610). The catalytic domain cleaves the β-1,4-bond in chitin (InterPro ID: IPR001223). The FN3 domain retains an optimal orientation between the binding and the catalytic domains (5). The secretion mechanism of ChiC from *P. aeruginosa* is unknown (5, 8). It is known that the N-terminal segment of 11 residues of ChiC gets cleaved off (5). The previous studies suggested the rChiC was not secreted from *E. coli* (8), implying that the secretion mechanism might not exist in *E. coli.*

In this study, we explored the purification of recombinant ChiC from *P. aeruginosa*. The pathogenic potential of *P. aeruginosa* makes expression of ChiC in its native host problematic (9). ChiC is also secreted slowly (i.e., over 4 days) from *P. aeruginosa* via an unknown mechanism, further complicating its expression and purification (5). The goal of past studies was cloning the *chiC* gene into a high-expression plasmid for *E. coli* to isolate high amounts of ChiC. Previously, pM3CRYY was created through the insertion of *chiC* gene into a pET28α plasmid (8). The expression of ChiC was also confirmed with SDS-PAGE (8, 10). In this study we set out to purify functional rChiC. Our goal was to obtain a high yield of recombinant ChiC by optimizing the expression of recombinant ChiC in *E. co*li BL21. We were able to purify denatured rChiC protein from the insoluble fraction of transformed *E. coli* BL21 cell lysates using immobilized metal (Ni^{2+}) affinity chromatography (IMAC) under denaturing conditions and refolded the enzyme through dialysis. Utilizing a chitin-binding column, we showed that the purified rChiC has chitin-binding activity. Overall, this study is a stepping stone for biotechnological applications of ChiC in agriculture.

METHODS AND MATERIALS

IPTG Induction. *E. coli* BL21 transformed with pM3CRYY, and *E. coli* BL21 transformed with pET-28α (negative control) were grown overnight in 5ml of LB+50 µg/mL kanamycin. The overnight cultures were diluted 1:25 and incubated at 37 °C, shaking at 200 rpm, until an OD_{600} of 0.7 was reached. For each subculture, uninduced controls were made and stored at -20 °C. Protein expression was induced with different IPTG concentrations, and incubated for 1 or 2 hrs at 37 °C or 20 °C (Tables 1S & 2S). The induced cultures were then normalized so that OD_{600} reached 0.7.

Cell Lysis Using Bead Beating. The uninduced and induced cultures were then centrifuged at 15000 rpm for 5 minutes to separate the extracellular supernatant and cell pellets. The pellet was resuspended in phosphate-buffered saline (PBS) and the cells were lysed by bead-beating for 60 seconds (3 cycles of 20 seconds) using MP FastPrep Beadbeater®. The cell lysates were centrifuged at 15,000 rpm for 5 minutes to separate the soluble and insoluble fractions.

Sample Preparation and SDS-PAGE. For each supernatant or soluble fraction, 2x Laemmli dye with 5% β mercaptoethanol (BME) was added. The pellet was resuspended in 2x Laemmli dye with 2.5% BME. The samples were then heated at 95 °C for 5 minutes to denature proteins. Before loading, each sample was centrifuged for 15 seconds at 15,000 rpm to collect condensations from the walls of the tube. The PageRuler ™ Unstained Broad Range ladder (ThermoFisher Scientific®) or Precision Plus Unstained Standards (Bio-Rad®) and each denatured stained sample was loaded per lane in an SDS-PAGE gel. Both 15-well precast 4-20% Mini-PROTEAN® TGX Stain-Free TM Protein Gels (Bio-Rad®) or TGX FastCast Acrylamide Gels (Bio-Rad®) were used. Gels were run at 200V in 1X SDS-Running Buffer.

Purification of rChiC under denaturing conditions using immobilized metal (Ni^{2+}) affinity chromatography (IMAC). For purification of rChiC, a culture (LB + 50 µg/mL kanamycin) of *E. coli* BL21 transformed with pM3CRYY was grown overnight. The

overnight culture was added to LB + 50 µg/mL kanamycin and grown to OD600 = 0.7 and then induced at 0.1 mM IPTG for 2 hrs at 37 °C. Subsequently, the cells were lysed using bead beating. Following QIAGEN® protocol for purification of His-tagged proteins under denaturing conditions, the ChiC protein were denatured and purified from the lysed cells (11). In brief, the column was equilibrated in the Basis buffer (100mM NaH2PO4, 10mM Tris base and 8M Urea), the sample was applied and allowed to bind at room temperature at pH 8. The column was washed with a wash buffer (Basis buffer at pH 6.3). Then, the protein was eluted using five fractions of Elution Buffer (Basis buffer at pH 4.5). Samples of the crude lysate, cleared lysate, washes and the elution fractions from column were prepared and run as described in *Sample preparation and SDS-PAGE*.

