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Expression and Purification of a Potato Type II Proteinase Inhibitor in *Escherichia coli* Strain BL21(DE3)

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Type II proteinase inhibitors (PI2) derived from potato tubers have demonstrated protective capabilities against microbes and insects through the inhibition of foreign proteases. This family of protease inhibitors also display anticancer activity and hold potential therapeutic applications in multiple cancer models. We report here the cloning of a genetically-engineered PI2 gene variant, $pi2_{E.coli}$*c.994_1110del encoding Pi2K, into different expression vectors encoding one of two affinity tags, a mannose-binding protein (MBP) or a hexahistidine tag (His-tag). Growth of *Escherichia coli* strains DH5α and BL21(DE3), transformed with the constructed plasmids, suggest that tagged Pi2K is not toxic. Purification using amylose resin and SDS-PAGE analysis of recovered fractions revealed that the MBP-Pi2K fusion protein is soluble and may be susceptible to degradation. These results provide a framework to explore the biochemical inhibitory activity of Pi2K to determine its specific activity and substrate range. Together, our data point to the capacity of *E. coli* BL21(DE3) strain for heterologous expression of soluble Pi2K and further studies of the biotechnological and therapeutic value of this proteinase inhibitor.

Proteins perform essential roles in cellular processes and are harnessed for a myriad of biotechnological and medical purposes (1). Proteinase inhibitors, a class of proteins found in all forms of life, often play a protective role (2). In plants, protease inhibitors are produced to defend against foreign proteolytic enzymes from microbes and insects (2). Specifically, a family of type II proteinase inhibitors (PI2) derived from potato tubers is well-characterized, acting against trypsin and chymotrypsin, two digestive enzymes in the gastrointestinal tract of many vertebrate predators (3). Interestingly, as a medical tool, PI2 blocks protein activation linked to human skin carcinoma and acts as a hunger suppressant by increasing cholecystokinin levels (4-6). Other potato proteinase inhibitors have been linked to anticancer activity on human colorectal cancer cells (7). These usages have further garnered interest in the economical production and characterization of PI2. These studies hold important implications for cancer prevention, as well as commercial applications in pest control (8-10).

The canonical PI2 protein contains two domains, both of which belong to the potato type II proteinase inhibitor family (PF02428) (11) and share homology with protease inhibitors from other plants (12). Each domain is characterized by eight cysteine bonds that form four disulphide bridges. PI2 family members otherwise vary in protein sizes and protomer composition (3, 9, 13). Keil *et al.* isolated one of the first genomic clones of a potato PI2 (GenBank accession# X04118.1) (14). The gene (herein *pi2*) contains two exons flanking a 117 bp intron. The processed mRNA transcript codes for a 16.7 kDa protein (herein Pi2K), containing 154 amino acids (14).

To date, large-scale production of Pi2K has been unsuccessful. Protein production in eukaryotic systems is limited by difficulties with overexpression, as well as ethical and financial constraints. Progress has therefore been made to utilize eukaryotic gene expression in prokaryotic systems, particularly in *Escherichia coli* (15). A challenge to eukaryotic protein synthesis in *E. coli*, however, is the improper folding of proteins, which results in aggregate formation and inclusion bodies (16). The difficulty of heterologous recombinant protein expression is further compounded by differences in genetic parameters between eukaryotes and prokaryotes (17). In particular, cysteine residues may pose a challenge to proper folding and may result in insoluble protein aggregation, since the prokaryotic system lacks the optimal oxidizing environment in the endoplasmic reticulum to promote disulphide bond formation (13, 18). These factors all contribute to the difficulty in producing Pi2K in an *E. coli* system.

To overcome this challenge, Prezewski *et al.* and Geum *et al.* studied a modified *JEMI-pi2* gene that closely resembles

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the potato wild type gene reported by Keil *et al.* (14, 19, 20). Both genes are incompatible for expression in *E. coli* due to the lack of eukaryotic splicing machinery to remove the 117 bp intron prior to translation (21). The non-spliced mRNA introduced frameshift mutations and a premature stop codon. Furthermore, *JEMI-pi2* was not optimized for codon usage or GC content for expression in *E. coli* (21). The difference in GC content can contribute to variations in DNA stability, among other parameters. To overcome these incompatibilities, a modified PI2 gene variant, *synPI2* (herein named $pi2_{E.coli}$*c.994_1110del*), was engineered into the pCR2.1-TOPO-1-1eta_M13R plasmid storage vector (21). The modified $pi2_{E.coli}$*c.994_1110del* is an intron-less coding sequence, with optimized codon usage and GC content for expression in *E. coli* (21).

