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Development of a recombinant Chitinase C chitinolytic activity assay using the DNS method for detection of reducing sugars

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SUMMARY Chitin is a polymer consisting of N-acetyl-D-glucosamine (GlcNAc) monomers. Chitinolytic organisms such as various *Pseudomonas* spp. are promising in pest biocontrol research due to their ability to break down chitin-containing exoskeletons of insects. Previous studies have designed a *chiC* expression plasmid, pM3CRYY, which encodes *chiC* from *Pseudomonas aeruginosa* PAO1. Purified recombinant chitinase C (rChiC) expressed in *E. coli* BL21 (DE3) pM3CRYY A3 has been shown to possess a functional chitin binding domain (CBD). However, the chitinolytic activity of rChiC in this model has not been explored. Our study aims to develop a functional assay to measure chitinolytic activity of rChiC expressed by *E. coli* BL21 (DE3) pM3CRYY A3. We used dinitrosalicylic acid (DNS) reagent to assay for GlcNAc with spectrophotometry, as DNS reagent detects reducing sugars such as GlcNAc at 540 nm. Our substrate was chitosan, a popular chitin derivative in chitin-requiring experiments due to its solubility in acetic acid. *Streptomyces griseus* positive control chitinase and rChiC exhibited chitinolytic activity against chitosan, which was inhibited by heat denaturation of the chitinases. A small amount of chitinolytic activity was also detected in *E. coli* BL21 (DE3) pM3CRYY A3 culture supernatant, which suggests rChiC may be secreted by *E. coli*. Confirmation that rChiC is functional highlights the potential to develop an eco-friendly insecticide that can be upscaled through an *E. coli* expression system.

INTRODUCTION

Chitin, which contains N-acetyl-D-glucosamine (GlcNAc) monomers, is one of the most abundant biopolymers on Earth (1, 2). Chitinases are glycosyl hydrolases that degrade chitin directly to chitooligomers (3). A wide range of organisms are capable of chitinolytic activity, including bacteria, fungi, plants, and mammals (4). A growing body of literature on the usage of chitin and chitinases for biomedical, agricultural, and cosmetic purposes has emphasized the potential of chitinases as biopesticides because insect exoskeletons and fungi cell walls contain chitin (2). Chitinolytic bacteria are a promising replacement for the more harmful practices of applying toxic chemicals in synthetic pesticides and fungicides (1). *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 encodes the 55 kDa chitinase C (ChiC) protein in *chiC* (2, 5). ChiC has three domains: with a fibronectin-like type III domain, a catalytic domain, and a chitin-binding domain (CBD) (6). Previous studies have designed a *chiC*-encoding expression plasmid, pM3CRYY, and expressed it in *E. coli* BL21 (DE3) by induction with isopropyl-ß-D-thiogalactopyranoside (IPTG) to produce recombinant ChiC (rChiC) (7). While ChiC demonstrates chitinolytic activity in *P. aeruginosa*, whether rChiC expressed by *E. coli* possesses chitinolytic activity has yet to be explored (4). Previous studies have found that purified rChiC from *E. coli* retains a functional CBD (8, 9, 10). Lin et al., Kim et al. and Mohebat et al. have isolated rChiC from cell lysates using nickel-based immobilized metal affinity chromatography (IMAC) (8, 9, 10). These researchers subsequently evaluated the chitin binding ability of rChiC using a chitin binding assay, confirming that the CBD of rChiC is functional (8, 9, 10). However, since rChiC possesses a CBD and catalytic domain that are distinct from each other, presence of a functional CBD does not confirm whether rChiC has chitinolytic activity.

Chitinolytic activity has been assessed in chitinolytic microorganisms including *Pseudomonas fluorescens, Stenotrophomonas rhizophila* G22, and *Stenotrophomonas*

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maltophilia (11, 12, 13). One common methodology used to detect the presence of reduced sugars such as GlcNAc uses 3,5-dinitrosalicylic acid (DNS) (11, 12, 13). Reducing sugars reduce one of the NO_2 nitro groups in DNS to NH_2, an amine group. The product of this reaction, 3-amino,5-nitrosalicylic acid, can be detected via spectrophotometry at an absorbance of 540 nm (14, 15, 16). This study confirms CBD functionality in rChiC and explores whether rChiC possesses chitinolytic activity that is detectable by DNS assay.

