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# Cloning, Purification, and Biochemical Characterization of Human Prolyl Endopeptidase

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# Cloning, Purification, and Biochemical Characterization of Human Prolyl Endopeptidase

By

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**Cloning, Purification, and Biochemical Characterization of Human Prolyl Endopeptidase**

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# ABSTRACT

*Eurygaster integriceps* Puton, more commonly known as Sunn pest, is regarded as one the most harmful pests of wheat, specifically durum wheat and bread wheat (Javahery, 1995). They primarily infest the wheat fields of Central and Western Asia, as well as areas of Northern Africa and Eastern Europe. The pest parasitizes the wheat grains by injecting a prolyl-endylprotease (spPEP), a proteolytic enzyme, which degrades the gluten proteins, enabling it to eat (Darkoh et al., 2010). Even minimal damage of wheat grains by the Sunn pest (2-3%) can reduce the grain crop to being unusable in baking (Hariri et al., 2000). The impact of the pest in these regions has been extremely detrimental to their respective local economies, and more importantly, their overall livelihood. Within these locations, wheat is the main source of human food, with over 100 million tons of wheat based products harvested annually (Javahery, 1995).

To tackle this dilemma, potential inhibitory peptides to the spPEP are being considered. Previously, it has been shown that peptides isolated from *Lactobacillus* hydrolysates of caseins in bovine milk can inhibit mammalian PEP in colon cells (Juillerat-Jeanneret et al., 2011). While these peptides are potential inhibitors of the spPEP, recombinant versions must be created and tested to ensure that they are specific to spPEP.

To conclude whether these proposed inhibitory proteins can be used as a bio-pesticide or even function to negate the detrimental effects of spPEP and recover compromised wheat grains for human consumption, the inhibitors must have a specificity for spPEP while not having an impact on the mammalian PEP homologue. The focus of this proposed research project was to clone the human prolyl-endylpeptidase (hPEP) into an expression vector and then transform hPEP construct into the same expression system as that used for the spPEP. Following a confirmation of the desired enzyme activity, it was then expressed in a large culture volume and partially purified. As a result of this project, future studies to compare the effects of potential inhibitors on hPEP and spPEP will be possible.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **[S]** | Substrate concentration |
| **A260** | Absorbance at 260 nm |
| **A280** | Absorbance at 280 nm |
| **A410** | Absorbance at 410 nm |
| **APS** | Ammonium persulfate |
| **BLAST** | Basic local alignment search tool |
| **bp** | Base pair |
| **BSA** | Bovine serum albumin |
| **CFU** | Colony forming units |
| **dCTP** | Deoxycytidine triphosphate |
| **dGTP** | Deoxyguanosine triphosphate |
| **dNTP** | Deoxynucleotide triphosphate |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **Gly-Pro-pNA** | glycyl-proline-*para*-nitroanilide |
| **GPpNA** | Gly-Pro-pNA |
| **hPEP** | Human prolyl-endylpeptidase |
| **IMAC** | Immobilized metal affinity chromatography |
| **IPTG** | Isopropyl thiogalactosidase |
| **kD** | kilo-Dalton (1000 g/mol) |

| | |
|---|---|
| **LB** | Lysogeny Broth or Luria-Bertani medium |
| **Ni-NTA** | Nickel-nitrilotriacetic acid, Nickel charged affinity resin |
| **OD600** | Optical Density at 600 nm |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **pLIC or LIC vector** | pNYCOMPS-LIC-FH10T+ |
| **SDS PAGE** | Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis |
| **spPEP** | Sunn Pest prolyl-endylprotease |
| **TAE** | Trizma base, glacial acetic acid, EDTA |
| **TEMED** | Thermo Scientific Pierce Tetramethylethylenediamine |
| **TF** | Trigger factor |
| **$V_o$** | Velocity |
| **x-int** | x intercept |
| **y-int** | y intercept |

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# INTRODUCTION

## *Eurygaster integriceps* Puton

*Eurygaster integriceps* Puton, more commonly known as Sunn pest, is a heteroperous insect, belonging to the order Hemiptera within the family Scutelleridae that resides in Northern Africa, Europe, as well as Western and Central Asia. It primarily consumes the wheat grains of *Triticum aestivum* and *Triticum turgidum* (Javahery, 1995). This is problematic, as these grains are vital for bread production in the region. With as little as 2% of grain contamination by Sunn pest, entire yields of crops can be rendered useless (Hariri et al., 2000).

When feeding, the Sunn pest injects the grain with an enzyme known as Prolyl Endoprotease (spPEP) which degrades the gluten proteins, rendering the grain no longer functional for bread production. While the damage is only localized to the grain that the Sunn pest is eating, the damage is significantly propagated when the grain is milled (Darkoh et al., 2010 and Vaccino et al., 2006).

## PEP (Prolyl Endylpeptidase)

Prolyl endylpeptidase, otherwise referred to as prolyl oligopeptidase (POP), is an enzyme that has been found to be universal to all organisms

(Szeltner and Polgar, 2008, and Rawlings and Barrett, 1994). PEPs belong to the S9 family of enzymes; they are ubiquitous serine proteases, by activity which is mediated by an α/β hydrolase domain (Rawlings and Barrett, 1994). This domain contains a conserved catalytic triad, Ser-Asp-His, that is believed to be involved in the entry and cleaving of the substrate (Polgar, 2000). PEP isoforms have been identified in bacteria, fungi, insects, and animals. Depending on the organism, PEPs can vary in function.

For reference, the Km and Kcat in the literature for porcine PEP are 5.9 ± 0.5 μM and 32.5 ± 1.2 s-1, respectively (Szetlner et al., 2002).

## Human PEP

When attempting to identify a potential inhibitor to spPEP, human PEP (hPEP) must be considered; belonging to the prolyl oligopeptidase family, hPEP shares similar conserved regions. Using the UCSF Chimera software (Petterson et al., 2004) to compare the amino acid sequence of spPEP to the known structure of human PEP (Yandamuri et al., 2014) generated a 3-dimensional structure (Fig. 1) that demonstrates the high level of conservation between mammalian and spPEP. Dysfunctional PEP has been linked to a variety of neurological diseases in mammals, including Alzheimer's disease (AD), in which decreased PEP activity may lead to neuronal degeneration (Laitinen et al., 2001). More current research has controversially suggested that PEP inhibition is linked to Amyloid-β

accumulation, another potential factor in AD (Rossner et al., 2005). In previous research, hPEP has been shown to be selectively inhibited by peptides containing less than 30 amino acid residues; those greater than 30 did not bind (Polgar, 2000). Because of this, the hypothesis for this study is that recombinant peptides designed to be larger than 30 amino acids should bind and inhibit spPEP without affecting hPEP.

## Peptides from casein as potential inhibitors of PEP

In recent studies, peptides from Lactobacillus hydrolysates of caseins in bovine milk have been shown to inhibit hPEP in human colon cells (Juillerat-Jeanneret et al., 2010). Since hPEP and spPEP share homology, this suggests that recombinant peptides will also inhibit spPEP. It is suspected that the inhibition of PEP is size specific. Previous research has demonstrated that a partial digestion of whole casein, resulting in a pool of peptides of varying sizes, yielded an 87% inhibition of purified spPEP (Hargrove, 2013).

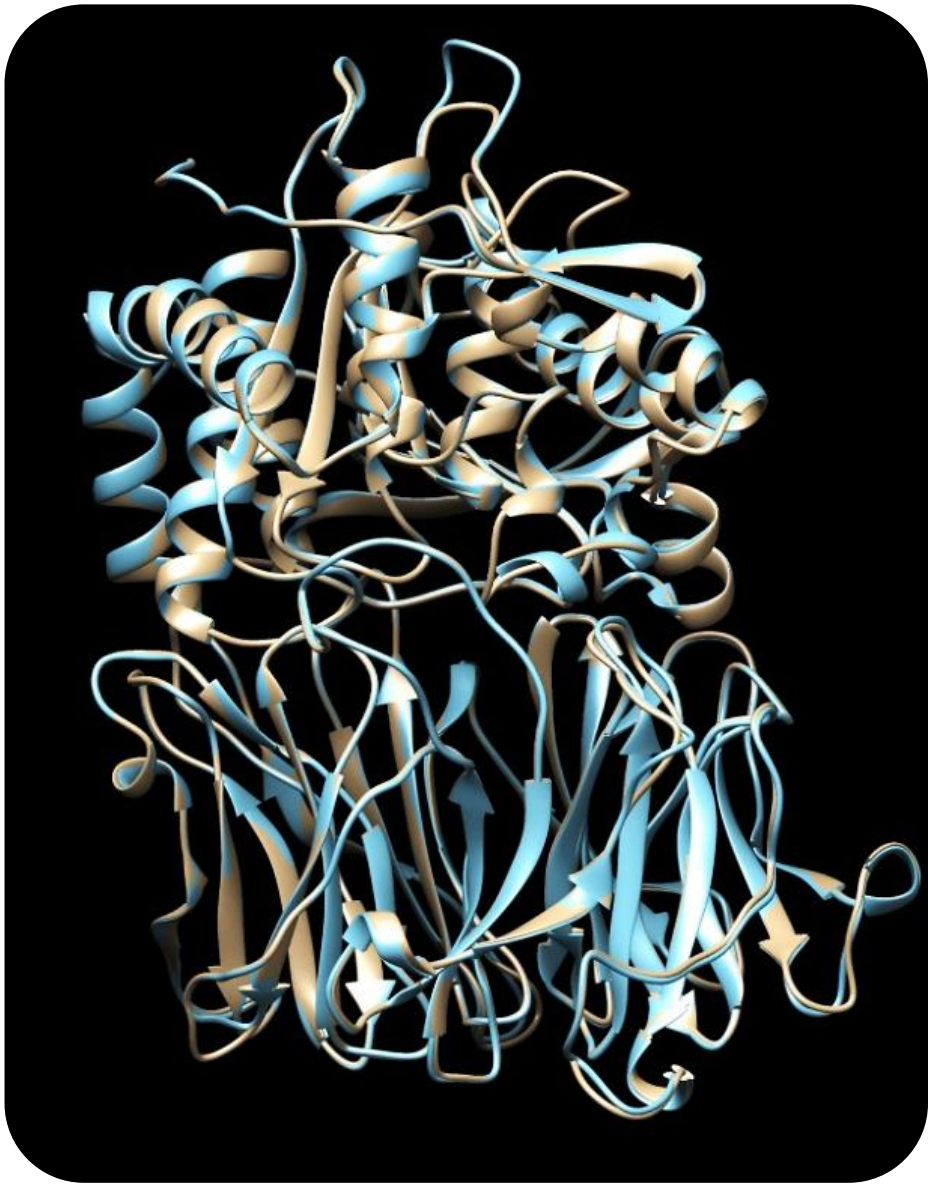

Figure 1: The predicted structural alignment of spPEP (blue) to the known crystalized structure of porcine PEP (tan) (Yandamuri et al., 2014) using UCSF Chimera (Petterson et al., 2004).

# MATERIALS AND METHODS

Inserting hPEP into pLIC Vector and Transformation of JM109 *E. coli* strain**.**

**Transformation of JM109 with LIC Vector**

Commercially competent *E. coli* JM109 cells (≥ 1 x $10^8$ cfu/µg DNA, Promega, Corp.) (genotype: F′ (traD36, proAB+ lacI q, lacZ∆M15) endA1 recA1 hsdR17(rk – ,mk + ) mcrA supE44 λ- gyrA96 relA1 ∆(lac-proAB) thi-1 lon) were obtained from the -80°C freezer and placed immediately in ice. Once thawed, the tubes were flicked gently to mix the cells. 10 ng of vector pNYCOMPS-LIC-FH10T+ (pLIC) (Arizona State DNA Repository) DNA was transferred to chilled five 17 x 100mm round-bottom polypropylene culture tubes. To each tube, 50 µL of the cells was added. These were then gently flicked and placed on ice for 10 minutes. The cells were then heat-shocked at 42°C for 45-50 seconds. The tubes were returned immediately to ice for 2 minutes. 400 µL of SOC medium (Fisher Scientific) was added to each tube, which was then incubated at 37°C for one hour with shaking at 200 rpm. Aliquots from each tube of 100 µL and 50 µL were spread in duplicate on kanamycin/chloramphenicol plates (50 µg/mL kanamycin, 34 µg/mL chloramphenicol). The plates were then incubated overnight at 37°C.

## Purification of LIC vector

Eight kanamycin/chloramphenicol cultures were inoculated using individual colonies and incubated overnight at 37°C. For purification, a plasmid miniprep (ZymoPURE™ Plasmid Miniprep, cat. D4200/1, Zymo Research) was performed per manufacturer's protocol on each culture. The purified plasmids were then stored at −20°C.

## Polymerase Chain Reaction of hPEP transformants

A PCR was performed using the gene specific primers listed in Table 1. JM109 *E. coli* cells were previously transformed with hPEP (obtained from GE Healthcare Dharmacon, Lafayette, CO). Ten individual bacterial colonies were selected and used as a template for a reaction. Colonies selected were first transferred to a grid plate using a pipette tip. The tip was then swirled in the PCR reaction buffer, transferring the remaining cells to the buffer. GoTaq DNA polymerase (Promega Corp., Madison, WI) was used for amplification. The PCR was performed using an iCycler thermal cycler (Bio-Rad Laboratories), using the reagents and parameters for the reaction listed in Table 2 and Table 3, respectively. Additionally, to optimize the annealing step, a temperature gradient (55-63°C) was established on the thermal cycler. Following amplification,

agarose gel electrophoresis (1% w/v) was used to screen the PCR products for verification.

