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Investigation of MBP tag removal from recombinant potato type II proteinase inhibitor and testing of serine protease inhibition

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SUMMARY Potato type II proteinase inhibitor (PI2) is a small, disulfide-rich serine protease inhibitor that requires careful folding to retain its functionality. Due to the promising applications of PI2 in medicine, cancer treatment and biotechnology, researchers have been interested in optimizing its large-scale production. However, as a result of the small size and complex conformation of PI2, large affinity tags such as maltose-binding protein (MBP) complicate its ability to be purified and can potentially interfere with its inhibitory function. The present study investigates whether the removal of the MBP fusion tag affects the inhibitory function of PI2. MBP-PI2 recombinant protein was expressed in *E. coli* SHuffle (C3028) and purified by amylose gravity affinity chromatography. A subsequent factor Xa cleavage optimization assay suggested inefficient MBP tag removal from recombinant MBP-PI2, indicating the possibility that PI2 inhibits factor Xa, and therefore MBP-tagged PI2 may be sufficiently functional. Further investigation of MBP-PI2 functionality in a trypsin inhibition assay appeared to show trypsin cleaving MBP-PI2. Subsequent bioinformatic analysis predicted potential non-specific cleavage of MBP and PI2, suggesting that trypsin is unsuitable for PI2 functional assays. Nonetheless, these results provide insight into the functionality of MBP-PI2, allowing further exploration into the therapeutic potential of the protein.

INTRODUCTION

Contained within Russet Burbank potato tubers is a 16 kDa, disulfide-rich protein called potato type II proteinase inhibitor (PI2) which has been used as a treatment option for cancer and as a weight loss therapeutic (1, 2). By inhibiting proteases, PI2 can effectively inhibit tumor growth and metastasis (3), making it a desirable protein to purify in an industrial setting. The protein is especially effective at inhibiting serine proteases, such as trypsin and chymotrypsin (1). However, PI2 contains eight disulfide bridges, and with its resistance to proper folding in reductive cytoplasm (4), a protocol for its expression and purification has yet to be developed. One method to increase the solubility of proteins of interest, such as PI2, is to fuse them with maltose binding protein (MBP) (4, 5). MBP is a popular recombinant protein tag which is over two times larger than PI2 itself, at 42.5 kDa (6). While MBP is widely used as a fusion tag it has been shown to hinder the proper folding process of various proteins of interest (6, 7). Additionally, some contaminating enzymatic activity has been found to exist when MBP remains attached to recombinant proteins (8). Though MBP may enhance the solubility of recombinant PI2, there remains the potential for the activity of MBP-tagged PI2 to be compromised.

The pMAL-c2X plasmid used in the present experiment was originally designed and constructed by Walker *et. al* (9). It contains an ampicillin resistance gene, a *lacI* gene encoding a transcription factor involved in the metabolism of lactose (10), β-galactosidase-α (β-gal-α), and an MBP gene, along with several restriction enzyme sites for cloning in recombinant genes (9). Notably, it contains a factor Xa site between the MBP tag gene and the recombinant gene insertion site to cleave the recombinant protein from MBP after purification. Lapointe *et. al*. (5) identified pMAL-c2X as a suitable plasmid for PI2 expression and thus constructed the pMAL-c2X-LLMZ16 (pMAL-c2X-PI2) plasmid. The present study used the pMAL-c2X and pMAL-c2X-PI2 plasmids to express MBP and MBP-PI2, respectively, in *Escherichia coli* (E. coli) SHuffle (C3028) cells.

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SHuffle cells are a unique strain of *E. coli* that have an oxidizing cytosolic environment, allowing proteins with disulfide bridges to properly fold (11). Additionally, this strain contains the chaperone protein DsbC, a disulfide bond isomerase, that further facilitates the folding of disulfide-rich proteins like PI2 (11). Grewal *et al*. were able to express MBP-tagged PI2 in this particular strain of *E. coli* (4). Although the authors suggested that PI2 was purified in its correctly folded state, the inhibitory function of PI2 was not determined. Here, we investigated the MBP-PI2 generated from the pMAL-c2X-PI2 vector to assess its functionality compared to untagged PI2.

To accurately assess the functionality of a recombinant protein, cleavage of the affinity tag is required, to ensure no contaminating protein activity. Factor Xa is a trypsin-like serine protease and is commonly used to remove fusion tags, such as MBP, from recombinant proteins of interest (12, 13). This protease cleaves the peptide bonds contained in basic amino acid residues (14). More specifically, factor Xa will cleave the carboxyl side of these proteins at a site consisting of isoleucine-(glutamic acid or aspartic acid)-glycine-arginine (12). Likewise, trypsin is another serine protease with high cleavage specificity. Compared to factor Xa, which cleaves arginine residues, trypsin is a serine protease that cleaves either arginine or lysine residues at the carboxyl side of proteins (14). Trypsin inhibitors are a class of compounds which inhibit trypsin by competitively and irreversibly binding the active site of trypsin (15). PI2 has been found to be an efficient trypsin inhibitor, and thus its ability to inhibit trypsin may be used as an indicator of its functionality (1).

In this study we expressed, purified, and qualitatively assessed the activity of MBP-tagged PI2 to elucidate if the MBP fusion protein impacts the functionality of PI2. We hypothesized that both MBP-tagged PI2 and untagged PI2 (uPI2) would inhibit serine protease activity, but that MBP-PI2 would be less effective due to the presence of the MBP tag. To address this research question, chemically competent SHuffle cells were transformed with the pMAL-c2X and pMAL-c2X-PI2 plasmids and MBP-PI2 expression was induced with isopropyl β-d-1-thiogalactopyranoside (IPTG). Amylose affinity chromatography was subsequently performed to purify MBP-PI2. We also attempted to optimize MBP tag cleavage from MBP-PI2 in a factor Xa cleavage assay to generate uPI2. However, it was not possible to generate a sufficient amount of uPI2 to be used in subsequent functionality tests. As such, we only assessed the functionality of MBP-PI2 in a trypsin inhibition assay. The present study provides important insight into the overarching research question of whether the MBP protein tag interferes with the functionality of the PI2 protein.

