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Purification of Chitinase C in transformed *E. coli* BL21 (DE3) preserves a functional Chitin-binding Domain

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SUMMARY Conventional pesticides play an integral role in commercial agriculture, however, many of these pesticides also have a negative impact on human health, leading to increased interest in finding safe alternatives. Chitin, a biopolymer commonly found in the exoskeleton of insects, has received significant attention as a potential target for the development of biopesticides. Chitinolytic enzymes are naturally produced by many bacterial species such as *Pseudomonas aeruginosa*. In particular, the 55kDa chitinolytic enzyme chitinase C (ChiC), secreted by *Pseudomonas aeruginosa* PAO1, has demonstrated insecticidal activities in previous studies. ChiC has been extensively characterized in its native host and previously transformed into other bacteria such as *Escherichia coli* using the pM3CRYY *chiC* expression plasmid to isolate ChiC for purification and mechanistic studies. We hypothesized that soluble protein purification of ChiC would allow for the preservation of a functional chitin-binding domain. Purification was performed using immobilized metal affinity chromatography and dialysis, and the activity of the chitin-binding domain in purified ChiC was tested using chitin resin affinity purification. The successful purification of ChiC with a functional chitin-binding domain highlights the potential of the ChiC expression system in *E. coli* BL21(DE3), which may be further investigated to elucidate the possible functionality of the whole protein.

INTRODUCTION

Industrialization of agriculture has led to the proliferation of synthetic pesticide use in conventional agriculture and these products have also been associated with negative health and environmental outcomes (1, 2). There is currently active research on alternative forms of pest control that are more specific in target and application. One such target for alternative pest controls is chitin, a biopolymer widely found on the exoskeletons of insects (3). Notably, many bacterial species such as *Pseudomonas aeruginosa* and *Bacillus thuringiensis* can degrade chitin through the production of chitinase and utilize it as a carbon and energy source (3, 4). The synthesis of chitinases is often regulated through catabolite repression; expression and secretion of chitinase is repressed when the bacteria are grown in rich medium with alternative carbon sources such as glucose but induced when the strains are grown in minimal medium supplemented with only chitin (5). The exact mechanisms involved in the catabolite repression of the chitinolytic pathways are not extensively understood and differences in the systems are common between closely related organisms (5).

The gene *chiC,* native to *Pseudomonas aeruginosa* PAO1, codes for the extracellular chitinolytic enzyme chitinase C (ChiC) (3). Previous studies have shown that expressed ChiC is accumulated in the cytoplasm and then secreted into extracellular space after several days of cell growth (6). The secreted ChiC was found to have an N-terminal segment of 11 residues

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cleaved off compared to intracellular ChiC (3). This extracellular 55-kDa enzyme is composed of 483 amino acids distributed within three different domains: a carbohydrate-binding module, a glycoside hydrolase family (GH18) domain, and the fibronectin type-III domain (3). Each domain plays a role in the degradation of chitin: the carbohydrate-binding module binds to carbohydrates and has *O*-glycosyl hydrolase activity, the GH18 domain catalyzes random cleavage of β-1, 4 bonds within chitin chains, and the fibronectin domain binds and interacts with other proteins (3).

Previous research has detailed the isolation and transformation of chitinases to various heterologous hosts; this process has established a better understanding of the characteristics and production of various chitinases (3, 7). Moreover, these studies have reported on the antifungal and insecticidal activities of chitinases, indicating their potential as alternatives to prevailing pesticide use (4). When *chiC* was expressed in heterologous hosts *Pseudomonas putida* and *Escherichia coli* (*E. coli*), trace amounts of ChiC were detected in the culture medium, indicating that the primary structure encoded in *chiC* does not contain all the functions required for secretion (3). Folders *et al*. have concluded that the secretion of ChiC into the extracellular space may act through a novel secretion pathway distinct from known secretion systems (3). Despite the secretion pathway currently being inactive, the heterologous non-pathogenic host models still allow for intracellular expression of ChiC.

Previous studies have cloned *chiC* from P. aeruginosa PAO1 into vector pCR2.1 to produce the plasmid pGKMS21 (8). The *chiC* from pGKMS21 was then amplified and inserted into an empty pET-28a(+) vector, resulting in the recombinant expression vector pM3CRYY (9). ChiC expression after transforming pM3CRYY into non-pathogenic *E. coli* BL21 (DE3) was also verified by Rocha *et al.* in 2022 (9). However, prior to this study, the chitinolytic activity of ChiC produced using transformed *E. coli* BL21 (DE3) + pM3CRYY remained uncertain. We hypothesized that the purification of chitinase C using immobilized metal affinity chromatography (IMAC) would allow for the preservation of functional ChiC. In this study, we purified ChiC expressed in *E. coli* BL21 (DE3) + pM3CRYY using IMAC and tested the functionality of the chitin binding domain using chitin resin affinity purification.