METHODS AND MATERIALS

Preparation of growth media and antibiotics. Luria-Bertani (LB) broth was prepared by dissolving 10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract in distilled water (dH_2O) and sterilized by autoclaving (17). Stock 25 mg/mL kanamycin solution was prepared by dissolving 0.125 g of kanamycin sulfate in 5 mL dH_2O. The solution was sterilized by a 0.22 µm filter and stored at 4°C (18).

rChiC expression in *E. coli* BL21 (DE3). A single colony of A3 *E. coli* BL21 (DE3) pM3CRYY transformant was used to inoculate 5 mL of LB broth supplemented with 25 µg/mL kanamycin and grown overnight at 37°C with 200 rpm shaking (7, 19). The overnight culture was subcultured at a 1:100 dilution in 200 mL of LB broth supplemented with 25 µg/mL of kanamycin and incubated at 37°C with 200 rpm shaking (7, 19). Once an OD_{600} of approximately 0.7 was reached, IPTG was added to a final concentration of 1 mM and the induced culture was incubated at 37°C with 200 rpm shaking for 20 to 24 hrs (20). After IPTG induction of *chiC*, the culture was centrifuged at 5,000 rpm for 10 min in a Beckman Coulter Avanti-J30I centrifuge to separate the cell pellet and extracellular supernatant. The supernatant and cell pellet were stored at -20°C overnight until cell lysis. The same protocol was repeated using a single colony of *E. coli* BL21 (DE3) pET-28a transformant. Supernatants were prepared for SDS-PAGE analysis by combining with 2X Laemmli Sample Buffer with 10% beta-mercaptoethanol (BME) (Bio-Rad) at a 1:1 ratio (21).

Cell lysis and rChiC protein extraction. Cell pellets of IPTG-induced pM3CRYY A3 and pET-28a cultures were resuspended in 12.5 mL of pH 7.5 lysis buffer. Lysis buffer was prepared with 50 mM Na_2HPO_4, 300 mM NaCl, 1% Triton X-100, 10% glycerol, 0.01% BME, 1 mg/mL lysozyme, and 1 tablet/12.5 mL of Roche cOmplete™ Mini Protease Inhibitor Cocktail Tablet (8). Once resuspended in lysis buffer, cell pellets were incubated and stirred at 4°C for 30 min (8). After incubation, cell lysates were filtered by passing each sample through a 26-gauge needle five times. Filtered lysates were centrifuged at 5,000 rpm for 10 min. The resulting whole cell lysate pellets and supernatants were stored at -20°C overnight until protein purification. Aliquots of the whole cell lysates and supernatants from both cultures were prepared for SDS-PAGE analysis by combining the samples with 2X Laemmli Sample Buffer and 10% BME (Bio-Rad) at a 1:1 ratio (21).

Purification of rChiC using immobilized metal affinity chromatography (IMAC). The 6xHis-tagged rChiC was purified from the supernatant of the filtered and centrifuged pM3CRYY whole cell lysate using the NEBExpress Ni resin (22, 23). The supernatant was loaded onto a column with 0.5 mL of Ni resin and 10 mL of binding buffer (50 mM Na_2HPO_4, 300 mM NaCl; pH adjusted to 7.5) (22, 23). Before collecting the flowthrough, the samples were incubated in the column for 10 min to allow the 6xHis-tagged rChiC to interact with the Ni resin (22, 23). After collecting the flowthrough, the resin was first washed with 5 mL of 10 mM imidazole buffer followed by 5 mL of 25 mM imidazole buffer (22, 23). rChiC was eluted from the resin using 5 mL of 500 mM imidazole buffer and stored in 0.5 mL aliquots (8). The samples from the binding buffer wash and each of the imidazole washes were prepared for SDS-PAGE analysis by combining the samples with 2X Laemmli Sample Buffer and 10% (BME) (Bio-Rad) at a 1:1 ratio (21). The same protocol was followed for pET-28a supernatant as a negative control.

Dialysis and storage of purified rChiC. IMAC elution fractions were pooled and dialyzed in a 15mm Spectra/Por 4 12-14 kD MWCO membrane for 24 hrs, stirring in 2 L of 1X PBS at 4°C. The dialyzed rChiC was stored in 1 mL aliquots at -70°C in 1X PBS with 3% glycerol.