**Table 1:** Forwards and reverse gene specific primers were used for the amplification of hPEP.

| Primer Name | Sequence | $T_m$ |
|---|---|---|
| **hPEPLICfor** | 5'-TATTTTAATCCTACGTAATGCT GTCCCTTCAGTACCCCGAC-3' | 79.2°C |
| **hPEPLICrev** | 5'-CCCTCAATATTATACGGGTCATTAT GGAATCCAGTCGACGTTCAGGCA-3' | 83.7°C |

**Table 2:** PCR reaction Mix. Reaction volumes were prepared at 50 µL for each tube. A total of 8 reactions were performed using separate bacterial colonies as the template.

| **Reagents** | **Volume (µL)** |
|---|---|
| 10X GoTaq Buffer | 5 |
| 10µM dNTP Mix | 2.5 |
| Forward Primer 10 µM | 2.5 |
| Reverse Primer 10 µM | 2.5 |
| GoTaq Polymerase | 0.5 (1.5 U) |
| di$H_2O$ | 37 |

**Table 3:** PCR parameters for amplification of hPEP With Temperature Gradient

| Step | Temperature (°C) | Time | |
|---|---|---|---|
| Initial Denaturation | 95 | 4 min | |
| Denaturation | 95 | 30 sec | 35 Cycles |
| Annealing | 55-63 | 30 sec | |
| Extension | 68 | 3 min | |
| Polishing<br>Extension | 72 | 10 min | |
| Hold | 4 | ∞ | |

## Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for all visualization of DNA plasmids, restriction enzyme products, and PCR amplicons. For comparison, 5 µL Bionexus Hi-Lo™ DNA marker was added to one or two lanes in each gel. Bromophenol blue containing loading dye was added (5 µL) to 10 µL of each PCR product and these were loaded into individual lanes. The gel was run with 1X TAE buffer (48.4 g Trizma base + 20 mL 0.5M EDTA, pH 8.0 + 11.4 mL glacial acetic acid) at 100 V for approximately 40 minutes. For visualization, ethidium bromide was added during the preparation of the gel. All gels were analyzed using a Typhoon FLA 9500 spectrophotometer (GE Healthcare). The volume remaining from the samples that were confirmed to contain hPEP were pooled and run on a separate preparative gel.

## Purification of PCR amplicons and restriction enzyme products

The bands corresponding to the amplified PCR products as well as products from restriction enzyme digestion were cut from the agarose gel and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Corp.) as per manufacturer's protocol.

## Measurement of DNA Concentration and Purity

Following purification of the DNA samples, the concentration was then measured using a Cary®50 UV-Vis spectrophotometer (Agilent Corp.). The machine was zeroed using the same eluent that was used during the plasmid purification, nuclease free water. The absorbance was then measured at $A_{260}$ and $A_{280}$. For purity, the [$A_{260}/A_{280}$] was determined.

To calculate the concentration of DNA, the following formula was used:

$$_{ds}DNA\ concentration = 50\ \frac{\mu g}{mL}\ x\ OD_{260}\ x\ dilution\ factor^{*}$$

*Using a 1 cm pathlength, the optical density at 260nm ($OD_{260}$) is equal to 1.0 for a 50µg/mL solution of dsDNA (Barbas et al., 2007).

## Concentrating pLIC by Sodium Acetate and Ethanol Precipitation

To perform the SnaBI restriction enzyme digestion of the pLIC to prepare it for the future insertion of hPEP, approximately 10 µg DNA was used. The measured pLIC was initially too dilute to accomplish the digestion at the desired volume, and therefor was concentrated. One tenth the pLIC volume of 3 M Na-Acetate and 2x the pLIC volume of ice-cold 100% ethanol was added. This was mixed by flicking and left to incubate at -80°C for 30 minutes. After being allowed to chill, it was centrifuged at max speed for 15 minutes. The supernatant was

carefully decanted from the pellet. The remaining ethanol was evaporated and the pellet was re-suspended in 10 µL nuclease-free $H_2O$.

## Restriction Enzyme Digestion of LIC Vector

A SnaBI (New England Biolabs, Ipswich, MA) restriction digestion was performed to linearize the LIC vector. The pLIC vector contains two restriction sites for SnaBI, located at 5313 bp and 6840 bp (Figure 2). The restriction digestion was prepared using 10 µg of pLIC DNA and 20 U SnaBI in a 50 µL reaction. The reaction components are listed in Table 4. The mixture was incubated at 37°C for a minimum of 2 hours. 10 µL of the digested and 20 µL undigested products were electrophoresed on an analytical 1% agarose gel for comparison. The remainder of the digested pLIC was run on a preparative gel.

## $T_4$ DNA Polymerase Ligation

The pLIC and hPEP were each treated with 3 U $T_4$ DNA polymerase (3U/µL) to create complimentary overhangs on both the vector and the insert (Sambrook et al., 2001). In one tube, 500 ng hPEP was treated in the presence of dCTPs, producing an overhang on the insert. In a second tube, 500 ng pLIC was treated with $T_4$ DNA polymerase in the presence of dGTPs to produce a complimentary overhang on the vector to the insert. The reaction components for the treatment are listed in Table 5. Both tubes were incubated for 30 minutes at room temperature. They were then incubated at 75°C for 20 minutes to inactivate

the polymerase. The insert and vector were then mixed at Vector:Insert molecular ratios of 1:1, 1:2.5, 1:5, and 1:5 in the presence of 50 μM EDTA. The mixtures were incubated at 75°C for 5 additional minutes and then mixed by flicking. They were allowed to anneal overnight at 4°C.

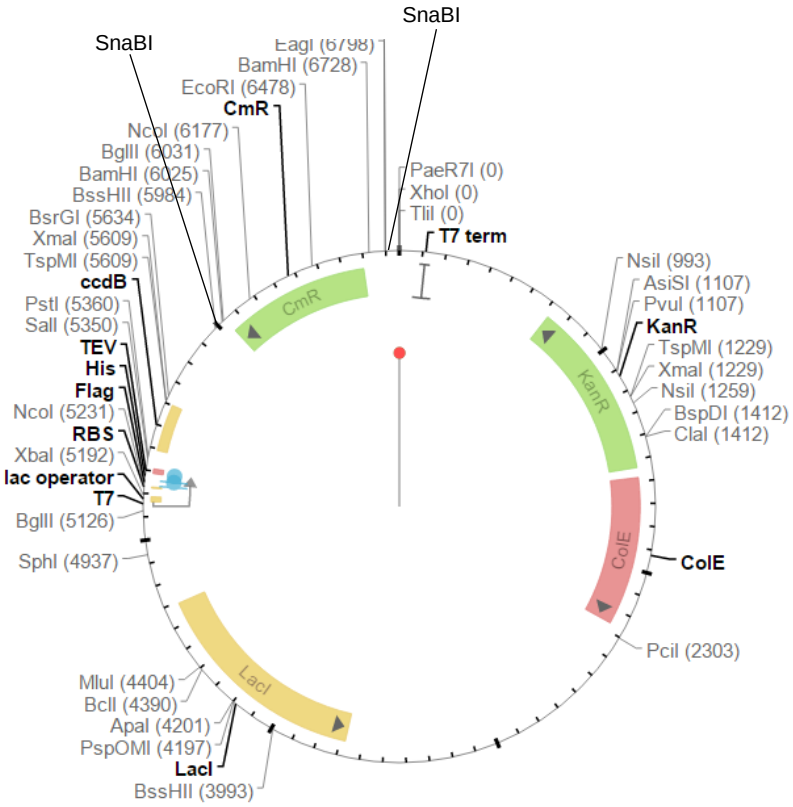


Figure 2: pNYCOMPS-LIC-FH10T+ (pLIC) (Arizona State DNA Repository) plasmid map, 6840 bp. A double digestion was performed using 20 Units of SnaBI, cutting out the chloramphenicol resistance segment of the vector (CmR).

**Table 4**: The reaction mixture for the digestion of pLIC is shown below. As a control, a sample of pLIC was also used in a reaction mix lacking the digestion enzyme.

| Reaction components | Uncut | Cut |
|---|---|---|
| SnaBI (4U/µL) | N/A | 5 µL (20 Units) |
| 10x CutSmart® Buffer (NEB) | 2 µL | 5 µL |
| pLIC DNA | 1 µL | 8 µL |
| di$H_2O$ | 17 µL | 32 µL |
| Total Volume | 20 µL | 50 µL |

**Table 5:** $T_4$ DNA Polymerase exonuclease activity was used to create complimentary overhangs on hPEP and pLIC.

| Reaction components | hPEP | pLIC |
|---|---|---|
| Sample DNA | 500 ng | 500 ng |
| 10x Buffer | 3 µL | 3 µL |
| $_d$CTP | 100 µM | - |
| $_d$GTP | - | 100 µM |
| $T_4$ Polymerase | 3 U | 3 U |
| $_dH_2O$ | 4 µL | 3 µL |
| Total Volume | 30 µL | 30 µL |

## Transformation of JM109 With pLIC-hPEP

The tubes containing the ligation reactions were briefly centrifuged to collect the contents at the bottom. From each of the tubes, 2 µL were used to transform commercially competent *E. coli* JM109 cells (≥ 1 x $10^8$ cfu/µg DNA, Promega, Corp.) via heat-shock as described previously. A tube containing JM109 cells was taken through the transformation protocol as a negative control. After the transformation, the cells were plated in duplicate at aliquots of 100 µL and 50 µL. The remaining volume was centrifuged, decanted, re-suspended, and then plated. To select for transformants, cells were plated in the presence of kanamycin (50mg/mL). Three control plates were also made. Two, containing only LB agar, were plated with the non-transformed JM109 cells. The final control plate was prepared with 50 µg/mL kanamycin and was plated with the same cells.

## Confirmation of Transformation by PCR and Gel Electrophoresis

Eighteen colonies were selected at random to be PCR screened for verification of hPEP insert as an indication of successful ligation and subsequent transformation. The colonies selected were also spot inoculated on a grid plate for later use. The previously described PCR reaction mix components and hPEP forward and reverse primers were used for the reaction. The parameters for the

thermal cycler are listed in Table 6. The amplified products were run on a 1% agarose gel for analysis.

**Table 6:** Parameters for PCR screening using colony picks from pLIC-hPEP transformed JM109 plates.

| Step | Temperature (°C) | Time | |
|---|---|---|---|
| Initial Denaturation | 95 | 4 min | |
| Denaturation | 95 | 30 sec | 35 Cycles |
| Annealing | 60 | 30 sec | |
| Extension | 72 | 3 min | |
| Polishing Extension | 72 | 5 min | |
| Hold | 4 | ∞ | |

## Maxiprep of pLIC-hPEP for sequencing

A 100-200 ng/µL plasmid concentration was required prior to sending the sequencing to Eurofins MWG Operon LLC. To achieve the required concentration, four colonies that were shown positive for hPEP were selected from the grid plate and used to inoculate individual Erlenmeyer flasks containing 150 mL Super Broth II (Per liter: 32 g Tryptone, 20 g yeast extract, 5 g NaCl (s), and 1 g Trizma base (Sigma-Aldrich Co., St. Louis, MO, USA)). These were incubated overnight at 37°C with aeration. To purify the plasmid, a maxiprep (ZymoPURE™ Plasmid Maxiprep Kit, Cat. D4202, Zymo Research) was performed on each 150 mL culture. The concentration and purity of each plasmid sample was determined by measuring the $A_{260}$ and $A_{280}$, as described previously. The samples were then diluted to 100-200 ng/µL and sent off to Eurofins MWG Operon LLC. (Louisville, KY) for both forward and reverse sequencing using the previously described hPEP primers.

## Sequence Analysis

The resulting sequencing data was received as abi compatible files. CodonCode Aligner (Version 6.0.1., CodonCode Corporation, 2015) was used to remove any vector and non-reliable (low signal) sequence data. BioEdit (Hall T. A., 2013) was used to align the sequences via the sequence-nucleic acid-reverse compliment tool. A contig sequence was then built using the BioEdit Cap Contig

program on the aligned sequences. The contig sequence was submitted as a query to NCBI nucleotide-BLAST (Altschul et al., 1990).

