Overexpression of recombinant proteins in *Escherichia coli* can result in biologically inactive aggregates of misfolded proteins known as inclusion bodies. The fusion of thioredoxin A (TrxA) to target proteins has been used in commercial plasmids to increase the solubility of overexpressed protein which can reduce the formation of inclusion bodies. The objective of this study was to create recombinant plasmid vectors that would overexpress TrxA fused to proteinase inhibitor II (PI2), a protein domain containing 8 disulphide bonds known that is known to form inclusion bodies. We hypothesized that the co-expression of TrxA in either cis or trans with PI2 would increase levels of soluble PI2 when overexpressed in *E. coli*. We designed oligonucleotide primers to amplify the PI2 gene from plasmid pE32PI2 using polymerase chain reaction. We found that the addition of dimethyl sulfoxide at concentrations between 0.25% and 1.0 % was required for PCR amplification of the PI2 gene from pE32PI2. In this study the PI2 gene was successfully amplified by PCR using DMSO as an additive. The expected DNA band sizes were confirmed by gel electrophoresis.

*Escherichia coli* overexpression vectors have been instrumental in the biotechnology and pharmaceutical industry in the mass production of protein. One complication that can arise with high-level protein expression is the production of misfolded protein (2). Protein misfolding can result in the formation of protein aggregates known as inclusion bodies. Misfolded protein shows a reduction in biological activity (2). Purification and refolding of misfolded protein can be attempted but these steps are complex and time-consuming, reduce the overall purification yield, and are often unsuccessful (3). Finding conditions that result in expression of soluble, active protein is often most desirable.

Thioredoxin (trx) is a biological disulfide reductase, which has been used as a recombinant fusion partner to increase the solubility of overexpressed proteins in *E. coli* (4,5). Although the precise mechanism is unclear, thioredoxin is proposed to affect the redox state of the cell environment. PI2 originates from eukaryotic cells, which are known to have different redox states compared to prokaryotes (10). This difference in redox state causes the formation of abnormal disulfide linkages in overexpressed proteins within *E. coli* (10). Coproduction of thioredoxin enhances protein solubility by altering the redox state of *E. coli*, through the reversible oxidation of cysteine thiol groups (11).

Proteinase inhibitor 2 (PI2) is an endo-acting peptide bond hydrolase, found in a variety of tomato and potato plants (6,7). PI2 has been shown to form inclusion bodies when overexpressed in *E. coli*. PI2 is composed of 2 domains which contain a total of 8 disulfide bonds (8). Thus, it was chosen to investigate the influence trxA would have on a protein containing a high number of disulfide bonds relative to the size of its tertiary structure. Previous work has attempted to generate various PI2 constructs to investigate the effects of trxA on the solubility of PI2, but our analysis indicated that PI2 was absent in these putative clones (1, 2).

The aim of our study was to generate a pET32a(+)–PI2 plasmid construct and a pET32a(+)–PI2/Δtrx plasmid construct to evaluate the effects of Trx on the solubility of PI2. Primers were designed with linkers encoding restriction sites to enable directional cloning of PI2 from pE32PI2 into pET32a(+). Putative clones were isolated and subjected to colony PCR, which revealed fragments of similar size to PI2, however, subsequent analysis did not confirm PI2 nucleotide sequence.

**MATERIALS AND METHODS**

**Strains and Plasmids.** The pET32a(+), obtained from Novagen, is the template vector used in this study. JP061, JP062, JP063, JP064, CD111, CD112, CD113, and CD114 plasmid constructs (TABLE 1) were preserved in *E. coli* DH5α strains as part of the Ramey strain collection (Department of Microbiology and Immunology, University of British Columbia). Cells containing each of the plasmids were grown overnight at 37°C at 180 RPM in Luria-Bertani (LB) liquid medium (pH 7.0) containing tryptone (1.0%), yeast extract (0.5%), and NaCl (1.0%).

**Plasmid Isolation.** Plasmid DNA was isolated using the Invitrogen PureLink HQ Mini Plasmid DNA Purification Kit (Cat# K2100-01). DNA concentration and purity of the plasmid products were assessed by measuring the absorbance at 260 nm and 280 nm using a Beckman Coulter DU530 spectrophotometer.