Chitin binding assay. 160 µL of chitin resin beads (New England Biolabs) were incubated overnight with 40 µL of purified rChiC. A pH 8.5 wash buffer (20 mM Tris-HCl, 0.5 M NaCl) in 10 mL dH_2O (24). The rChiC incubated with the chitin resin beads was suspended in 150 µL of wash buffer three times and spun down at 8,000 rpm for 10 s after each wash. Each of the wash fractions was suspended in 2X Laemmli Sample Buffer with 10% BME (Bio-Rad) at a 1:1 ratio (21). Subsequently, 20 µL of the resin beads were treated with 20 µL 2X Laemmli Sample Buffer with 10% BME (Bio-Rad) to denature the protein and elute it from the beads. The prepared wash and elution fractions were analyzed using SDS-PAGE. To serve as the negative control, the same protocol was followed for 40 µL of 0.5 mg/mL bovine serum albumin (BSA).

SDS-PAGE analysis. Samples prepared in 2X Laemmli Sample Buffer with 10% (BME) (Bio-Rad) were denatured at 95°C for 5 min and then centrifuged at 8,000 rpm for 20 s (21). 10 µL of each denatured sample were loaded per well in an SDS-PAGE gel, along with 10 µL of PageRuler ™ Unstained Broad Range ladder (ThermoFisher Scientific) in a separate well (21). The 15-well gels, either the 4–20% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad) or gels prepared from TGX Stain-Free™ FastCast™ Acrylamide Solutions (Bio-Rad), were used (21, 25). SDS-PAGE was performed using the Bio-Rad protein electrophoresis chamber system. The gels ran at 200 V for 45 min in 1X SDS-running buffer (21). Finally, the gels were visualized using the ChemiDoc Imaging System (Bio-Rad) (21).

DNS assay. DNS reagent was prepared by dissolving 0.5 g 3, 5-dinitrosalicylic acid in 25 mL dH_2O (13). Then, 0.8 g NaOH and 15 g potassium sodium tartrate tetrahydrate were dissolved in 10 mL dH_2O and added to obtain a final solution volume of 50 mL (13). Finally, the solution was heated at 60°C for 10 min (13, 15). If left at room temperature (RT) for too long, white precipitate formed in the solution and was dissolved by re-heating.

Chitosan was dissolved in 1% (w/v) acetic acid solution to form a 2% (w/v) chitosan solution. Chitosan solution was stirred at RT for 24 hrs so that it could fully dissolve (26). A GlcNAc standard curve was prepared in 1% acetic acid with GlcNAc concentrations of 2000, 1000, 500, 250, 125, 6.25, 3.125, and 0 µg/mL.

Streptomyces griseus (S. griseus) chitinase in lyophilized powder form was dissolved in 50 mM KPO_4 to a final concentration of 4.8 mg/mL. *E. coli* pM3CRYY A3 and pET-28a culture supernatants were collected from cultures that were cultured to OD_{600} 0.6-0.8 and then induced with IPTG for 24 hrs (7, 19, 20). *S. griseus* chitinase and rChiC were denatured in PCR tubes at 100°C for 5 min in a thermocycler to serve as negative controls for chitinolytic activity.

After sample preparation, all samples except standard curve samples were incubated at a 1:1 ratio with 2% chitosan in a final volume of 0.3 mL for 1 hr at 37°C in 1.5 mL microfuge tubes. 2% chitosan only was included to measure background signal. All samples and standards were transferred into glass test tubes and combined with DNS reagent at a 1:1 ratio with a final volume of 0.4 mL. Samples were heated in a boiling water bath for 5 min, then cooled until they reached RT (14). DNS reacted with chitosan to produce a thick, gelatinous, cloudy substance so samples were transferred to microfuge tubes and centrifuged in a mini centrifuge for 5 s to sediment the DNS-chitosan reaction product. For each sample, 80 µL of clear supernatant was transferred in duplicate to a microplate and absorbance was measured at 540 nm.