METHODS AND MATERIALS

Bacterial strains and plasmids. Competent *E. coli* SHuffle strain (C3028) were used for all protein expression experiments. SHuffle cells were streaked on lysogeny broth (LB) agar plates from glycerol stocks provided by the Straus Lab at UBC. The uncloned pMAL-c2X plasmid was provided by the Eltis Lab at UBC, and the pMAL-c2X-PI2 plasmid was provided by Lapointe *et al.* (5). pMAL-c2X and pMAL-c2X-PI2 (Fig. S1) were maintained in *E. coli* DH5α cells and streaked on LB agar plates containing 100 µg/mL ampicillin (VWR, Cat no. 0339-25G) from glycerol stocks. Each DH5α transformant was grown at 37°C overnight in 5 mL of LB media containing 100 µg/mL ampicillin, and both plasmids were isolated using the BioBasic EZ-10 Spin Column Plasmid DNA Miniprep Kit according to the manufacturer protocol for low-copy plasmids. Plasmids were sent to GeneWiz for Sanger sequencing using the universal forward M13 primer. The presence of the *pi2* gene within the isolated pMAL-c2X-PI2 plasmid was confirmed by using the NCBI BLAST algorithm to align the resulting pMAL-c2X-PI2 sequence against the *pi2* insert sequence provided by Lapointe *et al*. (5) (Table S1). The identity of the pMAL-c2X vector was also confirmed by aligning the resulting pMAL-c2X and pMAL-c2X-PI2 sequences against the original pMAL-c2X vector sequence (Table S1).

Preparation of competent SHuffle cells. Competent SHuffle cells were kindly provided by MICB 401 team 2beta and were prepared according to a procedure adapted from Chang *et al.* (16). Following an overnight culture, SHuffle cells were subcultured and grown at 30°C to an OD_{600} of 0.4. OD_{600} readings were measured using a Pharmacia Biotech Ultrospec 3000 UV-Visible Spectrophotometer. The cells were then centrifuged at 4000 rpm in 4°C for 10

minutes and the resulting pellet was resuspended in ice-cold 0.1M $CaCl_2$. After incubating the resuspension on ice for 30 minutes, the centrifugation process was repeated and 5 mL ice-cold $CaCl_2$ with 15% glycerol was used to resuspend the pellet. Competent cells were aliquoted and stored at -80°C for future transformation experiments.

Transformation of pMAL-c2X and pMAL-c2X-PI2 into SHuffle. 96.0 ng and 51.9 ng of pMAL-c2X and pMAL-c2X-PI2, respectively, were added to 50 µL of competent SHuffle cells. Cells were incubated with the plasmids on ice for 15 minutes, then heat-shocked for 30 seconds at 42°C and placed back on ice for 2 minutes. Pre-warmed LB broth was added to each transformation for a final dilution of 1:10, then incubated in a shaking incubator at 30°C for 1 hour. 25 µL of each transformant was plated on LB agar plates containing 100 µg/mL ampicillin and grown overnight. One colony from each transformant plate was picked and grown overnight in 5 mL of LB broth containing 100 µg/mL ampicillin. Each overnight culture was mixed with sterile glycerol for a final glycerol concentration of 25% v/v, then stored at -80°C for future use.

Induction of protein expression. Transformed SHuffle cells were inoculated into 5 mL of LB broth containing 100 µg/mL ampicillin. 5 mL of each overnight culture was inoculated into 500 mL of LB broth containing 100 µg/mL ampicillin and grown at 30°C until the sample reached an OD_{600} of 0.6. Each culture was then induced with 500 µL of 1M IPTG and grown in a 30°C shaking incubator at 200 rpm overnight. Following incubation, 1.5 mL aliquots of each induction culture were centrifuged for 10 minutes at 10,000 rpm and 4°C. The pellets were resuspended in column buffer (pH 7.4, 20 mM Tris, 200 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5 mM D-maltose) and lysed using the MP FastPrep-24 homogenizer for 40 seconds at 4 m/s. Lysate samples were analyzed by SDS-PAGE (as described below) under reducing and non-reducing conditions, using β-mercaptoethanol (BME) (Bio-Rad, Cat no. 161-0737) as the reducing agent. The remaining induction culture samples were centrifuged at 4000 x g and 4°C for 30 minutes and the resulting pellets were stored at -80°C for future purification experiments.

SDS-PAGE. Samples were added to 2X Laemmli sample buffer (Bio-Rad, Cat no. 161-0737) with 5% BME to a 1X dilution and heated at 95°C for 5 minutes. Proteins were separated by SDS-PAGE at 200V using Bio-Rad 4-20% Mini-PROTEAN® TGX Stain-Free™ Precast Gels (Cat no. 456-8095), and 10X Tris/Glycine/SDS electrophoresis buffer (Bio-Rad, Cat no. 161 0732) diluted to 1X in distilled water. Protein band molecular weights were compared to a Precision Plus Protein™ Unstained Standard (Bio-Rad, Cat no. 161-0363EDU). 2 mg/mL Bovine serum albumin (BSA) Standard (GBiosciences, Cat no. 224B-B) (66 kDa) was run as a reference for the band corresponding to MBP-PI2 (66.5 kDa). Unstained gels were imaged using a Bio-Rad ChemiDoc MP Imaging System, using the Blot/UV/Stain-Free Sample Tray. All gels were subsequently stained in Coomassie stain (40% ethanol, 10% glacial acetic acid, 0.1% Gibco™ Coomassie Brilliant Blue R-250), de-stained in tap water overnight, and imaged using the White Sample Tray.

Amylose gravity affinity chromatography. Harvested cell pellets from induced SHuffle transformants were resuspended in 10 mL of column buffer containing 400 µL of 25X cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, Cat no. 04693159001). Cells were lysed using the MP FastPrep-24 homogenizer at 4 m/s for 1 minute. Lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C and the resulting soluble protein fraction was collected and sterilized through a 0.22 µm filter for subsequent purification.

