

Subcloning *emoA* into pET32a(+), pET32a(+) Δ *trxA*/+*fre* and pET32a(+) Δ *trxA* to Assess the Relative Effect of Thioredoxin A and Flavin Oxidoreductase on *emoA* Solubility

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The Novagen pET32a(+) system is widely used for high-level expression of recombinant proteins. Key to its success is the thioredoxin (TrxA) protein that allows the peptide product to stay in a soluble state. Flavin oxidoreductase (*fre*) is another oxidoreductase which could be used to increase protein solubility of highly expressed peptides. To investigate the solubility-enhancing effects of *fre* in comparison to *trxA*, EDTA monoxygenase A (*emoA*), a relatively insoluble protein, was incorporated into the original pET32a(+) vector as well as a genetically modified pET32a(+) vector with *fre* as the replacement for *trxA*. The new constructs were attempted by isolation of *emoA* by gel purification, T4 DNA ligation to insert the PCR product into the vector and calcium chloride transformation of the new constructs into DH5 α *E. coli*. None of the isolated transformants were shown to be the desired vectors in the verification stage after transformation. As such, this report troubleshoots the problems faced and guides future experiments to get the appropriate clones.

The use of peptides pervades the pharmaceutical, agricultural and household industries. As the demand for peptides rises to meet consumer demands, there will be need for more efficient and productive systems to answer this call. Plasmid expression systems are one of many means of peptide synthesis. They are widely used in the synthesis of heterologous proteins and usually genetically engineered to utilize the enzymes and nucleotides of its bacterial host to produce high levels of a desired protein product. In a bacterial culture, this design allows many copies of a protein to be produced in parallel.

As efficient as it may be, there are a few drawbacks to high-level expression - one of which is the formation of inclusion bodies (4). One of many expression vectors in Novagen's pET system, the pET32a(+) high expression vector is fused with a thioredoxin (*trxA*) tag to increase the solubility of its expressed protein. Co-expression of thioredoxin by the expression vector has shown to increase the solubility of heterologous proteins (8). By acting as a reducing agent, thioredoxin alters the oxidation state of the product, disrupting disulfide bonds to increase the solubility of the product (8). Another protein that functions as a similar oxidoreductase is flavin oxidoreductase (*fre*). This project was originally developed by previous researchers to test the idea that *fre* may substitute for *trxA* in conferring an increase in solubility of expressed proteins (3).

In previous studies, two important constructs were

made which carried over to use in this study. First is the pET32a(+) Δ *trxA* plasmid, which had *trxA* removed by restriction with NdeI (7). The second construct is pET32a(+) Δ *trxA*/*fre*, which incorporates the *fre* gene from pES1. This was done by PCR amplification of *fre*, excision from pES1 by NdeI and ligation into pET32a(+) Δ *trxA*. Initial attempts to insert *emoA* into pET32a(+) Δ *trxA* were unsuccessful due to the lack of transformants (7), and further effort to subclone *emoA* was sidelined by disruptions due to hairpin formation when amplifying *emoA* from pEmoA (2).

The goal of this study was to proceed in subcloning *emoA* from plasmid pEmoA into three pET32a(+) plasmids - Novagen pET32a(+) containing the *trxA* gene, the second with *fre* in place of *trxA* (pET32a(+) Δ *trxA*/*fre*) and a control plasmid with *trxA* deleted (pET32a(+) Δ *trxA*). In doing so, the solubility conferred by *trxA* and *fre* to the expressed *emoA* protein can be compared. Unfortunately, owing to difficulties in subcloning and ligating *emoA* into the desired plasmids, these constructs were not created.