METHODS AND MATERIALS

Preparation of LB liquid media and agar plates. 10 mL of 50 mg/mL Kanamycin stock solution was prepared from 0.5 g Gibco ™ Kanamycin Sulfate (Lot 1913841) and sterile dH_2O. Sterile 1mL aliquots were made by filtering into 1.5mL microfuge tubes and stored at -20°C. 500mL bottles of 1% NaCl LB media were prepared using 5g tryptone, 2.5g yeast extract, dH_2O, and 5g NaCl. 500mL bottles of 0.5% NaCl LB media were also prepared with the same measurements except with 2.5g NaCl. LB agar was prepared by adding 3.25g agar to 250mL LB media. Kanamycin LB media or agar was prepared by adding 1μL of Kanamycin stock solution for every mL of cooled media or molten agar.

Preparation of experimental materials and reagents. 0.1M $CaCl_2$ and 0.1M $CaCl_2$ + 15% Glycerol buffer solutions were adapted from Chang et al. (2017)'s methods on preparing competent cells (10)**.** 100mL of 0.1M $CaCl_2$ was prepared with 1.11g $CaCl_2$ and dH_2O, and 50mL of 0.1M $CaCl_2$ + 15% glycerol was prepared with 0.55g $CaCl_2$, 12.5mL 60% glycerol, and dH_2O. Extraction of plasmid vectors, pET-28a (+) and pM3CRYY, were performed using the protocol from the EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic). 10% polyacrylamide gels for SDS-PAGE were prepared using the TGX Stain-Free™ FastCast ™ Acrylamide Kit, 10% (BioRad) protocol. 100mM isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution was provided by the MICB 471 teaching team, diluted to 0.1mM as a working solution.

Growth and transformation of *E. coli* BL21 (DE3). *E. coli* competent cell preparation with 0.1M $CaCl_2$ wash and cell transformation of pM3CRYY into *E. coli* BL21(DE3) procedures were adapted from Chang *et al.* with 1 minute heat-shock at 42°C (10). *E. coli* BL21 (DE3) + pET-28a (+) was transformed as a negative control for downstream application.

Sanger Sequencing of pM3CRYY plasmid. Internal primers were designed in ApE (Table 1) and ordered from IDT. IDT custom primers and isolated pM3CRYY plasmid were prepared following GeneWiz guidelines and sent for Sanger sequencing. T7 universal promoter (forward) and terminator (reverse) primers provided by GeneWiz were also requested for Sanger sequencing. Plasmid map of pM3CRYY and reference sequence was constructed by restriction cloning simulation following protocol from Rocha *et al.* using SnapGene 6.1.2. Sequencing results were aligned and compared against the pM3CRYY reference sequence in SnapGene 6.1.2**.** Reads were trimmed to exclude low-quality regions.

TABLE. 1 Internal Primers Sequences for Sanger Sequencing.

Primer Name	Primer Sequence
Internal Forward Primer	5’ - AAG CAC TTC ATC GTC AGC ATG - 3’
Internal Reverse Primer	5’ - GCG GAA CTC CCA GTT GTA GC - 3’

IPTG Induction of transformed *E. coli* BL21 (DE3) + pM3CRYY. A single *E. coli* BL21 (DE3) + pM3CRYY colony was inoculated into 20mL LB broth and 20µL of 50 mg/mL Kanamycin stock solution and incubated overnight at 37℃ in a shaking incubator. 5mL overnight culture and 245mL LB broth + Kanamycin into a 1L Erlenmeyer flask for IMAC Purification. 2mL overnight culture and 98mL LB broth + Kanamycin into a 500mL Erlenmeyer flask for various induction time collections (2 hrs, 4 hrs, 24 hrs, 48 hrs, 72 hrs, and 96 hrs post-induction times) These 2 subcultures were placed in a shaking incubator at 37℃ 200 rpm until OD_{600} reaches 0.6 to 0.8. Then, 100mM IPTG stock solution was added to a final IPTG concentration of 0.1mM. Induced flasks were incubated at 25℃ 200 rpm in the shaking incubator. *E. coli* BL21 (DE3) + pET-28a was inoculated in 5mL LB broth and 5µL Kanamycin and incubated overnight at 37℃, 200 rpm in a shaking incubator as a negative control for downstream application. After 48hrs of IPTG induction, the 250mL *E. coli* recombinant BL21 (DE3) + pM3CRYY cell culture was centrifuged for 6 min at 5000 rpm using floor centrifuge (Beckman Coulter) and the supernatant was removed. The cell pellet stored in -70℃ for purification.

Sample Collection of insoluble and soluble ChiC. 1mL culture samples from the 500mL Erlenmeyer flask containing IPTG induced *E. coli* BL21 (DE3) + pM3CRYY were collected at 2 hr, 4 hr, 24 hr, 48 hr, 72 hr, and 96 hr post induction. OD_{600} readings of each sample were recorded using Ultrospec 3000 (Biochrom). Samples were centrifuged for 1 min at 13000 rpm, and the cell pellet and supernatant were separated and stored in -70℃ until all samples were collected and ready to run SDS-PAGE. Cells were lysed by resuspending the pellet in BugBuster® HT Protein Extraction Reagent (Millipore). For every 500µL of sample collected, 10µL per 1 OD unit of BugBuster reagent was added. Cells were then incubated for 45 minutes at room temperature. The cells were centrifuged for 1 min at 13000 rpm. The supernatant containing the soluble protein was collected. The pellet was resuspended in NP + 8M urea pH 8 with the same volume of BugBuster reagent added. Samples were incubated for 40 mins. Samples were centrifuged for 1 min at 13000 rpm and the supernatant was collected.