Amylose affinity chromatography was carried out at 4°C using gravity flow columns. Amylose resin (New England Biolabs, Cat no. E8021S) was added to each column such that the settled resin bed volume was 1.5 mL, equaling 1 column volume (CV). 5 CVs of distilled H_2O were used to remove residual ethanol from the resin, and 5 CVs of column buffer were used to equilibrate the resin. Each soluble lysate was added to their respective column and incubated on an orbital shaker for 1 hour. Following incubation, the columns were washed with 15 CVs of column buffer. The MBP-β-gal-α and MBP-PI2 proteins were eluted using 5 CVs of elution buffer (pH 7.4, 20 mM Tris, 200 mM NaCl, 10% glycerol, 1 mM EDTA, 10

mM D-maltose). The collected fractions were analyzed by SDS-PAGE as described above. The elution fractions for each respective protein were pooled and concentrated with 30K MWCO Pierce™ Protein Concentrator PES (Thermo Scientific, Cat no. PI88529). Concentrated proteins were quantified using the NanoDrop™ 2000c Spectrophotometer (Thermo Scientific) (Table S2) and by Pierce™ Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific) (Fig. S2). Proteins were aliquoted and stored at -80°C for use in downstream functional assays.

Factor Xa cleavage assay. Factor Xa (New England Biolabs, Cat no. P8010S) was added to purified MBP-PI2 (0.208 mg/mL) in two separate reactions: in ratios of 1:10 (0.0208 mg/mL factor Xa) and 1:50 (0.00417 mg/mL factor Xa). Factor Xa was added to purified MBP-β-gal-α (0.208 mg/mL) in a ratio of 1:10 as a negative control. Elution buffer was added such that the total volume of each reaction was 60 µL. Reactions were incubated on ice over 1 minute, 15 minutes, 1 hour, 3 hour, and 24 hour time points. At each time point, 10 µL of each reaction was immediately added to 10 µL of 2X Laemmli sample buffer with 5% BME and heated at 95°C for 5 minutes. 15 µL of each prepared sample was analyzed by SDS-PAGE according to the protocol previously described.

Trypsin inhibition assay. According to results obtained from a trypsin activity optimization assay (Fig. S3), 0.13 mg/mL of trypsin (MP Biomedicals, Cat no. 9002-07-7), trypsin inhibitor (Sigma-Aldrich, Cat no. 9035-81-8), and BSA were added to the appropriate control and experimental reaction vessels. Controls containing trypsin, trypsin inhibitor, and elution buffer were incubated for 30 minutes at room temperature. BSA was added to the appropriate reaction vessels to produce a final volume of 50 µL and the reaction was incubated for an additional 30 minutes. MBP-β-gal-α and MBP-PI2 were each incubated in equal concentrations with trypsin for 30 minutes at room temperature. BSA was added to produce a final volume of 100 µL, and the reaction was incubated over 0 minutes, 1 minute, 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hour, and 3 hour time points. Following each time point, 10 µL of each reaction was added to 10 µL of 2X Laemmli sample buffer with 5% BME. 15 µL of each prepared sample was analyzed by SDS-PAGE according to the protocol previously described.

RESULTS

MBP-PI2 was expressed in SHuffle cells. SHuffle cells transformed with pMAL-c2X-PI2 were grown in 500 mL cultures and MBP-PI2 expression was induced with 1 mM IPTG. As negative controls, MBP-PI2 expression was compared to untransformed SHuffle cells and uninduced pMAL-c2X-PI2 transformants. An identical procedure was performed with SHuffle cells transformed with pMAL-c2X to express MBP-β-gal-α. To confirm that MBP-PI2 was expressed in SHuffle cells, 1 mL of each IPTG-induced culture was lysed by homogenization and the whole-cell, soluble, and insoluble lysates were analyzed by SDS-PAGE (Fig. 1). Consistent with the findings of Grewal *et al.* (4), MBP-β-gal-α and MBP-PI2 overexpression was indicated by the high intensity bands at 50.5 kDa (Fig. 1A) and 66.5 kDa (Fig. 1B), respectively. These bands appeared less intense in the uninduced lysate conditions, thus confirming that overexpression was a result of induction with IPTG. A greater band intensity at 50.5 kDa than 66.5 kDa indicated that MBP-β-gal-α was expressed at higher levels compared to MBP-PI2. A large amount of MBP-β-gal-α and MBP-PI2 was found to be insoluble after pelleting the cell debris, as indicated by the intense bands at 50.5 kDa and 66.6 kDa in the insoluble fractions. Nonetheless, the visible bands observed in the soluble fractions suggest that protein expression levels were sufficiently high for large-scale purification. In addition, lysates were prepared for SDS-PAGE in reducing and non-reducing conditions, using BME as a reducing agent. If the eight disulfide bonds of PI2 greatly contribute to the structure of the protein, the MBP-PI2 protein band at 66.5 kDa would be expected to shift downward in the non-reducing condition. Interestingly, no gel shift was observed in the non-reducing condition compared to the reducing condition. The 66 kDa BSA standard, however, contains 17 disulfide bonds (17) and saw a considerable shift to approximately 45 kDa, indicating that the BME indeed provided a reducing environment. Together, these results