We hypothesize that the optimized $pi2_{E.coli}$*c.994_1110del* may be expressed as soluble protein from the expression vectors of pMAL-c2X and pET-30b. The pMAL-c2X expression vector encodes a maltose-binding protein (MBP) affinity tag and solubility enhancer, promoting proper folding of its fusion protein and facilitating purification (22). pET-30b carries a N-terminal His•Tag®/thrombin/S•Tag™/enterokinase configuration, which serves a similar role to MBP. However, the latter produces a smaller tag than MBP, and thus may increase solubility of the fusion partner without interfering with protein function (23).

In this paper, we describe the cloning of $pi2_{E.coli}$*c.994_1110del* into the expression vectors of pET-30b and pMAL-c2X, followed by soluble protein expression and purification from *E. coli* BL21(DE3). This strain has demonstrated high-level induction and expression of genes, with high transformation efficiency desirable for protein expression. This research provides a solid foundation for the future studies to optimize isolation of Pi2K and its activity with respect to protein inhibition.

MATERIALS AND METHODS

Competent *E. coli* DH5α and BL21(DE3) strains. *E. coli* strains DH5α and BL21(DE3) (herein BL21) were cultured from frozen stocks provided by the UBC Department of Microbiology and Immunology. Cells were rendered chemically competent using transformation storage solution (TSS) buffer, and stored at -80°C (24).

Isolation of expression vectors. Drs. Eltis and Fernandez (University of British Columbia) generously provided the pMAL-c2X and pET-30b expression vectors, respectively. All plasmids were propagated in *E. coli* strain DH5α. Cultures were incubated overnight at 37°C in Luria broth (LB) media containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml), for propagation of pMAL-c2X and pET-30b respectively. Plasmid isolation was performed using PureLink® Quick Plasmid DNA Miniprep Kit (Invitrogen, cat# K210010), following manufacturer protocol. DNA concentrations of the isolates were determined using the NanoDrop 200c Spectrophotometer (ThermoScientific).

Plasmid construction. The plasmid pCR2.1-TOPO-$pi2_{E.coli}$*c.994_1110del* was confirmed by Sanger sequencing (21). Primers for Gibson assembly were designed using the NEBuilder Assembly Tool (New England Biolabs). $Pi2_{E.coli}$*c.994_1110del* was cloned as a MBP-fusion (herein MBP-Pi2K) in pMAL-c2X and histidine tag-fusion (herein His-Pi2K) in pET-30b using Gibson assembly (New England Biolabs cat# E2611). $Pi2_{E.coli}$*c.994_1110del* was amplified from pCR2.1-TOPO- $pi2_{E.coli}$*c.994_1110del* using the primers found in Table 1 (21). PCR amplification followed one cycle of 94˚C for 2 min; 35 cycles of 94˚C for 30 s, 60.4˚C for 30 s, and 72˚C for 1 min; one cycle of 72˚C for 5 min; and held at 4˚C (21).

TABLE 1 Gibson primers for amplification of $pi2_{E.coli}$*c.994_1110del* from the pCR2.1-TOPO storage vector. The $pi2_{E.coli}$*c.994_1110del*-specific portion of the primer sequence (bolded) allows PCR amplification of the target insert, while the remaining bases allow insertion into the expression vector. PI2F_pMAL required two spacer bases (underlined) for insertion into the proper reading frame.

Primer	Sequence (5′ to 3′)
PI2F_pMAL	GATCGAGGGAAGGATTTCAG<u>AT</u>**ATGGACGTTCACAAAGAAG**
PI2R_pMAL	GGTCGACTCTAGAGGATCCG**TTACATCGCCGGGTACATG**
PI2F_pET	ACAAGGCCATGGCGATATCG**ATGGACGTTCACAAAGAAG**
PI2R_pET	GTCGACGGAGCTCGAATTCG**TTACATCGCCGGGTACATG**