## Transformation of BL21(DE3)+pTF-S with the pLIC-hPEP Construct and Subsequent Expression

### Inducing the Competency of BL21(DE3) + pTF-S

The host that was used for the expression of hPEP was BL21(DE3)pTF-S *E. coli* (Genotype: *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS; λ DE3 = (λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5)* ). Prior to transforming the host with the hPEP construct, the cells were made competent using the calcium chloride/Tris buffer method (Mendel and Higa, 1970). Ten milliliters of LB broth (34 µg/mL chloramphenicol) was aliquoted into two 50 mL conical tubes. A glycerol stock of BL21(DE3)+pTF-S (Dareddy, V., 2012) cells were thawed on ice and then were mixed by flicking. Ten microliters of the stock was used to inoculate one of the conical tubes containing the LB/chloramphenicol. This was incubated overnight at 37°C with aeration. The following day, 10 µL of the culture was aliquoted into the remaining conical tube. This was cultivated at 37°C with aeration. The optical density at 600 nm was measured intermittently using a DU® 800 spectrophotometer (Beckman Coulter, Brea, CA, USA) until the culture reached an $OD_{600}$ of ~0.6. The culture was

chilled on ice for 10 minutes and centrifuged for 5 minutes at 4000 x g, 4°C. The supernatant was aseptically decanted from the pellet. 25mL of sterile, ice-cold $CaCl_2$ in Tris buffer (pH 8.0) was added to the pellet, which was then vortexed to resuspend. The cells were centrifuged using the same conditions as before. At this point, the cells were handled gently due to them being fragile from treatment. The supernatant was decanted from the tube carefully as to not dislodge the pellet. Five milliliters of ice-cold $CaCl_2$-Tris buffer was added and the tube was gently swirled to resuspend the pellet. The cells, now competent for transformation, were dispensed into 1.5 mL microcentrifuge tubes, at 500 µL aliquots. Five hundred microliters of 70% glycerol was added to each tube followed by flash-freezing using liquid nitrogen and then stored at -80°C.

**Transformation of BL21(DE3)+pTF-S cells with pLIC-hPEP**

A stock of freshly competent BL21(DE3)+pTF-S (Dareddy, 2012) cells was removed from -80°C storage and thawed on ice. While thawing, three 17x100mm round-bottom polypropylene culture tubes were labeled “1”, “2”, and “3”. Two microliters of the hPEP construct was added to Tube 2, while 5 µL was added to Tube 3. Tube 1 served as a control, to which no plasmid DNA was added. 50 µL of the BL21 cells was aliquoted into each tube. Each tube was subjected to the heat-shock method of transformation and plated as previously described in the transformation of JM109 with pLIC-hPEP. These plates consisted of LB agar, with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol to select for transformants.

Aliquots of 100 µL from Tube 1 were added to three plates containing only LB agar. The plates were incubated overnight at 37°C. Transformants were grid-plated and then screened by PCR and 1% agarose gel electrophoresis. 10 mL cultures (LB broth with chloramphenicol and kanamycin) were grown using the colonies that were confirmed to contain the hPEP construct. Glycerol stocks were prepared for each culture, flash-frozen using liquid $NO_2$, and then stored at -80C°.

**Expression of hPEP**

To express hPEP, a glycerol stock of the BL21(DE3)+pTF-S + pLIC-hPEP was first thawed on ice. A loop-full of the stock was used to inoculate 100mL of LB broth with 50 mg/mL kanamycin and 34 µg/mL chloramphenicol. This was incubated overnight at 37°C with aeration. The following day, 60 mL of the culture was used to inoculate 6 liters of 2x LB media containing the previous kanamycin and chloramphenicol concentrations. The 6L growth was performed using the BioFlow 110 Modular Fermentor & Bioreactor (New Brunswick Scientific Co., INC., Edison, NJ, USA). Heat was applied to the vessel by a heat jacket, the output of which was monitored and regulated by the system using a thermistor. The growth was kept at a constant 30°C. For aeration, $O_2$ was added through a 0.22 µm filter by an air pump with the output for the mixer rotor kept at a constant 200 rpm. Prior to inoculation, a 3 mL sample was taken out as a blank. Once inoculated, the $OD_{600}$ was measured every 30 minutes until it reached approximately 0.6. At this point, 10 mL removed from the culture was

incubated overnight at 37°C to serve as an uninduced sample. The expression of the remaining culture was induced by adding IPTG to a final concentration of 4 mM. The induced sample was allowed to continue growing overnight in the fermenter under the same conditions.

To harvest the induced cells, the culture was syphoned into 1 L centrifuge bottles and centrifuged in a swinging bucket rotor at 1250 x g for 30 minutes. The supernatant was decanted from each pellet. The pellets were stored overnight at -20°C to assist with cell lysis. The following day, the pellets were resuspended in 50 to 75 mL of 0.1M sodium phosphate, pH 8.0, buffer. The pellets were pooled into a single 1 L bottle. Phosphate buffer was added to bring the total volume to 500 mL. Ten milliliters of phosphate buffer was added to two vials of CelLytic™ Express (Sigma-Aldrich Co., St. Louis, MO, USA). These were added to the pooled lysate, which was then shaken vigorously and stored at -4°C for 4 hours. The pooled lysate was then pulse sonicated using a 60 Sonic Dismembrator (Fischer Scientific, Lafayette, CO, USA) at 1 second intervals for 1 minute to aid in lysis. The lysate was divided evenly into three 250 mL centrifuge bottles and centrifuged at 9300 x g for 30 minutes. The supernatant from each bottle was collected in a 1 L Erlenmeyer flask. TWEEN® 20 (Sigma-Aldrich Co., St. Louis, MO, USA) was added to the clarified lysate to a final concentration of 0.1% (v/v). A magnetic stir bar was placed in the flask followed by 5 mL Ni-NTA resin (Thermo Fisher Scientific, Waltham, MA, USA), pre-equilibrated with the 0.1M

phosphate buffer. The resin-lysate mixture was left spinning slowly overnight at 4°C.

## Purification of hPEP with Ni Affinity Chromatography

The resin-lysate mixture was removed from 4°C and centrifuged at 9,300 x g to pellet the resin. The supernatant was carefully poured away from the pellet to remove as much as possible without losing any of the resin. A slurry was made of the enzyme-Ni resin which was transferred to an open column for column chromatography. An ÄKTA Protein Purification System (GE Healthcare Bio-Sciences, Pittsburgh, PA) was used in manual mode for the chromatography. The resin was washed using 120 mL of 0.1M phosphate buffer (pH 8.0) containing 0.1% TWEEN-20 to remove unbound contaminants. The enzyme was eluted using a linear gradient of increasing imidazole from 0 to 0.25 mM over 400 mL total volume. Fractions of 5 mL were eluted at a flow rate of 1 mL/min.

## Concentration of Enzyme

The enzyme fractions were pooled and concentrated using a Centricep® Centrifugal filter device with an Ultracel® 50K membrane (EMD Millipore Corp., Billerica Massachusetts, USA). Fifteen mililiters of the sample was added to the device and centrifuged at 1,500 x g for 2 hours. The filtrate was decanted, and then more sample was added up to 15 mL total, followed by another round of

centrifugation. This was repeated as necessary to concentrate protein fractions for enzyme kinetic assays.

**Gly-Pro-pNA Assay**

All obtained hPEP lysates were assayed to measure activity. The assay was performed in a 96-well microtiter plate, using a SoftMax® Pro 5 plate reader (SN# SMP500-05066-QAQD, Molecular Devices, LLC, Sunnyvale, CA). The components of the assay are listed in Table 7. Final concentrations of the individual components were 1x PBS, 0.3 mM GPpNA, and 0.1 M DTT in a 200µL microtiter assay. The absorbance at 410 nm was used to detect hydrolysis of the GPpNA. An absorption coefficient of 8800 L/mol•cm was used to convert the absorption to µmol of substrate cleaved. The assay was measured in kinetic mode using a SoftMax® Pro 5 plate reader (Molecular Devices, LLC) set at 37°C, collecting a measurement every 9 seconds over an hour.

Due to low activity levels in one of the enzyme batches, different amounts of enzyme were also tested to determine optimum enzyme amounts to add to each assay. Table 8 lists the components of each of these assayst.

To determine Km and Vmax of the different samples of hPEP, standard assays were performed varying the amount of substrate present (Table 9). For the first enzyme batch, the final concentrations of substrate used were 0.3 mM, 0.15 mM, 0.075 mM, 0.03 mM, 0.015 mM, and 0.0075mM. To determine the Km

and Vmax for the hPEP from the second enzyme batch, the final concentrations of the substrate were 0.3 mM, 0.15mM, 0.1 mM, 0.075 mM, 0.05 mM, and 0.0 mM. The data for the assays were analyzed and used to produce a graph demonstrating the concentration of pNA released vs Time. Using linear regression, the slopes produced by the different substrate concentrations were recorded. These slopes correlate to the velocity ($V_o$) of the different reactions. Plotting the $V_o$ versus concentration of substrate ([S]) produced a Michaelis-Menton plot of the data. Plotting the inverse ($1/V_o$ vs $1/[S]$) produced the Lineweever-Burk Plot. The Km's were calculated using linear regression to calculate the x-intercepts (x-int) for each assay. The Km is equal to 1/[X-int]. The Vmax can be calculated similarly by taking 1/[y-int] (Segel, 2014).

**Table 7:** The standard reaction mixture components and volumes for a GPpNA microtiter assay are shown. The reaction volume for all wells was 200 µL and consisted of 100 µL enzyme

| | **1 Well** | **50 Wells** | **100 Wells** | **150 Wells** |
|---|---|---|---|---|
| **10x PBS** | 20 µL | 1 mL | 2 mL | 3 mL |
| **3mM GPpNA** | 20 µL | 1 mL | 2 mL | 3 mL |
| **1M DTT** | 2 µL | 100 µL | 200 µL | 300 µL |
| **diH2O** | 58 µL | 2.9 mL | 5.8 mL | 6.7 mL |
| **Total Volume** | **100 µL** | **5 mL** | **10 mL** | **15 mL** |
| **Enzyme** | 100 µL per Well | | | |
| **Total Well Volume** | **200 µL** | | | |

**Table 8:** A GPpNA assay was performed using 3 different volumes of hPEP to determine the lowest amount of enzyme that can be used while still achieving high activity. hPEP was varied in increments of 50 µL. The volume of di$H_2O$ was adjusted to maintain a well volume of 200 µL.

| 10x PBS (µL) | 3mM GPpNA (µL) | 1M DTT (µL) | di$H_2O$ (µL) | hPEP (µL) |
|---|---|---|---|---|
| 20 | 20 | 2 | 108 | 50 |
| 20 | 20 | 2 | 58 | 100 |
| 20 | 20 | 2 | 8 | 150 |

**Table 9:** GPpNA assays were conducted using hPEP isolated from two different expression experiments (A) and (B). The assays were run at 410 nm and the absorbance was measured every 9 seconds for 1 hour. The substrate concentration was varied, allowing for the determination of Km and Vmax by taking the slopes of the linear portions of graphs.

| A | | | | | | | B | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hPEP (µL) | di$H_2O$ (µL) | 1M DTT (µL) | 10X PBS (µL) | 0.3 mM GPpNA (µL) | 3 mM GPpNA (µL) | Final [GPpNA] (mM) | hPEP (µL) | di$H_2O$ (µL) | 1M DTT (µL) | 10X PBS (µL) | 1 mM GPpNA (µL) | 3 mM GPpNA (µL) | Final [GPpNA] (mM) |
| 150 | 8 | 2 | 20 | - | 20 | 0.3 | 50 | 108 | 2 | 20 | - | 20 | .3 |
| 150 | 18 | 2 | 20 | - | 10 | 0.15 | 50 | 118 | 2 | 20 | - | 10 | .15 |
| 150 | 23 | 2 | 20 | - | 5 | 0.075 | 50 | 108 | 2 | 20 | 20 | - | .1 |
| 150 | 8 | 2 | 20 | 20 | - | 0.03 | 50 | 113 | 2 | 20 | 15 | - | .075 |
| 150 | 18 | 2 | 20 | 10 | - | .015 | 50 | 118 | 2 | 20 | 10 | - | .05 |
| 150 | 23 | 2 | 20 | 5 | - | .007 | 50 | 128 | 2 | 20 | 0 | - | 0 |

## Bradford Assay

To determine total protein content of each cell lysate, a Bradford assay (Bio-RAD., Herculus, CA, USA) was performed. Known concentrations of BSA from 0.6 µg/mL to 10.0 µg/mL were prepared with ultrapure BSA (1.0 mg/mL, Fischer Scientific, Lafayette, CO, USA). These known concentrations were assayed to plot the standard curve. BSA was added to the microtiter plate in the following amounts: 0.6 µg, 0.8 µg, 1.00 µg, 2 µg, 4 µg, 6 µg, 8 µg, and 10 µg. The total volume of each BSA sample was brought to 100 µL with 0.1M sodium phosphate buffer (pH 8.0). Dilutions were made for each of the cell lysates to 1/100 and 1/1000. From both dilutions of each sample, volumes of 10 µL, 5 µL, and 2 µL were added to the microtiter plate in duplicate. The total volume was brought to 100 µL with 0.1M buffer. Ninety microliters of the 0.1M buffer and 10 µL di$H_2O$ were added to one well as a blank. To all wells, 100 µL of 2x Bradford reagent (Bio-RAD, Herculus, CA, USA) was added and mixed by pipetting up and down carefully to prevent introducing air bubbles. In instances where air bubbles occurred, the plate was centrifuged for 1 minute at 3000 x g. The absorbance at 595 nm was measured using a SoftMax® Pro 5 plate reader (SN# SMP500-05066-QAQD, Molecular Devices, LLC, Sunnyvale, CA). The total protein concentration for each sample was calculated using the linear portion from the standard curve and correcting for dilution.

