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Effect of Temperature, Inducer Concentration, and *Escherichia coli* Cytosolic Redox State on MBP-PI2 Expression

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Type II proteinase inhibitors (PI2) found in potato (*Solanum tuberosum*) have been observed to confer protective enzymatic activity towards foreign proteases and were shown to have potential in applied contexts including anticancer and hunger suppression. Previous studies in our laboratory have synthesised, cloned, expressed and purified a PI2 variant fused to maltose binding protein, termed MBP-PI2. We studied MBP-PI2 expression levels with respect to growth temperature (30°C vs 37°C), isopropyl β-D-1-thiogalactopyranoside (IPTG) inducer concentration, and cytosolic redox state using *Escherichia coli* strains Origami 2 (DE3) and BL21 (DE3) SDS-PAGE analysis of whole cell lysate show that the highest level of MBP-PI2 expression occurred in strain BL21 (DE3) with a 2 hour induction period. Expression was not detected in strain Origami 2 (DE3). Similar expression levels of MBP-PI2 were observed at 30°C vs 37°C and 0.2 mM vs 1 mM IPTG. Our study acts as a framework for future experiments exploring the production and application of MBP-PI2.

Proteinase inhibitor II (PI2) is a native protein found within most solanaceous plants that functions as a serine protease inhibitor, inhibiting the activity of enzymes such as trypsin and chemotrypsin (1). PI2 primarily functions to protect its host from foreign proteolytic enzymes originating from other organisms such as insects and microbes (2). PI2 has been shown to have applied potential due to its anti-cancer and hunger suppressant properties (3-5). PI2 was shown to aid in the prevention of radiation and UV damage by inhibiting proteases involved in the propagation of cellular damage in both plants and murine cell lines (3, 4). PI2 can act as a hunger suppressant by increasing the concentration of cholecystokinin (CCK), a hormone which signals satiety, in our bodies (5). There has been much interest in the expression and isolation of PI2 in high quantities (3-5).

PI2 is a two domain, 16.5 kDa, protein with a reaction center in each domain (6). Each domain contains 8 cysteine residues (6). These cysteine residues form 8 disulfide bonds per functional protein and are essential for the correct protein folding of its two reaction centers (2, 6). PI2 requires an oxidizing environment, such as the endoplasmic reticulum, to facilitate proper disulfide bond formation and folding of the protein into its native structure (6). To date, recombinant PI2 has been expressed and isolated from *Escherichia coli* strain BL21 (DE3) which has a reduced cytoplasmic environment (6). It is possible that the reductive environment in the cytoplasm of *E. coli* BL21 strain (DE3) may not support the formation of disulfide bonds which may cause the protein to fold incorrectly. This could result in its aggregation as insoluble, non-functional proteins, referred to as inclusion bodies (7). To address the challenge of disulphide bond formation in *E. coli* strain Origami 2 (DE3) strain was developed by Novagen. *E. coli* strain Origami 2 (DE3) bear mutations in glutaredoxin (*gor*) and thioredoxin (*trxB*), reductase genes which creates an oxidative cytosol (8) which has been shown to improve the soluble expression of some proteins with disulfide bonds (9).

Previous experiments in our laboratory have synthesised and cloned a *pi2* variant fused into plasmids encoding maltose binding protein or hexa-histidine fusion tags (10). Following amylose resin chromatography and resolution by SDS-PAGE, Lapointe *et al.* observed bands migrating at approximately 66 kDa, corresponding to their predicted molecular mass of MBP-PI2 (2). Optimal expression conditions for MBP-PI2 have not been established.