Sample Preparation and SDS-PAGE. SDS-PAGE gel samples were prepared by adding 1:1 ratio of sample and 2x reducing Laemmli loading dye. The samples were heated at 95℃ for 5 minutes. 10µL of Precision Plus Protein™ Ladder and 10µL of the sample was loaded. The gel was run at 200V for 40 mins in 1x Tris/Glycine/SDS running buffer diluted from the 10x stock. Gels were then visualized on ChemiDoc MP Imaging System (BioRad) with Stain-Free Gels setting.

Purification of ChiC using nickel resin immobilized metal affinity chromatography. ChiC collection and purification procedures were adapted and modified from Siwayaprahm *et al.* and Barrick Lab (11, 12)**.** Binding buffer, 10 mM imidazole wash buffer and 200 mM imidazole elution buffer were prepared and adapted from NEBExpress Ni Resin Batch Binding Typical Protocol, replacing sodium phosphate with Tris-HCl/Trizma-Base (13). Lysis base buffer is prepared with 50mM Tris, 300 mM NaCl, 1% Triton X-100, 10%

Glycerol. *E. coli* lysis buffer was prepared with 40mL lysis base buffer, 4uL β-mercaptoethanol, and 40 mg lysozyme. The entire procedure except for SDS-PAGE was performed in a cold cabinet at 4°C. *E. coli* recombinant BL21 + pM3CRYY cell pellet was thawed and resuspended using 40 mL of *E. coli* lysis buffer. Cell lysate was stirred for 30 minutes. NEBExpress Ni Resin was washed with 5x column volume of dH_2O and then washed using 15mL of equilibrium buffer. After 30 minutes stirring, the cells were centrifuged for 10 minutes at 12000 rpm. The supernatant was transferred to a 50mL centrifuge tube. Some of the supernatant was collected for downstream SDS-PAGE. A pellet left after centrifugation was also collected for downstream SDS-PAGE. 1mL of NEBExpress Ni Resin was added to the supernatant. The NEBExpress Ni Resin + supernatant was left in the cold cabinet for 30 minutes on a shaker, followed with centrifugation for 5 minutes at 5000 rpm. Flowthrough was aspirated and some was collected for downstream SDS-PAGE. The NEBExpress Ni Resin was resuspended in equilibrium buffer and transferred back to the column and was washed with 10mL of 10mM Tris-based imidazole wash buffer to remove unbound or insignificant bound proteins. A sample of the wash after passing through the column was collected for downstream SDS-PAGE. ChiC bound to the NEBExpress Ni Resin was eluted with 10mL of 200mM Tris-based imidazole buffer. Each mL of ChiC elution was collected in a separate 1.5mL microcentrifuge tube. SDS-PAGE was performed with the pellet lysate, supernatant before binding, flowthrough after binding, 10mM imidazole wash, and elution samples for each fraction.

Dialysis of purified ChiC. A total of 4L of 20mM Tri-HCl 300mM NaCl dialysis buffer was prepared at pH 7.4. Dialysis tubing (12,000 to 14,000 Dalton MWCO) was cut to the proper length and soaked in dialysis buffer. Purified ChiC samples were added to the dialysis tubing and sealed tightly. The dialysis tubing was stirred in 2L of dialysis buffer for 4 hrs at 4°C and replaced with a fresh 2L dialysis buffer and left overnight stirring at 4°C. Imidazole-free ChiC was collected from the dialysis tubing and stored at -70°C for downstream application.

BCA Assay. 1mL standards of 2000, 1000, 500, 250, 125, 62.5, 31.25, and 0 µg/mL were prepared by serial dilution from 2mg/mL Bovine serum albumin (BSA) from Pierce BCA Protein Assay Kit (ThermoScientific). Working reagent was prepared by combining 50 parts of BCA Reagent A and 1 part of BCA Reagent B. 10µL of standard or purified ChiC samples were added into a 96-well plate in duplicates. 190µL working reagent was added to each well containing either the 10µL of standards or samples. The plate was covered with sealing film and incubated at 37°C for 25 minutes. The plate was read at 562 nm absorbance using a microplate reader (BioTek). A standard curve was generated with the average blank-corrected 562 nm measurement for each BSA standard vs its concentration in µg/mL and was used to determine the concentration of the purified ChiC sample.

Chitin resin affinity chromatography of ChiC chitin-binding domain. A column buffer was prepared according to NEB Chitin Resin protocol (14). 100µL of Chitin Resin (NEB) was added to a 1.5 mL microfuge tube. The resin was washed with 1mL dH_2O 3x by centrifuging for 1 minute at 8000 rpm and aspirating the supernatant to wash off ethanol. The resin was then washed with 1mL column buffer 3x by centrifuging for 1 minute at 8000 rpm the supernatant to balance the resin. ChiC or BSA as negative control was added to the Chitin Resin and incubated for 30 minutes at room temperature. The chitin resin was washed 3x using PBS by centrifuging at 8000 rpm for 1 minute and aspirating the supernatant. Each wash was collected in separate microcentrifuge tubes for SDS-PAGE. SDS loading dye, containing 950µL Laemmli dye + 50µL β-mercaptoethanol, was added to the chitin resin to elute the ChiC. SDS-PAGE was performed to visualize protein binding.