## SDS-PAGE Analysis

To determine the purity of expressed hPEP, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used on both lysates demonstrating enzyme activity. A standard Laemmli SDS-PAGE (He, F., 2011) was performed using a 10% resolving gel and a 4% stacking gel. These gels were handcrafted using the components listed in Table 10. Ten micrograms total protein for each hPEP sample (as determined using the standard curve from the Bradford assay) was added to 1.5 mL microfuge tubes. Five microliters of sample loading dye was added to each, followed by denaturation at 95°C for 2 minutes. A 10 µL sample of Precision Plus Protein™ Standards (Bio-RAD., Herculus, CA, USA) was subjected to denaturation as well. The samples and standard were loaded into individual lanes and electrophoresis was performed at 100 V for 1 hour. For visualization, the gel was stained in a solution containing 0.25% Coomassie Blue (Bio-RAD., Herculus, CA, USA). The staining process took place overnight at room temperature while rocking. The gel was destained using 50% methanol, and 10% glacial acetic acid in $H_2O$.

**Table 10**: Standard Laemmli SDS PAGE Gel Recipe (He, F., 2011). The components for preparing the resolving and stacking parts of an SDS PAGE gel are shown. Both were prepared in different 50 mL conical tubes.

| | | |
|---|---|---|
| **10% Resolving:** | 29.2% acrylamide + 0.8% bis | **5 mL** |
| | 1.5 M Tris-HCl, pH 8.86 | **3.75 mL** |
| | di$H_2O$ | **6.25 mL** |
| | 10% SDS | **150 µL** |
| (When ready to polymerize) | 10% APS | **70 µL** |
| | TEMED | **7 µL** |
| **4% Stacking:** | 29.2% acrylamide + 0.8% bis | **2 mL** |
| | 1.5 M Tris-HCl, pH 8.86 | **3.78 mL** |
| | di$H_2O$ | **9.1 mL** |
| | 10% SDS | **150 µL** |
| (When ready to polymerize) | 10% APS | **70 µL** |
| | TEMED | **14 µL** |

# RESULTS

The overall goal of the project was to clone and express hPEP in the same host as spPEP. The first part of the project included the cloning, purification, and restriction digestion of the pLIC vector and the subsequent ligation of it to PCR amplified hPEP; this construct was cloned into JM109 *E. coli* cells. The second objective of this project was cloning and transforming the pLIC-hPEP construct into the final *E. coli* expression host BL21(DE3)+pTF-S cells. The final part of the project involved the expression and partial purification of hPEP and the subsequent analysis of the enzyme's kinetics.

## PCR Amplified hPEP Products

Gene specific primers were used to amplify hPEP, using 10 colonies transformed with hPEP as the template for the reaction. A temperature gradient from 55-63°C was established for the annealing step of the reaction. The amplified products were loaded on a 1% agarose gel for analysis (Figure 3). All lanes demonstrated the expected ~2133 bp band except gel A-lane 6, corresponding to a 63°C annealing temperature. The hPEP products were

pooled and loaded on a preparative 1% agarose gel to purify the amplified hPEP products (Figure 4). The resulting band was cut out and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Corp.). The weight of the gel slice corresponding to hPEP was 441mg.

## Purification of pLIC

The pLIC was transformed into a JM109 *E. coli* cell line. The plasmid was then purified using a ZymoPURE™ Plasmid Miniprep (cat. D4200/1, Zymo Research). The purified pLIC and hPEP product were loaded onto a 1% agarose gel (Figure 5). The concentration and purity of both samples were determined by measuring the $A_{260}$ and $A_{280}$ with a Cary®50 UV-Vis spectrophotometer (Varian, Inc.). The final measurements, included calculated purity and concentration, are listed in Table 11. The average concentration for the hPEP samples was 28.0 ng/µL, whereas the average for the pLIC was 46.25 ng/µL.

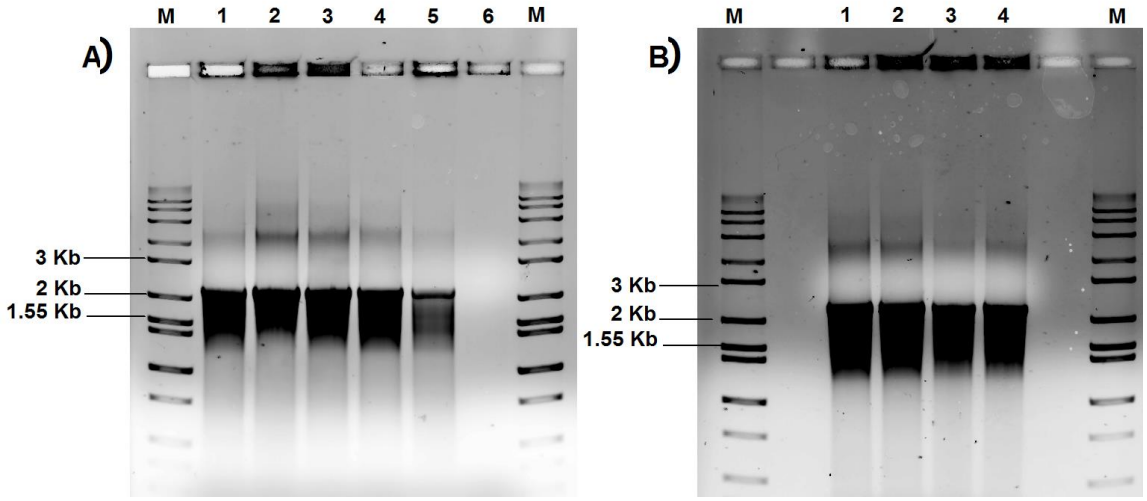


**Figure 3: Amplified hPEP products from PCR using individual colonies as template on a 1% agarose gel.** Ten colonies transformed with hPEP were selected as the template for the reaction. A temperature gradient from 55-63°C was established for the annealing step of the reaction. On the first gel (**A**), lanes 1 through 5 exhibited a band of ~2133bp. The band in lane 5 was noticeably lighter than the others. For the different samples, the annealing temperatures from the gradient are as follows: Gel **A** Lane 1 – 57.3°C, lane 2 – 58.4°C, lane 3 – 59.5°C, lane 4 – 60.6°C, lane 5 – 61.8°C, lane 6 - 63°C; Gel **B** Lane 1 - 55°C, lane 2 - 55°C, lane 3 - 55°C, lane 4 – 56.3°C. **M** represents Bionexus Hi-Lo™ DNA marker.

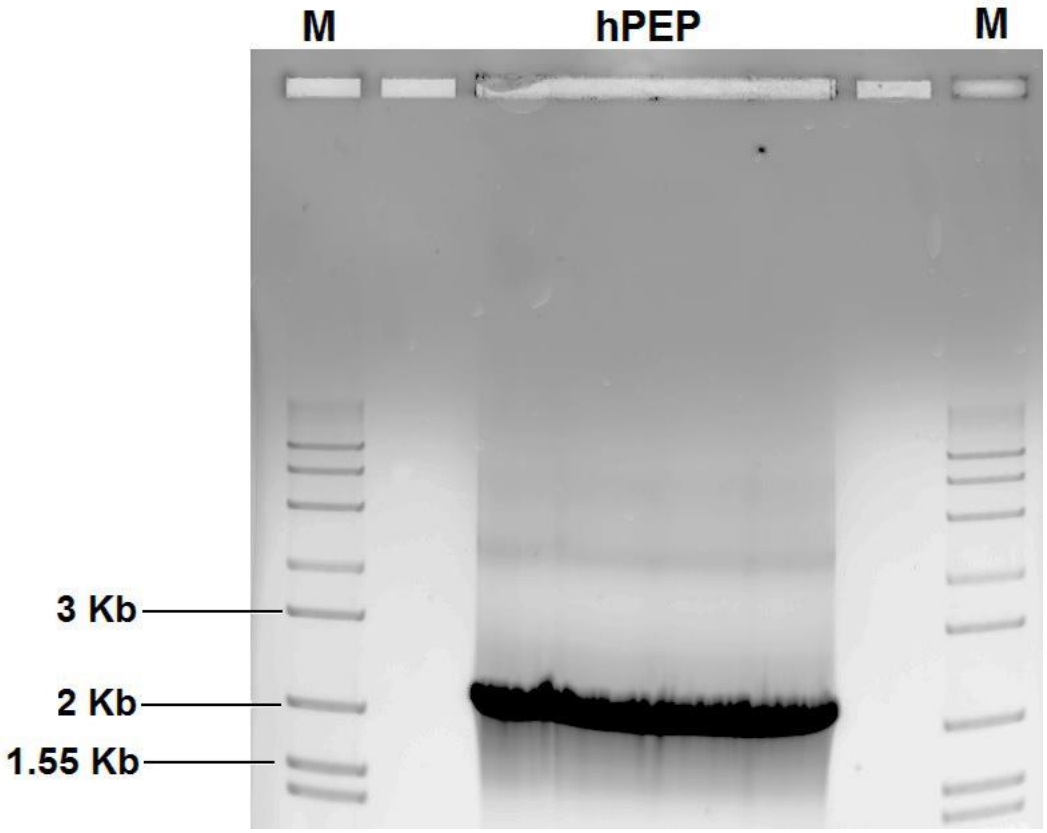


**Figure 4: Preparative gel of pooled samples of amplified hPEP.** The remaining volume from the samples confirmed to contain hPEP (those that exhibited a ~2133 bp band on the analytical gels) were pooled. This was run on a 1% agarose gel. A single band of (~2133 bp) is visible under the lane marked **hPEP**. This band was excised and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Corp.). **M** represents Bionexus Hi-Lo™ DNA marker.

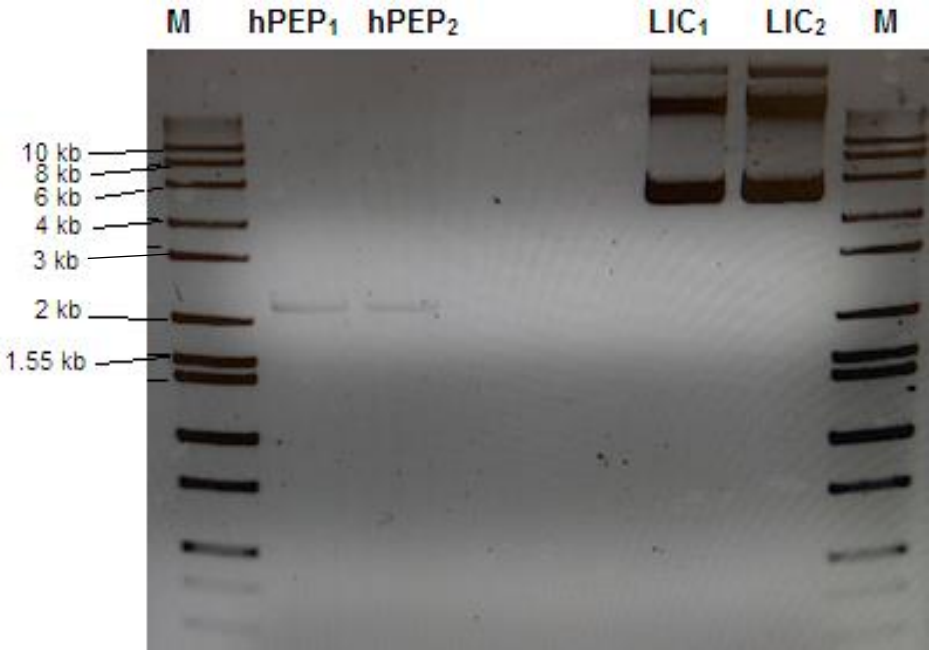


**Figure 5: Results of the clean up of LIC vector and the gel purification of hPEP.** The bands were separated on a 1% (w/v) agarose gel in 1X TAE buffer at 100V for 40 minutes. The positions of both hPEP are at the desired approximate 2133 bp. The pLIC bands are consistent with that of undigested plasmids, each band resulting from the different plasmid conformations. **M** represents Bionexus Hi-Lo™ DNA marker.

**Table 11:** The concentration and purity of the cleaned up LIC vector and amplified hPEP are shown below.

| **Sample** | **$A_{260}$** | **$A_{280}$** | **Concentration (ng/µL)** | **Purity ($A_{260}/A_{280}$)** |
|---|---|---|---|---|
| **$hPEP_1$** | 0.56 | 0.31 | 28.0 | 1.81 |
| **$hPEP_2$** | 0.56 | 0.30 | 28.0 | 1.87 |
| **LIC $Vector_1$** | 1.0 | 0.55 | 50.0 | 1.81 |
| **LIC $Vector_2$** | 0.85 | 0.47 | 42.5 | 1.81 |

## SnaBI Restriction Digestion of pLIC

A restriction digestion was performed on the pLIC vector using SnaBI enzyme. pLIC contains two restriction sites for SnaBI, resulting in two linear fragments when loaded on an agarose gel. This is demonstrated in Figure 6, where a sample of the SnaBI digestion of pLIC was loaded side-by-side to an undigested sample of pLIC for comparison. The digested sample was comprised of two primary fragments: ~1527 bp and ~5313 bp. The remaining volume of the digested sample was loaded onto a preparative 1% agarose gel (Figure 7). The ~5313 bp fragment was excised and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Corp.). The gel slice was measured to be 649 mg. Following the purification of the digested pLIC, a sample was loaded onto a 1% agarose gel to verify that no other bands were present. Figure 8 demonstrates a single band at ~5313 bp. No other bands were visible.

