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Expression, purification, and functional characterization of potato proteinase inhibitor type II (PI2)

Anna Gao, Hasti Haghdadi, Nicholas Wilkinson, Parsa Khatami

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY Proteinase inhibitors regulate the activity of proteinases, which catalyze the breakdown of other proteins. They are commonly found in plant species to confer protection against external stressors but may also play a role in human health. As such, researchers have aimed to optimize the large-scale recombinant production of proteinase inhibitors with potential clinical applications. One example is potato proteinase inhibitor type II (PI2), which inhibits the activity of serine proteases such as trypsin and has anticarcinogenic and appetite suppressing properties. PI2 requires diligent formation of its multiple disulfide bonds to maintain its functionality. As such, previous studies have used SHuffle *Escherichia coli* strain, which has a favorable cytosolic environment for disulfide bond formation, to produce maltose-binding protein tagged PI2 (MBP-PI2). The present study investigates whether PI2 expressed in SHuffle *E. coli* cells is functional by developing an assay to evaluate its trypsin inhibition activity. Results from this assay revealed that MBP-PI2 produced by SHuffle cells may be capable of inhibiting trypsin activity and that this functionality may be conferred by PI2 rather than the attached MBP tag. Thus, SHuffle *E. coli* strain may be a suitable expression host for large-scale production of functional PI2 for clinical applications. Additionally, the developed trypsin inhibition assay may be adapted for exploration into functionality of other serine protease inhibitors.

INTRODUCTION

Plant species often express proteinase inhibitors in their seeds and storage organs in response to stressors such as insect attacks or viral infections to facilitate defense mechanisms (1-3). However, some of these proteinase inhibitors also have potential therapeutic benefits for humans and should therefore be further studied. One such protein is the potato proteinase inhibitor type II (PI2). PI2 is a 16 kDa dimeric serine proteinase inhibitor expressed primarily in potato tubers (4). This protein provides protection against insect attacks, wounding, viral infection, and other environmental stressors by regulating the activity of proteinases, which catalyze the breakdown of other proteins (4). PI2 also exhibits potential clinical applications as it possesses anticarcinogenic and appetite suppressing properties (5, 6). The anticarcinogenic properties stem from the ability of PI2 to inhibit trypsin, which prevents degradation of the extracellular matrix surrounding cancerous cells and thereby delays metastasis (5). Additionally, PI2 is able to suppress appetite by inhibiting proteases that prevent the production of the appetite suppressing hormone cholecystokinin (6). Given the ranking of cancer as a leading cause of death worldwide and the rise of obesity as a global epidemic, the therapeutic properties of PI2 have led to extensive research into the recombinant expression of the protein in various strains of *Escherichia coli* for potential large-scale production (7-9).

PI2 possesses two functional domain binding sites, with one capable of binding and inhibiting chymotrypsin and the other capable of binding and inhibiting both trypsin and chymotrypsin (4). Structurally, the protein contains eight key disulfide bonds and their proper

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Address correspondence to: Anna Gao, anna.gao78@gmail.com

formation during the folding process of PI2 is driven by the oxidation of thiol groups on cysteine residue pairs (10). Disulfide bond formation is an enzyme catalyzed process involving both membrane bound and soluble enzymes, such as thiol-disulfide oxidoreductases (11). In this process, two electrons are transferred from the sulfur atoms contained within the cysteine residues to other molecules in the cytosol of high reduction potential (11). As such, the proper formation of disulfide bonds relies on the surrounding environment being oxidative in nature (11). Therefore, the expression host utilized for the recombinant production of PI2 must provide an oxidative environment for proper protein folding. *E. coli* is the most popular host for recombinant protein production, and as such, numerous genetic modifications have been conducted on the bacteria. These modifications have led to the generation of different strains, each possessing attributes enhancing recombinant protein production under certain circumstances. One such strain is SHuffle Express (C3028), which was engineered by Lobstein and colleagues at New England Biolabs (12). SHuffle is engineered to possess an oxidative cytosolic environment and to cytoplasmically overexpress the disulfide bond isomerase DsbC (12). DsbC helps refold incorrectly oxidized proteins, which together with the oxidative cytosol, allows SHuffle cells to express large amounts of disulfide bond-containing recombinant proteins such as PI2 (12).

Previously, Grewal *et al.* found that PI2 bound to maltose binding protein (MBP-PI2) could be expressed in the *E. coli* strain SHuffle (13). Furthermore, results from a limited proteolysis analysis of MBP-PI2 purified from SHuffle cells suggested that the protein was folded into a uniform and three dimensional structure rather than forming random aggregates (13). However, the functionality of PI2 was not investigated. Thus, the aim of this paper was to characterize the functionality of PI2 when produced by SHuffle cells under optimized conditions. We hypothesized that SHuffle cells would be able to express functional PI2 due to their oxidative cytosolic environment and their expression of DsbC as these features could support proper protein folding.

METHODS AND MATERIALS

Bacterial strains and plasmids. The SHuffle Express (C3028) strain of *E. coli* was provided by the Straus Lab at the University of British Columbia (UBC) and streaked onto an LB agar plate (15 g tryptone, 7.5 g yeast extract, 15 g NaCl, 7.5 g agar, 1.5 L distilled H_2O) for overnight growth at 30°C. The expression vector, pMAL-c2X, was provided by the Eltis Lab at UBC. The pMAL-c2X-LLMZ16 construct containing an optimized *pi2* gene variant, hereby referred to as pMAL-c2X-PI2, was provided by Lapointe *et al*. (8). Both plasmids were maintained in DH5α *E. coli* cells which were streaked onto LB agar containing ampicillin (100 µg/mL) and grown overnight at 37°C. 5 mL of LB broth containing ampicillin was subsequently inoculated with a single colony from each plate of plasmid-containing DH5α cells and grown overnight at 37°C and 200 rpm. The pMAL-c2X and pMAL-c2X-PI2 plasmids were then isolated from DH5α cells using the BioBasic EZ-10 Spin Column Plasmid DNA Miniprep Kit (Cat no. BS414) and following the manufacturer protocol for purification of low copy plasmid DNA. To confirm their identity, isolated plasmids were sent to GENEWIZ/Azenta Life Sciences (Seattle, WA) for Sanger sequencing using the universal M13F primer (5'-GTAAAACGACGGCCAG-3'). Each plasmid sequence was subsequently aligned in NCBI BLAST against the optimized *pi2* gene variant sequence provided by Lapointe *et al*. (8). Simplified vector maps for both pMAL-c2X and pMAL-c2X-PI2 were created based on the confirmed identity of each plasmid.

Preparation of competent SHuffle Express *E. coli* cells. SHuffle cells were made competent following the protocol outlined by Chang *et al*. (14). Cells were subcultured at a 1/100 dilution and grown to an OD_{600} of 0.4 and placed on ice for 20 minutes. The cells were centrifuged at 4000 rpm at 4°C for 10 minutes and the resulting pellets were resuspended in 20 mL ice-cold 0.1 M $CaCl_2$ and placed on ice for 30 minutes. The centrifugation process was repeated, and the pellets were each resuspended in 5 mL of ice-cold 0.1 M $CaCl_2$ with 15% glycerol. Competent cells were divided into several 100 µL aliquots that were then used for transformation or stored at -70°C.

Transformation of competent SHuffle cells with pMAL-c2X and pMAL-c2X-PI2. The pMAL-c2X (hereby referred to as empty vector or EV) and pMAL-c2X-PI2 plasmids were transformed into competent SHuffle cells following the protocol outlined by Chang *et al*. (14). 75 ng of purified EV or pMAL-c2X-PI2 was added to 100 µL of competent SHuffle cells and placed on ice for 30 minutes. Cells were subsequently heat shocked at 42°C for 30 seconds then placed on ice for 2 minutes. 900 µL of LB broth was added to each sample for a final dilution of 1/10 and the cells were incubated at 30°C for 1 hour at 200 rpm. Transformants were plated on LB agar containing ampicillin and incubated overnight at 30°C.