pMAL-c2X vector was linearized by digestion with FastDigest EcoRI (ThermoFisher Scientific, cat# FD0274) and pET-30b vector was linearized by digestion with FastDigest BamHI (ThermoFisher Scientific, cat# FD0054), and the amplified $pi2_{E.coli}$*c.994_1110del* was inserted into the vector using Gibson reaction (New England Biolabs cat# E2611). The Gibson reaction was performed using a 1:3 pmol ratio of vector:insert, with 50 ng of linearized vector and following manufacturer protocol. Cloning reactions were transformed into *E. coli* DH5α and selected on LB plates containing ampicillin (100 µg/ml) for pMAL-c2X construct and kanamycin (50 µg/ml) for pET-30b construct. Colonies containing pMAL-c2X-$pi2_{E.coli}$*c.994_1110del* and pET-30b-$pi2_{E.coli}$*c.994_1110del* were screened by colony PCR using, respectively -21M13 and M13R primers, and T7 Forward and Reverse primers (Table S1). The correct insertion of $pi2_{E.coli}$*c.994_1110del* into pMAL-c2X and pET-30b was confirmed by Sanger sequencing. The confirmed constructs were named pMAL-c2X-LLMZ16 and pET-30b-LLMZ16, respectively.

Sequence management. Sequence data were aligned using Multiple Sequence Alignment (MUSCLE) muscle3.8.31 (25). Primer reads were merged to form consensus sequences with no observed nucleotide discordance in the overlapping region of reads. To identify potential mutations, custom Basic Local Alignment Search Tool (BLAST) analysis against the empty expression vectors and the $pi2_{E.coli}$*c.994_1110del* sequence was performed (26). Sequences were visualized using Aliview (27).

Protein expression. MBP-Pi2K and His-Pi2K were expressed in *E. coli* BL21. BL21 was transformed with pMAL-c2X-LLMZ16 and selected on LB plates containing ampicillin (100 µg/ml). BL21 was also transformed with pET-30b-LLMZ16 and selected on LB plates containing kanamycin (50 µg/ml). Overnight starter

cultures were grown in LB medium containing ampicillin (100 µg/ml) or kanamycin (50 µg/ml), as appropriate, for 18 hours at 37°C. 50 ml expression cultures were grown in LB medium with the appropriate antibiotic and 2% glucose by seeding with a 1% inoculum from the starter culture. Cells were grown to an optical density at 600 nm of 0.6 and then induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for overnight growth at various temperatures. Cells were harvested by centrifugation at 2,000 x g for 30 minutes and stored at -80˚C. Separate 1.5 ml aliquots of each expression cultures were saved for expression testing. Note, no uninduced sample was included in this study.

To test for expression, 1.5 ml cell pellets were resuspended in 400 µl of lysis buffer, composed of 20 mM Tris (pH 7.4), 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, and 1 mM dithiothreitol (DTT). Cells were lysed by probe sonication (at power level 1) using four 10 second rounds. The insoluble cellular fraction was pelleted by centrifugation 16,000 x g for 30 minutes. The soluble supernatant fraction was collected and the remaining insoluble pellet was solubilized in 20 mM sodium phosphate (pH 8), 0.5 M NaCl, and 8 M urea, and centrifuged at 16,000 x g for 20 minutes. The presence of MBP-Pi2K and His-Pi2K in either the soluble or the insoluble fraction was visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. A Precision Plus Protein Unstained Standard (Bio-Rad, cat# 1610363) was included as a ladder.

MBP-Pi2K protein purification. Frozen cell pellet was thawed on ice in 10 ml of column buffer, composed of 20 mM Tris (pH 7.4), 200 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, and containing cOmplete™, Mini, EDTA-free protease inhibitor cocktail tablet (Roche). Cells were lysed by sonication (at power level 1) using four 10 second rounds. The insoluble cellular fraction was pelleted by centrifugation 16,000 x g for 30 minutes, and the supernatant was collected.

Purification was carried out using an amylose resin affinity column (New England Biolabs, cat# E8021). The column was equilibrated with the column buffer, and the supernatant was incubated with the resin for 1 hour at 4˚C while gently rocking. Following incubation, the column was washed with 10 column volumes (CV) the column buffer and eluted with 5 CV of column buffer containing 10 mM maltose. Fractions were analyzed by 10% SDS-PAGE, to determine the presence of MBP-Pi2K. A Precision Plus Protein Unstained Standard (Bio-Rad, cat# 1610363) was included as a ladder. The appropriate fractions containing the MBP-tagged fusion protein were concentrated by centrifugation in a 30K Amicon® Ultra centrifugal filters (EMD Millipore) at 2,000 x g using successive 5-minute rounds. The protein was simultaneously buffer exchanged to remove maltose and DTT. The protein was quantitated using the NanoDrop 200c Spectrophotometer (ThermoScientific) and the measured concentration was adjusted by MBP-Pi2K's extinction coefficient (82250 M^{-1} cm^{-1}) determined using the Protein Parameters tool from SIB's ExPaSy Bioinformatics Resource Portal (28).