MATERIALS AND METHODS

Verification of plasmids. To select for cells containing plasmids, stocks of pEmoA, pET32a(+) Δ *trxA*, pET32a(+) Δ *trxA*/*fre* and pET32a(+) were independently plated onto Luria-Bertani (LB) plates (10g NaCl, 10 g tryptone, 5 g yeast extract, 20 g agar, pH to 7.0, add distilled water to 1L) containing 100 μ g/mL ampicillin. pEmoA was isolated from DH5 α *E. coli* cells using the Fermentas GeneJET miniprep kit (catalog # K0503) and 1 μ g of this product was digested with 5 units of Fermentas EcoRI in 1X EcoRI buffer (catalog #

R0101S) for 1 hour at 37°C to cleave the *emoA* insert from the plasmid. The fragments from this digestion are separated on a 1% agarose gel (Bio Rad Molecular Biology Agarose, catalog # 161-3101) by electrophoresis and post-stained in a 1% ethidium bromide bath to allow identification in UV by the Alpha Imager Gel Doc. The same procedure was carried out for the verification of these plasmids: pET32a(+)*ΔtrxA*, pET32a(+)*ΔtrxA/fre* and pET32a(+) with the following exceptions. pET32a(+) was digested with 5 units of New England Biolabs (NEB) NdeI (catalog # R0111S) in 1X accompanying NEBuffer 4. pET32a(+)*ΔtrxA/fre* was digested with 5 units of NEB NdeI and PflMI (catalog# R0509S) simultaneously in 1X NEBuffer 3 and 100 μg/mL Bovine Serum Albumin (BSA) (catalog # B9001S). All other reaction conditions were maintained as in the digestion of pEmoA. Unless otherwise stated, only 1% agarose gels were used in this study.

Gel extraction and subcloning of *emoA* into pET32a(+)*ΔtrxA*, pET32a(+)*ΔtrxA/fre* and pET32a(+). Post-verification, the *emoA* fragment in the gel was isolated using the QIAquick Gel Extraction Kit (catalog # 28706) and subcloned into plasmids pET32a(+)*ΔtrxA*, pET32a(+)*ΔtrxA/fre* and pET32a(+) by way of complementary base pairing. 2μg of individual plasmid vectors were linearized by digestion with 5 units of Fermentas EcoRI (catalog # ER0275) and dephosphorylated in 3 units of Fermentas Antarctic Phosphatase in 1X Antarctic Phosphatase Reaction Buffer (catalog #M0289S) for 75 minutes. The dephosphorylated vectors were individually ligated with the isolated *emoA* fragment using 10 units of Invitrogen T4 DNA ligase (catalog # 15224) and 5X ligation buffer at an insert to vector ratio of 2:1. The ligation reaction was done overnight (20 hours) at room temperature.

Making chemically competent cells for Calcium Chloride transformation. DH5α *E. coli* was streaked out and grown on an LB plate overnight at 37°C. An isolated colony was picked and grown in a 5 mL LB culture in a shaker at 37°C (at 160 r.p.m.) overnight. The next day, the culture is diluted in LB broth as much as 1:80 up to 500 mL. The culture was grown at 37°C with shaking until the culture achieves an OD₆₀₀ of 0.4. The cells were then spun down in a rotary centrifuge in 4°C cold, sterile 250 mL bottles at 700 r.p.m., 4°C for 10 minutes. After the first round of centrifugation, the cells were resuspended in 500mL ice-cold 50mM calcium chloride (CaCl₂) and incubated on ice for 20 minutes. In the second centrifugation step, the cells were again spun at 7000 r.p.m., 4°C for 10 minutes. The final resuspension step was done with a sterile solution consisting of 0.5 mL 2M CaCl₂, 1.5 mL 100% glycerol and 10 mL sterile distilled H₂O. 500 μL aliquots of competent cells were measured into pre-chilled microcentrifuge tubes and stored at -80°C.

Transformation of recombinant pEmoA plasmids into DH5α *E. coli*. To check for the success of the ligation, 100 ng of plasmid pET32a(+)*ΔtrxA/+emoA*, pET32a(+)*trxA/fre/+emoA* and pET32a(+)*+emoA* were individually transformed into 100 μL of DH5α *E. coli* competent cells. The competent cells were first placed on ice to thaw, then plasmid was added to the cells and allowed to incubate on ice for 30 minutes. The cells were then heat shocked in a 42°C water bath for 45 seconds and immediately placed on ice for 2 minutes. 100 μL of room temperature LB broth was added to the cells, and then placed in a shaking incubator at 37°C, 200 r.p.m. for one hour. Subsequently, the outgrown sample was diluted 1:200 and then plated onto LB plates, as well as LB plates containing 100 μg/mL ampicillin.