RESULTS

A 55 kDa protein was isolated from *E. coli* BL21 (DE3) pM3CRYY A3. Presence of a His-tagged 55 kDa protein, presumably rChiC, was confirmed in pM3CRYY whole cell lysate. A 55 kDa band was present in the last IMAC imidazole elution fraction of the pM3CRYY sample but not the pET-28a sample (Figure 1). In both the pM3CRYY and pET-

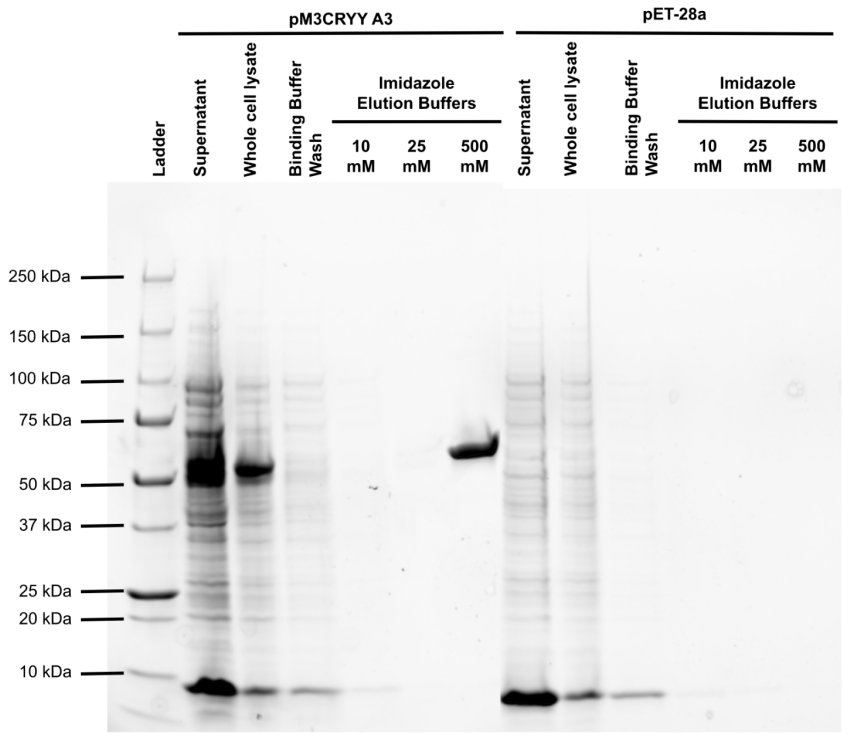

FIG. 1 *E. coli* BL21 (DE3) pM3CRYY A3 expresses a His-tagged 55 kDa protein when induced with IPTG. *E. coli* was cultured overnight, sub-cultured to OD 0.7, and induced with IPTG for 23 hrs prior to cell harvesting and lysis. rChiC was purified from cell lysate using IMAC, with all fractions analyzed by SDS-PAGE. *E. coli* BL21 (DE3) pET-28a cell lysate was included as a negative control. A 55 kDa band was observed in the pM3CRYY A3 500 mM imidazole eluent, while bands in the pET-28a control were present in the binding buffer wash only.

28a samples, no bands were observed in the binding buffer, wash fraction, nor the first two imidazole fractions.

Purified rChiC contained a functional chitin-binding domain. A chitin binding assay was performed to confirm the presence of a functional chitin-binding domain in rChiC. An approximately 55 kDa band was observed in the input and eluent lanes of the rChiC sample (Figure 2). An approximately 66 kDa band was only observed in the input lane of the BSA negative control sample (Figure 2).