RESULTS

Construction of tagged Pi2K proteins. To produce plasmids that would express *Pi2* as an MBP and histidine tagged fusion protein, $Pi2_{E.coli}$*c.994_1110del* was amplified from pCR2.1-TOPO-$pi2_{E.coli}$*c.994_1110del* and cloned into pMAL-c2X and pET-30b respectively, using Gibson

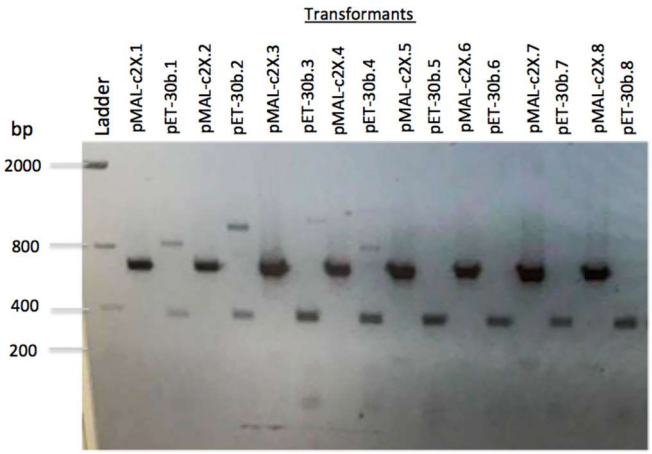

FIG. 1 Agarose gel analysis of colony PCR screening of DH5α pMAL-c2X-LLMZ16 and DH5α pET-30b-LLMZ16 transformants. Amplicons were separated by 1.5% agarose gel electrophoresis and visualized with SYBR Safe. An E-gel Low Range Quantitative DNA ladder was included.

assembly. Transformants for both pMAL-c2X-LLMZ16 and pET-30b-LLMZ16 were obtained. Screening by colony PCR revealed all tested pMAL-c2X-LLMZ16 transformants contained an approximately 700 bp sequence, corresponding to the proper insertion of $pi2_{E.coli}$*c.994_1110del* (Fig. 1). Three of the screened transformants were chosen at random for plasmid isolation and sequencing. All tested pET-30b-LLMZ16 transformants contain empty vector, as evident by prominent bands just below 400 bp. However, several transformants also contained bands above 800 bp, at varying sizes (Fig. 1). Proper insertion of $pi2_{E.coli}$*c.994_1110del* into pET-30b was predicted to produce an amplicon of approximately 830 bp. Thus, three transformants that showed bands just above 800 bp were chosen for plasmid isolation and sequencing.

Bidirectional Sanger sequencing confirmed that all six plasmids contained $pi2_{E.coli}$*c.994_1110del*. All $pi2_{E.coli}$*c.994_1110del* were confirmed to be within the correct reading frame. Sequence analysis revealed 100% nucleotide concordance with $pi2_{E.coli}$*c.994_1110del* in two pMAL-c2X-LLMZ16 isolates (pMAL-c2X-LLMZ161 and pMAL-c2X-LLMZ162) and one pET-30b-LLMZ16 isolate (pET-30b-LLMZ167) (Fig. 2). A single base transition mutation was introduced in each of the other three constructs. All mutations resulted in an amino acid substitution within Pi2K. These errors were likely introduced by the Taq polymerase during PCR amplification of the target insert.