RESULTS

Sanger sequencing confirms the presence of a ~1.5 kB DNA fragment corresponding to *chiC* in the pM3CRYY plasmid transformed into *E. coli* BL21 (DE3). To ensure that the obtained plasmid contains a functional *chiC* insert, isolated plasmid and designed internal primers flanking the gene were sent to GeneWiz for Sanger Sequencing. Sequences extended from Universal T7 Promoter and Terminator primers (T7F, T7R) and Internal Forward and

Reverse primers (IF, IR) were aligned with the pM3CRYY reference sequence using SnapGene (Figure 1B). Alignment results reveal an average of 99% sequence identity match; two insertions were found in the sequence extended from the IF primer. One insertion and one mismatch were found in the sequence extended from T7F primer. However, none of the changes are repeated in the overlap between the sequences produced by each primer.

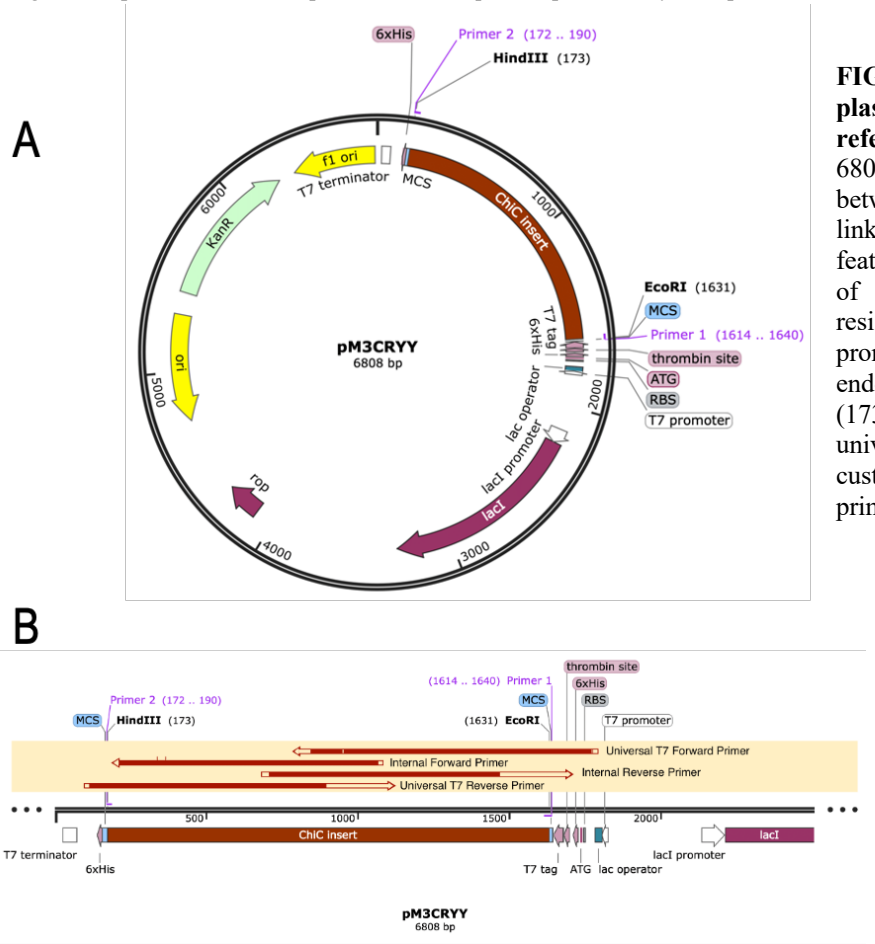

FIG. 1 Sanger sequencing of pM3CRYY plasmid and alignment to pM3CRYY reference sequence. (A) Plasmid map of 6808 bp pM3CRYY, showing *chiC* gene in between T7 promoter and T7 terminator linked with 6X Histidine tag. Other key features indicated on the map are: ori, origin of DNA replication, KanR, kanamycin resistance gene, *lacI*, lac repressor gene, lacI promoter, lac operator, and restriction endonuclease sites EcoRI (1631) and HindIII (173). (B) Sequencing results with T7 universal forward and reverse primers and customized internal forward and reverse primers to verify *chiC* gene in pM3CRYY.

Gel electrophoresis of *E. coli* BL21 (DE3) + pM3CRYY cell lysates demonstrates low solubility of ChiC in lysis buffer. To verify the applicability of nickel resin immobilized metal affinity chromatography (IMAC) for native ChiC purification, the solubility of ChiC was tested using sequential extraction with lysis buffer (Figure 2). As expected, negative control *E. coli* + pET28a (+) empty vector does not produce a significantly darker band at 55 kDa for both soluble and insoluble cell lysates. The soluble cell lysates produced increasingly darker bands between 2- and 4-hours post-induction with the darkest band visible at 24 hours post-induction. Interestingly, ChiC concentration in the soluble cell lysates from 48-96 hours post induction samples were significantly lower than the 24 hours post-induction sample. Although some lanes were merged, the insoluble cell lysate sample section (lane 9 to 15) saw a rise in ChiC concentration, indicated by the darkest bands at 55 kDa, which began at 2 hours post-induction and peaked in density at 24 and 48 hours post-induction with a moderate drop at the 72 and 96 hours post-induction measurements.