Protein refolding by dialysis. The elution fractions from the purified protein were mixed and diluted in 1:8 ratio with the Basis Buffer from QIAGEN® protocol (10). The diluted purified rChiC from Elution #2 and #3 were pooled and added to the dialysis bag and placed in the Basis Buffer. The dialysis was performed in Basis Buffer with 8 M urea and pH 4.5. The urea concentration in the basis buffer was reduced from 8 mM to 6 mM, to 4 mM and then continuously halved till 0.25 mM and then reduced to approximately 0 mM. The concentration of urea in the sample was reduced using urea-free Basis Buffer, and the sample-containing bag was retained for 2 hrs in each dilution step except for the 1 mM to 0.5 mM dilution, which remained for 12 hrs.

Yield of recombinant Chitinase C from transformed *E. coli* culture. The concentration of the refolded rChiC was then measured using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific®). The theoretical extinction coefficient of 96260 M^{-1}, cm^{-1} at 280 nm, as determined by Expasy ProtParam tool, was used to calculate the protein concentration. We found the concentration of the 1:8 diluted and refolded rChiC to be 0.117 mg/ml. Bovine serum albumin (BSA) control was diluted to achieve similar concentration of rChiC. The rChiC concentration was calculated to be 11.7 g of rChiC per 1 L of transformed *E. coli* BL21 culture.

Limited proteolysis. After dialysis refolding, 100 µl of rChiC sample was incubated with 0.1 µg/ml trypsin, and 10 µl samples were removed and added to 10 µl of 2x Laemmli + 5% BME at times 0, 30 sec, 1 min, 5 min, and 15 min. The same process was carried out for another 100 µl of rChiC sample; however, the samples were added to 10 µl of 2x Laemmli without BME. The samples were then heated at 95°C for 5 min and analysed using SDS-PAGE.

Chitin-binding column chromatography. rChiC and BSA each with a concentration of 11.7 mg/ml were added to a tube containing chitin beads (Bio-Rad®). The samples were then centrifuged and supernatants in each tube were collected, as crude lysates. After washing the samples with Tris-Buffered Saline (TBS), they were centrifuged again and the supernatants were collected. The washing step was repeated two more times. The rChiC and BSA were then eluted by 2x Laemmli buffer followed by centrifugation for obtaining the supernatants. The samples of the crude, three washes, elution fraction, along with rChiC and BSA input proteins were prepared and resolved on an SDS-PAGE as described in *Sample preparation and SDS-PAGE*, without the addition of BME. The protein band intensity for each lane was normalized using the input protein bands as 100% intensity by ImageJ software.

RESULTS

2-hours IPTG induction at 37°C leads to over-expression of rChiC protein. To identify the set of conditions that result in high expression of rChiC in *E. coli* BL21 transformed with pM3CRYY, different incubation times, temperatures, and IPTG concentrations were tested (Table S1 & S2). The conditions studied were temperatures of 20 °C and 37 °C, IPTG concentrations of 0, 0.1, 0.5, 1, 10 mM, and induction times of 0, 1, and 2 hrs. We then performed SDS-PAGE for the whole cell lysates and supernatants to determine the size and amount of protein within the cells and also secreted from the cells respectively. The IPTG induced cells produce thicker and darker bands at ~55kDa compared to the controls

for whole cell lysate proteins (Fig. 1A). The IPTG-induced cells did not secrete rChiC after 1 or 2 hrs induction, as shown by the lack of bands at ~55kDa compared to the controls (Fig. 1B). Comparing the different sets of conditions in Figure 1A suggests that *E. coli* BL21 transformed with pM3CRYY expresses most rChiC after 2 hr IPTG induction at 37 °C. We then measured the expression of rChiC at different IPTG concentrations for 2 hr induction at 37 °C (Fig. 1C). The rChiC expression was similar among the IPTG concentrations of 0, 0.1, 0.5, 1, 10 mM (Fig. 1C). As a result, the rChiC expression was not enhanced noticeably by increasing IPTG concentration above 0.1mM; consequently, 0.1 mM IPTG can be used for cost efficiency. These data show that the *E. coli* BL21 transformed with pM3CRYY is expressing the most rChiC when induced with 0.1mM IPTG for 1 hr or 2 hr at 37°C within the set of conditions studied.