RESULTS

Restriction enzyme analysis of plasmid. DH5α *E. coli* containing plasmids SW051, SW054 and SW055 (pET32a(+)*ΔtrxA/fre*) were cultured on LB plates with ampicillin and 1.5 μg, 0.337 μg and 0.2 μg plasmid was harvested respectively by miniprep. Multiple restriction enzyme digests of SW 051, 054 and 055 did not give

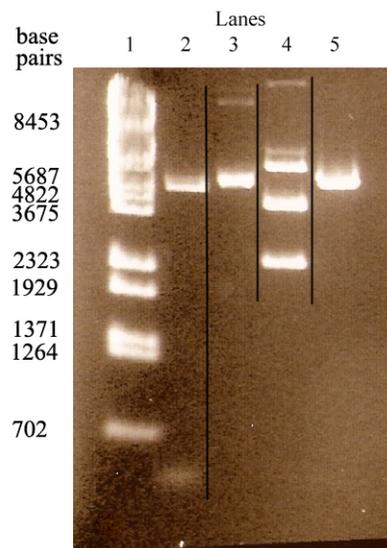


FIG 1. Verification of plasmids by restriction digest. Lane 1: lambda DNA digested with Bst-PI. Lane 2: pET32a(+) was digested with NdeI to produce two fragments, one at 300 bp corresponding to the *TrxA* fragment and the remainder of the pET vector at 5600 bp. Lane 3: pET32a(+)*ΔtrxA/fre* (SW051) was digested with NdeI and PflMI, but two bands were seen at 9000 bp and 5700 bp respectively instead of the expected four. Lane 4: pEmoA digested with EcoRI to give the *emoA* fragment at 2300 bp, the remainder of the plasmid at 5700 bp, as well as two other nicked and open-circle forms of the plasmid at approximately 6200 bp and 9000 bp. Lane 5: pET32a(+)*ΔtrxA* (SW057) digested with EcoRI to linearize the 5500 bp plasmid.

the desired amount of bands on the gel. While SW054 and SW055 digests produced inconsistent results with varying number of bands, SW051 Gave the best indication of the desired genotype because the size of the entire plasmid was the most accurate, although it did not give the *fre* insert at 320 bp (Fig 1). Furthermore, a larger amount of plasmid DNA was isolated from the SW051 than from SW054 and SW055.

DH5α *E. coli* SW057 (pET32a(+)*ΔtrxA*) was cultured and 1.6 μg plasmid DNA was harvested. Subsequently, the plasmid DNA was digested with EcoRI to produce a linear fragment. Agarose gel electrophoresis of this linear plasmid showed a band at 5500 bp, which corresponds to the correct fragment size. DH5α *E. coli* containing plasmid pET32a(+) was cultured and 1.2 μg of plasmid was recovered from miniprep. The identity of the plasmid was verified by digestion with NdeI to recover 2 fragments corresponding to the 5600 bp fragment without the *trxA* insert and the 320 bp *trxA* insert (Fig 1).

Using the same principle, the pEmoA plasmid was isolated from DH5α *E. coli* using plasmid miniprep, 2 μg of plasmid DNA was isolated from the miniprep and this was digested with EcoRI and verified with agarose

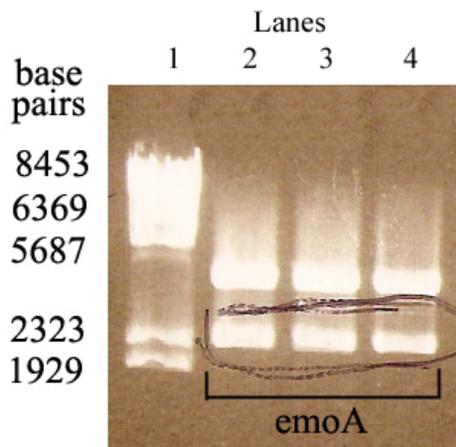


FIG 2. Restriction digest of pEmoA with EcoRI to isolate *emoA*. Lane 1: lambda DNA cut with Bst-PI. Lanes 2-4: pEmoA digested by EcoRI, to isolate *emoA* from pEmoA, a large amount of plasmid DNA was harvested by miniprep. The 2300 bp band, consistent with the *emoA* insert, was purified from the 1% agarose gel.