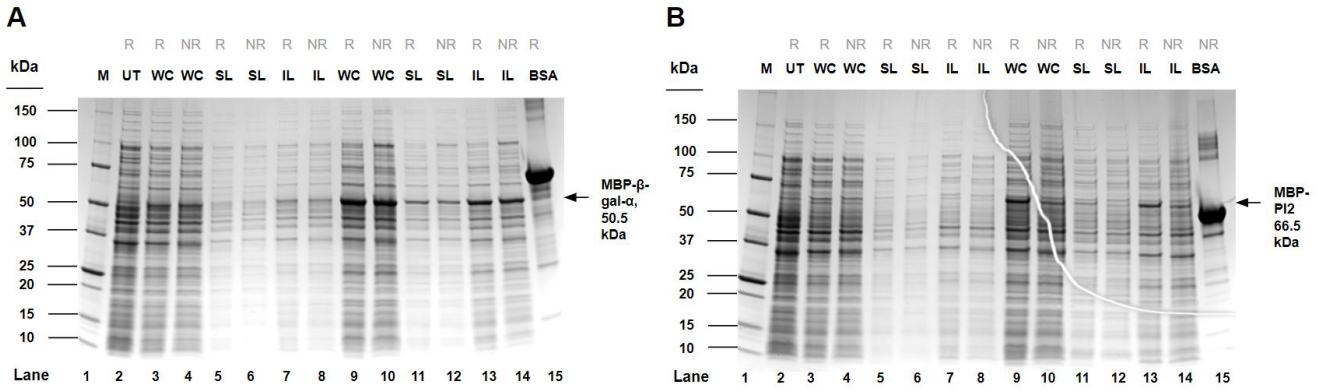

FIG. 1 MBP-PI2 expression in *E. coli* SHuffle cells. Coomassie blue-stained SDS-PAGE showing IPTG-induced and non-induced conditions in reducing (R) and non-reducing (NR) conditions. A) MBP expression via empty pMAL-c2X vector. B) MBP-PI2 expression via pMAL-c2X-PI2 vector. Lane 1: Protein ladder (M). Lane 2: Untransformed (UT) SHuffle C3028 protein lysates. Lanes 3-8: Uninduced cells. Lanes 3-4: Whole cell (WC) lysates. Lanes 5-6: Soluble Lysates (SL). Lanes 7-8: Insoluble Lysates (IL). Lanes 9-14: Induced cells. Lanes 9-10: WC lysates. Lanes 11-12: SL. Lanes 13-14: IL. Lane 15: BSA (66 kDa). Arrows indicate the predicted molecular weights for MBP-β-gal-α (50.5 kDa) and MBP-PI2 (66.5 kDa).

replicate the previous findings of Grewal *et al*. that MBP-β-gal-α and MBP-PI2 can be expressed at sufficiently high levels in SHuffle cells.

MBP-PI2 was purified in sufficiently high yield from SHuffle cells. Following the expression of MBP-PI2, the tagged protein was purified from SHuffle cells for downstream use in functional assays. MBP-β-gal-α was also purified for use as a negative control. MBP-β-gal-α and MBP-PI2 were purified from 250 mL and 500 mL volumes of IPTG-induced SHuffle cell, respectively, by amylose affinity chromatography using gravity columns. All fractions from purification were analyzed by SDS-PAGE (Fig. 2). Purification of MBP-β-gal-α (Fig. 2A) and MBP-PI2 (Fig. 2B) produced prominent 50.5 kDa and 66.5 kDa bands, respectively, in all seven elution fractions. Consistent with Grewal *et al.*, these bands indicate that MBP-β-gal-α and MBP-PI2 were bound to, and eluted from, the amylose column. Based on the elution fractions, MBP-PI2 appeared to have a lower yield than MBP-β-gal-α, despite having double the volume of IPTG-induced cell culture. These bands were also observed in the flow-through and wash fractions, indicating some loss of the target protein during the purification process. Nonetheless, all elution fractions contained very few contaminating protein bands, suggesting a high purity for both proteins. Elution fractions 2 to 5 (Fig. 2) contained the highest concentrations of the target proteins, and all seven elution fractions were pooled and concentrated. Protein yield was determined by the NanoDrop™ 2000c Spectrophotometer to be 0.363 mg/mL for MBP-β-gal-α and 0.250 mg/mL for MBP-PI2 (Table S2B). Due to inconsistencies between A280 readings, a secondary BCA assay was conducted, which resulted in concentrations of 0.252 mg/mL for MBP-β-gal-α and 0.753 mg/mL for MBP-PI2 (Fig. S2C). These results demonstrate that MBP-β-gal-α and MBP-PI2 were purified from IPTG-induced SHuffle cells in sufficiently high yield and purity, allowing for its use in downstream functional assays.

Factor Xa protease did not efficiently cleave the MBP fusion tag from MBP-PI2. To generate uPI2 for downstream functional assays, a factor Xa time-course cleavage assay was conducted. The reactions were analyzed by SDS-PAGE to determine the optimal MBP tag cleavage conditions (Fig. 3). A 1:50 ratio of factor Xa to MBP-PI2 was tested as per the product specifications, as well as a 1:10 ratio to potentially improve cleavage efficiency. Factor Xa was incubated with MBP-β-gal-α as a negative control, in which cleavage should not occur. The band at approximately 27 kDa represents one of the two factor Xa disulfide-linked chains that is reduced during sample preparation with BME (Fig. 3). Assay results suggest that MBP tag removal from MBP-PI2 by factor Xa was inefficient and did not generate a sufficient yield of uPI2 for downstream functional assays. If tag cleavage occurred,