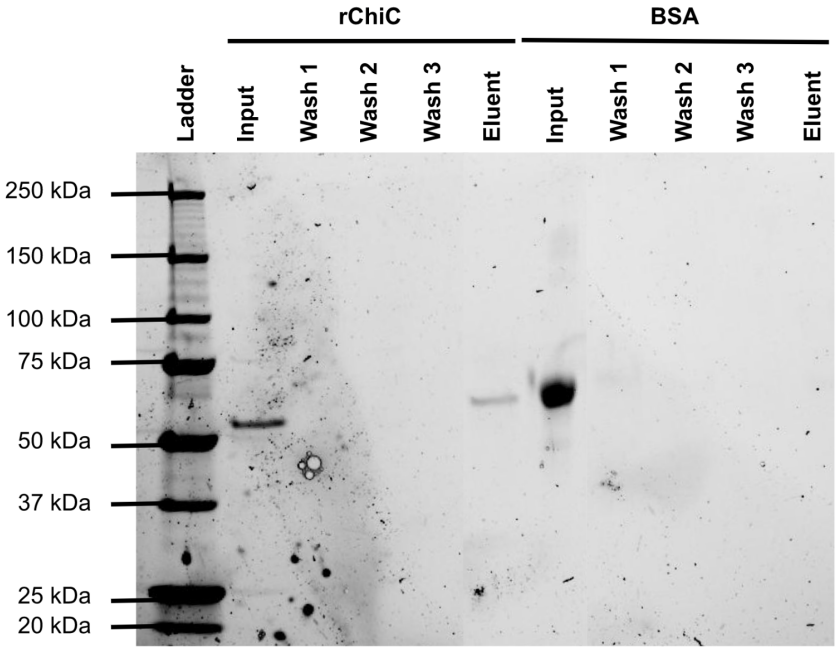

FIG. 2 Purified rChiC binds to chitin resin beads. A chitin-binding assay was performed on the purified rChiC sample using 0.5 mg/mL BSA as a negative control. Samples were incubated with chitin resin beads overnight. Input, supernatant, wash, and eluent samples were analyzed by SDS-PAGE. A 55 kDa band was present in the input and eluent samples of rChiC. A 66 kDa band was present only in the input sample of BSA control.

Purified rChiC and IPTG-induced pM3CRYY culture supernatant had chitinolytic activity against chitosan. To determine the chitinolytic activity of purified rChiC and IPTG-induced pM3CRYY supernatant, a DNS assay was performed. GlcNAc standard curves

showed that DNS reagent was sensitive to changes in GlcNAc concentration with signal showing a strong, positive, linear correlation to increasing GlcNAc (Figure 3). Final GlcNAc concentrations of approximately 600 and 150 µg/mL were measured in *S. griseus* chitinase and rChiC samples respectively (Figure 4A). Final GlcNAc concentrations decreased to approximately 100 and 0 µg/mL in heat-denatured *S. griseus* chitinase and rChiC samples respectively (Figure 4A). The IPTG-induced pET-28a supernatant serving as a negative control produced a baseline signal of approximately 220 µg/mL (Figure 4B). In pM3CRYY A3 supernatant, the signal was greater at approximately 330 µg/mL (Figure 4B).

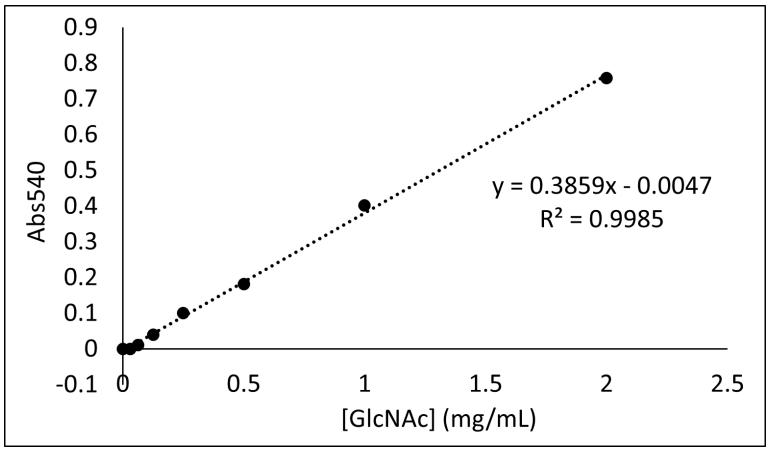

FIG. 3 DNS reagent is linearly sensitive to changes in GlcNAc concentration. A standard curve of GlcNAc was prepared in 1% acetic acid with GlcNAc concentrations of 2000, 1000, 500, 250, 125, 6.25, 3.125, and 0 µg/mL. The DNS assay was performed by adding an equivalent amount of DNS reagent to standards and incubating in a boiling water bath for 5 min. Samples were allowed to cool to RT and absorbance was measured at 540 nm. Values represent the average absorbance of two measurements taken per sample (n=2). A positive linear relationship ($r^2 > 0.998$) was observed between GlcNAc concentration and A_{540}.