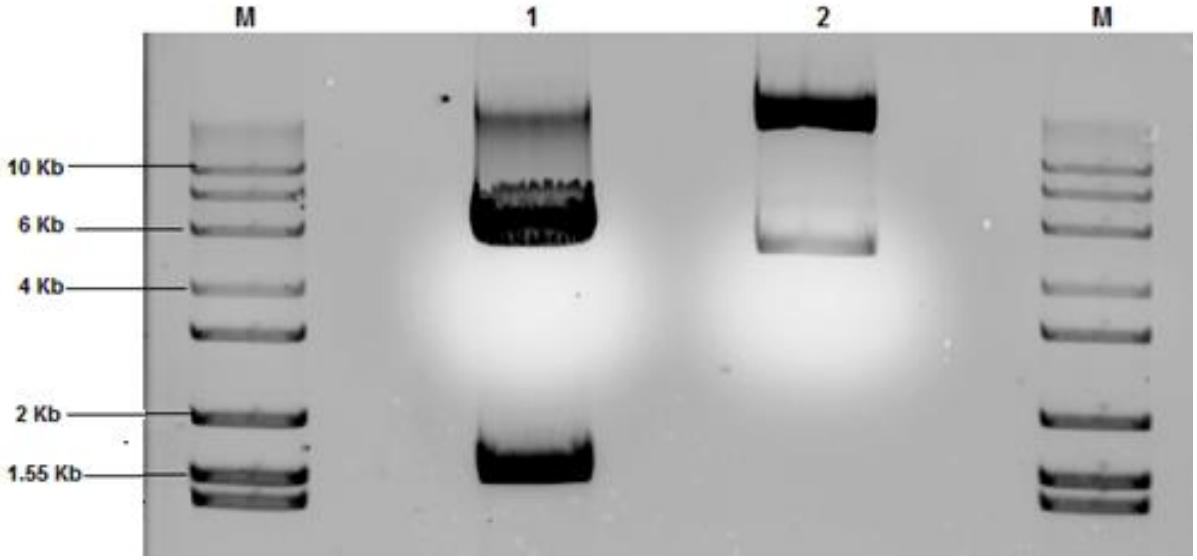


**Figure 6: Analysis of double SnaBI digestion of pLIC on 1% agarose gel.** The restriction digestion was prepared using 8 µL of pLIC DNA and 5 µL SnaBI in a 50 µL reaction. The mixture was incubated at 37°C for a minimum of 2 hours. Digested and Undigested were run side-by-side for comparison. Lane (1) shows pLIC digested with SnaBI; two primary bands are visible: (A) the 5313 bp fragment and (B) the 1527 bp fragment. Lane (2) shows the undigested plasmid. M represents Bionexus Hi-Lo™ DNA marker.

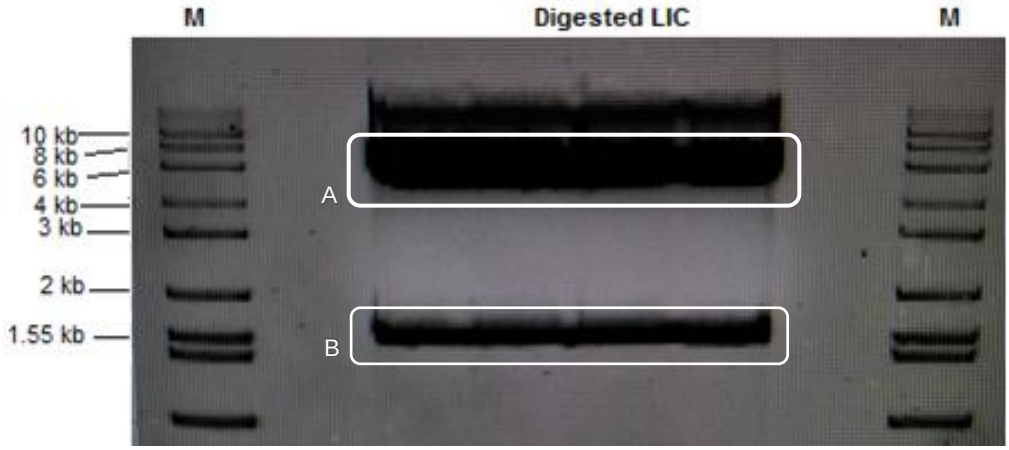


**Figure 7: Preparative agarose gel (1%) of double SnaBI digestion of pLIC.** In order to purify and excise the desired pLIC 5313 bp band (A), a 1% agarose preparative gel was run using the remainder of the digestion reaction mix. (B) The lower band represents the resulting dropout from the double digestion of pLIC (1527 bp). **M** represents Bionexus Hi-Lo™ DNA marker.

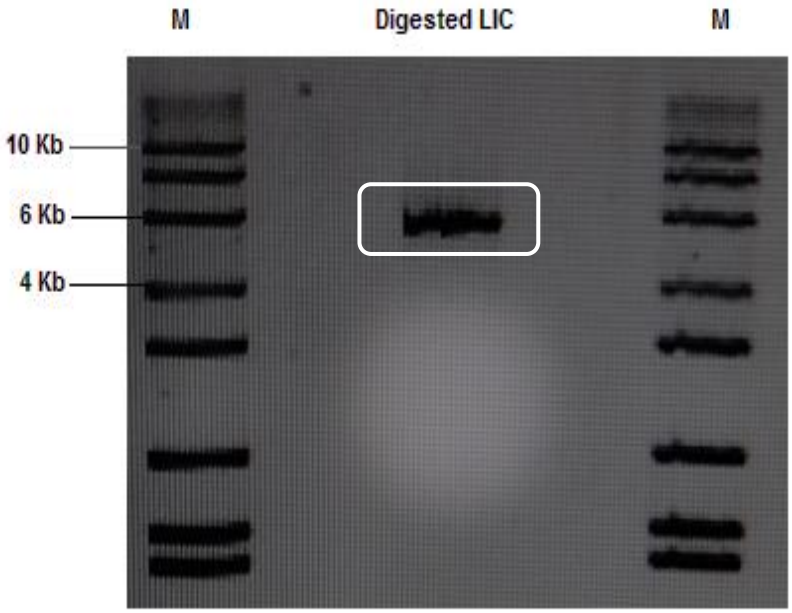


**Figure 8: Cleaned up pLIC 5313 bp band on agarose gel (1%)**. The 5313 bp band was excised from the analytical gel (weighing 649 mg) and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Corp.). The product was then run on a 1% agarose gel for verification. The encircled single band above shows the desired 5313 bp band of the digested pLIC. **M** represents Bionexus Hi-Lo™ DNA marker.

## Transformation of JM109 with pLIC-hPEP Construct

Complimentary overhangs were created on hPEP and the linearized pLIC. These were mixed at Vector:Insert molecular ratios of 1:1, 1:2.5, 1:5, and 1:5 in the presence of 50 µMol EDTA. After the insert and vector were allowed to anneal overnight, the resulting pLIC-hPEP construct was used to transform a JM109 *E. coli* cell line. The cells were plated in duplicate at aliquots of 100 µL and 50 µL; the remaining volume was centrifuged, decanted, re-suspended, and then plated. The number of colonies that grew after the transformation were recorded and are listed in Table 12. The positive control plates grew lawns as expected, indicating the cells remained viable throughout the transformation. The negative control demonstrated no growth, indicating the selective agent (kanamycin) functioned as desired. The vector to insert ratio that demonstrated the highest number of transformants, and therefore most successful ligation independent cloning, was the 1:1 mol sample. 18 colonies were selected at random and screened by PCR for verification using the previously described hPEP forward and reverse primers. These samples were loaded onto a 1% agarose gel for analysis (Figure 9). Of the 18 colonies screened, the expected 2133bp band for hPEP was present in 11 samples. In the first gel (fig. 9, A), lanes 1 and 7 show bands at (~2133bp), while lanes 2 – 6 failed show the presence of amplified hPEP. On the second gel (fig. 9, B), lanes 1 and 4 contained bands at (~2133bp), whereas this band was absent in lanes 2, 3, 5,

and 6. They can likely be explained as being false positive transformants. In the preparation of the plates, only 10 of the plates were made by adding the antibiotic prior to solidification of the agar. The other 10 plates had the antibiotic spread over the surface, and as of such, certain areas of the agar surface may have not been treated with the antibiotic. Without the selective agent present throughout, it is possible that the colony that grew either kicked out the pLIC-hPEP or it never actually contained it in the first place. Within the final gel (fig 9, B), all seven sample lanes showed a band at (~2133bp).

**Table 12: CFU count of JM109 transformed with pLIC-hPEP.** 500 ng hPEP was treated with 1 µL $T_4$ DNA polymerase in the presence of 1 µL dCTP to create the overhang on the insert. To create the overhang on the vector, 500 ng pLIC was likewise treated with 1 µL $T_4$ DNA polymerase in the presence of dGTPs. The vector and insert were then mixed at varying molar ratios (1:1, 1:2.5, 1:5, and 1:5 in the presence of 50 µMol EDTA). These were then incubated at 75°C for 5 minutes, mixed, and stored over night at 4°C. The following day, these mixtures were used to transform JM109 cells via heat shock. The cells were plated at varying volumes (100 µL and 50 µL in duplicate, and the remaining volume was plated after being centrifuged, decanted, and resuspended). They were plated in the presence of kanamycin (50mg/mL) to select for transformants. The plates were then incubated at 37°C overnight. The table above shows the number of successful transformants per vector to insert ratio and per volume plated.

| **Sample (V:I)→ Volume ↓** | **1:1** | **1:2.5** | **1:5** | **1:5 + 50µMol EDTA** |
|---|---|---|---|---|
| 100 µL | 13 | 4 | 2 | 3 |
| 100 µL | 12 | 8 | 2 | 3 |
| 50 µL | 11 | No Growth | 1 | 1 |
| 50 µL | 7 | 2 | No Growth | 5 |
| Resuspended | 22 | 4 | 5 | 2 |
| Positive Control 1 LAWN | | Positive Control 2 LAWN | | Negative Control No growth |

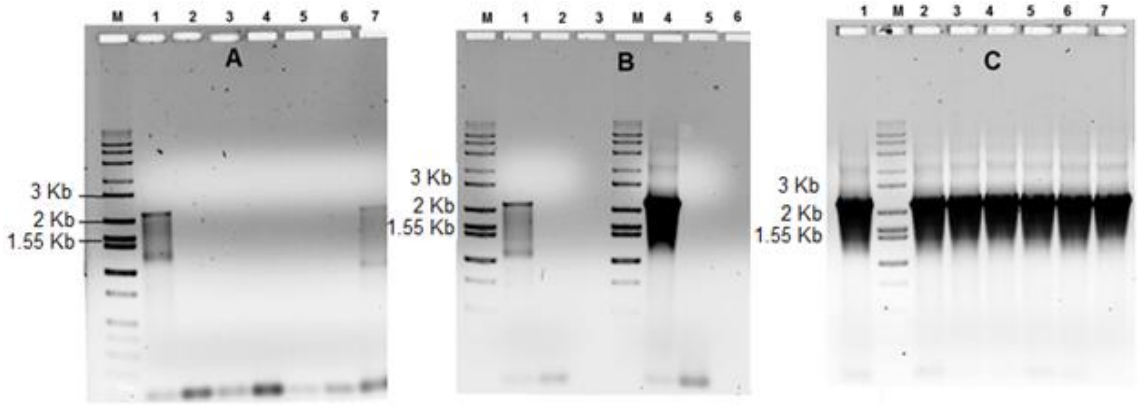


**Figure 9: PCR screening using colony picks from pLIC-hPEP transformed JM109 plates as DNA template and analysis by 1% agarose gel electrophoresis.** Colonies were selected at random to be PCR screened for verification of hPEP insert as an indication of successful ligation and subsequent transformation. The colonies selected were also spot inoculated on a grid plate for later use. The previously described hPEP forward and reverse primers were used for the reaction. The amplified products were run on 1% agarose gels for analysis. Of the 18 colonies screened, the expected 2133 bp band for hPEP was present in 11 samples. **(A)** In the first gel, lanes 1 and 7 show bands at (~2133bp), while lanes 2 – 6 failed show the presence of amplified hPEP. **(B)** On the second gel, lanes 1 and 4 contained bands at (~2133bp), whereas this band was absent in lanes 2, 3, 5, and 6. **(C)** Within the final gel, all seven sample lanes showed a band at (~2133bp). **M** represents Bionexus Hi-Lo™ DNA marker. Interestingly, sample lanes B4 and C1-7 exhibited much darker bands than the others, indicating excessive template DNA being used during the PCR amplification.

## Cloning and Purification of pLIC-hPEP

Four colonies on the grid-plate corresponding to successful transformants were used to inoculate separate Erlenmeyer flasks containing 150 mL Super Broth II. A ZymoPURE™ Plasmid Maxiprep Kit (Cat. D4202, Zymo Research) was performed on the cultures to purify the pLIC-hPEP construct. During the cell pelleting step of the maxiprep, it was noticed that the pellet for culture 4 was significantly smaller than the 3 other samples (2.4 cm compared to 3.2 cm). The concentration and purity of the plasmid from each sample was determined by measuring the $A_{260}$ and $A_{280}$ (Table 13). The acceptable $A_{260/280}$ measurement for a pure DNA sample is ~1.8 - 2.0 (Sambrook and Russell, 2001). Cultures 1 through 3 had DNA concentrations of 500, 640, and 740 ng/µL, respectively. Culture 4 had a much smaller concentration of 160 ng/µL. All four cultures were within the acceptable range for purity. This is likely related to the cell pellet size previously mentioned. The first three samples were diluted to 100-200ng/µL and sent off along with the previously described hPEP forward and reverse primers to Eurofins MWG Operon LLC for Sanger Sequencing.