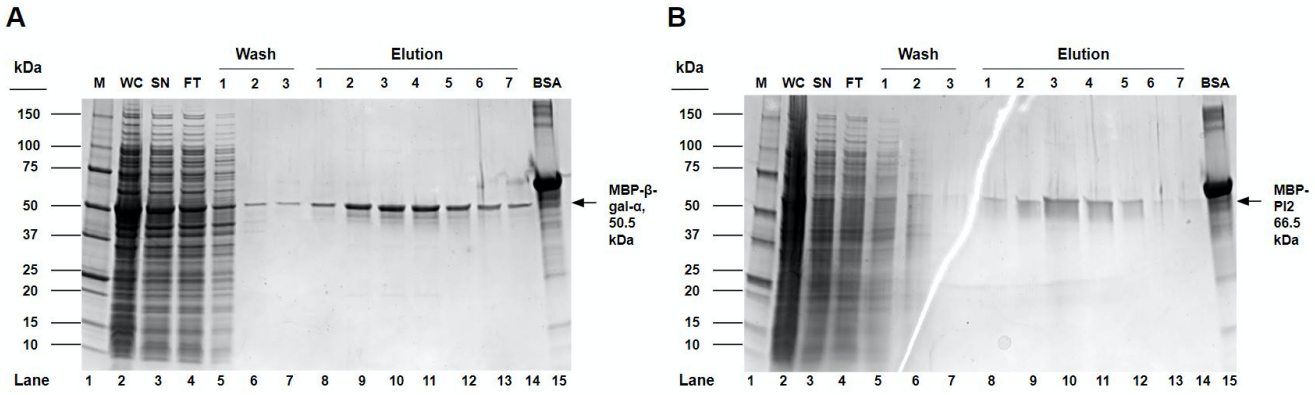

FIG. 2 MBP-PI2 purification from *E. coli* SHuffle cells by amylose affinity chromatography. Coomassie blue-stained SDS-PAGE gel showing IPTG-induced A) MBP-β-gal-α and B) MBP-PI2, purified via amylose column affinity chromatography. Lane 1: Protein ladder. Lane 2: Whole cell lysate (WC). Lane 3: Supernatant (SN). Lane 4: Flowthrough (FT). Lanes 5-7: Washes 1-3. Lanes 8-17: Elutions 1-7. Lane 15: BSA (66 kDa). Arrows indicate A) MBP-β-gal-α (50.5 kDa) and B) MBP-PI2 (66.5 kDa). Note: B) MBP-PI2 was derived from double the amount of induced culture in A).

prominent bands would be seen at 16 kDa for uPI2 and 42.5 kDa for MBP, both of which would increase in intensity over time. In addition, a band at 66.5 kDa for MBP-PI2 would be expected to decrease in intensity over time. However, only the 24 hour time point showed evidence of tag cleavage with a faint band at 16 kDa and an increase in band intensity at 42.5 kDa compared to the shorter time points. Very faint 16 kDa and 42.5 kDa bands were observed at the 1 min, 15 min, 1 hour, or 3 hour time points. There appears to be no difference in cleavage efficiency between the 1:10 and 1:50 ratios of factor Xa, as shown by the similar band intensities of MBP and uPI2. Ultimately, these results show that factor Xa did not generate a sufficient amount of uPI2.

MBP-PI2 does not inhibit trypsin, however, it may be cleaved by trypsin. The final aim was to investigate whether purified MBP-PI2 retained its functionality to inhibit serine proteases. Due to the insufficient amount of uPI2 generated in the factor Xa assay, the trypsin inhibition assay could only assess the functionality of MBP-tagged PI2. A trypsin inhibition time-course assay was conducted to test if MBP-PI2 would inhibit trypsin from cleaving BSA substrate. MBP-PI2 was incubated with trypsin for 30 minutes to allow for sufficient inhibition prior to the addition of BSA, then the reaction was analyzed at various time points by SDS-PAGE (Fig 4). As a negative control, trypsin was incubated with MBP-β-gal-α, which was not expected to inhibit trypsin. As a positive control, trypsin was incubated with trypsin inhibitor, which confirmed that BSA remains intact when trypsin is properly inhibited. The results showed that there was no difference between MBP-β-gal-α and MBP-PI2 in trypsin inhibition. As indicated by the increasing intensity of the band at approximately 60 kDa, trypsin continuously cleaved BSA over the course of the reaction regardless of the presence of MBP-β-gal-α or MBP-PI2, suggesting that neither MBP-β-gal-α or MBP-PI2 inhibit trypsin. It is possible that purified PI2 did not retain its inhibitory function. Unexpectedly, trypsin appears to have cleaved MBP-β-gal-α and MBP-PI2 at approximately the same site after the 30-minute incubation period. This was shown by the identical bands at approximately 45 kDa in both reactions at all timepoints, although this conclusion cannot be definitively made without running MBP-β-gal-α and MBP-PI2 alone on the gel. This highly selective trypsin cleavage activity raises further questions regarding the structural integrity of MBP- PI2 in the assay and will be discussed below.

DISCUSSION

Previously, Grewal *et al*. was able to express and isolate MBP-PI2 from SHuffle cells, which have a unique oxidative cytosolic environment that provides optimal conditions for disulfide bond formation and PI2 protein folding (4). However, due to the small size and complex conformation of PI2, large affinity tags such as MBP may interfere with its proper folding and ability to inhibit serine proteases (8, 18, 19). Thus, our study aimed to investigate whether

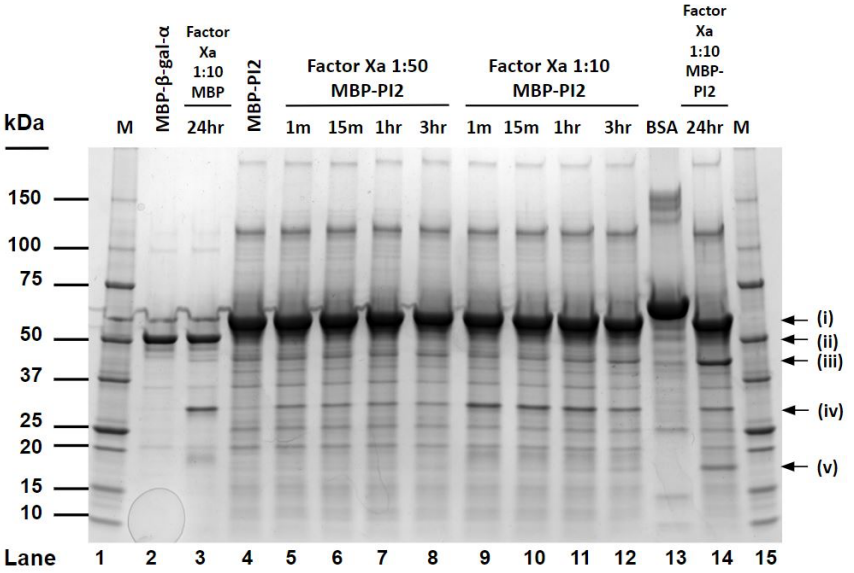

FIG. 3 Factor Xa cleavage optimization time-course assay with MBP-PI2. Coomassie blue-stained SDS-PAGE gel showing assay results for controls and two ratios of factor Xa to MBP-PI2 (1:50 and 1:10) at various incubation times. Lanes 1 & 15: Protein ladder. Lane 2: MBP only. Lane 3: Factor Xa with 1:10 MBP. Lane 4: MBP-PI2. Lanes 5-8: Factor Xa with 1:50 MBP-PI2 at various incubation times. Lanes 9-12 & 14: Factor Xa with 1:10 MBP-PI2 at various incubation times. Lane 13: BSA (66 kDa). Arrows indicate i) MBP-PI2 (66.5 kDa), ii) MBP-β-gal (50.5 kDa), iii) MBP (42.5