Optimization of induction to maximize soluble MBP-PI2 expression. A single colony of SHuffle cells transformed with EV or pMAL-c2X-PI2 were inoculated in 5 mL of LB broth containing ampicillin and grown overnight at 30°C and 200 rpm. Cells transformed with pMAL-c2X-PI2 were subcultured at a 1/100 dilution in 250 mL of LB broth containing ampicillin, and 2% glucose. Glucose was included to suppress the lac promoter and the expression of maltose genes on the host chromosome as the proteins expressed could degrade amylose in the downstream protein purification step. Cells transformed with the EV were subcultured in 50 mL under the same conditions. Both subcultures were incubated at 30°C and 200 rpm until they reached an OD_{600} of 0.6. Subsequently, pMAL-c2X-PI2-containing cells were induced with either 0.1 mM or 1 mM of IPTG then incubated shaking overnight at room temperature (RT) or 30°C. All four induction conditions for pMAL-c2X-PI2-containing cells (i.e., 0.1 mM IPTG and RT, 1 mM IPTG and RT, 0.1 mM IPTG and 30°C, 1 mM IPTG and 30°C) were duplicated as technical replicates and completed using 25 mL of culture. 25 mL of uninduced pMAL-c2x-PI2-containing cells served as a negative control and were thereby incubated shaking overnight at RT or 30°C without the addition of IPTG. Additionally, 25 mL of EV-containing cells were induced with 1 mM IPTG then incubated shaking overnight at RT or 30°C to confirm that the EV is capable of expressing MBP-ß-gal-α only and not MBP-PI2.

After the overnight incubation, two 1.5 mL aliquots of each culture flask were collected for testing protein expression. The remaining cells were pelleted by centrifugation at 2000 x g for 15 minutes then stored at -70°C. To obtain the whole cell extract of each culture, one aliquot was centrifuged at 16,000 x g for 10 minutes at 4°C. The resulting pellet was resuspended in 400 µL of lysis buffer (20 mM Tris (pH 7.4), 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol) and incubated on a shaker at 250 rpm and 4°C for 10 minutes. To obtain the soluble cell extract of each culture, the second aliquot was centrifuged at 16,000 x g for 10 minutes at 4°C and the resulting pellet was resuspended in 400 µL of lysis buffer. The resuspended cells were then lysed with sand and the MP Biomedicals FastPrep-24 homogenizer using 2 x 20 second rounds. The insoluble cell fraction and sand were pelleted by centrifugation at 16,000 x g for 10 minutes at 4°C and the soluble supernatant fraction was collected. The prepared whole cell extracts and soluble fractions were separated by SDS-PAGE and subsequently visualized with stain-free gel imaging to examine protein expression in each condition. MBP-PI2 band intensities from the soluble fraction sample of pMAL-c2X-PI2-containing cells induced under the four different conditions were normalized to the total intensity of the sample using densitometry analysis in ImageJ to control for inconsistencies in sample loading. More specifically, the relative density of the MBP-PI2 band was calculated as a ratio of the MBP-PI2 band over a consistent protein band across the gel (i.e., control band). The relative densities of the MBP-PI2 bands from the technical duplicates were then averaged. Finally, the optimal IPTG concentration and induction temperature was determined by comparing the average normalized MBP-PI2 band intensity in the soluble fraction sample from each of the four induction conditions to one another.

Protein purification. Using the method described above, 100 mL of pMAL-c2X-PI2-containing and EV-containing SHuffle cells were induced to express MBP-PI2 and MBP-ß-gal-α respectively at the experimentally determined optimal induction parameters (1 mM IPTG and overnight incubation at RT and 200 rpm). Uninduced pMAL-c2X-PI2-containing cells served as a negative control for MBP-PI2 expression. 1.5 mL aliquots were taken from each culture to prepare whole cell lysates and soluble fraction samples as previously described

and to subsequently confirm expression of MBP-PI2 or MBP-ß-gal-α using SDS-PAGE. The remaining cells of the induced cultures were pelleted by centrifugation at 2000 x g for 15 minutes. The resulting pellet for the MBP-ß-gal-α-expressing cells was stored at -70°C for potential future use. The resulting pellet for the MBP-PI2-expressing cells was used for MBP-PI2 purification by resuspending in 2.5 mL of ice-cold lysis buffer. Resuspended cells were lysed with sand and the soluble fraction was collected as previously described. The soluble fraction was diluted with 12.5 mL of lysis buffer to achieve a 1/6 sample dilution for subsequent purification.

Protein purification steps were adapted from the pMAL™ Protein Fusion & Purification System Instruction Manual (15). A 1.5 mL amylose resin (New England BioLabs, Cat no. E8021S) gravity flow column was used to carry out MBP-PI2 purification at 4°C. The resin storage buffer was collected, and the resin was washed with 5 column volumes or CVs (1 CV = 1.5 mL) of distilled water then equilibrated with 5 CVs of column buffer (identical to lysis buffer). The diluted soluble fraction sample was loaded onto the column and the collected flow-through was added back to the column to increase protein binding then re-collected. The column was then washed with 12 CV of column buffer and the washes were collected in two equal fractions. MBP-PI2 was eluted with 2 CV of elution buffer (20 mM Tris (pH 7.4), 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 10 mM maltose) and 10 elution fractions of approximately 300 µL were collected. The collected flow-through, wash fractions, and elution fractions were analyzed with SDS-PAGE as discussed below. Select elution fractions containing purified MBP-PI2 were either pooled together or kept separate and the MBP-PI2 concentration was quantified using the NanoDrop™ 2000c Spectrophotometer (Thermo Scientific) using the molar extinction coefficient of 82250 M^{-1} cm^{-1} determined by Lapointe *et al.* (8). Aliquots of the purified protein samples were stored at -70°C.

Due to time constraints, purified MBP-ß-gal-α was provided by another team of MICB 401 students to later use in a follow-up experiment. MBP-ß-gal-α was purified primarily using the steps outlined above. Minor procedural differences included: 1) incubating the column containing the soluble cell lysate on an orbital shaker for 1 hour to increase protein binding rather than reloading the lysate flow-through on the column, 2) using 15 CVs of column buffer for washing, 3) using 5 CVs of elution buffer for elution, and 4) concentrating the pooled elution fractions with the 30K MWCO PierceTM 159 Protein 160 Concentrator PES (Thermo Scientific, Cat no. PI88529).

Development of a trypsin inhibition assay. A two-step approach was used to develop a trypsin inhibition assay. First, an optimal concentration of trypsin (MP Biomedicals™, Cat no. ICN1011791) that would allow for visible fragmentation of bovine serum albumin (BSA) (Sigma Aldrich, Cat no. 10735078001) was determined. To do so, a 2-fold serial dilution of 1.24 mg/mL trypsin was prepared and incubated with BSA (1.36 mg/mL). Reactions consisting of trypsin only and BSA only were prepared as negative controls and for comparative purposes. Reaction mixtures were then incubated at 37°C for 15 minutes to activate trypsin. An additional non-incubated trypsin only reaction was included as a control to determine the molecular weight of inactive trypsin. Samples were then analyzed by SDS-PAGE.

Secondly, a suitable concentration of soybean trypsin inhibitor from Glycine max (Sigma-Aldrich, Cat no. T6522) was determined to inhibit trypsin activity at the two optimal concentrations identified in the previous stage. The first optimal concentration of trypsin (0.62 mg/mL) was incubated with a 2-fold serial dilution of 1.86 trypsin inhibitor solution and BSA (1.36 mg/mL). The second optimal concentration of trypsin (0.155 mg/mL) was incubated with a 2-fold serial dilution of 0.47 mg/mL trypsin inhibitor and BSA (1.36 mg/mL). Two negative controls were prepared: a BSA (1.36 mg/mL) only reaction, and a trypsin inhibitor (1.86 mg/mL) with trypsin (0.62 mg/mL) only reaction. A reaction of BSA (1.36 mg/mL) with trypsin (9.62 mg/mL) was also prepared as the positive control. All reactions were incubated at 37°C for 15 minutes. Samples were then analyzed by SDS-PAGE.

Characterizing the trypsin inhibitory function of PI2. Functionality of the purified MBP-PI2 was examined using the optimized trypsin inhibition assay. Elution fractions three and

four of MBP-PI2 were used to create a 2-fold serial dilution starting at 0.37 mg/mL or 0.53 mg/mL and were mixed with trypsin (0.155 mg/mL) and BSA (1.36 mg/mL). Both elution fractions were used in order to test a wider concentration range of MBP-PI2 for trypsin inhibition activity. Four negative control reactions were prepared: BSA only (1.36 mg/mL), trypsin only (0.155 mg/mL), MBP-PI2 only (0.37 mg/mL) from elution fraction three, and MBP-PI2 only (0.53 mg/mL) from elution fraction four.

To determine the source of inhibitory function by MBP-PI2 in a follow-up experiment, the functionality of MBP-ß-gal-α only versus MBP-PI2 was compared. A 2-fold serial dilution of MBP-ß-gal-α (0.22 mg/mL) or MBP-PI2 (0.19 mg/mL) was mixed with trypsin (0.155 mg/mL) and BSA (1.36 mg/mL). Four negative control reactions were prepared: BSA only (1.36 mg/mL), trypsin only (0.155 mg/mL), MBP-PI2 only from elution fraction three (0.19 mg/mL), and MBP-ß-gal-α only (0.22 mg/mL).