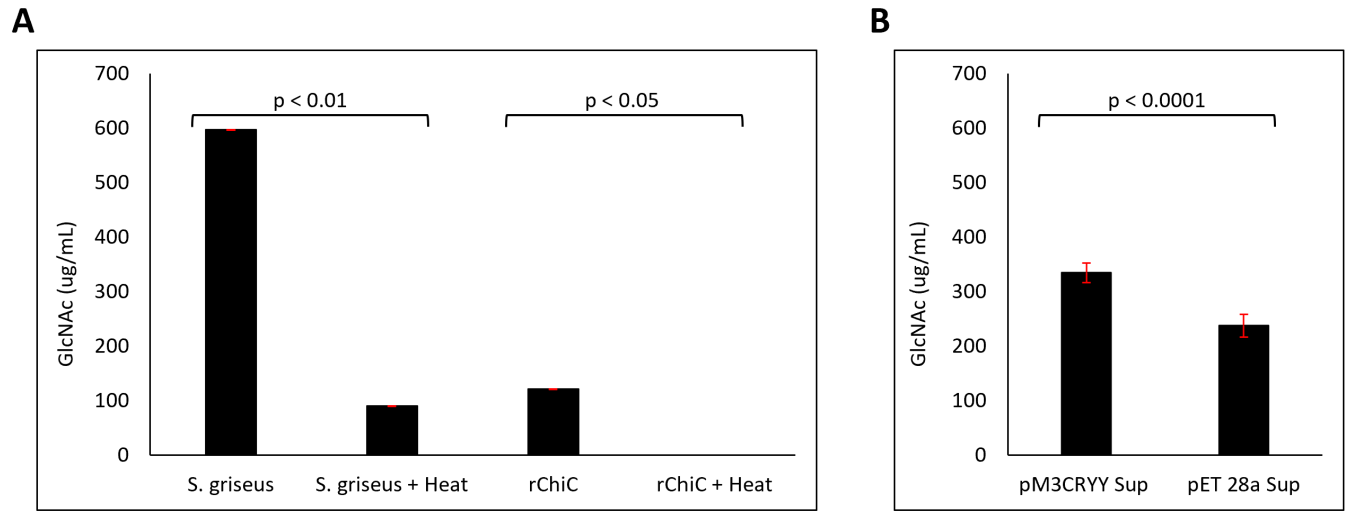

FIG. 4 rChiC-containing samples exhibit chitinolytic activity against chitosan. Samples were incubated with 2% chitosan in 1% acetic acid for 1 hr at 37°C. The DNS assay was performed by adding an equivalent amount of DNS reagent to GlcNAc standards and samples. Samples were incubated in a boiling water bath for 5 min. Samples were allowed to cool to RT and absorbance was measured at 540 nm. GlcNAc concentrations were calculated based on the standard curve. **(A)** Final GlcNAc concentrations in *S. griseus* chitinase and rChiC samples after chitosan incubation. Concentrations are calculated after background (chitosan only) signal subtraction. Negative controls were prepared by denaturing chitinase samples at 100°C for 5 min. Data are presented as the average of two technical replicates (n=2) with error bars representing standard deviation between duplicates. **(B)** Final GlcNAc concentrations in *E. coli* pM3CRYY A3 and pET-28a culture supernatants. Cultures were induced with IPTG for 24 hrs prior to supernatant collection. Data are presented as the average of three biological replicates (n=3) with error bars representing standard deviation between triplicates. P-values are from a one-tailed t-test that is either paired (A) or unpaired, equal variance (B).

rChiC is present in IPTG-induced pM3CRYY culture supernatant. After detecting chitinolytic activity in the pM3CRYY supernatant, we assessed whether rChiC was present in the IPTG-induced pM3CRYY culture supernatant with IPTG-induced pET-28a culture supernatant included as a negative control. SDS-PAGE analysis of the IPTG-induced pM3CRYY supernatant demonstrated the presence of two faint bands at approximately 50

and 60 kDa (Figure 5). Two bands at approximately 50 and 60 kDa were observed in the purified rChiC sample, with the band at 50 kDa appearing more intense (Figure 5). No bands were observed in the IPTG-induced pET-28a culture supernatant.