Expression of MBP- and histidine-tagged Pi2K. The protein was expressed with an N-terminal MBP- or His-tag to increase solubility and facilitate purification (22). The $pi2_{E.coli}$*c.994_1110del* fusion genes were heterologously expressed in *E. coli* BL21 at three temperatures following induction. Based on SDS-PAGE analysis of the soluble

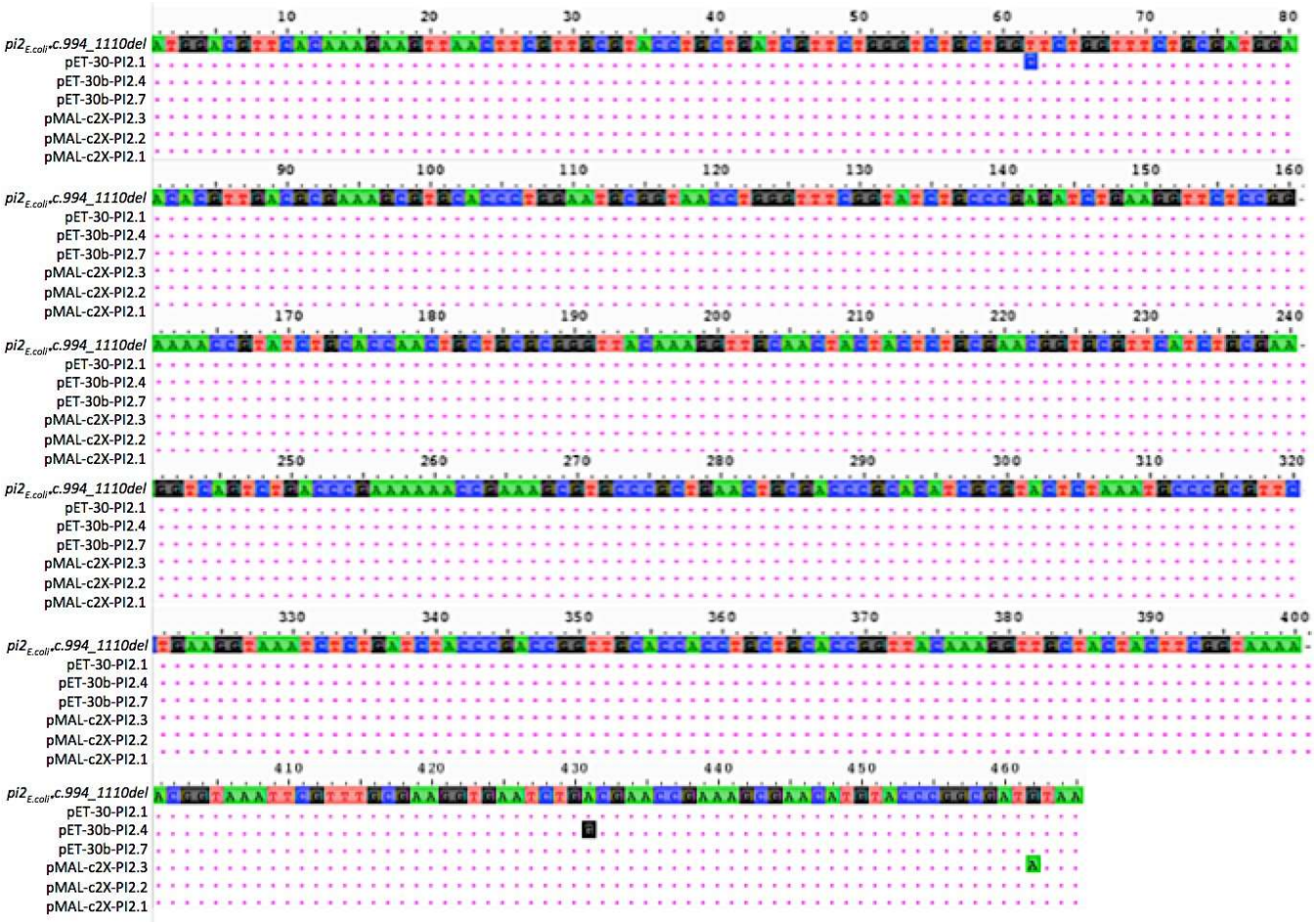

FIG. 2 Sequence analysis of pMAL-c2X-LLMZ16 and pET-30b-LLMZ16 isolates. Plasmids (n=6) were isolated from transformants after Gibson cloning, transformation into DH5α, and colony PCR screening. Bases matching the *pi2*$_{E.coli}$*c.994_1110del* sequence are represented by dots, and mismatches are represented by the base identity. Three base mutations were detected in three independent plasmids, resulting in single amino acid substitutions.

fractions, His-Pi2K was soluble across all tested temperatures, with negligible differences in the amount of protein observed in the gel (Fig. 3a). Bands were observed in the insoluble fractions, but these were fainter than those from SDS-PAGE analysis of MBP-Pi2K.