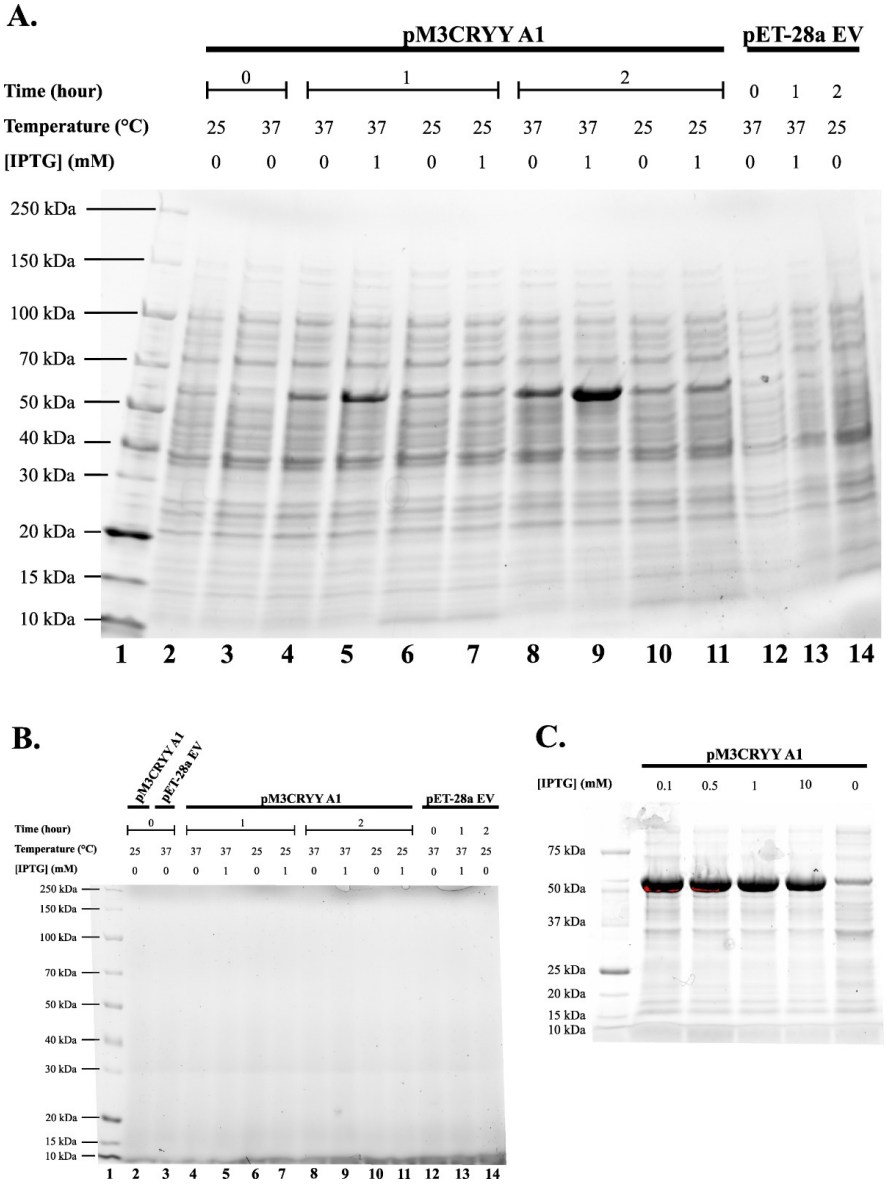

FIG. 1 2-hour IPTG induction at 37 °C causes over-expression of rChiC protein in *E. coli* BL21 transformed with pM3CRYY. *E. coli* BL21 transformed with pM3CRYY and *E. coli* BL21 transformed with pET-28α (negative control) were induced with IPTG at different concentrations (0, 0.1, 0.5, 1, and 10 mM) for 1 hr or 2 hrs at 37 °C or 25°C. Then, the whole cell lysates and extracellular supernatants of these induced samples were isolated. SDS-PAGE was performed to detect relative size and intensity/concentration of rChiC protein. **(A)** SDS-PAGE for whole cell lysate protein at different conditions. **(B)** SDS-PAGE for the extracellular supernatant protein at different conditions. **(C)** SDS-PAGE for whole cell lysate protein at different IPTG concentration after 2 hrs induction at 37 °C.

Over-expressed rChiC partitions in the insoluble fraction of the cell lysates. To investigate whether the expressed rChiC is a soluble or insoluble protein, we induced *E. coli* BL21 transformed with pM3CRYY and *E. coli* BL21 transformed with pET-28α (control) with 0 or 0.1 mM IPTG for 1 or 2h. Subsequently we lysed the cells by bead-beating and resolved the soluble and insoluble protein fractions using SDS-PAGE (Fig. 2). The IPTG induced *E. coli* BL21 transformed with pM3CRYY produces thicker and darker bands at 55kDa compared to the controls. In contrast to insoluble proteins, no band at 55kDa was observed in the soluble fraction of the lysates, suggesting that rChiC is not soluble in *E. coli*

BL21 cells under conditions tested (Fig. 2). This result indicates that the majority of the expressed rChiC in *E. coli* BL21 transformed with pM3CRYY are insoluble.