Our project aimed to further characterize expression of MBP-PI2. We hypothesized that expression of soluble PI2 would be higher in *E. coli* strain Origami 2 (DE3) compared to the BL21 (DE3) strain (12). An oxidative environment may promote the formation of disulphide bonds between the 16 cysteine residues in PI2 which may yield more natively folded protein (12). In addition, we tested the effects of temperature and isopropyl β-D-1-thiogalactopyranoside (IPTG) inducer concentration on MBP-PI2 expression levels. Our analysis of whole cell lysates using SDS-PAGE show that putative MBP-PI2 expression was observed in *E. coli* strain BL21 (DE3) but not in *E. coli* strain Origami 2 (DE3). Temperatures of 30°C and 37°C and IPTG inducer concentrations of 0.2 mM and 1 mM did not seem to impact expression levels of MBP-PI2 in *E. coli* strain BL21 (DE3).

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmid preparation, and antibiotic concentrations. *E. coli* strain BL21 (DE3) was cultured from frozen stocks provided by the UBC Department of Microbiology and Immunology. *E. coli* strain Origami 2 (DE3) was donated by the Straus lab from the University of British Columbia. *E. coli* strains BL21 (DE3) and Origami 2 (DE3) cultures were propagated in 5 mL LB medium liquid broth in a shaker at 37°C overnight and were grown up to an OD_{600} of 0.4 and put on ice.

Plasmids pMAL-c2X and pMAL-c2X-LLMZ16 were provided from the Eltis lab and Lapointe *et al.*, respectively. The plasmid storage strain (*E. coli* DH5α) was cultured on LB-Amp plates at 37°C for 24 hours. Once grown, an LB-Amp broth was inoculated with an isolated colony from the plate. The cells were incubated at 37°C overnight with shaking and were grown up to an OD_{600} of 0.4.

E. coli strain BL21 (DE3) cultures were plated and induced with IPTG in LB-Amp. *E. coli* strain Origami 2 (DE3) cultures were plated and induced with IPTG in LB-Amp-Tet. Working

concentrations of 100 μg/mL of ampicillin and 10 μg/mL of tetracycline were used.

Competent *E. coli* Origami 2 (DE3) and BL21 (DE3) strains. Cell cultures were centrifuged at 10,000 rpm and 4ºC for 5 minutes with Bio-Rad benchtop centrifugation for harvesting. The supernatant was subsequently removed and the cells were re-suspended in ice cold 0.1M $CaCl_2$ and left on ice for 30 minutes. After, the centrifugation steps were carried out again and the cells were suspended in 8 mL of 15% glycerol, 0.1 M $CaCl_2$ freezing solution. The competent cells were stored at -80ºC for future use.

Isolation of expression vectors. The plasmids were isolated from *E. coli* strain DH5α using an Invitrogen PureLink Quick Plasmid MiniPrep kit. An isolated pMAL-c2X-LLMZ16 plasmid was also sent to the Nucleic Acid Protein Service (NAPS) for sequencing. Prime pair PI2F_pMAL and PI2R_pMAL were obtained from Lapointe *et al.* (2). ExPasy Translate was used to align the gene into the correct reading frame and for translation of the nucleotide sequence into an amino acid sequence before confirming the identity of the plasmid and insert using the protein Basic Local Alignment Search Tool (BLAST).

Transformation of *E. coli* Origami 2 (DE3) and BL21 (DE3) strains. Competent cells were taken out of -80°C storage and thawed on ice for 25 minutes. 5 μL (75 ng) of plasmid was mixed into 50 μL of competent cells. Mixing was carried out by gently flicking the bottom of the tube 10 times. The mixtures were incubated on ice for 25 minutes. Each transformation tube was heat shocked at 42°C for 45 seconds. Tubes were put back on ice for 2 minutes. 500 μL of LB media (without antibiotic) were added to both mixtures and grown in a shaking incubator for 45 minutes at 37°C. 50 μL of *E. coli* BL21 (DE3) and Origami 2 (DE3) cultures were plated in duplicate on LB-Amp plates and LB-Amp-Tet plates, respectively, at 37°C for 24 hours. Overnight cultures of *E. coli* BL21 (DE3) and Origami 2 (DE3) strains were created by inoculating 5 mL of liquid media with the appropriate antibiotics from a single colony of each transformed strain at 37°C for 24 hours. Overnight cultures were used to create a 50% glycerol stock solution of each transformed *E. coli* strain which was stored at -80°C.