the removal of the MBP fusion tag affects the inhibitory function of PI2. Here, we were able to reproduce the main findings of Grewal *et al*. (4) through expression (Fig. 1) and purification (Fig. 2) of MBP-PI2 from SHuffle cells. However, inefficient fusion tag cleavage by factor Xa (Fig. 3) suggested that PI2 inhibited the factor Xa serine protease. Furthermore, a trypsin inhibition assay demonstrated that MBP-PI2 may have been non-functional, and that trypsin was unexpectedly cleaving MBP-PI2 (Fig. 4).

Protein expression. Following overnight induction of protein expression in SHuffle cells, our SDS-PAGE gel analysis confirmed the expression of MBP-β-gal-α and MBP-PI2 (Fig. 1). These results were consistent with those obtained by Grewal *et al*., who demonstrated that the oxidative cytosolic environment and presence of DsbC within SHuffle cells may be important for the proper folding of PI2 (4). However, contrary to what was expected, MBP-PI2 appeared to be expressed at lower levels compared to MBP-β-gal-α. Both proteins were expected to be expressed at similar levels, but the putative MBP-β-gal-α bands showed greater intensity compared to the putative MBP-PI2 bands following an overnight induction (Fig. 1). This observation could be due to a combination of leaky expression during uninduced conditions, and that PI2 may be toxic to SHuffle cells. During recombinant protein expression, the activity of correctly folded proteins may inhibit essential pathways within the cytoplasm of the expression host (20). Leaky or basal expression of a toxic protein can especially lead to reduced growth rates and lower yields of the protein of interest (20). Together with the results obtained from Fig. 1, it is possible that the production of PI2 was toxic to SHuffle cells, which led to slower growth rates and decreased expression in comparison to MBP-β-gal-α. Thus, the use of an alternate SHuffle strain, such as C3030, may reduce leaky expression and potentially improve the expression of MBP-PI2 (20).

In addition to discrepancies in protein expression, putative band intensities for MBP-β-gal-α and MBP-PI2 were found to be greater in the insoluble lysate fractions as opposed to the soluble lysate fractions (Fig 1). This was unexpected because MBP is a fusion protein that is known to enhance solubility and promote folding of its fused protein (21). However, it is possible that MBP-β-gal-α and MBP-PI2 were inefficiently folded during expression. When recombinant proteins are overexpressed in a heterologous host, they may become insoluble due to misfolding (22). Protein misfolding can be a result of failure to rapidly reach a native conformation or to interact with the appropriate folding chaperones (23). Furthermore, the likelihood of misfolding increases when strong promoters and high inducer concentrations are used during expression (23). Under such conditions, folding chaperones can be rapidly depleted, and the production of insoluble protein aggregates may exceed the assembly of properly folded soluble proteins (23). Thus, optimization of expression parameters (temperature, time of induction, and concentration of IPTG) may help with increasing the yield of soluble MBP-β-gal-α and MBP-PI2. Nonetheless, the distinct intensity of the putative

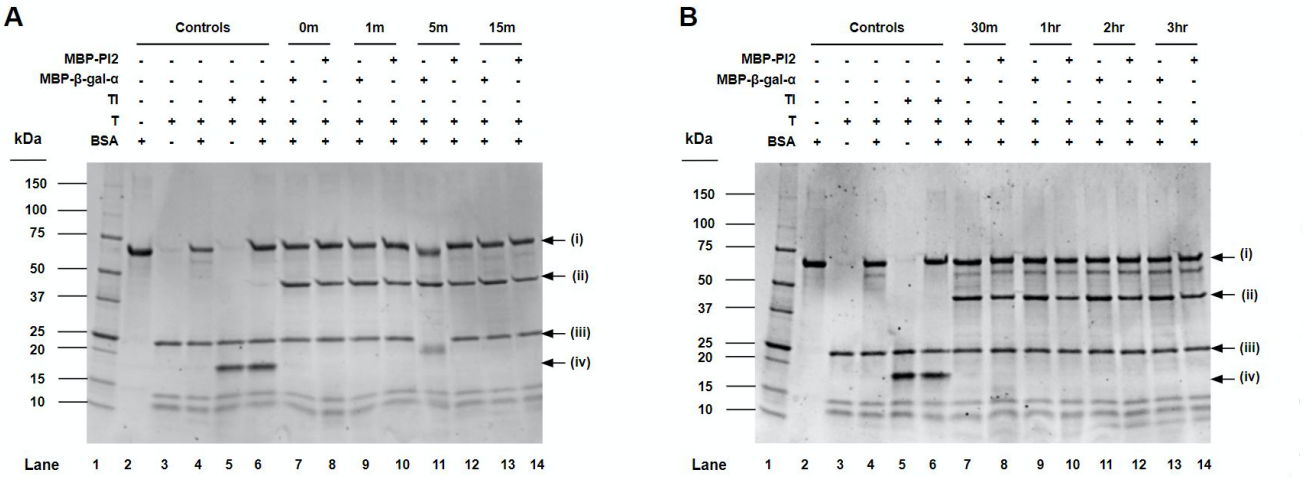

FIG. 4 Trypsin inhibition time-course assay with MBP-PI2. Coomassie blue-stained SDS-PAGE gel showing MBP and MBP-PI2 cleavage with trypsin at A) 0 min (immediate), 1 min, 5 min, 15 min and B) 30 min, 1 hr, 2 hr, 3 hr. "+" indicates the presence; "-" indicates the absence of reagents and proteins. Lane 1: Protein ladder. Lanes 2-6: Controls. Lanes 7-15: Samples. Lanes 5-6: Trypsin inhibitor added at a 1:1 ratio with trypsin. Arrows indicate i) BSA (66 kDa), ii) MBP-β-gal-α (50.5 kDa), iii) Trypsin (24 kDa), iv) Trypsin inhibitor (20.1 kDa).

bands observed after overnight induction are indicative of protein expression, and the visible bands observed in the soluble fraction suggest that protein levels were sufficiently high for large-scale purification.