**Table 13:** The concentration and purity of the pLIC-hPEP plasmid, post maxiprep, are shown below. Each of the samples was diluted by a factor of 20 to achieve accurate measurements. The absorbance values are shown below.

| Sample | $A_{260}$ | $A_{280}$ | Concentration (ng/µL) | Purity ($A_{260}/A_{280}$) |
|---|---|---|---|---|
| Colony 1 | 10.0 | 4.8 | 500 | 2.08 |
| Colony 2 | 12.8 | 6.8 | 640 | 1.88 |
| Colony 3 | 14.8 | 8.0 | 740 | 1.85 |
| Colony 4 | 3.21 | 1.58 | 160 | 2.03 |

## Sequence Analysis

The resulting sequencing data was sent as ab1 compatible files. The sequences were first cleaned up using CodonCode Aligner and then aligned with BioEdit to form a contig sequence. The contig sequence was subjected to a nucleotide-BLAST on the NCBI database. The BLAST result of the hPEP contig sequence yielded a 99.9% identity with *Homo sapiens* prolyl endopeptidase from the NCBI database. The contig sequence and the sequence corresponding to the highest identity match were aligned using http://www.fr33.net/translator.php to identify where disparities occurred between the two sequences. Figure 10 shows the nucleotide and amino acid sequence for the contig sequence (fig 10, A) and for the sequence of *Homo sapiens* prolyl endopeptidase from the NCBI database (fig 10, B). The alignment for the contig and hPEP cDNA from the NCBI database are shown in appendix A. Only two locations contained mismatches between the sequences. The first difference occurred at 1576 bp. Here, the sequence from the database contained a thymine, whereas the contig contained a cytosine. This change also resulted in a single amino acid variation between the query and subject sequences. The single nucleotide change resulted in the change of a positively charged Arg526 in the subject sequence to a polar Cys526 in the contig sequence. The second difference in sequences occurred at 2091 bp, where a guanine existed in the sequence from the database and adenine existed

in the contig sequence. This variation did not have an impact on the amino acid sequence, resulting in a conserved Ala697.

**A**

```
ATGCTGTCCCTTCAG TACCCCGACGTGTAC CGCGACGAGACCGCC GTACAGGATTATCAT GGTCATAAAATTTGT
 M  L  S  L  Q   Y  P  D  V  Y   R  D  E  T  A   V  Q  D  Y  H   G  H  K  I  C
GACCCTTACGCCTGG CTTGAAGACCCCGAC AGTGAACAGACTAAG GCCTTTGTGGAGGCC CAGAATAAGATTACT
 D  P  Y  A  W   L  E  D  P  D   S  E  Q  T  K   A  F  V  E  A   Q  N  K  I  T
GTGCCATTTCTTGAG CAGTGTCCCATCAGA GGTTTATACAAAGAG AGAATGACTGAACTA TATGATTATCCCAAG
 V  P  F  L  E   Q  C  P  I  R   G  L  Y  K  E   R  M  T  E  L   Y  D  Y  P  K
TATAGTTGCCACTTC AAGAAAGGAAAACGG TATTTTTATTTTTAC AATACAGGTTTGCAG AACCAGCGAGTATTA
 Y  S  C  H  F   K  K  G  K  R   Y  F  Y  F  Y   N  T  G  L  Q   N  Q  R  V  L
TATGTACAGGATTCC TTAGAGGGTGAGGCC AGAGTGTTCCTGGAC CCCAACATACTGTCT GACGATGGCACAGTG
 Y  V  Q  D  S   L  E  G  E  A   R  V  F  L  D   P  N  I  L  S   D  D  G  T  V
GCACTCCGAGGTTAT GCGTTCAGCGAAGAT GGTGAATATTTTGCC TATGGTCTGAGTGCC AGTGGCTCAGACTGG
 A  L  R  G  Y   A  F  S  E  D   G  E  Y  F  A   Y  G  L  S  A   S  G  S  D  W
GTGACAATCAAGTTC ATGAAAGTTGATGGT GCCAAAGAGCTTCCA GATGTGCTTGAAAGA GTCAAGTTCAGCTGT
 V  T  I  K  F   M  K  V  D  G   A  K  E  L  P   D  V  L  E  R   V  K  F  S  C
ATGGCCTGGACCCAT GATGGGAAGGGAATG TTCTACAACTCATAC CCTCAACAGGATGGA AAAAGTGATGGCACA
 M  A  W  T  H   D  G  K  G  M   F  Y  N  S  Y   P  Q  Q  D  G   K  S  D  G  T
GAGACATCTACCAAT CTCCACCAAAAGCTC TACTACCATGTCTTG GGAACCGATCAGTCA GAAGATATTTTGTGT
 E  T  S  T  N   L  H  Q  K  L   Y  Y  H  V  L   G  T  D  Q  S   E  D  I  L  C
GCTGAGTTTCCTGAT GAACCTAAATGGATG GGTGGAGCTGAGTTA TCTGATGATGGCCGC TATGTCTTGTTATCA
 A  E  F  P  D   E  P  K  W  M   G  G  A  E  L   S  D  D  G  R   Y  V  L  L  S
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 A  R  C  L  N   V  D  W  I  P   *
```

**Figure 10:** BLAST result of hPEP contig sequence yielded a 99.9% identity with *Homo sapiens* prolyl endopeptidase from the NCBI database. Changes in nucleotides were shown in **bold**. **A** is the contig (query) sequence, whereas **B** (see next page) is the subject sequence. The sequences were aligned to determine the location of mismatched bases (*http://www.fr33.net/translator.php*).

**B**

```
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 A  R  C  L  N   V  D  W  I  P   *
```

**Figure 10 (cont'd):** BLAST result of hPEP contig sequence yielded a 99.9% identity with Homo sapiens prolyl endopeptidase from the NCBI database. Changes in nucleotides were shown in **bold**. **A** (see previous page) is the contig (query) sequence, whereas **B** is the subject sequence. The sequences were aligned to determine the location of mismatched bases (http://www.fr33.net/translator.php).

## Transformation of Expression Host with pLIC-hPEP

Prior to transforming the pLIC-hPEP into the expression host BL21(DE3)+pTF-S *E. coli* cell line, the host had to be made competent by using the $CaCl_2$ Tris buffer method. A glycerol stock of the BL21s was used inoculate an overnight culture of LB broth containing 34 µg/mL chloramphenicol. 100 µL of the overnight culture was used to inoculate a fresh culture of LB+chlor. The optical density at 600nm was measured until it reached approximately 0.6 (Table 14). After achieving this OD, the cells were carried through the $CaCl_2$ Tris protocol and made competent. An aliquot of the competent cells was transformed with the pLIC-hPEP construct. For the transformation, two different amounts of plasmid DNA were used (2 µL and 5 µL). The cells were plated in duplicate at aliquots of 100 µL and 50 µL; the remaining volume was centrifuged, decanted, re-suspended, and then plated. The number of colonies that grew after the transformation were recorded and are listed in Table 15. Only the transformation involving 2 µL of plasmid DNA produced colonies. Increasing plasmid DNA to 5 µL likely resulted in decreased transformation efficiency. The colonies that grew were screened by PCR for hPEP. The PCR products were loaded onto a 1% agarose gel for confirmation (Figure 11). Out of the three colonies screened, only 1 showed a band of ~2133 bp. The colony that was shown to be a successful transformant was cloned and subcultured into several glycerol stocks

**Table 14:** The optical density at 600nm of BL21(DE3)+pTF-S was measured intermittently until the culture reached an approximate OD of 0.6.

| **BL21(DE3)+pTF-S** | |
|---|---|
| **Time** | **$OD_{600}$** |
| **Start** | **0.01** |
| **60 min** | **0.0479** |
| **183 min** | **0.0675** |
| **278 min** | **0.2664** |
| **317 min** | **0.568** |

**Table 15**: The number of colonies/potential transformants that grew following the transformation of BL21(DE3)-pTF-S cells with the hPEP construct. The BL21 cells grew lawns on each control plate, indicating the cells remained viable through the transformation protocol. Only cells transformed with 2 µL of construct produced colonies. Cells transformed with 5 µL of construct exhibited decreased transformation efficiency.

| Sample (V:I)→<br>Volume ↓ | 2 µL hPEP | 5 µL hPEP |
|---|---|---|
| 100 µL | 2 | No Growth |
| 100 µL | No Growth | No Growth |
| 50 µL | 1 | No Growth |
| 50 µL | No Growth | No Growth |
| Resuspended | No Growth | No Growth |
| Control 1 | Control 2 | Control 3 |
| LAWN | LAWN | LAWN |


**Figure 11: Confirmation of pLIC-hPEP construct in BL21(DE3)+pTF-S.**

## Expression and Purification of hPEP

Several expression studies were performed. The methods for optimized expression are as listed in the Material and Methods. Of the studies, two yielded sufficient activity for kinetic analysis. For these studies, six liter cultures of the BL21(DE3)pTF-S + pLIC-hPEP were grown in 2x LB + Kan + chlor. At an $OD_{600}$ of approximately 0.6, IPTG was added to a final concentration of 4 mM, inducing enzyme expression. The culture continued to grow overnight and was harvested the following day. The lysates collected from this were run over a Ni affinity column. The enzyme was eluted using an increasing gradient of imidazole. Fractions were collected in 5 mL increments and screened for activity using GPpNA assays. Fractions that demonstrated activity were pooled and concentrated using Centricep® Centrifugal filter devices with a Ultracel® 50K membrane.

## Determination of Km and Vmax of hPEP

GPpNA assays were conducted using concentrated hPEP isolated from two different expression experiments. The substrate concentration was varied, allowing for the determination of Km and Vmax by taking the slopes of the linear portions of graphs. First, the amount of pNA in µMol released was plotted versus time (Figure 12). The slopes were determined by linear regression. The magnitude of the slope is the velocity ($V_o$) at which pNA is released in µMol/min.

Plotting $V_o$ Vs substrate concentration ([S]) produces the Michaelis-Menton plot (Figure 13, A) whereas taking the inverse of the points ($1/V_o$ and 1/[S]) yields the Line-weever-Burk plot (Figure 13, B). Using linear regression analysis, the Km and Vmax of hPEP was determined to be 9.9 µM ± 0.7 and 4.0 µmol/min ± 1 respectively.

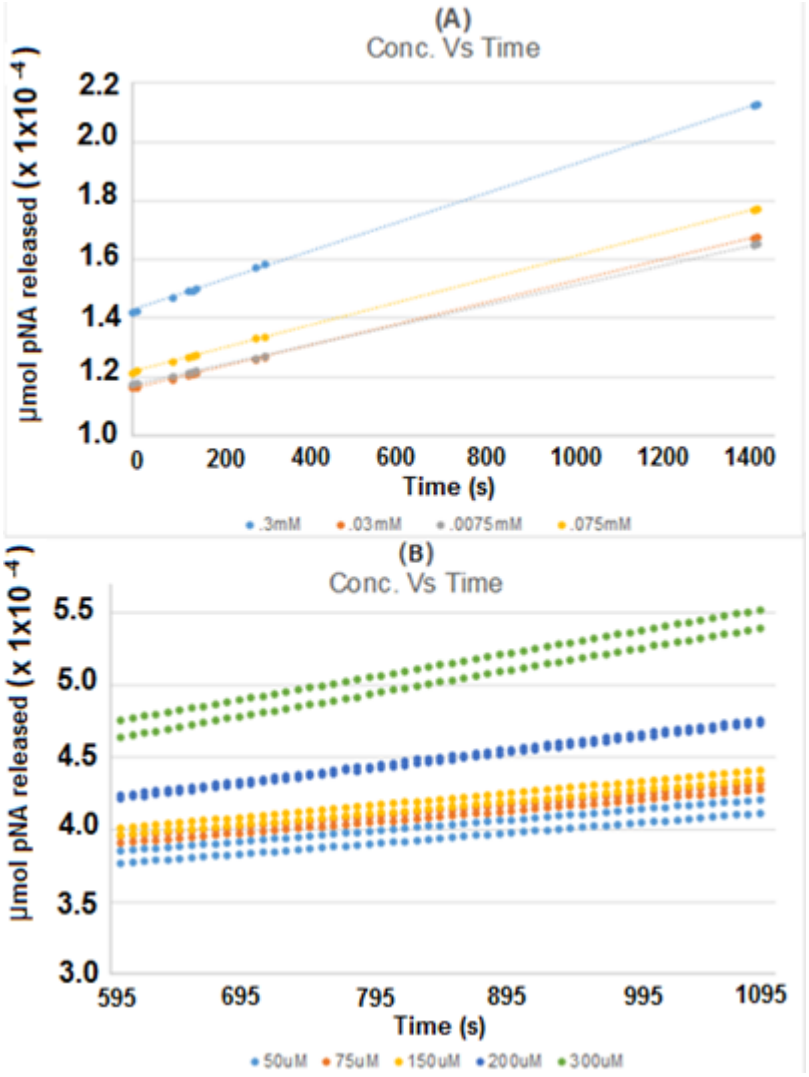


**Figure 12: Varied substrate for determining the $K_m$ of hPEP**. GPpNA assays were conducted using hPEP isolated from two different expression experiments (A) and (B). The assays were run at 410 nm and the absorbance was measured every 9 seconds for 1 hour. The substrate concentration was varied, allowing for the determination of Km and Vmax by taking the slopes of the linear portions of the graphs.