Nickel resin affinity chromatography produces a high yield of purified soluble ChiC protein. Purification of the native recombinant ChiC with a 6X Histidine tag on the C terminus was carried out via IMAC using nickel resin. SDS-PAGE analysis was used to separate and visualize a complex mixture of proteins. It also provided relative concentration on elution fractions containing purified ChiC as each lane was loaded with 10 µL samples from each 1 mL elution fraction (Figure 3). After cell lysis, some ChiC remained in insoluble form in the debris pellet (lane 1). Flowthrough (FT) was collected after gravity binding between soluble protein-containing supernatant (SN) and nickel resin. A smaller 55 kDa band

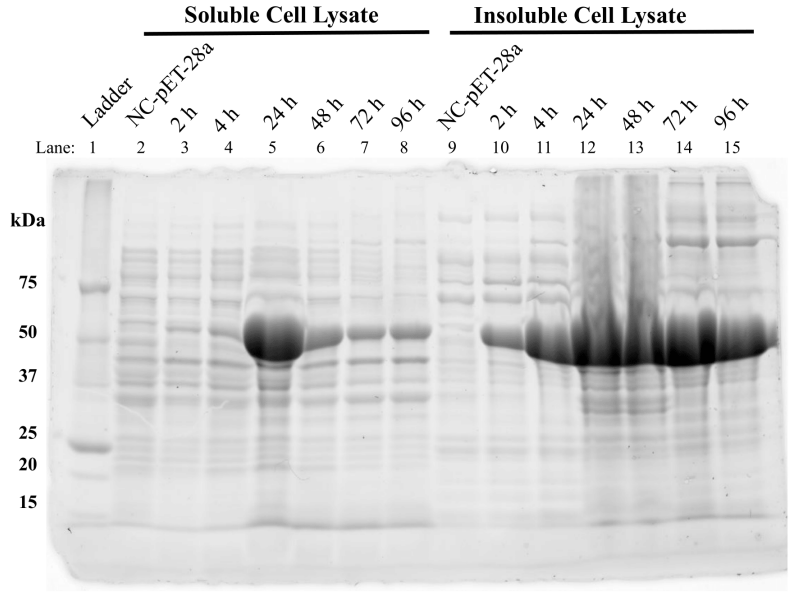

FIG. 2 10% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble and insoluble cell lysates after chemical lysis. 1 mL of uninduced *E. coli* BL21 (DE3) + pET28a (+) sample was collected after subculture and prior to induction as a negative control (NC). 1 mL *E. coli* BL21 (DE3) + pM3CRYY induced samples were collected at respective hours post-induction. Loading samples were composed of 5 µL 2x Laemmli loading dye + β-ME and 5 µL prepared sample (NC soluble cell lysate, NC insoluble cell lysate, induced soluble cell lysate, or induced insoluble cell lysate). Potential ChiC presence is corresponded by a band at approximately 55 kDa; this band was located in lanes 3-8, 10-15. Precision Plus Protein™ Unstained Protein Standards were used to provide reference for protein sizes.

in the FT is observed compared to the SN, indicating ChiC binding to nickel resin. Crude cell lysate samples (before purification) showed more intensive bands at various sizes than the elution fractions (purified ChiC) indicating improvement in purity via IMAC. 10 mL of 10 mM imidazole buffer was used for removing non-targeted proteins on the resin. Lane 4 had a faint band only at 55 kDa which suggested inadequate washing effect with imidazole buffer concentration of 10 mM. According to the 55 kDa bands' intensity, a higher concentrated sample (elution fractions 2-7) and a lower concentrated sample (elution fractions 8-10) were pooled together. BCA assay was performed to determine the concentrations of two pooled samples after removing imidazole by dialysis. The BCA assay standard curve generated with BSA had a high quality indicated by R^2 of 0.993, which indicated the data had a higher tendency to fit the linear regression line. IMAC purification of the 3.16g crude BL21(DE3) cell pellet yielded a total of 8.85mg of pure ChiC. This demonstrated that IMAC using nickel resin could be an efficient purification method to obtain pure recombinant ChiC.