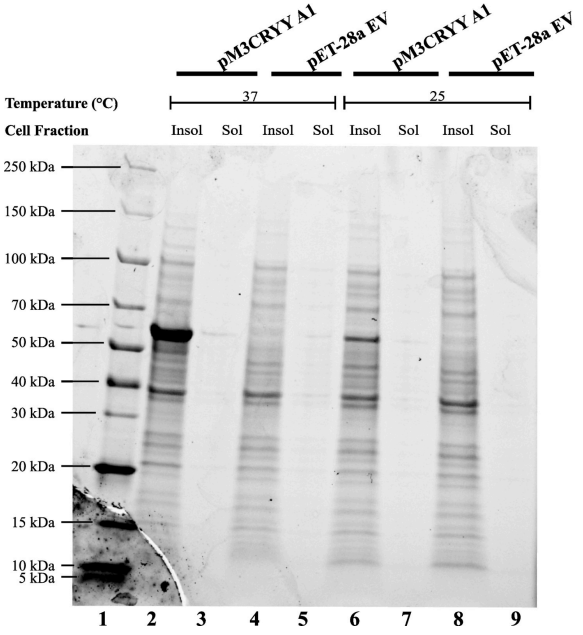

FIG. 2 Most of the expressed rChiC is in the insoluble fraction after 2-hr IPTG induction at 37 °C. Soluble and insoluble fractions within the cells were isolated from *E. coli* BL21 transformed with pM3CRYY and *E. coli* BL21 transformed with pET-28α (negative control) induced by 0.1mM IPTG for 1 or 2 hrs at 25°C or 37 °C. Bead-beating for lysing the cells was followed by SDS-PAGE to determine the relative amount of soluble and insoluble rChiC protein (55 kDa) within the cell.

Purification of rChiC using immobilized metal (Ni^{2+}) affinity chromatography under denaturing conditions. To obtain the highest yield of rChiC from the culture, we purified the 6X-His tag-containing rChiC under denaturing conditions using IMAC with Ni^{2+} resin. In this approach, we lysed the cells and denatured the proteins with 8M urea to solubilize all the proteins within the *E. coli* BL21 including rChiC. The supernatant of the centrifuged lysate was added to the IMAC column, then washed and eluted in five fractions. Aliquots from each step were collected throughout to be run on an SDS-PAGE gel. Minimal amounts of rChiC were observed in the wash fractions as shown by the band in lane 5, while most of it is retained on Ni^{2+} beads until the elution phase as shown by the dark bands in lanes 6 to 10 (Fig 3). Lanes 6 to 10 in Fig 3 show that rChiC is eluted with a high purity since there is no significant band in elutions with a different size than 55kDa (Fig. 3). These results indicate that solubilized rChiC from the whole cell lysate of *E. coli* BL21 transformed with pM3CRYY was purified by IMAC under denaturing conditions.

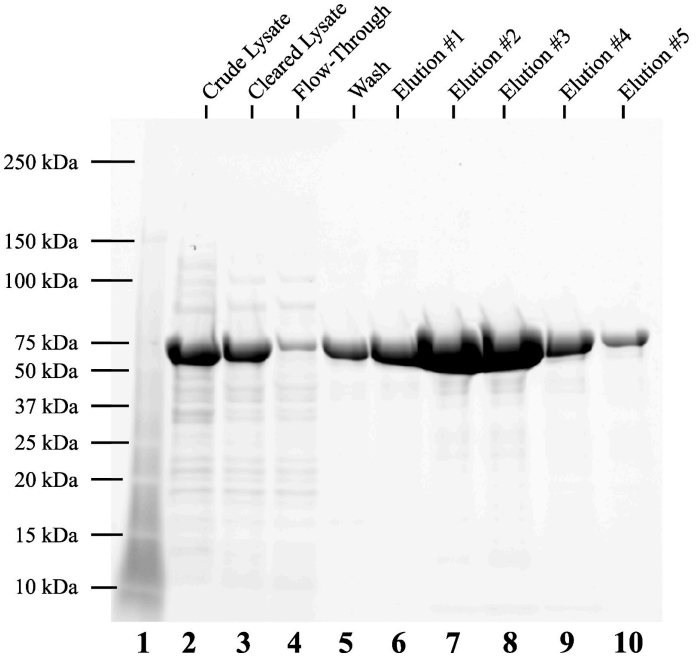

FIG. 3 rChiC is purified by immobilized metal (Ni^{2+}) affinity chromatography (IMAC) under denaturing conditions. 200ml of induced culture was used to purify rChiC with 6xHis tag using IMAC under denaturing conditions by 8M Urea. Following cell lysis, samples of purification steps including crude lysate, cleared lysate after centrifugation, flow-through from Ni-NTA column, wash and elutions fractions, were collected during purification. The samples were analyzed by SDS-PAGE.