gel electrophoresis to identify the 2300 bp *emoA* insert (Figure 2.). The *emoA* insert was purified from the agarose gel and used as vector for subcloning into SW051, SW057 and wild type pET32a(+)

Subcloning *emoA* into SW051, SW057 and pET32a(+) and transformation of DH5 α *E. coli*. Plasmid DNA from SW057 was dephosphorylated with antarctic phosphatase and ligated with *emoA* to produce pET32a(+) Δ trxA/*emoA*. The successful transformation of DH5 α *E. coli* with this putative plasmid was verified by plating DH5 α cells on three LB plates, as well as three LB plates containing 100 μ g/ml of ampicillin. After 24 hours, all LB plates had a lawn of growth. *E. coli* grown on LB containing ampicillin had 83, 85 and 46 colonies. To verify the identity of the plasmid, miniprep and restriction digest (with EcoRI) performed on a single cultured colony from each ampicillin plate indicated the presence of 2 bands from plates 1 and 3, but the fragment sizes did not correspond to the correct sizes of pET32a(+) Δ trxA and *emoA* respectively (Fig 4, 5).

Construction of pET32a(+) Δ trxA/*emoA*, pET32a(+) Δ trxA/*fre/emoA* and pET32a(+)/*emoA*. The construction of pET32a(+) Δ trxA/*fre/emoA* and pET32a(+)/*emoA* with plasmid DNA from SW051, pET32a(+) and the isolated *emoA* fragment resulted in a similar phenomenon. Transformation of plasmid to DH5 α cells was successful, as observed by the lawn of growth on LB plates. On LB plates containing ampicillin, 62, 54 and 38 colonies were observed for *E. coli* with putative pET32a(+) Δ trxA/*emoA* while only 5, 4 and 8 colonies were observed for *E. coli* with putative pET32a(+) Δ trxA/*fre/emoA*. In both cases, restriction

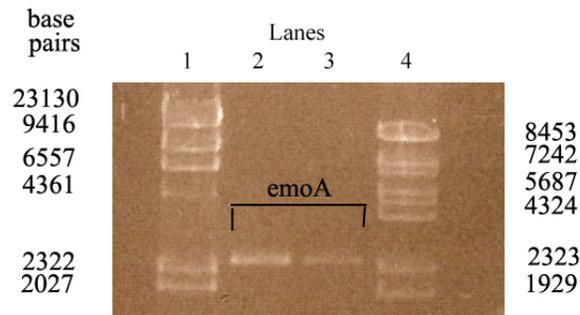


FIG 3. Post-gel purification, gel electrophoresis done using 3 μ L of product to verify the successful purification of *emoA*. Lane 1: Lambda DNA digested with Bst-PI. Lanes 2 and 3: the purified 2300 bp *emoA* insert. Lane 4: Lambda DNA digested with Hind III

digest with EcoRI produced fragments that did not correspond to the correct sizes of pET32a(+) Δ trxA, pET32a(+) Δ trxA/*fre* or the *emoA* insert (Fig 4, 5).

DISCUSSION

The expression vector pET32a(+) contains an EcoRI cut site in its multiple cloning site, which was used for the subcloning of *emoA* into plasmids from SW051, SW057 and pET32a(+). To test the activity of pflMI, multiple restriction reactions were done with SW051, SW054 and SW 055, but none of the reactions showed the presence of low molecular weight bands at 150 and 300 bp. Enzyme pflMI may have lost its restriction activity and as a result did not cleave the *fre* insert into two fragments. Despite this observation, an attempt to subclone *emoA* was made with SW051.

While the transformation of DH5 α *E. coli* with SW051, SW057 and pET32a(+) containing the *emoA* insert was successful, restriction digest of these plasmids with EcoRI did not show a 2300 bp band for the *emoA* fragment on the gel. As observed in the gel photographs, the outcomes of these digests with EcoRI are consistent and all show a band at the 5500-5800 bp range, corresponding to the plasmid itself. In addition, different supercoiled forms of the plasmid could have resulted in the consistent high molecular weight bands observed in some wells.