MBP-Pi2K was soluble under these expression conditions, as indicated by the prominent bands in the soluble fractions and the faint bands in the insoluble fractions. The solubility of this protein was maximal when expressed at 30˚C (Fig. 3b). Interestingly, the band corresponding to MBP-Pi2K appears to be more prominent in its fraction, compared to that corresponding to His-Pi2K (Fig. 3). Taken together, these results suggest that Pi2K was best expressed as a soluble protein when attached to an MBP affinity tag, using the pMAL-c2X-LLMZ16 construct. The MBP-Pi2K fusion protein was therefore selected for subsequent purification experiments.

Purification of MBP-Pi2K. Large-scale protein expression was carried out at 30˚C for optimal production of soluble proteins. The expressed protein was isolated by amylose resin affinity matrix in a gravity flow column. SDS-PAGE analysis showed a dominant band at 59.4 kDA corresponding to MBP-Pi2K, along with minor lower molecular weight bands, eluted in the column buffer supplemented with 10 mM maltose (Fig. 4). The five elution fractions were pooled and concentrated, yielding approximately 1 mg/ml of isolated protein. The other bands may indicate the presence of truncated MBP-Pi2K and early termination of translation in BL21. Note that without the inclusion of an uninduced sample control, bands cannot be conclusively identified as Pi2K fusion proteins. Protein contamination post-purification, or native *E. coli* BL21 proteins isolated during the purification process cannot be excluded.

DISCUSSION

Proteinase inhibitors are prevalent in all forms of life and perform protective roles, defending against foreign enzymes and microbes (29). Specifically, type II proteinase inhibitors in potato tubers inhibit digestive enzymes and exhibit anticancer activity (3-6). Keil *et al.* previously attempted to express and purify PI2 as

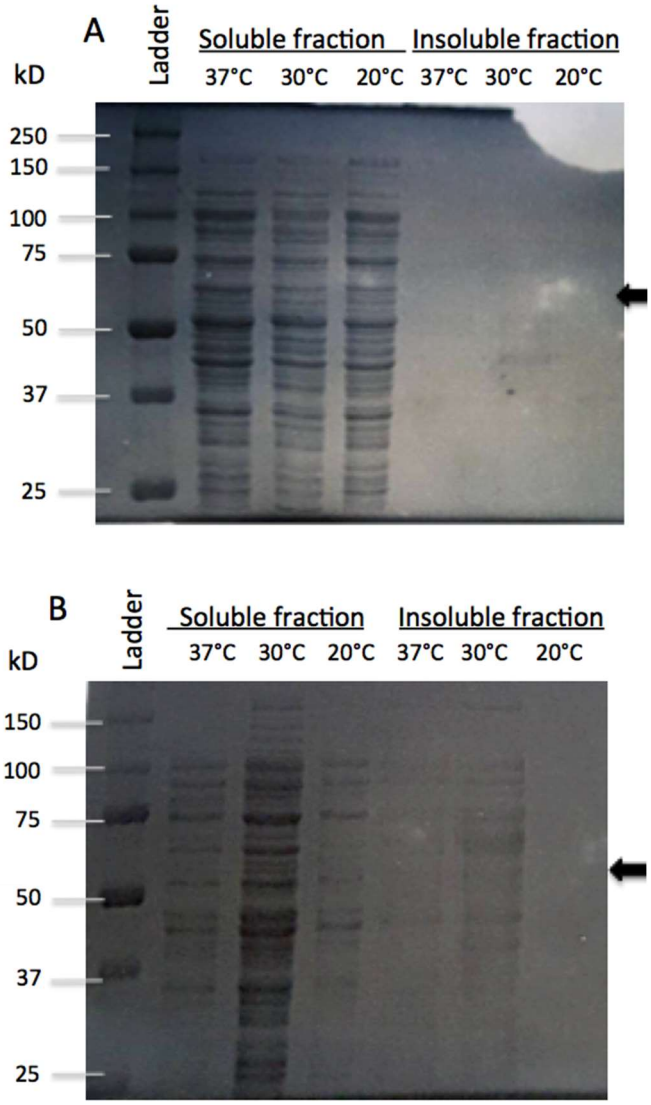

FIG. 3 SDS-PAGE gel analysis of expression of MBP-Pi2K and His-Pi2K fusion proteins. Fractions expressed at different temperatures were separated by SDS 10% polyacrylamide gel electrophoresis. All *Ladder* lanes indicate Bio-Rad Precision Plus protein standard. (a) Soluble and insoluble fractions of His-Pi2K expressed at various temperatures post-induction. (b) Soluble and insoluble fractions of MBP-Pi2K expressed at various temperatures post-induction. Arrows indicate the relative location of the band of interest (Pi2K, 59.4kDa) on the SDS-PAGE gel.