For both of the above experiments, a negative control was made to visualize fragmentation of BSA by trypsin using a sample consisting of BSA (1.36 mg/mL) and trypsin (0.155 mg/mL). As a positive control, inhibition of trypsin activity against BSA was visualized using a sample consisting of BSA (1.36 mg/mL), trypsin (0.155 mg/mL), and trypsin inhibitor (0.47 mg/mL). All reactions were then incubated at 37°C for 15 minutes. Samples were subsequently analyzed by SDS-PAGE. BSA and trypsin band intensities were quantified using ImageJ.

SDS-PAGE. Samples were mixed in equal volumes with BioRad 2X Laemmli Buffer (Cat no. 161-0737) containing 2-mercaptoethanol and heated at 95°C for 5 minutes. The proteins present in each sample were resolved on a 4-20% Bio-Rad Mini-PROTEAN® TGX StainFree™ Precast Gel (Cat no. #456-1096) with a Tris-Glycine buffer system and at 200V. Protein bands were compared to BioRad Precision Plus Protein™ Unstained Standards (Cat no. #161-0363) and 20 µg GBiosciences BSA Standard (Cat no. 224B-B).

RESULTS

The pMAL-c2X-PI2 plasmid construct contained the expected *pi2* gene variant. To confirm that the pMAL-c2X-PI2 plasmid in our possession correctly contained the pi2 gene insert prior to proceeding with our experiments, pMAL-c2X-PI2 and the empty expression vector pMAL-c2X were Sanger sequenced with the universal M13 forward primer. Previously, Fogarty *et al.* designed a *pi2* gene variant capable of expressing PI2 (7) that Lapointe *et al*. cloned into the pMAL-c2X expression vector (8). This resulting pMAL-c2X-PI2 plasmid construct also contains the *malE* gene, which encodes a 42.5 kDa maltose-binding protein (MBP) tag and the *lacZα* gene, which encodes an 8 kDa ß-gal-α protein fragment (8). Therefore, the *pi2* gene insert is between the *malE* gene and the *lacZα* gene of the plasmid, together encoding for a 66.5 kDa MBP-PI2-ß-gal-α protein hereby referred to as MBP-PI2. These three genes are all downstream of a *tac* promoter, which is inducible by IPTG (Fig. 1). Finally, this plasmid construct confers resistance to ampicillin by encoding an ampicillin resistance marker (Fig. 1). A BLAST alignment between our Sanger sequenced samples and the optimized *pi2* gene sequence provided by Lapointe *et al*. revealed that the expected *pi2* gene variant was present in pMAL-c2X-PI2 but absent in pMAL-c2X. Therefore, pMAL-c2X-PI2 would encode a 66.5 kDa MBP-PI2 protein while pMAL-c2X would encode a 50.5 kDa MBP-ß-gal-α protein upon induction with IPTG (Fig. 1). Simplified vector maps of both plasmids, adapted from Grewal *et al*. (13), were subsequently generated to display our alignment results (Fig. 1). Overall, pMAL-c2X-PI2 was confirmed to contain the correct *pi2* gene insert and could be used for subsequent MBP-PI2 expression.

Induction with 1 mM IPTG at room temperature maximized the expression of soluble MBP-PI2. Maximizing the amount of expressed soluble PI2 may improve the purified MBP-PI2 yield. This is due to the structure of PI2 containing multiple disulfide bonds, where induction of protein expression may result in random insoluble protein aggregates rather than the properly folded MBP-PI2 protein. Considering this possibility and given that the downstream protein purification step requires the soluble fraction of the cell lysate, four different induction conditions for MBP-PI2 expression were tested as described in the methods to identify the condition with the maximum expression of soluble MBP-PI2. SDS-

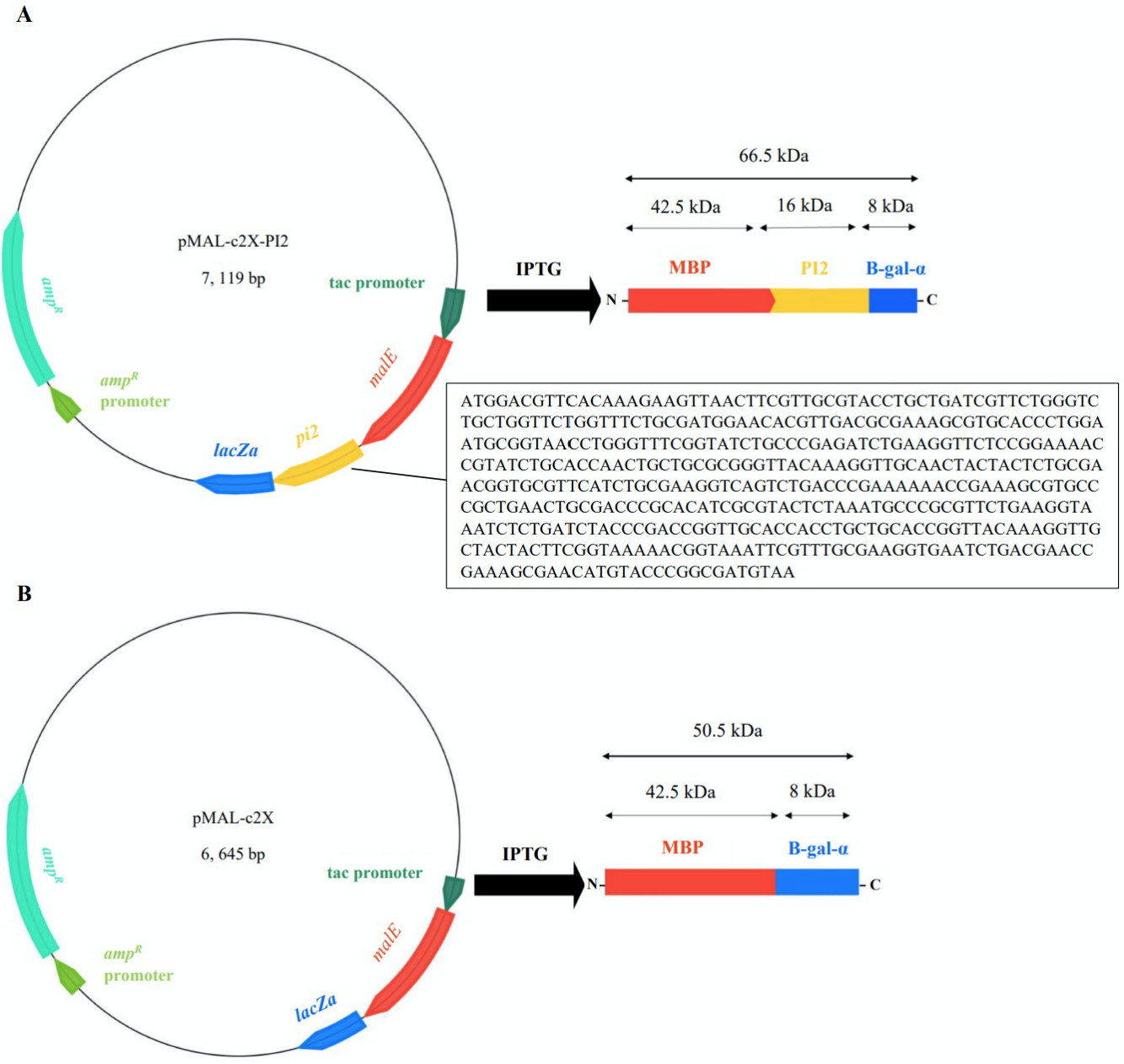

FIG. 1 Presence of the *pi2* gene was confirmed in the pMAL-c2X-PI2 plasmid construct. (A) The pMAL-c2X-PI2 vector contains the expected optimized *pi2* gene insert as indicated by the boxed nucleotide sequence for *pi2*. When induced with IPTG, the resulting fusion protein (66.5 kDa) is expected to consist of PI2 (16 kDa) flanked by a maltose-binding protein tag (42.5 kDa) at the N-terminus and ß-gal-α (8 kDa) at the C-terminus end. **(B)** The empty vector, pMAL-c2X, was confirmed to not contain the *pi2* gene insert, thereby encoding for MBP-ß-gal-α (50.5 kDa) when induced by IPTG.