A plausible explanation for this observation is that the subcloning of *emoA* into plasmids from SW051, SW057 and pET32a(+) was not successful. After DH5 α *E. coli* was transformed with these plasmids, plasmid DNA was digested with EcoRI. Incomplete restriction resulted in the plasmids having various physical states. Bands with the highest molecular weights could be a result of closed circular plasmids as they have the highest resistance moving through the agarose gel, while nicked or open circle plasmids, having less

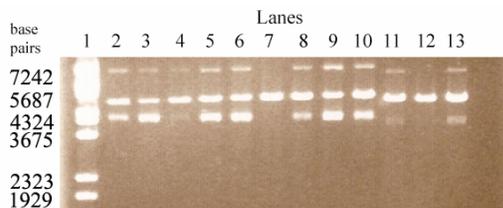


FIG 4. To confirm the identity of the new constructs, the putative vectors pET32a(+)/emoA and pET32a(+)/Δ*trxA*+emoA (SW057 +emoA) were digested with EcoRI to release the 2300 bp *emoA* fragment. As is shown, no bands were observed at 2300 bp. Lane1: lambda DNA digested with Bst-PI. Lanes 2 -10: pET32a(+)/emoA. Band sizes range from 4200 bp to 7300 bp. Lanes 11-13: pET32a(+)/Δ*trxA*+fre/emoA. Band sizes range from 4000 bp to 7000 bp.

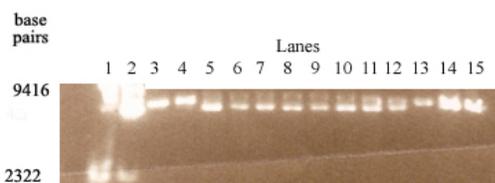


FIG 5. Lane 1: Lambda DNA digested with Hind III. Lane 2. Lambda DNA digested with Bst-PI. Lanes 3-8: putative pET32a(+)/Δ*trxA*/emoA (SW057+ *emoA*) digested with EcoRI. Lanes 9-15: putative pET32a(+)/Δ*trxA*+fre/emoA (SW051+emoA) digested with EcoRI. The bands in lanes 3-15 range from 6000-8000 bp. Again, no bands at 2300 bp were observed. The bands look much closer together than they did in the gel in figure 4 due to a larger bp range covered per unit distance.

resistance, move at a faster speed than the closed circular plasmids to give the bands seen in the 7000-8000 bp range. Finally, bands in the 5500 to 5800 bp range correspond to linearized plasmid. In Figure 4, a shift of similar magnitude can be seen in all bands in these three lanes compared to the bands in lanes 2-10. This is another piece of evidence that the observations of different fragment sizes might be just due to different forms of supercoiled, linear and relaxed plasmids, because if the bands had different numbers of bases, they would have had different shift patterns.

Unfortunately, due to time constraints, controls for the verification of new clones could not be completed. As well, subcloning and ligation of *emoA* into the target plasmids could not be repeated to test for different ligation conditions that could produce successful clones.

FUTURE EXPERIMENTS

To clarify the results of this transformation and further interpret the information on the photograph of these putative constructs, several controls should be

carried out. First, restriction with EcoRI should be performed with plasmids SW051, 057 and pET32a(+) alongside plasmids obtained after the transformation. This can be done on a large agarose gel containing these four groups: group I - uncut SW051, SW057 and pET32a(+). Group II - SW051, SW057, pET32a(+) cut with EcoRI. Group III - the uncut new constructs and Group IV – the new constructs cut with EcoRI. Comparing the difference in band sizes from such an experiment would show the physical differences in plasmids between the original and putative constructs, as well as the relative effect of EcoRI restriction on these plasmids.

Alternatively, a higher insert to vector ratio could be used, in addition to changing other variables such as temperature and duration of the ligation reaction. In this study, the ligation was allowed to go overnight (20 hours) at room temperature. If a lot more plasmid and *emoA* DNA is harvested, more experimental conditions could be tested. Gel electrophoresis can be done to compare the unligated constructs with constructs ligated under different insert to vector ratios, temperatures and duration.

If successful, the new constructs should be sequenced to determine their orientation and identity before the functions bestowed by the *trxA* and *Fre* protein in these constructs can be reasonably compared in further solubility assays.

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