Recombinant ChiC is refolded into a distinct tertiary structure using dialysis as suggested by limited proteolysis. After the purification of rChiC under denaturing conditions, we refolded the rChiC back into its active form using dialysis with decreasing concentration of urea. In order to confirm that the dialysis of the denatured rChiC leads to refolding of the protein, we used limited proteolysis to probe the tertiary structure. In this method, trypsin cleaves the protein at exposed arginine (Arg) and lysine (Lys) residues. Variable tertiary structures of a protein can lead to different availability of exposed Arg and Lys residues and thus different cleaving patterns on SDS-PAGE. The rChiC has 12 lysine and 29 arginine residues exposed in its primary structure where trypsin digestion produces short fragments that are not visible on the gel. For this test, after dialysis, two samples of rChiC (11.7 g/L) were treated with 0.1 µg/ml trypsin for different times of 0, 30 sec, 1 min, 5 min, and 15 min. Then, the trypsin-digested rChiC at each time point was added to 2x Laemmli dye (without BME). The trypsin-digested rChiC at different times was also added to 2x Laemmli dye + BME, which breaks the potentially reformed disulfide bonds and turns the rChiC into its primary structure. The fading of the band at 55 kDa from time 0 sec to 15 min, lanes 8 to 12, suggests that the trypsin is functionally active in cleaving the peptide bonds where lysine or arginine are present in the rChiC structure (Fig. 4). The bands in the BME-free sample have a unique pattern which is consistent throughout trypsin treatment time periods, as opposed to the BME-containing sample. This suggests that the rChiC was refolded into a uniform tertiary structure, which conceals some of the lysine and arginine residues from trypsin (Fig. 4). Therefore, trypsin analysis demonstrates that dialysis refolds the denatured rChiC into a tertiary structure.

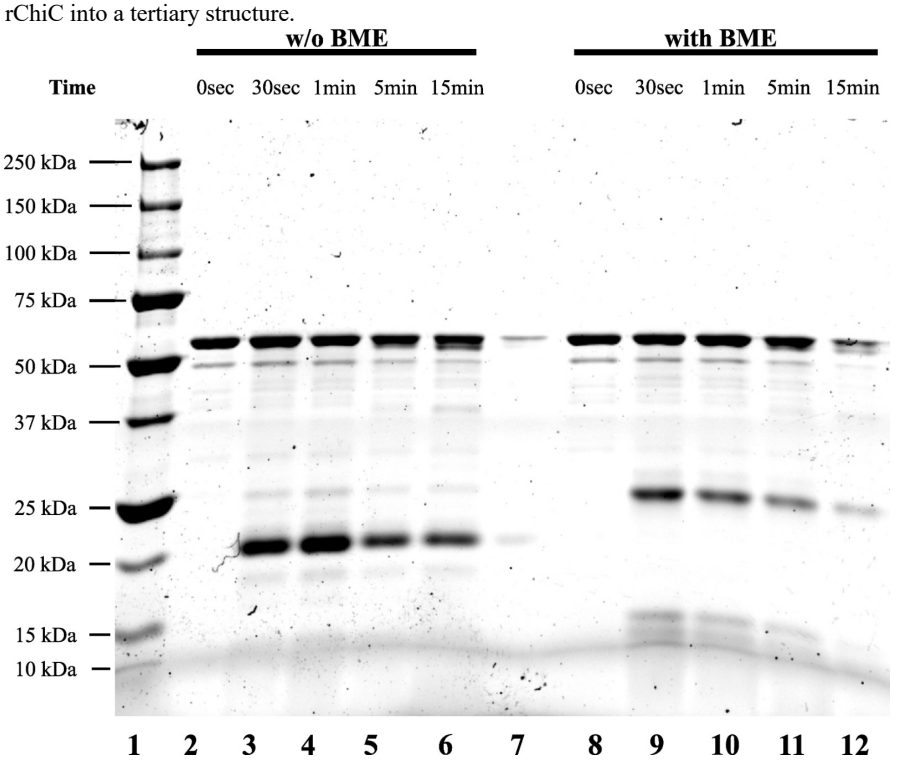

FIG. 4 Probing the tertiary structure of purified refolded rChiC using limited proteolysis by trypsin. We attempted to refold the purified rChiC by dialysis of urea from 8M to 0M. After refolding, two samples of 10 µl rChiC were treated by 0.1 µg/ml trypsin for 0 sec, 30 sec, 1 minutes, 5 minutes, and 15 minutes. One of the samples was mixed only with 2x Laemmli dye, and the other was mixed with 2x Laemmli + 5% BME immediately after reaching the desired time points. The samples were then analyzed by SDS-PAGE.