PAGE analysis of the whole cell and soluble fraction lysates from induced pMAL-c2X-PI2-containing cells revealed a band at 66.5 kDa consistent with the expected molecular weight of MBP-PI2, thereby confirming that pMAL-c2X-PI2 confers MBP-PI2 expression upon induction (Fig. 2A, 2B). SDS-PAGE analysis of lysates from uninduced pMAL-c2X-PI2-containing cells revealed the lack of a protein band at the molecular weight of MBP-PI2, as expected (Fig. 2A). Similarly, there were no MBP-PI2 bands apparent in the lysates from induced EV-containing cells (Fig. 2A). However, a band at 50.5 kDa consistent with the expected molecular weight of MBP-ß-gal-α was present in the lysates from induced EV-containing cells, thereby confirming that pMAL-c2X confers MBP-ß-gal-α expression upon induction (Fig. 2A).

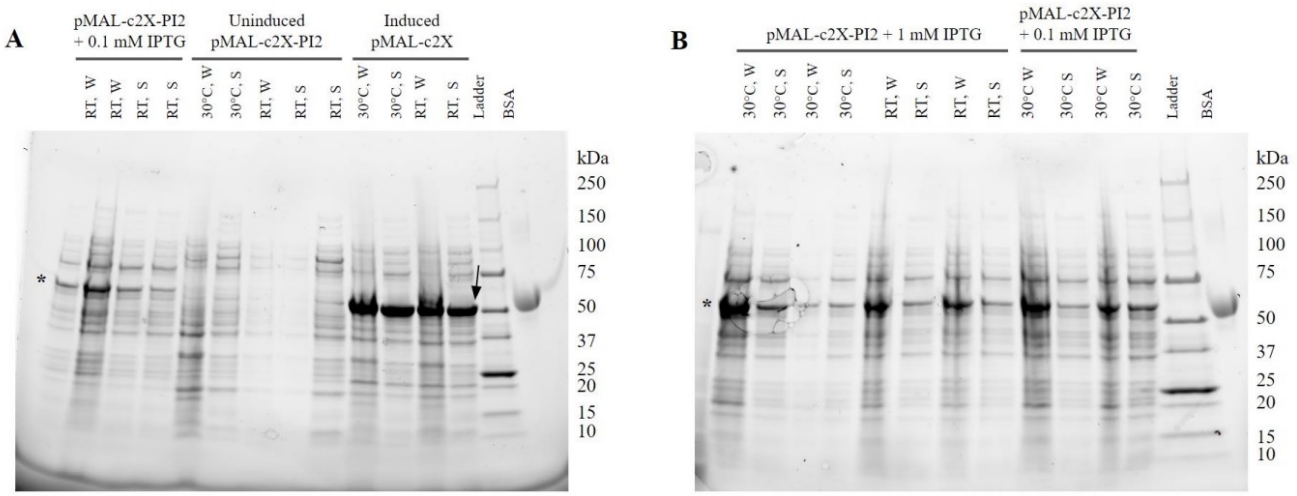

FIG. 2 SDS-PAGE analysis of MBP-PI2 expression identified 1 mM IPTG and room temperature to be optimal for induction. (A-B) SHuffle cells transformed with either pMAL-c2X-PI2 or pMAL-c2X were cultured overnight and induced with either 1.0 mM or 0.1 mM IPTG at either room temperature (RT) or 30ºC. Uninduced pMAL-c2X-PI2 transformed cells and induced pMAL-c2X transformed cells served as negative controls. Whole cell lysates (W) and soluble cell fractions (S) were extracted from the cultures and analyzed using SDS-PAGE, with each induction condition carried out in technical duplicates. A reference ladder and 2 mg/L bovine serum albumin (BSA) (66.5 kDa) were used as molecular weight standards. Bands of varying densities at the expected molecular weight of MBP-PI2 (66.5 kDa) were observed in all conditions, as indicated by the asterisk, except for the negative controls. **(A)** In the induced pMAL-c2X condition, bands at 50.5 kDa indicated the presence of MBP-ß-gal-α, as indicated by the arrow. Refer to Table S1 for the associated densitometry analysis.

Quantification using ImageJ of the MBP-PI2 band in the soluble fraction from each induction condition revealed that induction with 1 mM IPTG at RT had the most concentrated MBP-PI2 band and therefore the highest relative expression of soluble MBP-PI2 (Table S1). These results demonstrate that MBP-PI2 expression is inducible in SHuffle cells transformed with pMAL-c2X-PI2. Additionally, induction with 1 mM of IPTG followed by overnight incubation at RT can be implemented to increase purified protein yield for downstream analyses of MBP-PI2 functionality.

Two elution fractions of purified MBP-PI2 were chosen for subsequent protein functionality analysis. To characterize the functionality of PI2, MBP-PI2 was isolated and purified from SHuffle cells. The optimized induction condition of 1 mM IPTG at room temperature was first used to maximize expression of soluble MBP-PI2. SDS-PAGE analysis of the soluble fraction lysate from induced pMAL-c2X-PI2-containing cells confirmed MBP-PI2 expression by revealing a 66.5 kDa band that was absent from the soluble fraction lysates of both negative controls (Fig. S1). Moreover, a protein band for MBP-ß-gal-α was present in the soluble fraction lysate of induced EV-containing cells, as expected (Fig. S1). Subsequently, MBP-PI2 was purified from SHuffle cells using affinity chromatography and the protein content of each elution fraction was analyzed by SDS-PAGE (Fig. 3). The presence of lower molecular weight bands that were not MBP-PI2 within the different elution fractions indicated the presence of impurities or protein degradation. While the 66.5 kDa MBP-PI2 band was distinctly visible in all elution fractions, the third elution fraction contained the purest yet most concentrated MBP-PI2 due to the reduced presence of lower molecular weight bands (Fig. 3). Therefore, the third elution fraction was chosen for subsequent functional characterization of PI2. While smaller molecular weight bands were visible in elution fraction four (Fig. 3), this fraction was also chosen for subsequent functional characterization of PI2 due to its higher analyzable amount of protein evidenced by its higher band intensity of MBP-PI2 (Fig. 3). Notably, SDS-PAGE analysis also revealed that some MBP-PI2 was lost in the lysate flow-through (FT), which may account for reduced yield (Fig. 3). However, there was very minimal protein loss in the washes conducted during the purification steps (Fig. 3). Overall, MBP-PI2 was purified from SHuffle cells to be used for characterization of its functionality.

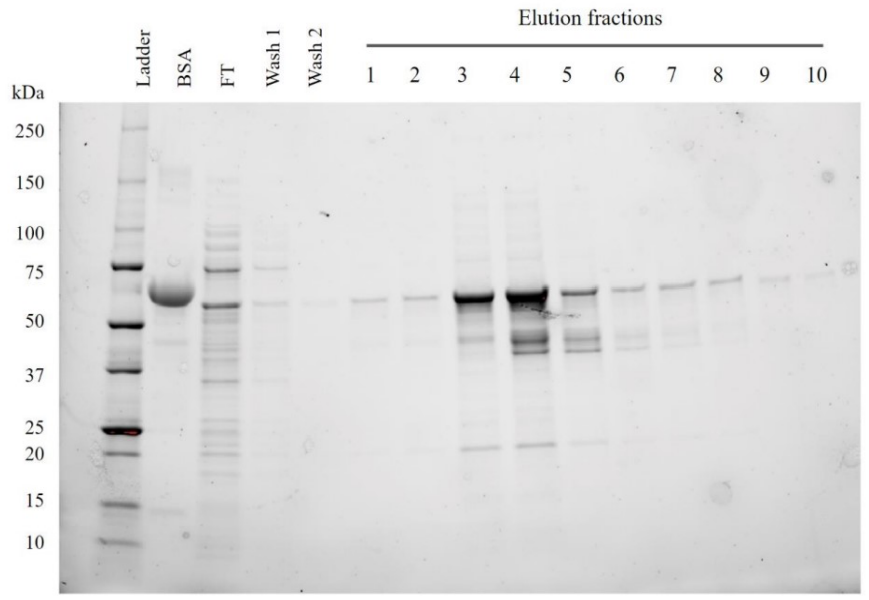

FIG. 3 Elution fractions three and four contained the highest yield of purified MBP-PI2. The soluble cell extract from induced pMAL-c2X-PI2-containing SHuffle cells was purified using affinity chromatography on an amylose resin column. The collected flow-through (FT), wash fractions, and elution fractions were analyzed with SDS-PAGE to analyze their protein content. A reference ladder and 2 mg/L bovine serum albumin (BSA) (66.5 kDa) were used as molecular weight standards. MBP-PI2 (66.5 kDa) was observed in all elution fractions. Lower molecular weight proteins appear to have been co-purified with MBP-PI2 in several elution fractions.