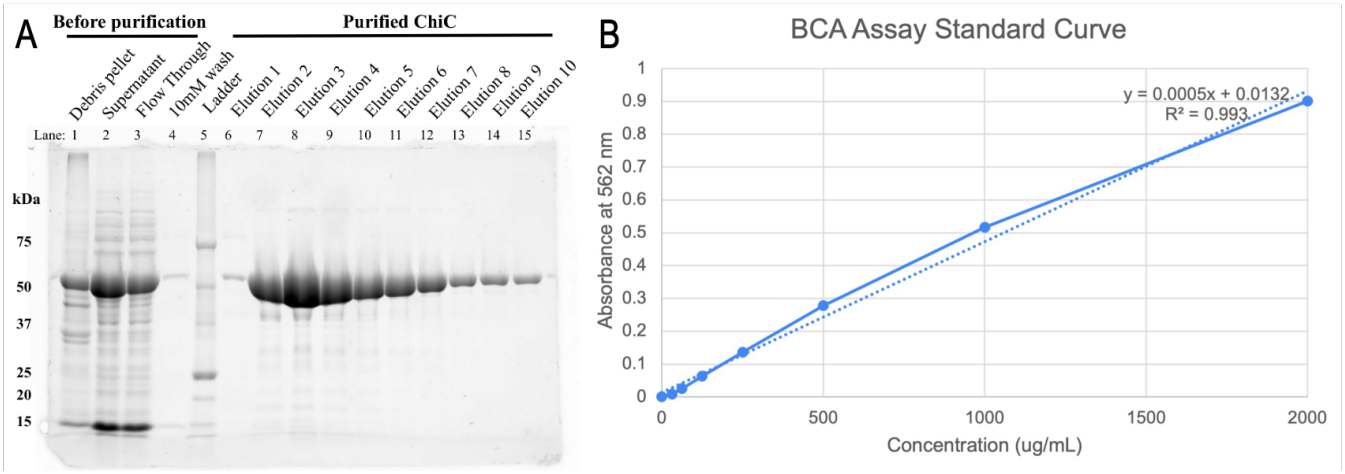

FIG. 3 10% SDS-PAGE result from IMAC purification and BCA assay quantification of purified ChiC. (A) 10% SDS-PAGE analysis of the recombinant protein ChiC purified from the 250mL culture medium, 48 hours expression (total 3.16 grams of cell pellet) via nickel resin affinity chromatography purification. 55kDa bands representing ChiC were observed in all samples. Precision Plus Protein™ Unstained Protein Standards were used to provide reference for protein sizes. (B) Purified ChiC elutions collected according to concentrations on the gel. Concentrations of the two pooled fractions were determined from the BCA assay's standard curve.

Chitin resin affinity binding confirms function of the chitin-binding domain in purified ChiC. To determine whether the chitin-binding domain (CBD) in purified ChiC is functionally active, we carried out a binding assay using chitin resin, which has strong specific

binding for CBD-fusion proteins. Properly folded ChiC proteins resulting from nickel resin affinity chromatography purification were expected to have a CBD that can bind to the chitin resin. Unused chitin resin in lane 1 represented a blank. In this assay, BSA and ChiC were incubated with chitin resin for 30 minutes and 3 wash samples were collected after centrifugations. BSA and ChiC references in lanes 2 and 7 indicate their correct protein sizes of 66kDa and 55kDa respectively (Figure 4). Small amounts of protein were detected in wash 1 for both proteins and most of the ChiC was detected in the chitin resin bound fraction (lane 11, Figure 4). In contrast, BSA, which does not have a CBD, was not detected in the chitin resin bound fraction (lane 6). These results suggest that the recombinant purified ChiC can bind to chitin via its properly folded CBD.

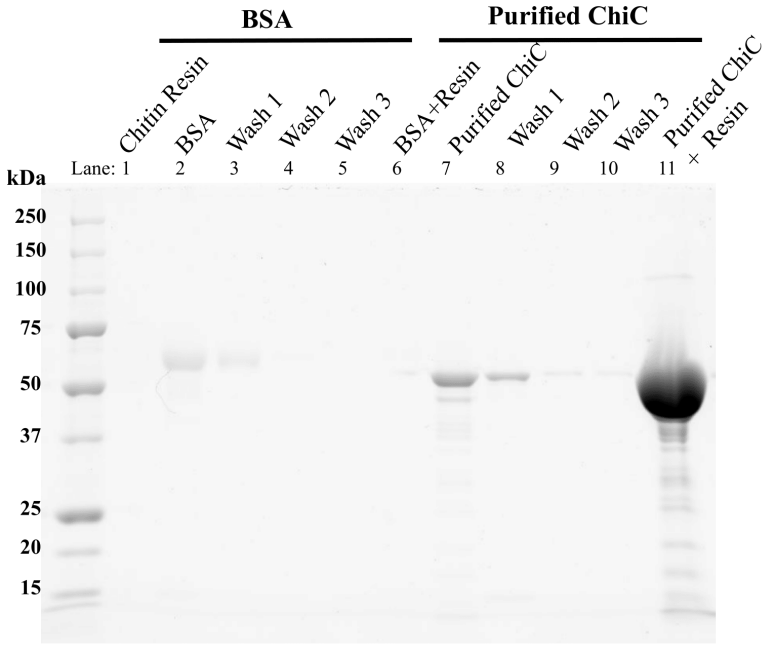

FIG. 4 10% SDS-PAGE gel of purified ChiC affinity-binding to chitin resin. 250µL of purified ChiC sample was incubated with 100µL of the chitin resin for chitin-binding domain verification. Bovine Serum Albumin (BSA) tested binding with chitin resin as a negative control. Precision Plus Protein™ Unstained Protein Standards were used to provide reference for protein sizes. Lane 1 was loaded with clean chitin resin as a blank. 3 times PBS washes for both BSA and purified ChiC were collected. Gel was shown to have purified ChiC at expected band size of 55 kDa and BSA at expected band size of 66 kDa.