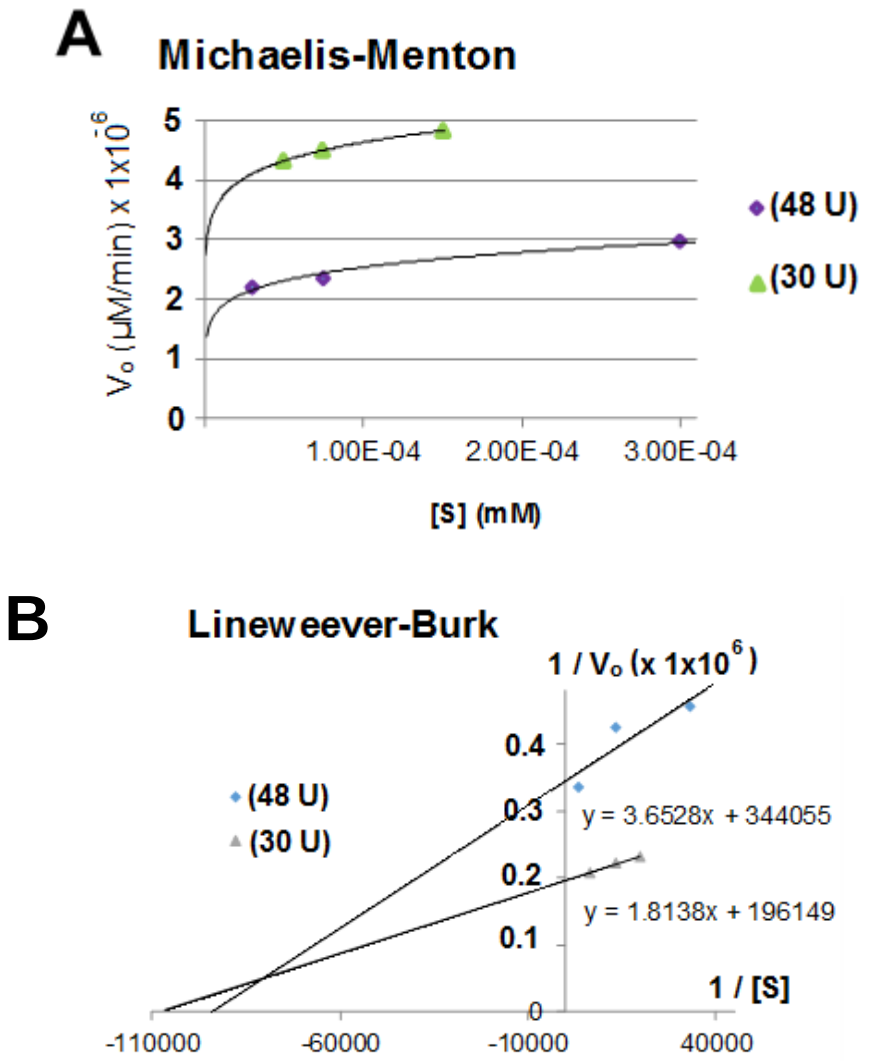


**Figure 13: Michaelis-Menton and Lineweever Burk plots from GPpNA assays of hPEP.** GPpNA assays were performed using two different batches of enzyme, containing 48 U and 30 U. The slopes of each line from Conc. Vs Time (A) and (B) were determined by linear regression. The magnitude of the slope is the velocity ($V_o$) at which pNA is released in µMol/min. Plotting $V_o$ Vs substrate concentration ([S]) produces the Michaelis-Menton plot, whereas taking the inverse of the points ($1/V_o$ and 1/[S]) gives the Line-weever-Burk plot. Using linear regression analysis, the Km's were estimated by determining 1/x-intercept.

## Determination of Total Protein in hPEP Samples for SDS-PAGE

To determine the total protein content within the cell lysates, a Bradford assay was performed. This was done by comparing the absorbance values of the lysates to that of BSA on linear portion of the curve and extrapolating the concentration. Known concentrations of BSA from 0.6 µg/mL to 10.0 µg/mL were used to plot the standard curve (Figure 14, A). The linear portion of the curve comprised 0.6 µg/mL to 2.0µg/mL BSA, shown in Figure 14, B. The total protein content of the concentrated hPEP samples from the two expression studies was measured to be 120 µg/µL and 70 µg/µL, respectively. A sample from two different concentrated flow-through volumes (produced while washing the nickel bound enzyme resin) were measured for total protein content, which was determined to be 22 µg/µL and 30 µg/µL, respectively.

For SDS-PAGE visualization, 20 µg of each sample was loaded. Lanes 1 and 3 were loaded with the flow-through from the two expression studies, while lanes 2 and 4 were loaded with the concentrated hPEP fractions. Lane 5 was loaded with a concentrated and purified sample of spPEP for reference. The resulting gel (Figure 15) demonstrated a band consistent with the expected 70 kD for hPEP in each of the sample lanes. The lane loaded with spPEP also showed a band at approximately 70 kD. In each lane containing hPEP, there is a high presence of contaminating proteins, indicating low purification.

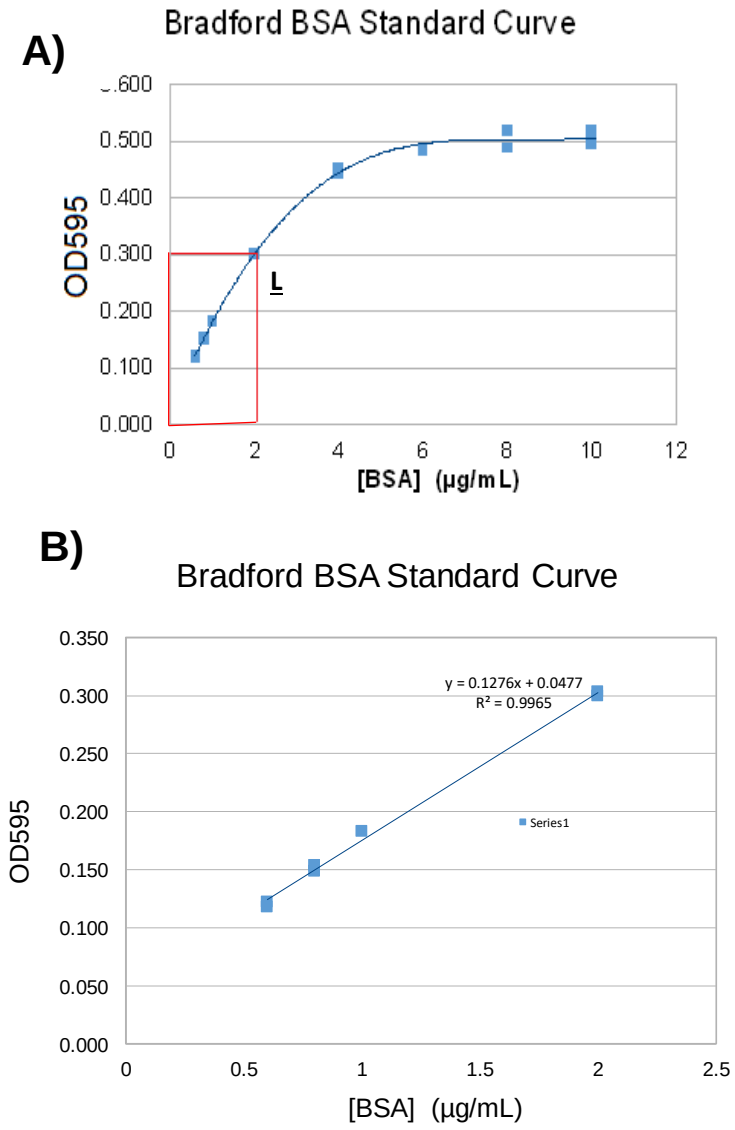


**Figure 14: Bradford assay standard curve for the absorbance of BSA.** The Bradford assay was performed to measure total protein content in the cell lysates. This was done by comparing the absorbance values of the lysates to that on linear portion of the curve and extrapolating the concentration. Known concentrations of BSA from 0.6 µg/mL to 10.0 µg/mL were used to plot the standard curve. The linear portion of the curve comprised 0.6 µg/mL to 2.0µg/mL BSA. The complete curve is shown in **(A)**. The linear portion shown within the red rectangle (**L**) is shown on larger scale **(B)**.

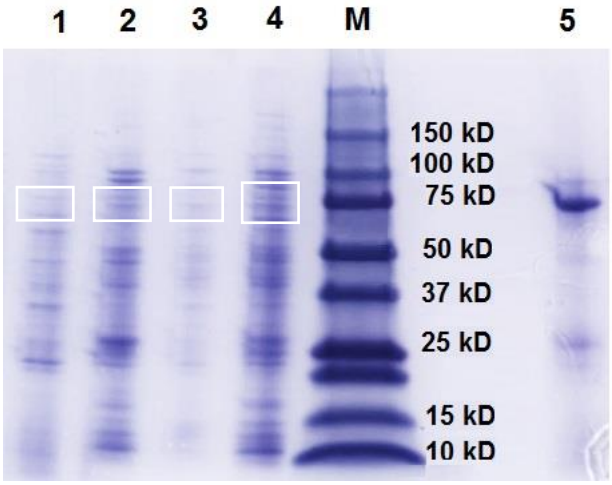


**Figure 15: The lysates and concentrated fractions of hPEP were analyzed using SDS-PAGE.** Lanes 1 and 3 correspond to the concentrated flow-through samples. Lanes 2 and 4 represent two different concentrated samples of pooled fractions that demonstrated hPEP activity. Potential bands that signify hPEP (80 kD) are shown within the white rectangles. Lane 5 contains a purified sample of spPEP for reference. **M** represents Precision Plus Protein™ Standards molecular marker (Bio-RAD, Corp.).

# DISCUSSION

The primary focus of this study was to clone and express recombinant hPEP in BL21(DE3)+pTF-S cells and perform subsequent kinetic studies on the expressed enzyme. The current experiment began with cloning hPEP and the pLIC vector into JM109 and then purifying them for ligation independent cloning. pLIC was digested using SnaBI and thereafter, the vector and hPEP were treated with $T_4$ DNA polymerase to allow for ligation. The pLIC-hPEP was then sequenced and aligned to the sequence published within the NCBI database.

Alignment showed 2 base substitutions and 1 amino acid change for the recombinant hPEP. These sequence variations may be due to differences in the specimen from which they were originally obtained or an error that occurred during sequencing or during amplification by polymerase reaction. To confirm that any mutation that took place, the sequencing could be replicated using multiple clones. If a change did indeed occur, this could be repaired using site directed mutagenesis.

While a change in a single amino acid could be problematic due to possibly changing the conformation of the overall enzyme, it is not expected this

amino acid variation had much impact on the results. hPEP demonstrated hydrolysis of the GPpNA in the assay, indicating that the catalytic triad within the enzyme remained intact.

**Troubleshooting-**

Expression studies of hPEP yielded significant amounts of active enzyme. The purification of hPEP was attempted several times by using a Ni-NTA column with standard protocol for immobilized metal affinity chromatography (IMAC), but the enzyme continued to come off the nickel during the early washes, as well as throughout the imidazole gradient. Modifications to the column purification were made, including adjusting pH, adding urea prior to the wash, and changing the nickel resin. The addition of urea increased the amount of enzyme that came off during the wash before adding imidazole. When reviewing the sequencing data, the 10x his-tag was found to be present on the N-terminus of the enzyme. With this his-tag, the enzyme was expected to stick to the nickel resin well enough to purify by IMAC, but it continued to come off in the wash during every attempt. It is possible that the his-tag was shielded by a hydrophobic region of the enzyme and therefore only bound weakly to the nickel if at all.

The SDS-PAGE gels had multiple bands throughout, including a few that are consistent with the theoretical molecular weight of hPEP (Figure 15). The gels were inconclusive due to the large presence of contaminating proteins that

came off the column with the hPEP. To correct for this, the enzyme in study must first be purified and then analyzed by SDS-PAGE.

**Future Studies-**

To solve the problem of hPEP not sticking to the nickel resin, a new recombinant hPEP will be designed to have a 10x his-tag on the C-terminus. Following similar steps as detailed in this study, the recombinant hPEP will be cloned into the expression host. Future expression studies using the recombinant hPEP will be performed, which is expected to then be able to be purified using Ni-NTA IMAC. The purified enzyme will be used for inhibition studies for side-by-side comparison of hPEP to spPEP.