A trypsin inhibition assay was developed. As PI2 is a known trypsin inhibitor, a trypsin inhibition assay was developed to assess the functionality of our purified MBP-PI2. Trypsin functions by hydrolyzing peptide bonds at the C-terminal end of its substrate to cause fragmentation (16). Given that trypsin can use BSA as a substrate, we first determined the minimum concentration of trypsin needed to result in visible fragmentation of BSA on an SDS-PAGE gel. From a 2-fold serial dilution of trypsin, 0.16 mg/mL to 1.24 mg/mL of trypsin resulted in two distinct bands at approximately 60 kDa and 50 kDa that originated from fragmentation of the 66.5 kDa BSA (Fig. 4A). Decreasing the concentration of trypsin resulted in an increase in BSA band intensity and the disappearance of the two BSA fragmentation bands, as expected (Fig. 4). Interestingly, among the two trypsin-only controls, we noted that the trypsin (observed at approximately 23 kDa) underwent autolysis when activated at 37°C for 15 minutes, as evidenced by the comparison to the larger and higher intensity trypsin band in the lane without incubation at the activation temperature (Fig. 4A). From the range of trypsin concentrations determined to cause visible BSA fragmentation, the lowest concentration of 0.16 mg/mL was chosen for subsequent assay development steps. Additionally, a concentration of 0.62 mg/mL was also chosen due to its more distinct fragmentation of BSA to evaluate if a higher concentration of trypsin would be necessary in the assay.

Next, the concentration of trypsin inhibitor (TI) needed to inhibit the determined optimal trypsin concentrations was identified. For both trypsin concentrations, using TI at 3 times the trypsin concentration (i.e. highest tested TI concentration for each trypsin concentration) resulted in a BSA band at approximately the same intensity as the BSA only control and the disappearance of the 60 kDa and 50 kDa BSA fragmentation bands (Fig. 4B). Dilutions of TI beyond 3-times the 0.62 mg/mL trypsin concentration were insufficient at inhibiting trypsin activity, as the fragmented bands reappeared and the BSA band intensity decreased in a TI concentration-dependent manner (Fig. 4B). In contrast, there appeared to be stronger BSA band intensities within the serial dilution of TI incubated with 0.16 mg/mL trypsin, which persisted for both the original and first serial dilution of TI concentrations, indicating that the lower concentration trypsin is more strongly and consistently inhibited by TI (Fig. 4B). Notably, for all TI concentrations, the band intensity of trypsin was greater than the negative control, suggesting that the TI may have inhibited autolysis activity of trypsin across all TI concentrations (Fig. 4B).

From these results, it appears that the smaller concentration of trypsin (0.16 mg/mL) was sufficient at causing visible BSA fragmentation and could also be most clearly inhibited by the 0.47 mg/mL concentration of TI (Fig. 4). Together, three main findings from this set of experiments allowed us to proceed with an optimized trypsin inhibition assay. Firstly, a negative control would consist of BSA (1.36 mg/mL) with trypsin (0.16 mg/mL) that

exhibited clear fragmentation of BSA. Secondly, a positive control would consist of BSA (1.36 mg/mL), trypsin (0.16 mg/mL), and trypsin inhibitor (0.47 mg/mL) that would show inhibition of trypsin activity against BSA. Lastly, inhibition of trypsin activity would be assessed by either the lack of BSA fragmentation at 60 kDa and 50 kDa or an increase in BSA and trypsin band intensity.

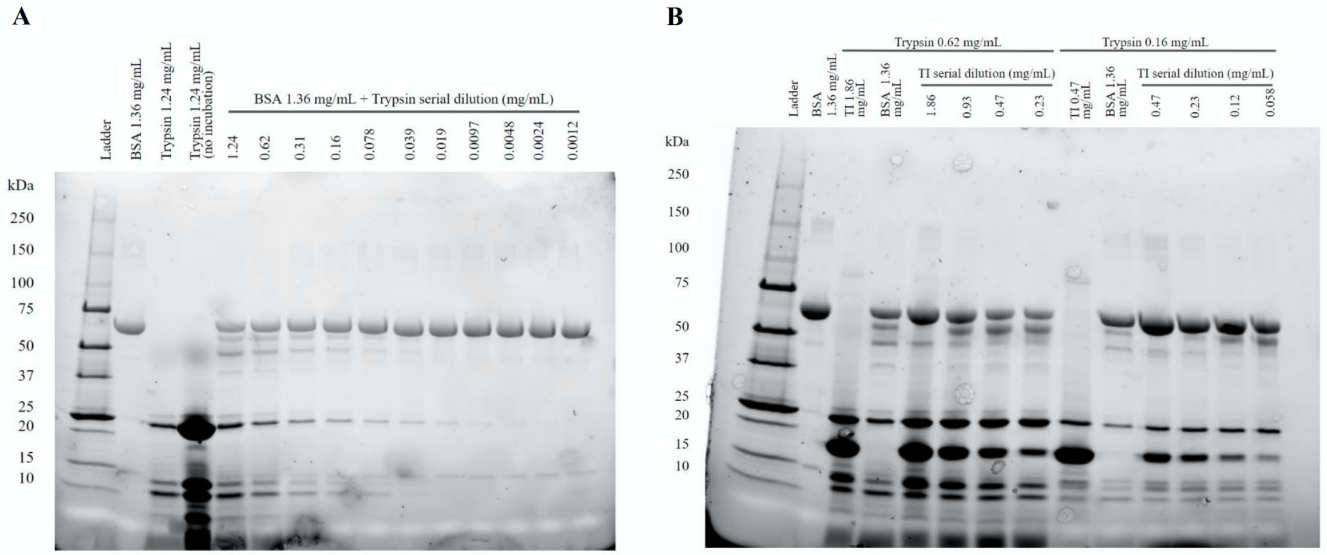

FIG. 4 A trypsin inhibition assay was developed. (A) To visualize fragmentation of bovine serum albumin (BSA) by trypsin, a 2-fold serial dilution of trypsin starting from 1.24 mg/mL was incubated with BSA (1.36 mg/mL) at 37°C for 15 minutes before analysis with SDS-PAGE. Trypsin autolysis was evaluated using trypsin-only controls either with or without incubation. **(B)** To identify the concentration of trypsin inhibitor (TI) needed to suppress activity of trypsin at 0.62 mg/mL or 0.16 mg/mL, two sets of TI 2-fold serial dilutions were made starting at 1.86 mg/mL and 0.47 mg/mL respectively and incubated with trypsin and BSA (1.36 mg/mL) at 37°C for 15 minutes. BSA (1.36 mg/mL) and trypsin (0.62 mg/mL or 0.16 mg/mL) reactions served as negative controls, which featured BSA fragmentation represented by 60 kDa and 50 kDa bands. TI (1.86 mg/mL or 0.47 mg/mL) and trypsin (0.62 mg/mL or 0.16 mg/mL) reactions served as molecular weight references for TI (18 kDa) and trypsin (23 kDa) respectively, and all reactions were analyzed by SDS-PAGE. **(A-B)** A reference ladder and 1.36 mg/mL BSA (66.5 kDa) were used as molecular weight standards.

MBP-PI2 appears to have PI2-specific trypsin inhibitory activity. Our trypsin inhibition assay was used to assess the functionality of our purified MBP-PI2 from elution fractions three and four (Fig. 3). To confirm the presence of our purified MBP-PI2, negative controls consisting of only MBP-PI2 were visualized and both lanes featured a band at the expected MBP-PI2 size (Fig. 5A). Considering that multiple concentrations of TI were capable of inhibiting trypsin activity (Fig. 4B), we analyzed a range of MBP-PI2 concentrations using a 2-fold serial dilution of each MBP-PI2 elution fraction (Fig. 5A) to identify which concentrations of MBP-PI2 were capable of trypsin inhibition. Unexpectedly, there was the appearance of two faint bands below BSA at all concentrations of MBP-PI2, including the positive and negative controls (Fig. 5A). Given that these bands aligned with the location of fragmented BSA previously observed at 60 kDa and 50 kDa, we instead conducted a quantitative assessment of the BSA and trypsin band intensity using densitometry with ImageJ to determine if MBP-PI2 was functional. BSA band intensities of the positive control and all reactions containing MBP-PI2 were quantitatively comparable and qualitatively similar on the SDS-PAGE gel (Table S2, Fig. 5), while the negative control featured an approximate 40% relative reduction in BSA band intensity (Fig. 5B, Table S2). This suggests that fragmentation of BSA by trypsin was inhibited in the presence of both the known TI from soybean and our purified MBP-PI2. In parallel with these results, trypsin band intensity was also increased in the positive control and MBP-PI2 reactions (Table S2). Therefore, it was likely that the trypsin was also inhibited from undergoing autolysis in the presence of the inhibitors. Additionally, the functionality of MBP-PI2 appeared to be concentration-dependent, with higher MBP-PI2 concentrations leading to an increase in BSA and trypsin band intensity (Fig. 5B, Table S2). Taken together, these findings suggest that MBP-PI2 may inhibit trypsin.