soluble protein (14). However, isolation of this Pi2K is fraught with complications due to the eight disulphide bonds present in the two domains of Pi2K. The non-optimized codons and the 117 bp intron also contribute to the difficulty in expressing this protein. Here, we sought to isolate soluble Pi2K using two different expression vectors. We found that Pi2K was expressed as a soluble fusion protein in *E. coli* BL21 (Fig. 3). Purification of the Pi2K fusion protein resulted in concentrated, isolated protein. These findings suggest that Pi2K can be expressed as soluble protein under the specified conditions, and provide a framework for future biochemical characterization.

Previous studies have reported difficulties in expressing and isolating Pi2K as soluble protein (19-21). To express and purify Pi2K as a soluble protein, we constructed a fusion protein with an MBP affinity tag on the N-terminus of Pi2K, as well as a His-tagged Pi2K. The constructs were obtained by Gibson assembly, inserting the codon-optimized $pi2_{E.coli}$*c.994_1110del* insert into the expression vectors of pMAL-c2X and pET-30b (21). MBP affinity tags have been shown to enhance solubility and facilitate isolation by amylose resin (22). Growth of BL21 containing either plasmid suggested that Pi2K was likely not toxic to the host *E. coli* cell. This approach allowed for the expression of soluble MBP-Pi2K and His-Pi2K. MBP-Pi2K appeared to be more highly expressed and soluble than His-Pi2K (Fig. 3), informing subsequent purification experiments. Variation in expression levels may be due to differences in the T7 and Ptac promoter expression systems used by plasmid vectors in this study.

Purification of MBP-Pi2K resulted in isolated protein, as well as minor contaminants or truncated proteins (Fig. 4). Using the Protein Parameters tool from SIB's ExPASy Bioinformatic Resource Portal, instability indices for the MBP-Pi2K, MBP and Pi2K proteins were determined to be, respectively, 27.33, 20.40 and 45.02 (28). These values suggest that the Pi2K protein is natively an unstable protein, while MBP is highly stable. The inherent instability in the Pi2K primary sequence may render it susceptible to protease activity, but the fusion to the affinity tag may confer some degree of stability. Hence, the addition of a stable tag such as MBP may have enhanced solubility and enabled extraction of intact, properly-folded Pi2K (22).

SDS-PAGE analysis indicated the presence of minor protein bands at a smaller size than Pi2K, suggesting contaminants or truncated proteins. Degradation of full-length MBP-Pi2K may have occurred due to membrane-bound and cytoplasmic proteases. BL21 is deficient in Lon and OmpT proteases, but other proteolytic enzymes remain active and may result in protein fragmentation (30). MBP-Pi2K degradation may have occurred during the harvesting and lysis stages, despite the addition of a protease inhibitor cocktail prior to lysis, albeit at non-optimized concentrations. Proteolysis may also be attributed to Pi2K instability, given that it is a cysteine-rich protein forming eight disulphide bonds. The presence of active reductases, such as TrxB and Gor in BL21, may keep the 16 cysteine residues in their reduced form, preventing proper