MBP-PI2 protein expression. 100 mL expression of both cultures containing either transformed or empty vector (EV) plasmids were grown overnight in LB medium supplemented with antibiotics. Cells were grown to an OD_{600} of 0.5 and induced with a working concentration of 0.2 mM IPTG at 37°C. Cells were harvested by centrifugation at 2,000 x g for 30 minutes and stored at -80˚C. 2 mL aliquots of each expression culture were saved for expression testing. These aliquots of induced cells were centrifuged at 16,000 x g for 20 minutes. The resulting cell pellet was suspended in 75 μL of of NuPAGE® LDS Sample Buffer (4X) (ThermoFisher Scintific, catalogue #NP0007) and the crude cell lysates were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% Tris-Glycine gels (ThermoFisher Scientific, catalogue #XP0010C). A SeeBlue® Plus2 Pre-stained Protein Standard (ThermoFisher Scientific, catalogue # LC5925) and BioRad Low Molecular Weight Protein Standard (Bio-Rad, catalogue #1610304) were included as ladders.

Optimizing MBP-PI2 protein expression. 100mL cultures were grown in 2% glucose LB medium supplemented with antibiotics. 2% glucose was added to repress the maltose genes on the chromosome of the *E. coli* host, one of which is amylase which can degrade the amylose on an affinity resin. This was performed in the case that time permitted the subsequent amylose resin purification of the MBP-PI2 protein. These cells were grown to an OD_{600} of 0.5 and induced under a variety of conditions including: temperature (30°C vs 37°C), inducer concentration (0.2 mM vs 1 mM IPTG) and induction time (1 hour vs 2 hour). 2 mL aliquots of each culture were saved for analysis by SDS-PAGE. The aliquots of induced cells were centrifuged at 16,000 x g for 20 minutes. The resulting cell pellet was suspended in 75 μL of of NuPAGE® LDS Sample Buffer (4X) (ThermoFisher Scintific, catalogue #NP0007) and the crude cell lysates were visualized by SDS-PAGE on 4-12% Tris-Glycine gradient gels (ThermoFisher Scientific, catalogue #XP04125BOX). A SeeBlue® Plus2 Pre-stained Protein Standard (ThermoFisher Scientific, catalogue # LC5925) and BioRad Low Molecular Weight Protein Standard (Bio-Rad, catalogue #1610304) were included as ladders.

RESULTS

SDS-PAGE analysis of MBP-PI2 with crude cell lysates. *E. coli* BL21 (DE3) and BL21 Origami (DE3) transformed with pPI2-MBP were grown to 0.5 OD units and induced with either 0.2 mM or 1 mM IPTG. Cultures were also split and grown at 30°C and 37°C to study the effect of temperature on PI2-MBP expression. Whole cell lysate pf cells harvest at 1 hour and 2 hours post induction were resolved by SDS-PAGE and visualized by staining of Coomassie blue. Prominent bands at roughly 66 kDa for the IPTG induced lanes from *E. coli* strain BL21 (DE3) were observed (Fig. 1b). The bands migrating at ~ 66 kDa were more apparent following 2 hour induction (Fig. 1b). Prominent bands migrating near 66 kDa were not observed in the induced lanes of *E. coli* strain Origami 2 (DE3) (Fig. 1a). In the absence of IPTG induction prominent bands migrating at ~ 66 kDa were not observed (Fig. 1). Taken together these data shows over expression of a protein migrating at ~ 66 kDa corresponding to the predicted molecular mass of PI2-MBP in E. coli strain BL21 (DE3) but not in strain BL21 Origami (DE3). Further, temperature and inducer concentrations did not seem to impact expression levels of MBP-PI2 in *E. coli* strain BL21 (DE3).