Purification of MBP-β-gal-α and MBP-PI2. MBP-β-gal-α and MBP-PI2 were purified from SHuffle cells (Fig. 2). Purification results indicate improved elution fraction purity compared to the experiment conducted by Grewal *et. al* (4). This is likely due to some experimental conditions being altered compared to Grewal's experiment. Here, we used a reduced volume of the amylose resin to decrease the availability of sites for non-specific binding of MBP-β-gal-α to amylose. The addition of 0.5 mM D-maltose to the column buffer likely improved purity by competing with lysate impurities for binding sites on the amylose column. In future studies, these changes will be helpful in optimizing strategies for the purification of PI2. Purification analysis indicated an identifiable amount of MBP-β-gal-α and MBP-PI2 lost in the supernatant, flowthrough, and wash fractions. However, given that yield was particularly high for this sample, this is an acceptable occurrence. Given the poor resolution of the bands for MBP-PI2 (Fig. 2B), it is possible that contaminating protein interfered with MBP-PI2 binding to the amylose column. It is also possible that MBP-PI2 has some affinity for other contaminating *E. coli* proteins, which were associated with the recombinant protein during purification.

Factor Xa cleavage assay. The factor Xa cleavage assay showed inefficient MBP tag cleavage (Fig. 3). The manufacturer's protocol states that a 1:50 ratio of factor Xa to fusion protein substrate should result in full cleavage in 6 hours or less (12). Our results showed only partial cleavage of MBP-PI2 after 24 hours, even at a ratio of 1:10, providing further evidence to a uniquely poor cleavage efficiency in the assay.

PI2 is a serine protease inhibitor (1) and factor Xa is a serine protease (14). The pMAL-c2X-PI2 plasmid was previously designed so that PI2 could be separated from MBP by means of a factor Xa cleavage site (Fig. S1). Therefore, the original design of the plasmid is flawed due to the presence of a cleavage site for a protease that is potentially inhibited by PI2. The factor Xa serine protease is unable to effectively cleave at the cut site due to the target protein being a serine protease inhibitor. This provides evidence that contradicts our hypothesis that MBP interferes with the inhibitory function of PI2. The ineffective cleavage in the factor Xa assay suggests that MBP-PI2 retains its serine protease inhibitory function.

Factor Xa cleaves basic amino acid residues at a preferred cleavage site of Ile-(Glu or Asp)-Gly-Arg with the most common secondary cut site being Gly-Arg (12). Looking at the

sequencing results of the pMAL-c2X-PI2 plasmid and the corresponding amino acid sequence, the preferred cleavage site is present along with one secondary cleavage site (Fig. S4). If cleavage had occurred at a secondary site, it would explain some of the consistent bands seen at various lengths less than 20 kDa in the gel (Fig. 3).

All trypsin-like serine proteases, including factor Xa, have very similar active sites which consist of a catalytic triad of histidine, aspartic acid, and serine residues (24). Additionally, these types of proteases have a similar mechanism of action regarding the hydrolytic reactions they catalyze. While the proteases can catalyze specific reactions, their inhibitors, including PI2, are less specific in their inhibition (25). Studies attempting to design a serine protease inhibitor that is specific to a single serine protease have been investigated (24). Although existing literature has found PI2 to be highly effective against trypsin (1), this lack of specificity shows how PI2 can display broad inhibitory effects against other trypsin-like serine proteases such as factor Xa.

Trypsin inhibition assay. Contrary to the results of the factor Xa optimization assay, our trypsin inhibition assay demonstrated that purified MBP-PI2 failed to inhibit trypsin from cleaving BSA (Fig. 4). The results showed no difference in BSA cleavage between MBP-β-gal-α and MBP-PI2 conditions, suggesting that PI2 did not retain its functionality to inhibit serine proteases. These findings were unexpected, considering MBP-PI2 had previously appeared to effectively inhibit factor Xa, also a serine protease, thereby preventing MBP tag removal. Moreover, trypsin appeared to cleave MBP-β-gal-α and MBP-PI2 at an unexpected site, yielding a fragment at approximately 45 kDa. These findings raised new questions regarding whether trypsin was cleaving MBP-PI2 at other sites as well.

To better understand why MBP-PI2 appeared to inhibit factor Xa (Fig. 3), but failed to inhibit trypsin (Fig. 4), we performed a basic bioinformatic analysis of the pMAL-c2X and pMAL-c2X inserts using ExPASy (Swiss Institute of Bioinformatics). The ExPASy Translate Tool was used to predict the MBP-β-gal-α and MBP-PI2 open reading frames, then translate the nucleotide sequences into amino acids (Fig. S4). The amino acid sequences were inputted into ExPASy PeptideCutter, adjusting the search parameters to only include sites with >70% probability of cleavage by factor Xa and trypsin proteases (Table S3). As expected, ExPASy PeptideCutter predicted one factor Xa cleavage site in MBP-PI2 yielding an estimated 17.4 kDa fragment, which is similar in mass to 16 kDa PI2. One factor Xa cleavage site was also predicted in MBP-β-gal-α at the very end of the amino acid sequence. Given that factor Xa is predicted to cleave MBP-PI2 at the correct site for tag removal, there was unlikely any non-specific cleavage of PI2. As such, we expect that PI2 remained intact during the factor Xa optimization assay. Therefore, purified MBP-tagged PI2 demonstrated functionality in serine protease inhibition.