DISCUSSION

Our study aimed to purify ChiC from *E. coli* BL21 (DE3) cells transformed with pM3CRYY and to determine whether purified ChiC proteins retain their functionality. The presence of a functional *chiC* insert in the pM3CRYY plasmid expression vector was confirmed using Sanger sequencing. We then isolated soluble ChiC from the *E. coli* BL21 (DE3) + pM3CRYY transformants using lysis buffer and centrifugation and purified the isolated soluble ChiC using nickel resin IMAC. Finally, we confirmed the function of the chitin binding domain in purified ChiC using chitin resin affinity chromatography.

It is important to ensure that the isolated pM3CRYY contains a functional *chiC* gene for downstream applications of ChiC purification and to test protein functionality. Sanger sequencing results confirmed the presence of a *chiC* insert in the plasmid with an 99% average sequence identity with pM3CRYY as reference sequence despite sequencing errors. Several insertion and mismatch errors are found at the ends of the sequencing reads where the chromatogram does not have clear distinct peaks and thus unreliable to confirm as true mutations. If a true mutation in the plasmid is present, it is expected to observe a mutation at the same position in all the different reads. However, there is no mutation observed at the same position on the high quality sequencing reads of T7R and IR primers that have clear and distinct peaks, which further suggests that these insertions/mismatches are due to sequencing errors. As a result, it is very unlikely that these are true mutations that will change the reading frame and affect the protein structure.

SDS-PAGE of cell lysates and supernatant after a 4-day IPTG induction at 25°C confirmed successful expression of ChiC, but not secretion, by *E. coli* BL21(DE3) + pM3CRYY (Figure S2). *E. coli* BL21 (DE3) cells were incubated at a lower temperature at 25°C, rather than the typical incubation temperature of 37°C, to promote proper folding (16). The *E. coli* BL21(DE3) transformants were incubated for four days, as opposed to the 1-hour

and 2-hour induced treatment groups by previous papers (9), to allow for potential secretion as previous literature has observed ChiC secretion after 3-4 days of growth (3). However, even after incubation, the majority of the ChiC remained in the cell lysates with only trace amounts of the protein increasing in the supernatant from 1-day to 3-day post induction. To test for cell lysis, we developed a growth curve using a microplate reader. Growth patterns of induced *E. coli* BL21 (DE3) + pM3CRYY indicate that the stationary phase, characterized by an equilibrium between the number of dividing and dying cells, is achieved at 36 hours post induction which does not correspond to when supernatant ChiC began to appear (Fig. 1S) (17). In our results, the 96-hour supernatant contained less ChiC than the 72 hour SN sample. Without duplicating the experiment (or duplicate sampling) and testing stages later than 96 hours, we are unsure if the small difference in band darkness and thickness between the 72 and 96 hours is an accurate representation of the ChiC concentration in the entire cell culture. Therefore, it remains inconclusive whether the presence of ChiC in the supernatant is from cell lysis or secretion. Further investigation through the optimization of *E. coli* BL21 (DE3) recombinant protein secretion systems is suggested to confirm the origin of ChiC in the supernatant.

E. coli BL21 (DE3) transformed with an empty pET28a (+) vector offers better performance as a negative control than uninduced *E. coli* BL21 (DE3) + pM3CRYY. In Fig 2S, a prominent protein band corresponding to ChiC is visible in the uninduced *E. coli* BL21 (DE3) + pM3CRYY, suggesting that there is expression of *chiC* independent of IPTG induction. This is likely due to leaky expression of the lac operon, where transcriptional control is not 100% efficient so basal level expression is present (17). This uncontrolled expression may also be further exacerbated by the lac repressor protein not binding to the operator with 100% efficiency or the presence of lactose analogs in the components of LB media. Alternatively, the presence of ChiC may also be due to the basal level of expression of T7 RNA polymerase, typical of the *E. coli* BL21 (DE3) cell line, which transcribes genes under the T7 promoter control (https://www.thermofisher.com/ca/en/home/technical-resources/technical-reference-library/protein-expression-support-center/bacterial-expression-support/bacterial-expression-support-getting-started.html). In subsequent SDS-PAGE procedures, an uninduced sample of the *E. coli* BL21 (DE3) transformed with the empty vector pET-28a (+) was used to avoid leaky expression of *chiC*. One could also use *E. coli* BL21 (DE3) pLysS cell line that contains T7 lysozyme to reduce leaky expression.

The optimal condition for obtaining the highest yield of soluble, native ChiC for downstream purification was at 24 hours post-induction. Soluble ChiC is preferred for purification because insoluble ChiC is in a partially or misfolded form that has to be refolded into its active conformation after purification based on denaturation (18). Notably, more insoluble than soluble ChiC was observed when comparing samples from the same time point except at 24 hours post-induction, suggesting that ChiC from BL21 (DE3) + pM3CRYY is mainly insoluble and partially soluble. The significant decrease in solubility level from 24h to 48h might be due to protein aggregation and formation of insoluble inclusion bodies. When the foreign recombinant polypeptide is expressed in high level in *E. coli*, it is likely to alter original intracellular conditions (eg. pH, osmolarity, folding mechanism), as well as increases in availability for interactions between similar regions of hydrophobic stretches in polypeptide. These factors lead to protein instability and aggregation (19).