EV lanes were also analysed with a previous SDS-PAGE analysis and are indicative of plasmids without the *pi2* gene insert. Prominent bands were observed at approximately 51 kDa in the induced and non-induced EV lanes for both *E. coli* strains BL21 (DE3) and Origami 2 (DE3) (Fig. S1). The approximate 51 kDa bands were observed to be more prominent for this SDS-PAGE analysis than the approximate 66 kDa band observed on the *E. coli* strain BL21 (DE3) IPTG induced lane (Fig. S1).

DISCUSSION

Recombinant expression of proteins containing disulphide bonds in *E. coli* is complicated by the redox state of the cytoplasm (6). In this study, we sought to characterize expression of a serine protease inhibitor, PI2, which contains 8 disulphide bonds. Following eluication of the nucleotide sequence and corresponding amino acid sequence of pi2 by Keil *et al.* (13) several studies have reported challenges with expression and isolation PI2 as a soluble protein (10, 14, 15). Thus, we set out to test expression conditions of PI2 fused to MBP. The MBP affinity tag was chosen as it was observed in previous studies to enhance solubility and facilitate protein isolation through amylose resin column purification (11).

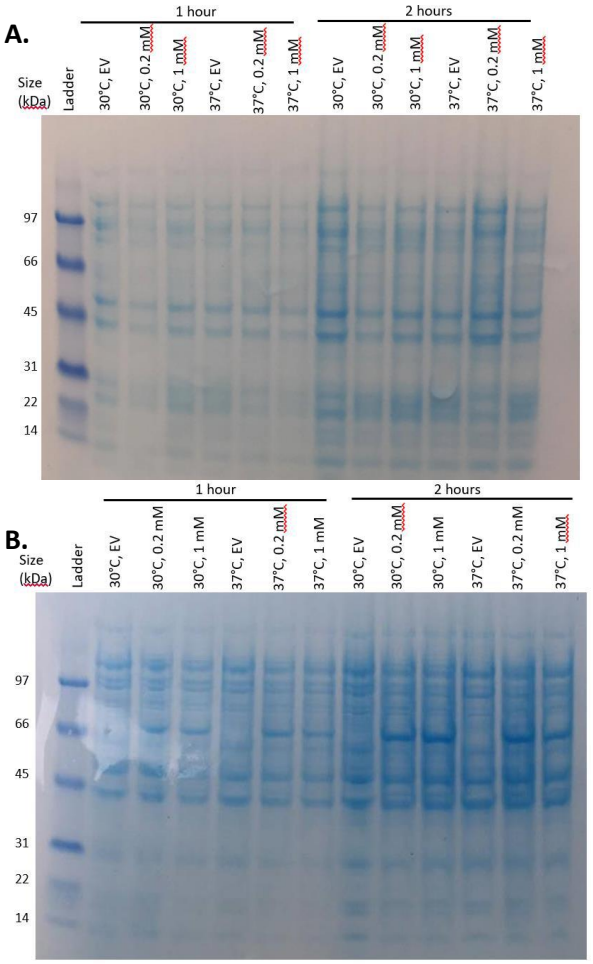

FIG. 1 SDS-PAGE analysis of MBP-PI2 protein in various conditions. **(A)** 2 mL of IPTG induced *E. coli* strain Origami 2 (DE3) cells at 2 time points (1 hour and 2 hours) were measured. The different lanes correspond to the different conditions the strain was subjected. Crude cell lysates were ran on the 10% SDS-PAGE gel. **(B)** 2 mL of IPTG induced *E. coli* strain BL21 (DE3) cells at 2 time points (1 hour and 2 hours) were measured. The different lanes correspond to the different conditions the strain was subjected. Crude cell lysates were run on the 10% SDS-PAGE gel.

Previous work with the pMAL-c2X-LLMZ16 plasmid have not reported the downstream fusion of *lacZα* to the *pi2* gene (2, 11). The resulting MBP-PI2 fusion protein is expressed with the β-gal-α protein fused at its C-terminus (11). The MBP-β-gal-α fusion protein is 50.8 kDa, and the PI2 protein is 16 kDa (6, 11). Thus, the molecular weight of the MBP-PI2 fusion protein is approximately 66 kDa and not the previously mentioned 59.4 kDa (2). A proposed MBP-PI2 fusion construct was developed for visualization (Fig. S2). PI2 has also been mistakenly classified as a homodimer from a previous study carried out from our laboratory (10). However, Li *et al.* has characterized PI2 as a single protein with two domains (6).