However, ExPASy PeptideCutter predicted 20 trypsin cleavage sites in MBP-β-gal-α and 27 cleavage sites in MBP-PI2 (Table S3). Trypsin is known to cleave with high specificity at the C-terminal end of lysine or arginine residues, unless a proline residue immediately follows on the N-terminus (26, 27). One of the 20 cleavage sites was likely present on the surface of MBP, resulting in the prominent 45 kDa cleavage fragment observed during the incubation of trypsin with MBP-β-gal-α and MBP-PI2. Further, MBP-PI2 contains 7 additional cleavage sites compared to MBP-β-gal-α. These 7 sites are likely present in PI2. Although further bioinformatic analysis is needed to confirm this, it is possible that one or more of the sites were exposed to the aqueous surroundings of the reaction. If this is the case, trypsin may have cleaved PI2. Given that cleaved PI2 is unlikely functional, this would explain the lack of serine protease inhibition by MBP-PI2 seen in the trypsin inhibition assay. Ultimately, trypsin was revealed to be a poor serine protease to be used for MBP-PI2 functionality tests.

Limitations Certain experiments had to be repeated multiple times due to the presence of contamination in some of the reagents, such as glycerol. The lack of adequate purification instrumentation restricted protocols to manual methods of purification for MBP-PI2 through amylose gravity affinity chromatography. This step in particular represents a limitation of the present study, as only a small amount of purified MBP-PI2 was generated during the single purification treatment, limiting the amount of MBP-PI2 that could be used in subsequent experiments. Future studies might seek to use the automated liquid chromatography methods,

such as AKTA Pure to yield a larger amount of higher purity MBP-PI2 for further experimentation. An additional solution to the problem of inadequate uPI2 would be to purchase uPI2 from commercial sources, so that there will be enough PI2 with which to conduct comparison assays against MBP-PI2. Furthermore, limited lab space prohibited the scaling up of protein expression and purification reactions, which may have reduced the yield of MBP-PI2.

Conclusions In summary, SHuffle cells were transformed with pMAL-c2X and pMAL-c2X-PI2 plasmids which, followed by IPTG induction, confirmed previous results that SHuffle cells enable MBP-PI2 expression. MBP-PI2 was also successfully purified by amylose affinity chromatography. The factor Xa cleavage assay results indicated that factor Xa inefficiently cleaved the MBP fusion tag from MBP-PI2, potentially due to its intrinsic inhibitory activity. Thus, the experimental aim of comparing the functionality of uPI2 and MBP-PI2 could not be addressed, given that uPI2 was not generated in sufficient amounts for downstream assays. Factor Xa appeared to be inhibited during MBP tag removal, but nevertheless, this may have demonstrated that MBP-PI2 is functional. The trypsin inhibition assay demonstrated that MBP-PI2 was ineffective at inhibiting trypsin from cleaving BSA. Multiple trypsin cleavage sites were bioinformatically predicted in MBP-β-gal-α, as well as additional sites in MBP-PI2. Trypsin cleavage may have compromised the structural integrity of PI2, thereby limiting its potential to properly function. Ultimately, the trypsin assay demonstrated that this protease was not ideal for testing MBP-PI2's complex functionality. This study was the first to identify critical inconsistencies regarding the ability to separate MBP from PI2 and has put forth foundational knowledge about this serine protease inhibitor.

Future Directions The present study allows for the possibility of further investigation through several avenues. Most importantly, future authors must do a profound literature search beforehand to ensure that all components of the project (including PI2, the affinity tag, the tag cleavage enzyme, and the functional assay enzyme) are compatible with each other. One key takeaway of our study was that trypsin was incompatible with functional assays due to its unexpected cleavage of MBP-β-gal-α and potentially PI2. Future groups seeking to investigate tagged recombinant PI2 functionality may wish to select a different serine protease that does not cleave PI2. One candidate protease would be thrombin. To assess the compatibility of thrombin with PI2, we conducted a simple bioinformatic analysis using ExPASy PeptideCutter. We found that the pMAL-c2X-PI2 insert contains zero thrombin cleavage sites (Table S3). Therefore, PI2 itself would be expected to remain intact, and MBP-PI2 can be more reliably tested in a thrombin inhibition assay.

However, MBP tag cleavage assays that aim to generate uPI2 using the pMAL-c2X-PI2 vector may become complicated. The present study found that factor Xa cleaved the MBP tag from MBP-PI2 because it is a serine protease and was likely inhibited by PI2. In future studies, the requirements for an MBP tag cleavage protease are that 1) it cannot be factor Xa, 2) it cannot be a serine protease, and 3) it does not have any cleavage sites within MBP-PI2. Since our current study has demonstrated expression and purification of PI2 fused to an MBP tag, future studies should continue using this system. However, a new vector must be designed to contain a tag cleavage site other than factor Xa. To do this, a pMAL-c6T backbone (New England Biolabs) could be used, which encodes an MBP tag and a Tobacco Etch Virus (TEV) protease cleavage site. TEV protease is often used for recombinant protein tag cleavage (28). It is a cysteine protease (29), and therefore will not be inhibited by serine protease inhibitors such as PI2. Additionally, ExPASy PeptideCutter predicts zero TEV protease cleavage sites in MBP-β-gal-α and MBP-PI2 (Table S3). If experiments using the pMAL-c6T backbone are successful, MBP-PI2 and uPI2 functionality could be compared in a thrombin inhibition assay. Possible thrombin substrates to be used in the assay include fibrinogen and protein C (30). If purified MBP-PI2 and uPI2 inhibit thrombin from cleaving its substrate, greater insight will be provided for the functionality of this therapeutic protein candidate.

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CONTRIBUTIONS

K.L. contributed to writing the abstract, methods, results, and future directions. H.L. contributed to writing the introduction and conclusions. B.S. contributed to writing the abstract, methods, acknowledgements, creating figures, and compiling references. E.V. contributed to writing the abstract, introduction, study limitations, future directions, and figure captions. Authors contributed equally to writing all other sections of the manuscript, including the discussion and supplemental materials. All authors were responsible for editing and making final revisions of the manuscript.

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