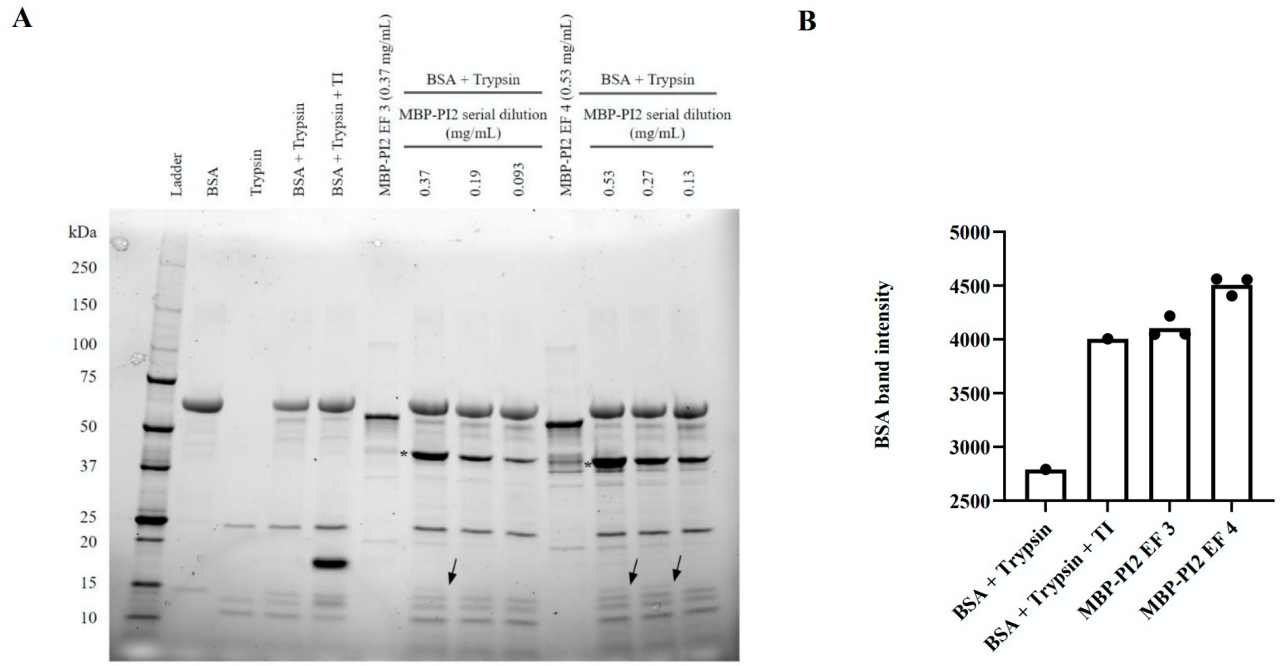

FIG. 5 MBP-PI2 may be functional at inhibiting trypsin activity. (A) To assess MBP-PI2 functionality, a 2-fold serial dilution of two concentrations of MBP-PI2 (starting from 0.53 mg/mL or 0.37 mg/mL) were incubated with trypsin (0.16 mg/mL) and bovine serum albumin (BSA) (1.36 mg/mL) at 37°C for 15 minutes, then analyzed by SDS-PAGE. BSA (1.36 mg/mL) and trypsin (0.16 mg/mL), which featured BSA fragmentation bands at 60 kDa and 50 kDa, served as a negative control. BSA (1.36 mg/mL), trypsin inhibitor (TI) (0.47 mg/mL), and trypsin (0.16 mg/mL), which did not feature BSA fragmentation, served as a positive control. A reference ladder and 1.36 mg/mL BSA (66.5 kDa) were used as molecular weight standards. Asterisks indicate the presence of MBP (42.5 kDa) bands and arrows indicate the presence of PI2 bands (16 kDa). **(B)** BSA band intensities were measured using ImageJ from the SDS-PAGE gel to indirectly assess BSA fragmentation. Average BSA intensity from each corresponding MBP-PI2 elution fraction is represented (n = 3). Negative control consists of the BSA + trypsin group (n = 1) and positive control consists of the BSA + trypsin + TI group (n = 1). Refer to Table S2 for the associated densitometry analysis.

To support these findings, we sought to identify if it was the PI2 domain of MBP-PI2 that conferred this trypsin inhibition activity observed in the previous experiment (Fig. 6A). Therefore, the trypsin inhibition assay was repeated with MBP-ß-gal-α and MBP-PI2 (Fig. 6A). In contrast to the positive control from Fig. 5A, there was a clear lack of BSA fragmentation bands at 60 kDa and 50 kDa in the positive control (Fig. 6A). Additionally, BSA fragmentation was clearly apparent in the negative control as represented by the smaller 60 kDa and 50 kDa bands under the originating 66.5 kDa BSA band (Fig. 6A). Therefore, in addition to BSA and trypsin band intensity, fragmentation of BSA was also used to assess whether there was trypsin activity in this experiment. Notably, while BSA fragmentation was seen below the BSA band in the MBP-ß-gal-α reaction with BSA and trypsin, there was no fragmentation in the MBP-PI2 reaction with BSA and trypsin (Fig. 6A). This suggests that PI2 may be responsible for the trypsin inhibitory activity of MBP-PI2. To further support our qualitative results, the BSA band intensity from both the MBP-ß-gal-α and MBP-PI2 reactions was quantified using ImageJ, which revealed that the BSA band intensity in the MBP-ß-gal-α reaction was lower than the MBP-PI2 reaction (Fig. 6B, Table S3). Additionally, the trypsin band intensity for the MBP-ß-gal-α reaction was reduced in comparison to the MBP-PI2 reaction, indicating trypsin autolysis (Table S3). Autolysis is a common property of exocrine proteases, such as trypsin, as their active state results in self-digestion (16). Together, these findings suggest that MBP-ß-gal-α does not inhibit trypsin activity on its own. Therefore, since inhibition of trypsin was evident in the MBP-PI2 reactions, we conclude that the PI2 domain of the recombinant MBP-PI2 protein may inhibit trypsin.

In addition, it is interesting to note that in the presence of trypsin and BSA, both MBP-PI2 and MBP-ß-gal-α appeared to have the MBP tag cleaved from the original protein (Fig.

5A, 6A). A clear serial dilution in band intensity was seen at approximately 42.5 kDa for both MBP-PI2 and MBP-ß-gal-α reactions, indicating that MBP-PI2 (66.5 kDa) had likely been fragmented into MBP (42.5 kDa) and PI2-ß-gal-α (24 kDa), and MBP-ß-gal-α (50.5 kDa) had been fragmented into MBP (42.5 kDa) and ß-gal-α (8 kDa) (Fig. 5A, 6A). Additionally, at concentrations of MBP-PI2 higher than 0.27 mg/mL, a faint band at approximately 16 kDa was visible, which is the size of PI2 (Fig. 5A). Though the mechanism of this fragmentation of MBP-PI2 and MBP-ß-gal-α is unclear, it may be a factor in the functionality of PI2. Nonetheless, these results support that SHuffle cells may be able to express a functional form of PI2.