**Restriction Digestion of Plasmids.** The identity of plJP061, plJP062, plJP063, plJP064, pCD111, pCD112, pCD113, and pCD114, and pE32PI2 plasmids were confirmed using: restriction digestion, PCR, and Sanger sequencing. To confirm the identity of the clones, each of the plasmid isolates from JP061, JP062, JP063, and JP064 were digested with 20 units of New England BioLabs EcoRI (Cat# R0101S) and 10 units of New England BioLabs NcoI (Cat# B0101S) with 5 µl of 10x NEBuffer EcoRI (Cat# B0101S). Plasmids CD111, CD112, CD113, and CD114 were digested with 15 units of New England BioLabs EcoRI (Cat# R0501S) and New England BioLabs Rsrl (Cat# B7004S) with 5 µl of 10x NEBuffer 4 (Cat# B7004S). The pE32PI2
plasmid isolates were digested using a series of single and double restriction enzyme reactions. For each of the single digests, 3 µl of pE32PI2 plasmid was mixed with 0.5 µl of the enzyme (NcoI, Hind III, EcoRI, XbaI), 1 µl of 10x NEBuffer 4 (Cat# B7004S), and 5.5 µl of sterile distilled water. For each of the double digests, 3 µl of pE32PI2 plasmid was mixed with 0.5 µl of each enzyme (NcoI+HindIII, NcoI+EcoRI, XbaI+EcoRI, HindIII+EcoRI), 1 µl of 10x NEBuffer 4 (Cat# B7004S), and 5 µl of sterile distilled water. Each of the reactions were performed in a final volume of 10 µl for 90 minutes at 37°C.

PCR Amplification of PI2. Attempts were made to amplify a 590 bp fragment encoding the PI2 gene from the putative clones pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, pCD114, and pE32PI2. Shown in TABLE 2 are the oligonucleotide primers that were designed to amplify the PI2 gene. The PI2 forward primer contains four extra nucleotides in order to engineer an NcoI restriction site, while the PI2 reverse primer introduces an EcoRI restriction site. All PCR reactions were carried out in volumes of 50 µl. Each reaction was set up to contain final concentrations of 1x PCR buffer, 1 mM MgSO\(_4\), 0.2 mM dNTPs, and 0.5 mM of both forward and reverse primers. Next, 100 ng of template plasmid was added to each reaction along with 0.5 µl of Taq Polymerase (Invitrogen, Cat# 10342053). PCR cycle conditions used are as follows: an initial denaturation step of 5 min at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 1 minute, and a final extension step at 72°C for 5 minutes. All PCR products were subsequently resolved on 1% agarose gel and visualized by exposure to UV light. In addition, varying amounts of dimethyl sulfoxide (DMSO) were added to reaction tubes during PCR optimization.

Confirmation of identities of putative clones. Our initial goal of this study was to confirm the identities of plasmids pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, and pCD114 (1, 2). We carried out restriction enzyme double digests of plasmids pJP061, pJP062, pJP063, and pJP064 using NcoI and EcoRI. Double digests were also performed on plasmids pCD111, pCD112, pCD113, and pCD114 using Rsll and EcoRI. Based on the previous study by Duronio (2), both pJP061 and pJP063 were expected to generate band sizes of 5900 bp and 590 bp, the latter of which corresponds to the size of the PI2 gene. pJP062 and pJP064 bear a deletion of the 300 base pair trx gene and were expected to generate bands of 5600 bp and 590 bp. pCD111, pCD112, pCD113,
pCD114, which contain the trx gene, were expected to generate similar band sizes to JP061 and JP063. While all of the clones generated the correct larger band sizes of either 5900 bp or 5600 bp, a band size of 590bp corresponding to PI2 was not found in any of the plasmids (Figure 1). To further investigate the identities of the clones, PCR was used to screen for the putative PI2 inserts using primers designed to flank the PI2 insert site. A band consistent with the expected PI2 size was not observed suggesting that the PI2 inserts may not have been present (data not shown). Each plasmid was successfully sequenced as suggested by the even distribution of peaks representing bases in the raw sequencing data (data not shown). Additionally, by using BLAST analysis, the sequenced region contained an unknown sequence that did not show any homology to PI2, which was expected to be present. Therefore, these data suggest that PI2 was not present in pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, and pCD114.

Restriction enzyme digest and characterization of pE32PI2 construct. Since little is known about the original pE32PI2 construct, a restriction enzyme digest was performed for characterization purposes. In Figure 2, the lane with uncut pE32PI2 shows two bands, suggesting that pE32PI2 may have two conformations, circular and supercoiled. Based on the single cut NcoI, the pE32PI2 plasmid appears to be 6600bp. The pE32PI2 plasmid was later sequenced using the PI2 reverse primer where it was determined that the plasmid contained the PI2 gene sequence (data not shown). PCR was conducted in which DMSO was added in varying concentrations as a PCR enhancing agent (Figure 3) (12). A DMSO concentration of 0.5% was found to be the only concentration to generate the expected band size of 590 base pairs. Bands corresponding to the sizes of the two conformations of the pE32PI2 plasmid are also apparent, which may be indicative of excessive template DNA in the PCR (Figure 3). An additional PCR was done in which DMSO concentrations of 0.25% to 1% successfully generated PI2 amplicons via PCR (Figure S1).