In this experiment, MBP-PI2 was expressed under different conditions in two different strains and analysis was done using SDS-PAGE of whole cell lysates. SDS-PAGE analysis of MBP-PI2 from *E. coli* strain BL21 (DE3) whole cell lysate resulted in the observation of a band at approximately 66 kDa band on the IPTG induced lane which corresponds to the predicted molecular mass of MBP-PI2 (Fig. 1).

Bands at approximately 66 kDa were not observed for the *E. coli* strain Origami 2 (DE3) in both the induced and non-induced lanes. Interestingly, this finding is contradictory to our initial hypothesis. The EV lanes for *E. coli* strain Origami 2 (DE3) show prominent bands at approximately 51 kDa indicating expression of the 50.8 kDa MBP-β-gal-α fusion protein (Fig. S1). We hypothesize that IPTG induction was adequate in *E. coli* strain Origami 2 (DE3) but the MBP-PI2 fusion protein did not fold correctly due to the oxidative cytosolic environment conferred by the cell, causing the aggregation of the MBP-PI2 protein and thus the absence of a band at roughly 66 kDa in the SDS-PAGE analysis of the whole cell lysate (Fig. S1). This is further supported by the SDS-PAGE analysis of the EV lanes for *E. coli* strain BL21 (DE3). The bands observed at approximately 51 kDa on the EV lanes were notably thicker than the bands observed at approximately 66 kDa band in the IPTG induced lane, potentially indicating the challenge of proper MBP-PI2 protein folding under reductive cytosolic conditions (Fig. S1). However, a specific reason remains to be elucidated as no previous studies linking PI2 formation to cytosolic redox environments has been found.

Other bands with varying sizes were also observed on the SDS-PAGE analysis due to our samples being crude cell lysates. Nonetheless, these results show that the bands migrating at approximately 66 kDa are likely to be MBP-PI2.

In conclusion, the results show that MBP-PI2 expression can be detected as a band migrating at approximately 66 kDa in SDS-PAGE analysis of crude cell lysates from *E. coli* strain BL21 (DE3) but not strain Origami 2 (DE3) (Fig. 1). Induction temperature levels of 30°C vs 37°C and IPTG concentration of 0.2 mM vs 1 mM did not seem to impact expression levels of *E. coli* strain BL21 (DE3).

FUTURE DIRECTIONS

The experiment only tested expression level of PI2-MBP in whole cell lysates using SDS-PAGE analysis. At this time it is not known if the putative PI2-MBP band represents soluble or functional forms of the protein. Follow-up experiments should investigate the structure and function of PI2-MBP using centrifugation to assess solubility, limited proteolysis to assess conformation, SDS-PAGE under reducing and non-reducing conditions to assess disulphide bond formation, and protease inhibition assays to test function. While MBP has been shown to enhance the solubility of proteins, the enzymatic

activity of PI2 has yet to be assessed when its N-terminus is linked to MBP (9). Future experiments may include a functional assay adapted from Kakade *et al.* to test the enzymatic activity of PI2 when expressed in this fusion construct (17). The functional assay could be paired with various *E. coli* strains to elucidate key strains that create an optimal condition for PI2 expression and isolation.

Further research of the oxidative environment conferred from *E. coli* strain Origami 2 (DE3) on PI2 folding is also suggested. Perhaps the expression or proper folding of PI2 is dictated by certain biological pathways which require a reductive, cytosolic environment. It may also be due to differential expression or activity of proteases in these two *E. coli* strains. Some proteases which target the MBP-PI2 protein may be more active in an oxidative cytosolic environment rather than a reduced cytosolic environment. Further understanding of this discrepancy may be key to optimize the isolation and expression of a soluble and enzymatically active PI2.

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