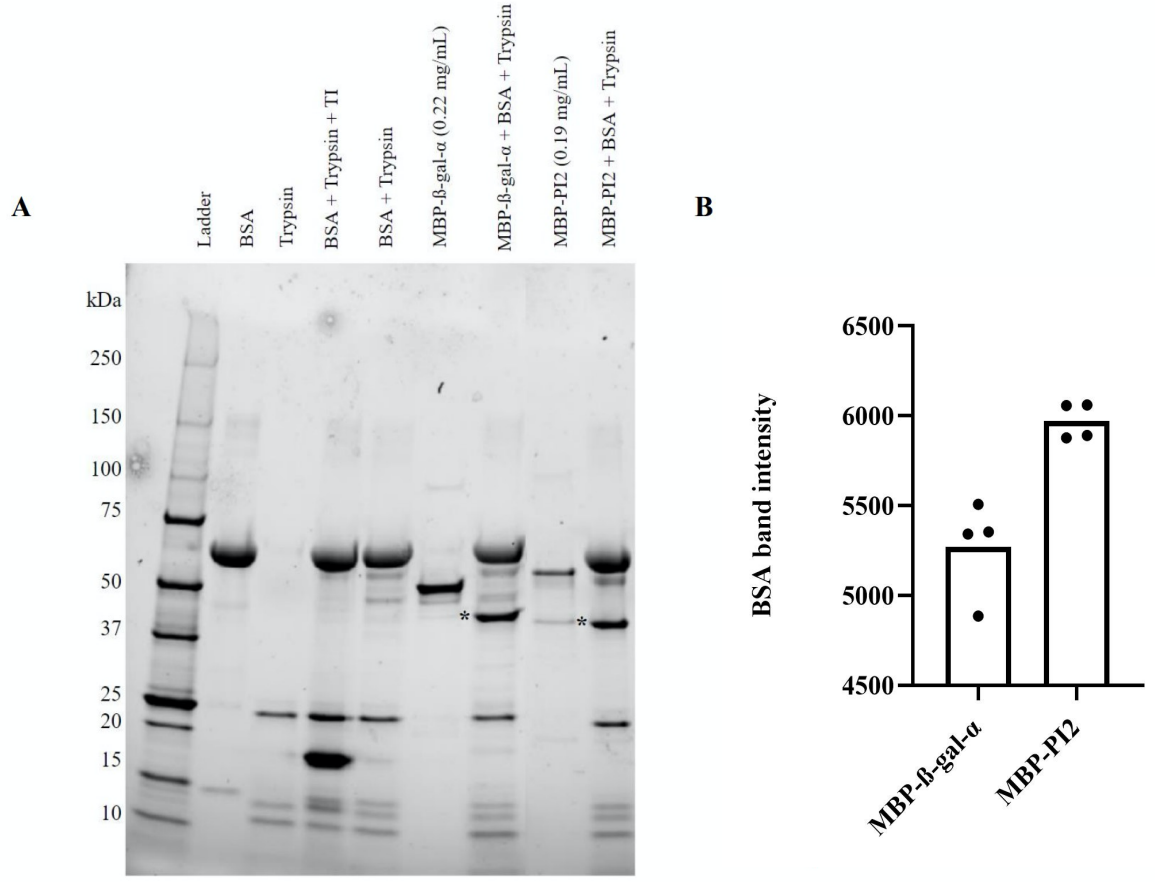

FIG. 6 The trypsin inhibition activity of MBP-PI2 may be facilitated by PI2. (A) To determine if the trypsin inhibitory activity of MBP-PI2 previously observed is due to the activity of PI2 and not MBP, either MBP-PI2 (0.19 mg/mL) or MBP-ß-gal-α (0.22 mg/mL) was incubated with trypsin (0.16 mg/mL) and bovine serum albumin (BSA) (1.36 mg/mL) at 37°C for 15 minutes then analyzed by SDS-PAGE. BSA (1.36 mg/mL) and trypsin (0.16 mg/mL), which featured BSA fragmentation, served as a negative control. BSA (1.36 mg/mL), trypsin inhibitor (TI) (0.47 mg/mL), and trypsin (0.16 mg/mL), which did not feature BSA fragmentation at 60 kDa and 50 kDa, served as a positive control. A reference ladder and 1.36 mg/mL BSA (66.5 kDa) were used as molecular weight standards. MBP-ß-gal-α (50.5 kDa) and MBP-PI2 (66.5 kDa) only samples served as molecular weight references for MBP-ß-gal-α and MBP-PI2 respectively. Asterisks indicate the presence of MBP (42.5 kDa). **(B)** BSA band intensities were measured using ImageJ from the SDS-PAGE gel to indirectly assess BSA fragmentation. Average BSA intensity from MBP-ß-gal-α and MBP-PI2 groups are represented (n = 4). The full SDS-PAGE gel image can be found in Fig. S2. Refer to Table S3 for the associated densitometry analysis.

DISCUSSION

Despite the many potential applications of PI2, recombinant expression of this protein has not yet been established. Previous research by Grewal *et al.* investigated the expression of MBP-tagged PI2 in various strains of *E. coli* and found that only in the SHuffle strain of *E. coli* was the protein expressed in measurable quantities (13). To follow up on this finding, the functionality of MBP-PI2 expressed in SHuffle *E. coli* cells was investigated in this study.

To begin this characterization of MBP-PI2 functionality, the effects of temperature and IPTG concentration on protein expression were investigated to determine optimal induction

conditions and thereby, maximize soluble MBP-PI2 yield. We found that expression of soluble protein was at its highest with the use of 1 mM IPTG concentration and incubation at room temperature (Fig. 2, Table S1). This finding contradicts research detailing the metabolic burden that greater IPTG concentrations exert on host cells, which can limit cell growth and protein production (18). The most likely explanation for our result is that both concentrations of IPTG tested (0.1 mM and 1 mM) were below the threshold concentration of IPTG that is toxic to SHuffle cells, and therefore an increase in inducer concentration led to an increase in protein expression. However, because only two IPTG concentrations were tested, we cannot fully discount the possibility that an IPTG concentration between 0.1 mM and 1 mM may lead to greater protein expression. The observed influence of temperature on induction, however, was in line with research analyzing expression levels of disulfide bond-containing proteins in SHuffle cells that found lower temperatures yielded higher amounts of isolatable protein (12).

Following induction optimization, MBP-PI2 was purified from SHuffle cells and, consistent with previous research by Grewal *et al.,* it was found to be purified along with a number of off-target proteins (Fig. 3) (13). As Grewal *et al.* proposed, it seems likely that these lower molecular weight bands are the result of protease degradation of MBP-PI2, and that the band present just above 37 kDa is likely MBP as it has a molecular weight of 42.5 kDa (13). This explanation is made more probable since we did not add protease inhibitors to our lysis buffer (i.e., column buffer) because protease inhibitors would have interfered with subsequent functional assays aimed at analyzing the function of PI2, which is itself a protease inhibitor.

To assess the functionality of PI2 as a serine protease inhibitor, we first developed the parameters for a trypsin inhibition assay that can determine whether PI2 can inhibit trypsin, a serine protease. BSA, which is a substrate of trypsin, was selected for the trypsin inhibition assay because trypsin cleaves BSA at the C-terminal end of its lysine and arginine residues, therefore allowing for visualization of cleaved polypeptide fragments directly below the BSA band itself (Fig. 4A). The BSA fragmentation visible in our negative control condition consisted of 60 kDa and 50 kDa degradation products below the 66.5 kDa BSA band following exposure to trypsin (Fig. 4A). As there was no knowledge of the functionality or potency of PI2 as a serine proteinase inhibitor when expressed by SHuffle cells prior to this study, the minimum trypsin concentration capable of causing BSA fragmentation was chosen to diminish the possibility of overwhelming our isolated MBP-PI2 protein with trypsin when completing the subsequent trypsin inhibition assay. Moreover, the determined optimal trypsin concentration value of 0.16 mg/mL is in line with previous research by Sasai *et al.* (16).

Following selection of the optimal trypsin concentration, we analyzed various concentrations of TI for their ability to prevent BSA fragmentation by trypsin (Fig. 4B). In addition to the absence of fragmentation bands located below BSA in treatments where trypsin cleavage of BSA was prevented by TI, we observed an increase in the band intensity of both BSA and trypsin (Fig. 4B). The latter phenomenon likely results from the prevention of trypsin autolysis, which commonly occurs when trypsin is active (16). Therefore, in addition to observing less BSA fragmentation, increasing BSA and trypsin band intensities in the presence of TI was used as a proxy for indicating trypsin inhibition.

Having optimized conditions for a trypsin inhibition assay, we were then able to assess the functionality of MBP-PI2 as a protease inhibitor. Every lane with trypsin and BSA exhibited some degree of BSA fragmentation, as indicated by the presence of polypeptide fragments underneath the 66.5 kDa BSA band at 60 kDa and 50 kDa (Fig. 5A). This likely indicates the presence of protease contaminants in our sample. However, the presence of MBP-PI2 resulted in BSA and trypsin band intensities similar to that observed in the positive control (i.e., trypsin is inhibited), and greater than that observed in the negative control (i.e., trypsin is active). Therefore, given our previous conclusion that increasing BSA and trypsin band intensities in the presence of a TI can indicate trypsin inhibition, MBP-PI2 appeared to have some degree of trypsin inhibitory function. To further support the results of our SDS-PAGE analysis, densitometry analysis indicated that MBP-PI2 was inhibiting trypsin in a concentration-dependent manner, with the greater MBP-PI2 concentration (elution fraction four) inhibiting a higher concentration of trypsin (Fig. 5B). Therefore, it seems probable that the protein is expressed in a functional form by SHuffle cells. This result may indicate the