Purified and refolded rChiC binds to chitin beads. To determine whether the chitin binding domain (CBD) of rChiC is functional, we added 1:1 ratio of either normalized rChiC or BSA (control) to chitin resin. The wash steps and the elution step were conducted using TBS and 2x Laemmli buffer, respectively. Aliquots from each step were collected throughout to be run on an SDS-PAGE gel. The band corresponding to rChiC (55 kDa) fades throughout the wash steps; however, it reappears after the elution step (Fig. 5A). The intensity of the input rChiC and BSA bands are normalized to 100% semi-quantitatively (Fig. 5B). 28% of the input protein was removed from the column as unbound protein, and 92% of the input rChiC was eluted following the elution buffer. On the other hand, 34% of the input BSA was unbound protein, and only 13% of the input BSA was eluted following the elution step (Fig. 5B). Unlike BSA, which lacks CBD, binding of rChiC to chitin prevented it from being

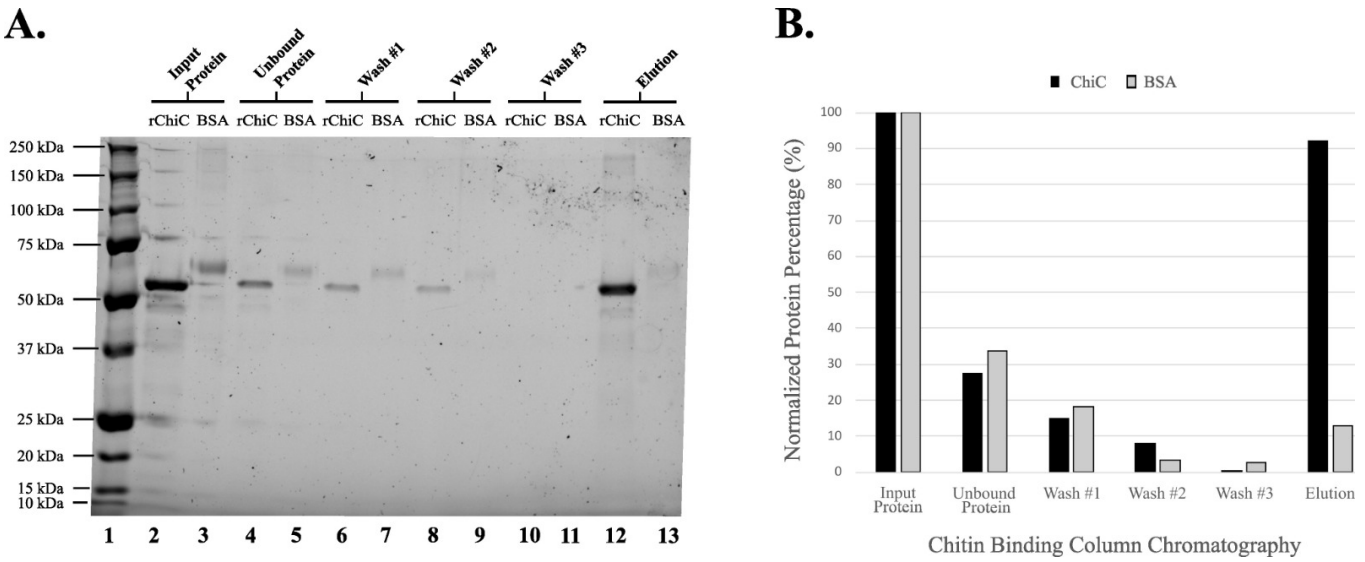

FIG. 5 The binding domain of refolded rChiC is functional as shown by chitin column chromatography. 150 µl of rChiC and BSA (control) with the same concentration, were each added to 150 µl of chitin beads. **A)** Samples for input proteins, unbound proteins, three washes, and elution were collected. These samples were loaded on SDS-PAGE gel. The bands corresponding to rChiC are 55 kDa, and to BSA are 67 kDa. **B)** Representative semi-quantitative graph for normalized intensity of rChiC and BSA at different stages of chitin column chromatography. Intensity of SDS-PAGE gel bands were quantified using ImageJ software. Each input protein band intensity was set to 100%, and other bands were normalized to their respective input protein, rChiC or BSA.

completely washed away, and since the elution buffer disrupts the interactions of the binding domain, the protein was eluted. Therefore, these results indicate that the CBD of rChiC is refolded appropriately into a functional domain.

DISCUSSION

Due to its ability to degrade the chitin in insect exoskeletons, ChiC from *P. aeruginosa* can be used as a pesticide. This study was conducted to use an *E. coli* system to express, and an IMAC system to purify functional rChiC cloned from *P. aeruginosa* in a time and cost-efficient manner. In this study, we determined the optimal condition for the *E. coli* expression of recombinant Chitinase C cloned from *P. aeruginosa*, purified and refolded a functional form of rChiC.