Nickel resin affinity column chromatography successfully purified 6xHis-tagged protein ChiC but requires further optimization. Lanes with eluted purified ChiC (lanes 6-15) have ChiC bands of similar intensity but less bands overall relative to the unpurified samples. This result demonstrates that the IMAC was successful overall in isolating the ChiC from the cell lysates. However, elution fractions 2 to 7 (lanes 7-12) were associated with more bands other than 55kDa, representing more impurities and indicating that further optimization should be investigated to refine the purification process. The remaining soluble fraction of ChiC in the FT could be further purified with a second affinity binding and following steps. The 10mM imidazole wash in lane 4 showed a very faint band at 55kDa, which was not ideal for removing other non-targeted proteins bound on the resin prior to the elution process (Figure 3A). To increase purity of the final product, it is recommended to increase the imidazole concentration in wash buffers. The use of Tris-based buffers instead of phosphate-based buffers might be another cause of high impurity presence (20). Dialysis was performed to

remove imidazole from final products and possible dimerization at 110 kDa were observed after dialysis (Figure 4S). Protein concentrations were determined by BCA assay and a total 8.85 mg of ChiC was yielded from a one-time binding process, demonstrating that the IMAC purification method under native conditions can produce high yield of this recombinant ChiC.

Chitin resin affinity chromatography can confirm the function of the chitin-binding domain but not the function of the overall protein. When the purified ChiC protein is incubated with the chitin resin, it is expected that the protein will bind to the chitin resin and will remain bounded after washes if it has a functional CBD. Bovine Serum Albumin (BSA), a protein concentration standard used in various laboratory applications, was included as a negative control since it does not contain a CBD, thus, it was washed off and unable to bind to the chitin resin (21). As expected, the majority of the ChiC proteins remained bound to the resin and only minimal amounts of protein were washed off. This demonstrated that our purified protein has a functional CBD enabling specific binding to the chitin resin. This also suggests that the purified protein is likely in a folded conformation with a high affinity to the chitin resin. It is possible that some protein is washed off due to not using enough chitin resin when incubating with such high concentration of purified ChiC as this resin is designed to purify low concentrations of CBD-containing protein from crude cell extract. To obtain purified ChiC from crude cell extract using chitin resin, we would need to redesign our recombinant *chiC* plasmid to include an intein-CBD tag, which would allow on-column cleavage by a thiol reagent (14). In our preliminary testing, lysozyme was observed to be able to bind to chitin resin (Figure S3). Literature has shown that chitin is also a substrate for lysozyme where it can cleave glycosidic bonds in chitin, although not as effectively as true chitinases (22). Therefore, if using crude cell extract with chitin resin, one should consider a method of lysis other than lysozyme to avoid unnecessary binding. From our results, it is inconclusive whether the GH18 and fibronectin type-III domains of our purified protein is functional and has a chitinolytic function. Cleavage of chitin mainly depends on the GH18 domain to catalyze random cleavage of β-1,4-bonds within chitin chains (3). Further investigation would be needed to evaluate the protein's chitinolytic ability; this can be achieved by performing dinitrosalicylic acid (DNS) assay or loading purified ChiC on colloidal chitin plates to observe clearance. When a positive result of the protein's chitinolytic is observed, one could consider generating ChiC on a larger scale as a biopesticide in agriculture, an alternative to synthetic pesticide.

Conclusions We aimed to determine whether the functionality of purified ChiC from *E. coli* BL21 (DE3) is preserved. SDS-PAGE of cell lysates after IPTG induction demonstrated that ChiC is partially soluble in *E. coli* BL21 (DE3), which led to the development of a purification protocol under native conditions. IMAC purification was performed with high binding affinity between the 6XHis-tag and Nickel resin and resulted in high yield of purified ChiC. Specific binding to the chitin resin revealed that purified ChiC retained its function in the CBD.

Future Directions Our findings have successfully developed a protocol for the purification of chitinase C in transformed *E. coli* BL21 (DE3) + pM3CRYY. However, while we have been able to confirm that this procedure preserves the functionality of the chitin-binding domain in purified ChiC, the impact of the purification process on the GH18 and fibronectin type-III domain remain unclear. Future experiments may build on our experiments to further elucidate the effects of nickel resin IMAC on ChiC functionality. For example, it may be possible to streak transformed *E. coli* BL21 (DE3) + pM3CRYY on LB agar plates with colloidal chitin to test for chitin degradation (3). Another possible experiment would be the use of insect feeding or contact assays to examine the efficacy of ChiC as an insecticide.

Alternatively, in contrast to previous experiments, our results note an increasing concentration of ChiC in the supernatant beginning at 24 hours post-induction (Figure 2S). As we were unable to assert whether this presence was due to cell lysis or potential secretion, future experiments can be directed in clarifying the source of supernatant ChiC. Confirmation of whether or not *E. coli* BL21 (DE3) can secrete ChiC will be pivotal to the design of an ideal host model for ChiC expression and secretion.

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