potential for large-scale production of recombinant MBP-PI2. Such production has potential applications in both agriculture and medicine, in the former case as a natural insecticide, and in the latter case as an antimetastatic drug or appetite suppressant (2, 5, 6). Additional potential applications of recombinantly expressed PI2 include use as a preservative for certain food items prone to proteinaceous decay, as a stabilizer for proteases in storage by preventing their autolysis, and as a biochemical tool used for the studying of enzyme structure (19). Additionally, our findings may serve as a general paradigm for using the SHuffle *E. coli* strain to recombinantly express other important disulfide bond-containing proteins in their active form. Notably, the probable functionality of MBP-PI2 observed in this assay is consistent with the limited proteolysis assay completed by Grewal *et al.*, which indicated that MBP-PI2 isolated from SHuffle cells was likely folded into its correct conformation (4). One unexpected finding from this assay was the cleavage of MBP-PI2 into MBP and PI2 in MBP-PI2 concentrations higher than 0.27 mg/mL, as evidenced by the presence of a band at 16 kDa representative of PI2, and a 42.5 kDa representative of MBP (Fig. 5A). The flexible linkage between MBP and PI2 consists of valine and aspartic acid residues (20). Given the composition of this linker sequence, and the fact that trypsin catalyzes peptide bond hydrolysis at the C-terminal end of arginine and lysine residues, it seems unlikely that this cleavage of MBP-PI2 is due to uninhibited trypsin (21). Instead, a more likely explanation is that there were other contaminating proteases from the elution buffer. These proteases may have been activated during the incubation period carried out for the activation of trypsin, and subsequently cleaved MBP-PI2 into its two component proteins.

Following the demonstration of MBP-PI2 functionality, we sought to determine whether trypsin inhibition could be attributed to PI2 alone, rather than to the MBP tag attached to it. To do so, the trypsin inhibition assay was repeated with both MBP-β-gal-α and MBP-PI2. Only the MBP-β-gal-α sample resulted in the appearance of BSA fragmentation bands at 60 kDa and 50 kDa along with decreased BSA band intensity, indicating trypsin inhibition was occuring with MBP-PI2 only and not MBP-β-gal-α (Fig. 6). These results indicate that trypsin inhibition was likely facilitated by PI2 only, and not by the attached MBP tag. Additionally, the presence of cleaved MBP from both the MBP-PI2-only and MBP-β-gal-α-only samples analyzed in the trypsin inhibition assay further reinforced the likelihood that protease contamination was present (Fig. 6). Notably, in this second trypsin inhibition assay, a PI2 band at 16 kDa was not apparent as a result of MBP-PI2 cleavage (Fig. 6A), however, this is likely due to the lower concentration of MBP-PI2 compared to what was used in our first trypsin inhibition assay. Nonetheless, despite the comparison between MBP-β-gal-α and MBP-PI2 pointing towards PI2 being functional, we cannot rule out the possibility that the MBP tag may still somehow be required for PI2 functionality. In other words, perhaps PI2 on its own is non-functional. Further research is required to rule out this possibility and advance our understanding of the functional characteristics of recombinantly expressed PI2.

Limitations While this study provides a preliminary characterization of PI2 and its functionality through the analysis of MBP-PI2 purified from SHuffle *E. coli* cells, a few key limitations exist. Though our findings indicate that the optimal induction of MBP-PI2 expression is at room temperature and with 1mM IPTG, we were unable to monitor and consequently report the precise temperature of the shaking incubator for the duration of the overnight incubation. This is partly due to the limitations with the shaking incubator itself but may also be due to the changes in the temperature of the room from extraneous factors. Additionally, due to time constraints, only 0.1 mM and 1 mM of IPTG were tested for optimal induction and therefore, a preferable IPTG concentration may still exist between those values. In regard to our developed trypsin inhibition assay, it is important to consider that the use of BSA as a substrate for trypsin made it difficult to visually identify trypsin as active or inactive as it was difficult to discern BSA fragmentation between conditions. Therefore, we needed to also rely on indirect measurements such as densitometry analysis of the BSA or trypsin band intensity to determine if there was inhibition of trypsin activity. Finally, while we demonstrated that it is likely the PI2 domain that is conferring the trypsin inhibitory activity seen by MBP-PI2, the validity of the comparison made between the MBP-PI2 and MBP-β-gal-α-only reactions may be compromised as the MBP-β-gal-α sample was provided by a different source where a slightly different purification protocol was used. For instance, based

on the band intensities on the SDS-PAGE gel, there appeared to be a discrepancy between the starting concentration of MBP-β-gal-α used in the reaction compared to our purified MBP-PI2, which may have affected the reactions during the 15-minute incubation at 37°C or in the visualization of fragmentation with SDS-PAGE.

Conclusions This study demonstrates that MBP-PI2 expressed by SHuffle *E. coli* cells may inhibit trypsin. and therefore, SHuffle E. coli may serve as a suitable expression host for large-scale production of PI2 for clinical use and agricultural applications. After determining the optimal induction conditions of room temperature and 1mM IPTG for MBP-PI2 expression, the purified MBP-PI2 was analyzed using our developed trypsin inhibition assay. Decreased BSA fragmentation and the inhibition of trypsin autolysis by MBP-PI2 rather than purified MBP-β-gal-α only samples suggest that trypsin inhibitory activity of MBP-PI2 may be specific to the PI2 domain. As this study is the first assessment and indicator of MBP-PI2 functionality when recombinantly expressed in SHuffle *E. coli*, it provides a foundation for future groups to further investigate SHuffle-expressed MBP-PI2 activity in order to establish the potential large-scale production of PI2 for clinical use.

Future Directions Given that our study elucidated the possibility of recombinantly producing functional PI2 using SHuffle cells and the several potential applications of PI2 as a recombinant protein, future studies may first consider further optimizing the expression and purification of MBP-PI2 using SHuffle cells. Although we identified induction with 1 mM IPTG at room temperature as the optimal conditions for MBP-PI2 expression, previous studies have identified a lower temperature, such as 16°C, that could further improve expression of poor folding proteins with many disulfide bonds in SHuffle cells (12). Therefore, the use of a shaking incubator capable of a lower temperature setting may improve conditions for inducing MBP-PI2 expression and consequently, protein functionality. Additionally, testing a wider range of IPTG concentrations via titration may reveal a concentration more optimal than 1 mM for protein expression. Moreover, further purification steps, such as size exclusion chromatography or using a centrifugal filter of the appropriate molecular weight cutoff, may improve the purity of isolated MBP-PI2 by removing the lower molecular weight bands that were observed in this study and by Grewal *et al*. in several MBP-PI2 elution fractions (13). While this further purification step was not pursued in this study to achieve the main goal of characterizing the activity of PI2, ensuring that the lower molecular weight proteins do not interfere with this activity may be a more accurate investigation into the functionality of PI2.

Additionally, although our trypsin inhibition assay used BSA as a substrate for trypsin, future studies may choose to use a different substrate to obtain clearer qualitative evidence of trypsin inhibition by PI2. For example, an alternative substrate may be BSA conjugated with fluorescein isothiocyanate (FITC) to measure fluorescence as the output of the assay instead (17). Digesting FITC-BSA with trypsin results in the release of fluorescently-tagged lysine residues (17). Therefore, by determining whether PI2 can inhibit trypsin from cleaving FITC-BSA and prevent the subsequent recovery of fluorescence from the liberated FITC, the functionality of PI2 can be further established. Lastly, given the interesting finding that MBP-PI2 appears to be cleaved into MBP and PI2 during the conditions of the trypsin inhibition assay, future studies may wish to identify the mechanism behind this cleavage by implementing techniques such as cryo-electron microscopy (cryo-EM). Gaining insight into this mechanism may reveal if the presence of MBP is somehow necessary for PI2 to inhibit trypsin. A comparison between MBP-PI2 and cleaved PI2 can also be carried out to investigate the implications of this cleavage mechanism and the functionality of PI2 on its own. To do so, the MBP tag would need to be removed from PI2 following purification. However, although MBP-PI2 consists of a Factor Xa cleavage site encoded by the pMAL-c2X-PI2 vector, it is likely not possible to cleave MBP at this site since Factor Xa is a serine protease that could be inhibited by functional PI2, which is a serine proteinase inhibitor. Therefore, future groups may need to construct a new plasmid for encoding PI2 such that it can be cleaved using another class of protease.

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CONTRIBUTIONS

A.G. and H.H. collaborated to design and execute all laboratory experiments in this study. In regard to the manuscript, N.W. completed the introduction, discussion, and references sections. N.W. also completed densitometry analyses and generated the majority of figures and tables. P.K. generated the first figure. A.G. and H.H. collaborated on the abstract, methods, results, limitations, conclusion, and future directions. A.G., H.H., and N.W. contributed equally to the editing of the manuscript and the final assembly of figures, tables, and their legends.

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