Optimizing expression and determining the solubility of over-expressed rChiC. In this experiment, we used SDS-PAGE to determine the optimal conditions that yield high-level expression of rChiC. We also explored the secretion and solubility of rChiC within *E. coli* BL21 transformed with pM3CRYY. Compared to other sets of studied conditions, induction with 0.1 mM IPTG at 37 °C for 2 hrs resulted in the highest level of rChiC expression (Fig. 1A & C). In a recent study, expression of rChiC was also obtained with the same conditions, except with 10 mM IPTG (8). Our results suggest that a high-level of rChiC expression can still be achieved with a lower concentration of IPTG (0.1 mM), which is more cost-efficient (13). Further, we observed that there is no secretion of rChiC into the extracellular environment after 2 hrs (Fig 1B), which aligns with past literature of gradual secretion of rChiC in *P. aeruginosa* (5). Most of the rChiC was insoluble within the induced *E. coli* BL21 transformed with pM3CRYY (Fig 2). High-level expression of proteins in *E. coli* BL21 usually results in inclusion bodies formed from aggregated proteins (12), likely explaining why the majority of the expressed rChiC is in the insoluble fraction of the cell lysate. This finding prompted us to focus on purifying rChiC from the insoluble fraction of induced *E. coli* BL21 transformed with pM3CRYY to obtain the highest yield.

IMAC purification and limited proteolysis to confirm refolding of rChiC by dialysis. We purified rChiC from the insoluble fraction using IMAC and then dialyzed the pure protein against decreasing concentrations of urea to attempt to refold the protein. To have a functional rChiC, we needed to refold it to its native form (14). Since ChiC is optimally active at pH of

4.5-5 (5), we performed dialysis at pH 4.5. We confirmed the refolding of rChiC by conducting limited proteolysis and chitin-binding experiments. The disadvantages of dialysis for refolding proteins include its long duration compared to other techniques, and the potential formation of aggregates of stable incorrectly folded intermediates (15). Thus, this method of refolding is not fully time-efficient, and dialysis can be optimized for a higher yield in a shorter duration. For instance, the time intervals or buffer compositions of the dialysis can be manipulated for optimization.

We showed that rChiC was folded by performing limited proteolysis to probe tertiary structure. The analysis of *P. aeruginosa* PAO1 ChiC nucleotide sequence, using NCBI BLAST shows that there is only one cysteine residue in the amino acid sequence of the protein (EIU3196988.1), which might form an intermolecular disulfide bond between two ChiC proteins. To find out whether the intermolecular disulfide bonds are formed during refolding, we added BME to the Laemmli dye in one of the samples in the limited proteolysis experiment. If the disulfide bond is present between the two proteins, we expect to see some of the bands in the sample with BME to be half in size compared to the ones in the samples without BME. If the disulfide bond is not present, we expect to see the same band pattern between the samples with and without BME. However, we made neither of those observations. The BME-free rChiC samples show a unique cleavage pattern by trypsin, which is consistent and stable as the trypsin digestion time is increased (Fig. 4). This finding suggests that the rChiC is refolded into a uniform tertiary structure, which shields some of the lysine and arginine residues from trypsin. However, considering the lack of intramolecular disulfide bonds in rChiC, it is unclear why the BME-containing sample is fragmented differently compared to the BME-free sample. While further analysis is required to explain this observation, BME structure can be a potential explanation. Having a thiol group, BME can act as a reducing agent (16), interacting with cysteine and possibly arginine. The guanidino group of arginine can be electrophilic (17), and might react with the thiol group of BME. These potential interactions might alter the protein structure or the stability of the protein fragments, resulting in various outcomes in trypsin limited proteolysis.

Confirming the functionality of the chitin-binding domain. To further ensure the refolding of rChiC, we performed chitin-binding column chromatography. We found the binding domain of the purified rChiC to be functional and folded properly as it was retained through the wash steps until the elution phase (Fig 5). This suggests that the employed refolding technique refolds the binding domain appropriately into a functional structure. Therefore, the correct folding of rChiC suggested by limited proteolysis is partially confirmed by our data from the chitin-binding column.

Yield of Chitinase C. In this study, the dialyzed protein was used for determining the rChiC concentration, in which the majority of the rChiC is expected to be refolded. The acquired yield (11.7 g of rChiC per 1 L of *E. coli* BL21 culture) was measured using a NanoDrop spectrophotometer and rChiC extinction coefficient. However, this method is ideally used for proteins in their primary structure since the folding alters the exposure of the amino acids to the emitted light. Consequently, the absorbance of the light would be lower in the sample containing folded rChiC compared to the sample with denatured rChiC. We expect the actual yield of refolded rChiC to be higher than what we have observed. We were not able to use more methods, such as BCA assay to confirm the yield of rChiC due to the limited time.