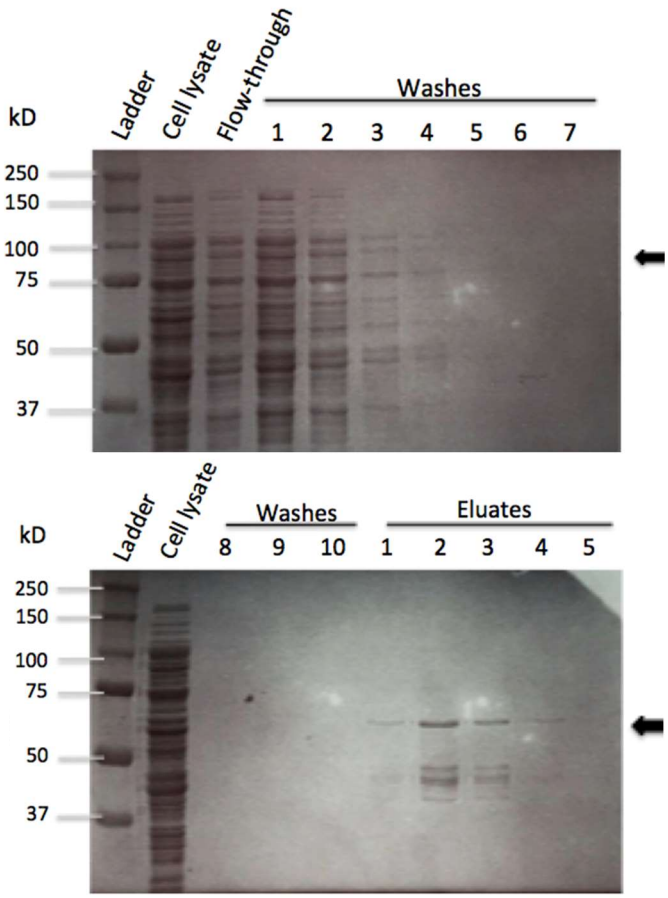

FIG. 4 SDS-PAGE gel analysis of purification of MBP-Pi2K fusion protein. Fractions recovered at different purification steps were separated by SDS 10% polyacrylamide gel electrophoresis. All *Ladder* lanes indicate Bio-Rad Precision Plus protein standard. Arrows indicate the relative location of the band of interest (Pi2K, 59.4kDa) on the SDS-PAGE gel.

protein folding and increasing the susceptibility of Pi2K to proteolysis. Additionally, the smaller bands seen on the gel (Fig. 4) may also correspond to incomplete translation of MBP-Pi2K, which would still have been capable of binding to the resin given that the MBP tag was on the N-terminus, not the C-terminus. Taken together, these results indicate that the purified protein likely contains MBP-Pi2K, as well as degraded proteins. Based on SDS-PAGE analysis (Fig. 4), the resulting isolated protein should be sufficiently pure for biochemical assays and characterization.

The results in this study illustrate the capacity of BL21 to produce soluble tagged Pi2K. This provides a solid foundation for future biochemical characterization of Pi2K and its activities. This holds important applications for the fields of crop control and medicine (4-8). The implications of proteinase inhibitors in health settings and for commercial applications are becoming more apparent in recent years. Current research on the mechanisms of action and sequence homology across different proteinase inhibitors can elucidate potential functions of these inhibitors (3, 9, 13). In particular, prevention of UV-induced AP-1 activation has been previously demonstrated by applying proteinase inhibitor I, InhI, and II, InhII to mouse epidermal JB6 cells (5, 6). The PI2 studied in this project, if shown to have enzymatic activity, may also confer protection against skin carcinoma. As PI2 holds high antimicrobial, insecticidal, and medicinal potential, a thorough understanding of its synthesis and specific activities may inform optimal production of this proteinase inhibitor.

FUTURE DIRECTIONS

Studies of Pi2K may benefit from optimization of protein purification, to increase the final concentration and protein yield. The specific inhibitory activities of Pi2K will need to be assessed using a trypsin inhibition assay, as well as other potential substrates such as AP-1. Protein function and structure may be influenced by the MBP tag through protein-protein interactions. Cleavage of the MBP affinity tag at the Factor Xa site may also modulate Pi2K stability and activity, though solubility of Pi2K may decrease and result in insoluble aggregates. Inclusion of a negative, uninduced sample control during protein expression experiments is also necessary to conclusively identify protein isolates as Pi2K fusion proteins.

Given the presence of active reductases within BL21, transformation and expression in a reductase-deficient *E. coli* strain such as Origami 2(DE3) is suggested as a future experiment. Origami 2(DE3) possesses a unique oxidative environment that facilitates formation of disulphide bridges. Mutations in Origami 2(DE3) strain *gor* and *trxB* reductase genes allow oxidation of the sulfhydryl group in the thiol side chains, promoting disulphide bond formation (31). This environment may also contribute to a decreased level of expression of endogenous *E. coli* proteins, such as proteases that inhibit expression of certain proteins. Down-regulation of such proteins, and other factors, may allow for high yields of recombinant proteins compared to other *E. coli* strains (32). Use of Origami 2(DE3) may thus allow expression of properly folded, stable, and active enzyme. Further experiments will be required to optimize the production of soluble, active Pi2K and to compare the activities of different preparations. This may elucidate key strains and conditions for optimal Pi2K activity and provide future insights into potential applications.

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