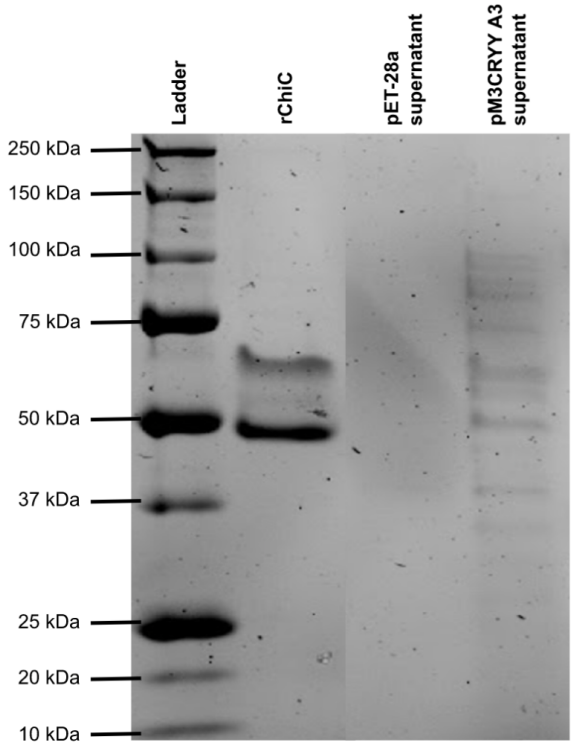

FIG. 5 Supernatant of *E. coli* BL21 (DE3) pM3CRYY A3 contains rChiC. Purified rChiC, IPTG induced pM3CRYY A3 and pET-28a supernatant for 24 hours were analyzed on an SDS-PAGE gel at 200V for 45 minutes. Purified rChiC (Lane 2) presented two distinct bands. One band at ~60 kDa and another at ~50 kDa. A faint band at ~50 kDa was detected in pM3CRYY A3 supernatant (Lane 4) and no band was identified in the negative control, pET-28a supernatant (Lane 3). The molecular weight of bands (kDa) in Lane 2-4 was determined based on the PageRuler Unstained Protein Ladder (Lane 1).

DISCUSSION

rChiC is expressed in an *E. coli* system. It is likely that the protein isolated with IMAC corresponds to rChiC because pM3CRYY includes a 6xHis tag, which is expected to bind tightly to Ni resin and elute from the column only with a high concentration of imidazole. Interestingly, rChiC was present in both the pM3CRYY supernatant and cell lysate. Considering that exo- and endo-chitinases have been identified in chitinolytic bacteria (4, 27, 28), this may indicate that rChiC exists in two forms: intracellular and extracellular. Alternatively, it is possible that rChiC is secreted by *E. coli* into the supernatant, or that *E. coli* cells are lysing during the culture period and releasing rChiC into the supernatant.

The CBD of rChiC retains functionality post-purification. The chitin binding assay indicates that rChiC binds to chitin resin beads, an observation that can be explained by binding between the chitin beads and the CBD of rChiC. The CBD is one of the three domains characterized in ChiC (4), and is demonstrated here to likely be present and functional in rChiC because chitin binding activity has been observed. This finding is supported by previous findings observing chitin binding activity in rChiC purified from the same *E. coli* expression system (8, 9, 10).

A DNS assay for detection of chitinolytic activity in rChiC has been developed. Here, we have shown that chitinolytic activity against chitosan in *S. griseus* chitinase and rChiC is detectable by measurement of GlcNAc concentration with the DNS assay and that a reduction in chitinolytic activity occurs when the chitinases are denatured with heat. This aligns with our hypothesis that rChiC expressed by *E. coli* retains chitinolytic activity post-purification. Using chitosan as a substrate, as previously done in experiments examining chitinolytic activity in *Chitiniphilus shinanonensis* and *Pseudomonas aeruginosa* 385 (29, 30), was a choice made due to the increased solubility of chitosan in acetic acid and greater measurable chitinolytic activity compared to if colloidal chitin is used. In contrast to chitosan, colloidal chitin is typically stored in sodium phosphate solution. However, it is insoluble at 37°C which makes it difficult to work with, and potentially means colloidal chitin as a substrate is less accessible to chitinases it is incubated with. Studies exploring chitinolytic activity in

Stenotrophomonas rhizophila G22 suggest that chitosan-specific activity may be a characteristic of rChiC from select bacteria like *P. aeruginosa* PAO1 (12). Perhaps rChiC is more effective at degrading chitosan than other forms of chitin. However, more research would have to be conducted using a wider variety of chitin derivatives as substrates in this assay to confirm this.