Chitinases, including ChiC, are also generated in other organisms (18). *Serratia marcescens*, which is a potent biological degrader of chitin, produces chitinase A (ChiA), chitinase B (ChiB), and chitinase C1 (ChiC1). A past study has analyzed the recombinant form of these chitinases in *E. coli*. They found that among the three enzymes, insoluble chitin was most degraded by ChiA, while soluble chitin was most degraded by ChiC1. This study suggested that chitin was efficiently degraded through the synergistic action of these enzymes (19). More specifically, they found a synergism between ChiA and either ChiB or ChiC, on chitin degradation. They also have different hydrolyzing activities, which is mainly due to structural differences in their catalytic domains. Specifically, ChiA and ChiB have catalytic domains of subfamily A, while ChiC1 has that of subfamily B (19). The catalytic clefts of chitinases in subfamily B (ChiC1) are likely shallower than those of chitinases in subfamily A. Further, the catalytic cleft of ChiA has a groove-like feature, which may confer ChiA endo-

enzymatic activity. ChiB, on the other hand, has exo-enzymatic activity (19). The structural differences and the endo- and exo- cleavage dynamic are suggested to play a role in the synergy among ChiA and either ChiB or ChiC (19). A past study showed that ChiC from *Pseudomonas* sp. strain TXG6-1 is in subfamily B. The TXG6-1 ChiC gene has high sequence homology with *P. aeruginosa* ChiC, suggesting that this ChiC is also in subfamily B (20).

The moderate activity and features of wild type chitinase might not meet the needs of biotechnological industries. The stability and activity of enzymes are essential for their use in industry. Thus, enhancing the activity and stability of chitinase is conducive to its agricultural applications. Past literature has shown that modifications of domains in enzymes can improve their properties. In a previous study, researchers designed a mutated form of a recombinant chitinase from *P. aeruginosa*, which lacked CBD domain (21). They found that the absence of CBD increased expression level and solubility of the enzyme, as well as its catalytic efficiency (21).

Conclusions Overall, our findings show that rChiC can be over expressed as an insoluble protein after 2 hr induction using 0.1 mM [IPTG] at 37 ℃. We were able to use IMAC to purify rChiC in its denatured form. The purified rChiC was then refolded using dialysis. Lastly, the refolded rChiC was shown to be able to bind to chitin, demonstrating functional activity of the chitin-binding domain.

Future Directions Future studies can be conducted to elucidate the structure of rChiC from *P. aeruginosa* using X-ray crystallography. The tertiary structure of rChiC would provide knowledge on exposed amino acids and domains, allowing for finer manipulation in biotechnological applications. The catalytic domain of the refolded rChiC needs to be tested for functionality. One option is a spectrophotometric assay via modified Schales' procedure where the reducing sugars produced from the breakdown of colloidal chitin is measured to assess enzymatic functionality. Another is a diffusion plate assay on colloidal chitin plates, quantifying the clearance zone from purified rChiC (22).

For acquired yield, it was measured using NanoDrop spectrophotometer, and it is preferred if another method, such as a Bradford assay is used to confirm the protein concentration. Furthermore, a higher refolded rChiC yield might have been achieved by optimizing the incubation times and pH of buffers used in the refolding method. To improve the rChiC yield and time-efficiency of purification, the refolding method could be revisited. Due to time constraints, we were unable to attempt refolding with other methods; however, future studies can investigate the efficiency of other refolding techniques. Further, future studies need to be conducted to determine whether the yield of 11.7 g rChiC per 1 L of transformed *E. coli* BL21 cell culture is sufficient for downstream biotechnological applications. Further, due to having limited resources, we were unable to have positive control in this study. To have more comparable results, future research can include a positive control with *P. aeruginosa* in their studies.

Future studies of ChiC from *P. aeruginosa* can investigate the potential synergistic effects of ChiC and other types of chitinases, such as ChiA, on the efficiency of chitin-degrading activity. Moreover, future explorations can investigate how modifying ChiC domains can enhance its chitin-degrading activity.

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CONTRIBUTIONS

Laboratory work. All the lab members were integral to all the components of the wet laboratory portion, but this will list the main contributors to each section. Zee and Pooya induced *E. coli* BL21 transformed with pM3CRYY at the different conditions and performed the solubility test of rChiC in the induced *E. coli* BL21. Negarin and Soroush executed Immobilized metal (Ni^{2+}) affinity chromatography (IMAC) and Limited proteolysis. Pooya and Soroush performed the protein refolding by dialysis. All members have contributions in the chitin column chromatography experiment.

Manuscript. Zee was responsible for writing the abstract, introduction, acknowledgment, and conclusion. He also made the figures. Negarin wrote the future directions, discussion, and citations. Pooya and Soroush worked on the results and materials sections. Everyone contributed significantly to other sections of the paper by editing each other's work.

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