Colony PCR and DNA Sequencing of Transformants Containing the Putative PI2 Insert. After the attempted ligation of the putative PI2 sequence into the pET32a(+), JP061, JP064, and CD111 plasmids constructs were introduced into competent BL21 (DE3) E. coli cells using CaCl2 heat shock transformation. Vector DNA that is incompletely digested will give rise to high background so therefore ligations of pET32a(+) without insert DNA were performed as controls. Transformants were obtained for all conditions, except for the condition in which ligation into pJP061 was attempted. Colony PCR amplified a product of 590 base pairs derived from the modified pET32a(+) and pJP064 amplified an approximately 590 bp product resembling PI2 (Figure 4A). Colony PCR of the pCD111 transformants amplified a product of approximately 200 base pairs, which was not expected (Figure 4B). For both gels, a positive control from Invitrogen containing template and primers known to amplify, and a negative control using sterile dH2O as template, indicated that the PCR reaction was successful. This data suggested that our modified pET32a(+) and JP064 constructs may have contained the correct PI2 sequence.
DISCUSSION

To investigate the increase in solubility of overexpressed protein observed with fusion to thioredoxin, constructs were generated with expression of TrxA in cis or trans with PI2. Expression of constructs were to be induced and inclusion body formation assessed to further explore the role of TrxA in the reduction of aggregates. Confirmation of previously constructed plasmids was required before expression could be induced. A double restriction digest with NcoI and EcoRI was prepared to identify the putative plasmids. A band of 590 bp (PI2) was absent suggesting that PI2 was not present. Band sizes of 5900 bp (pET32a) in lanes 1&3 and 5600 bp (pET32a-Δtrx) in lanes 2&4 of Figure 1 demonstrated that trx was present and absent in the respective constructs. PCR using primers designed to flank PI2 revealed that PI2 was absent in all of the constructs. The putative constructs were then sequenced for PI2. We found that the incorrect nucleotide sequences in plasmids pJP061, pJP062, pJP063, pJP064, pCD111, pCD112, pCD113, and pCD114 (1, 2).

Using the nucleotide sequence of PI2 a primer pair flanking the gene was designed for targeted amplification. It was found that 0.25-1% DMSO was necessary for the amplification of PI2 PCR products. DMSO facilitates strand separation by base pair disruption, and could reduce possible secondary structure in the PI2 nucleotide sequence. The products of PCR were resolved on a gel to determine if products of the expected size of PI2 were present. Fragments of desired size were observed, however there were also non-specific products on the gel. Due to the low DNA concentration of previous gel extractions, PCR products were directly digested with restriction enzymes and ligation into the target vector was attempted. Ligation products were transformed into E.coli and screened via colony PCR for insertion of PI2. Colonies were observed bearing PCR products of the correct size, however, sequencing results showed that incorrect sequences were present. A significant number of inserts were of 200-300 bp in size, smaller in comparison to the PI2 sequence. Ligation or amplification of the smaller non-specific PCR products may have been favored in the reaction. In addition, these plasmids containing the smaller PCR product insert may also have higher transformation efficiency again due to its smaller size. Another explanation that may explain the lack of transformants with the desired PI2 insert is due to PI2’s toxicity when expressed in the E. coli strain BL21. Basal level of the T7 RNA polymerase transcribes the target protein enough to hinder growth or cause cell death. Transformants with the PI2 recombinant vector would not develop into colonies and therefore be absent in colony screening. This would explain why previous studies came to the same conclusion, as only colonies without PI2 recombinant vectors would be generated. This could also explain why a large concentration of 590bp PCR products is not sufficient to produce transformants with the correct insert.

FUTURE DIRECTIONS

The amplification and isolation of PI2 to generate a pET32a(+)–PI2 construct proved to be challenging. To eliminate the problem of amplifying non-PI2 products in the PCR of pE32PI2 it would be helpful to sequence pE32PI2 to analyze potential non-specific primer binding. Primers should be redesigned with higher specificity to reduce non-specific products and allow for accurate sequencing. PCR amplification of PI2 from pE32PI2 and sequencing of the subsequent PCR products with newly designed primers should be performed to confirm PI2 isolation.

To solve the possible problem of PI2 transformants not generating colonies, basal T7 RNA polymerase should be inhibited. This could be solved by co-transformation of competent cells with pLys S/R. pLys S/R is a small plasmid that encodes a T7 lysozyme. T7 lysozyme binds onto the C-terminal domain of T7 RNA polymerase forming a complex and conformational change that causes T7 RNA polymerase to fall off the T7 promoter. This
inhibits basal level transcription of the toxic PI2 gene of interest. However, this is only effective on low or basal levels of T7 RNA polymerase. Therefore when IPTG is added and large amounts of T7 RNA polymerase is generated, T7 lysozyme is no longer active and transcription continues unhindered. Another strategy would be to transform the plasmid into an E. coli strain that lacks T7 polymerase (e.g. DH5α) as an intermediate cloning step. This may allow propagation and sequencing of the plasmid before testing expression in another E. coli host, such as strain BL21.

The difficulty in sequencing PI2 may be from secondary structures that form when strands are separated. Future experiments could combat this problem with the addition of DMSO, similar to the PCR protocol outlined.

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