Incubation time and temperature were altered during DNS assay optimization to characterize rChiC activity. Increasing incubation time from 30 min to 1 hr corresponded to an increase in final GlcNAc concentration (data not shown), suggesting that lengthening incubation time allows for the chitinase to degrade more chitosan. Samples were typically incubated at 37°C to promote chitinolytic activity, however they were also heated to 100°C to denature them. This denaturation resulted in a reduction in chitinolytic activity. The decrease in GlcNAc in heated samples suggests that signal in the 37°C incubated samples is attributed to enzymatic activity. rChiC is likely not functional at 100°C because ChiC has been shown to be non-functional at this temperature (12, 27).

rChiC may be secreted by *E. coli*. GlcNAc concentration in pM3CRYY supernatant was significantly greater than in pET-28a supernatant, suggesting that pM3CRYY supernatant demonstrates chitinolytic activity because it contains rChiC. Previous research has shown that ChiC is secreted in trace amounts and retains its functionality (4, 28). However, in *P. aeruginosa* 385, rChiC is localized in the cytoplasm favouring the alternative explanation that rChiC may be released into the supernatant when cell lysis is caused by the experimental conditions (4, 30). The mechanism leading to presence of rChiC in supernatant requires further analysis to distinguish whether rChiC is secreted into the supernatant or released by cell lysis. Additionally, two distinct bands were shown for purified rChiC (Lane 2) on the SDS-PAGE gel (Figure 5). This may be due to different degradation processes of rChiC or from different conformations of rChiC that migrate differently in the gel.

Limitations These findings are considered preliminary as only one biological replicate was examined when assessing chitinolytic activity using chitosan. This suggests that this experiment needs to be repeated to further support these data. Additional replicates can provide validity for DNS detection of chitinolytic activity through statistical evaluation. While additional consideration of incubation and temperature time points can assist with refining the optimal conditions for detecting chitinolytic activity, our study did not explore this due to time constraints.

Another limitation is that evaluating whether the signals generated by DNS assay are accurate as rChiC chitinolytic activity has not previously been tested. Furthermore, the DNS reagent has broad specificity to all reducing sugars. Therefore, the signals measuring chitinolytic activity against chitosan can be further validated using fluorogenic substrate and immunofluorescence staining. Immunofluorescence staining could potentially be used to detect chitinolytic activity in live cell populations.

Though chitinolytic activity was assessed by proxy of reduced monomers, whether GlcNAc is the resulting sugar product is unverified. Mass spectrometry can provide further confidence that GlcNAc was generated.

Lastly, although *E. coli* may secrete rChiC, our SDS-PAGE analysis alone is not able to exclude other plausible mechanisms. The present interpretation on mechanism of secretion or lack thereof cannot be determined without further analysis.

Conclusions This study has optimized a DNS assay for detection of chitinolytic activity against chitosan in rChiC. rChiC was isolated and purified, then confirmed to include a functional CBD. Then, it was demonstrated that rChiC has chitinolytic activity against chitosan. Heat denaturation of rChiC renders it non-functional. Furthermore, IPTG-induced pM3CRYY culture supernatant exhibits chitinolytic activity which suggests that rChiC may be secreted by *E. coli*. However, the mechanism by which rChiC appears in the supernatant has yet to be characterized.

Future Directions While we have developed an assay to detect chitinolytic activity, further protocols can be developed to measure rChiC more quantitatively. This includes the use of fluorogenic substrates to detect enzymatic activity. The fluorescent dye can be detected to directly quantify the amount of chitinolytic activity in the sample. Furthermore, mass spectrometry can be used to identify the peptides from the products of the DNS assay, as well as measure the quantity of the product through the observed peptide peaks. Finally, the enzyme kinetics and reaction mechanism of rChiC have yet to be characterized.

We have also conferred that rChiC is potentially secreted. However, incorporating proteins that are not secreted can also serve as negative controls to more confidently conclude that rChiC is secreted. Future studies can explore the mechanism of rChiC secretion in *E. coli* pM3CRYY which may reveal characteristics of the protein that are beneficial for further research.

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CONTRIBUTIONS

Maria J. Beletsky (MJB), Ashleen Kaur Khatra (AKK), Jenny Shee (JS), and Wei Chuan Kevin Wang (WCKW) contributed equally in the design and experiments performed for isolation and purification of protein by IMAC, chitin binding assay, and DNS assay development. AKK and MJB generated the respective figures for SDS-PAGE analysis, standard curve and chitinolytic activity. AKK and MJB wrote the methodology, results, and figure captions. JS and WCKW wrote the introduction, discussion, study limitations, conclusion, and future directions. All authors contributed equally to the formulation and final editing of this manuscript.

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