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# APPENDIX A

Alignment of Contig to hPEP cDNA

```
Contig   ATGCTGTCCCTTCAGTACCCCGACGTGTACCGCGACGAGACCGCCGTACAGGATTATCAT   60
hPEP     ATGCTGTCCCTTCAGTACCCCGACGTGTACCGCGACGAGACCGCCGTACAGGATTATCAT   60
         ************************************************************

Contig   GGTCATAAAATTTGTGACCCTTACGCCTGGCTTGAAGACCCCGACAGTGAACAGACTAAG   120
hPEP     GGTCATAAAATTTGTGACCCTTACGCCTGGCTTGAAGACCCCGACAGTGAACAGACTAAG   120
         ************************************************************

Contig   GCCTTTGTGGAGGCCCAGAATAAGATTACTGTGCCATTTCTTGAGCAGTGTCCCATCAGA   180
hPEP     GCCTTTGTGGAGGCCCAGAATAAGATTACTGTGCCATTTCTTGAGCAGTGTCCCATCAGA   180
         ************************************************************

Contig   GGTTTATACAAAGAGAGAATGACTGAACTATATGATTATCCCAAGTATAGTTGCCACTTC   240
hPEP     GGTTTATACAAAGAGAGAATGACTGAACTATATGATTATCCCAAGTATAGTTGCCACTTC   240
         ************************************************************

Contig   AAGAAAGGAAAACGGTATTTTTATTTTTACAATACAGGTTTGCAGAACCAGCGAGTATTA   300
hPEP     AAGAAAGGAAAACGGTATTTTTATTTTTACAATACAGGTTTGCAGAACCAGCGAGTATTA   300
         ************************************************************

Contig   TATGTACAGGATTCCTTAGAGGGTGAGGCCAGAGTGTTCCTGGACCCCAACATACTGTCT   360
hPEP     TATGTACAGGATTCCTTAGAGGGTGAGGCCAGAGTGTTCCTGGACCCCAACATACTGTCT   360
         ************************************************************

Contig   GACGATGGCACAGTGGCACTCCGAGGTTATGCGTTCAGCGAAGATGGTGAATATTTTGCC   420
hPEP     GACGATGGCACAGTGGCACTCCGAGGTTATGCGTTCAGCGAAGATGGTGAATATTTTGCC   420
         ************************************************************

Contig   TATGGTCTGAGTGCCAGTGGCTCAGACTGGGTGACAATCAAGTTCATGAAAGTTGATGGT   480
hPEP     TATGGTCTGAGTGCCAGTGGCTCAGACTGGGTGACAATCAAGTTCATGAAAGTTGATGGT   480
         ************************************************************

Contig   GCCAAAGAGCTTCCAGATGTGCTTGAAAGAGTCAAGTTCAGCTGTATGGCCTGGACCCAT   540
hPEP     GCCAAAGAGCTTCCAGATGTGCTTGAAAGAGTCAAGTTCAGCTGTATGGCCTGGACCCAT   540
         ************************************************************

Contig   GATGGGAAGGGAATGTTCTACAACTCATACCCTCAACAGGATGGAAAAAGTGATGGCACA   600
hPEP     GATGGGAAGGGAATGTTCTACAACTCATACCCTCAACAGGATGGAAAAAGTGATGGCACA   600
         ************************************************************

Contig   GAGACATCTACCAATCTCCACCAAAAGCTCTACTACCATGTCTTGGGAACCGATCAGTCA   660
hPEP     GAGACATCTACCAATCTCCACCAAAAGCTCTACTACCATGTCTTGGGAACCGATCAGTCA   660
         ************************************************************

Contig   GAAGATATTTTGTGTGCTGAGTTTCCTGATGAACCTAAATGGATGGGTGGAGCTGAGTTA   720
hPEP     GAAGATATTTTGTGTGCTGAGTTTCCTGATGAACCTAAATGGATGGGTGGAGCTGAGTTA   720
         ************************************************************
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Contig   TCTGATGATGGCCGCTATGTCTTGTTATCAATAAGGGAAGGATGTGATCCAGTAAACCGA   780
hPEP     TCTGATGATGGCCGCTATGTCTTGTTATCAATAAGGGAAGGATGTGATCCAGTAAACCGA   780
         ************************************************************
Contig   CTCTGGTACTGTGACCTACAGCAGGAATCCAGTGGCATCGCGGGAATCCTGAAGTGGGTA   840
hPEP     CTCTGGTACTGTGACCTACAGCAGGAATCCAGTGGCATCGCGGGAATCCTGAAGTGGGTA   840
         ************************************************************
Contig   AAACTGATTGACAACTTTGAAGGGGAATATGACTACGTGACCAATGAGGGGACGGTGTTC   900
hPEP     AAACTGATTGACAACTTTGAAGGGGAATATGACTACGTGACCAATGAGGGGACGGTGTTC   900
         ************************************************************
Contig   ACATTCAAGACGAATCGCCAGTCTCCCAACTATCGCGTGATCAACATTGACTTCAGGGAT   960
hPEP     ACATTCAAGACGAATCGCCAGTCTCCCAACTATCGCGTGATCAACATTGACTTCAGGGAT   960
         ************************************************************
Contig   CCTGAAGAGTCTAAGTGGAAAGTACTTGTTCCTGAGCATGAGAAAGATGTCTTAGAATGG   1020
hPEP     CCTGAAGAGTCTAAGTGGAAAGTACTTGTTCCTGAGCATGAGAAAGATGTCTTAGAATGG   1020
         ************************************************************
Contig   ATAGCTTGTGTCAGGTCCAACTTCTTGGTCTTATGCTACCTCCATGACGTCAAGAACATT   1080
hPEP     ATAGCTTGTGTCAGGTCCAACTTCTTGGTCTTATGCTACCTCCATGACGTCAAGAACATT   1080
         ************************************************************
Contig   CTGCAGCTCCATGACCTGACTACTGGTGCTCTCCTTAAGACCTTCCCGCTCGATGTCGGC   1140
hPEP     CTGCAGCTCCATGACCTGACTACTGGTGCTCTCCTTAAGACCTTCCCGCTCGATGTCGGC   1140
         ************************************************************
Contig   AGCATTGTAGGGTACAGCGGTCAGAAGAAGGACACTGAAATCTTCTATCAGTTTACTTCC   1200
hPEP     AGCATTGTAGGGTACAGCGGTCAGAAGAAGGACACTGAAATCTTCTATCAGTTTACTTCC   1200
         ************************************************************
Contig   TTTTTATCTCCAGGTATCATTTATCACTGTGATCTTACCAAAGAGGAGCTGGAGCCAAGA   1260
hPEP     TTTTTATCTCCAGGTATCATTTATCACTGTGATCTTACCAAAGAGGAGCTGGAGCCAAGA   1260
         ************************************************************
Contig   GTTTTCCGAGAGGTGACCGTAAAAGGAATTGATGCTTCTGATTACCAGACAGTCCAGATT   1320
hPEP     GTTTTCCGAGAGGTGACCGTAAAAGGAATTGATGCTTCTGATTACCAGACAGTCCAGATT   1320
         ************************************************************
Contig   AAATTGGATGGCTCTCATCCAGCTTTCTTATATGGCTATGGCGGCTTCAACATATCCATC   1440
hPEP     AAATTGGATGGCTCTCATCCAGCTTTCTTATATGGCTATGGCGGCTTCAACATATCCATC   1440
         ************************************************************
Contig   ACACCCAACTACAGTGTTTCCAGGCTTATTTTTGTGAGACACATGGGTGGTATCCTGGCA   1500
hPEP     ACACCCAACTACAGTGTTTCCAGGCTTATTTTTGTGAGACACATGGGTGGTATCCTGGCA   1500
         ************************************************************
Contig   GTGGCCAACATCAGAGGAGGTGGCGAATATGGAGAGACGTGGCATAAAGGTGGTATCTTG   1560
hPEP     GTGGCCAACATCAGAGGAGGTGGCGAATATGGAGAGACGTGGCATAAAGGTGGTATCTTG   1560
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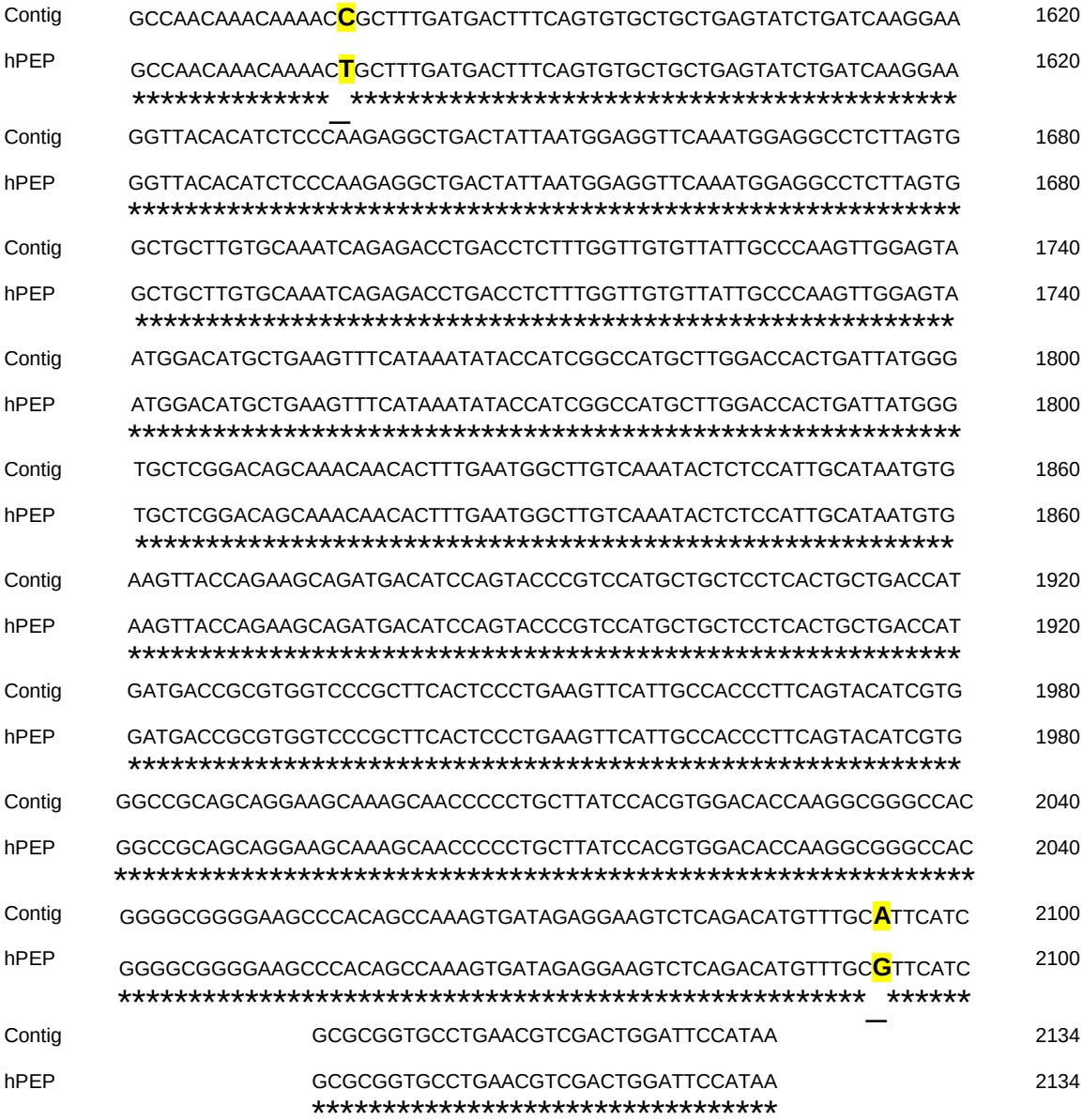
Contig GCCAACAAACAAAACCGCTTTGATGACTTTCAGTGTGCTGCTGAGTATCTGATCAAGGAA 1620
hPEP GCCAACAAACAAAACTGCTTTGATGACTTTCAGTGTGCTGCTGAGTATCTGATCAAGGAA 1620
Contig GGTTACACATCTCCCAAGAGGCTGACTATTAATGGAGGTTCAAATGGAGGCCTCTTAGTG 1680
hPEP GGTTACACATCTCCCAAGAGGCTGACTATTAATGGAGGTTCAAATGGAGGCCTCTTAGTG 1680
Contig GCTGCTTGTGCAAATCAGAGACCTGACCTCTTTGGTTGTGTTATTGCCCAAGTTGGAGTA 1740
hPEP GCTGCTTGTGCAAATCAGAGACCTGACCTCTTTGGTTGTGTTATTGCCCAAGTTGGAGTA 1740
Contig ATGGACATGCTGAAGTTTCATAAATATACCATCGGCCATGCTTGGACCACTGATTATGGG 1800
hPEP ATGGACATGCTGAAGTTTCATAAATATACCATCGGCCATGCTTGGACCACTGATTATGGG 1800
Contig TGCTCGGACAGCAAACAACACTTTGAATGGCTTGTCAAATACTCTCCATTGCATAATGTG 1860
hPEP TGCTCGGACAGCAAACAACACTTTGAATGGCTTGTCAAATACTCTCCATTGCATAATGTG 1860
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hPEP AAGTTACCAGAAGCAGATGACATCCAGTACCCGTCCATGCTGCTCCTCACTGCTGACCAT 1920
Contig GATGACCGCGTGGTCCCGCTTCACTCCCTGAAGTTCATTGCCACCCTTCAGTACATCGTG 1980
hPEP GATGACCGCGTGGTCCCGCTTCACTCCCTGAAGTTCATTGCCACCCTTCAGTACATCGTG 1980
Contig GGCCGCAGCAGGAAGCAAAGCAACCCCCTGCTTATCCACGTGGACACCAAGGCGGGCCAC 2040
hPEP GGCCGCAGCAGGAAGCAAAGCAACCCCCTGCTTATCCACGTGGACACCAAGGCGGGCCAC 2040
Contig GGGGCGGGGAAGCCCACAGCCAAAGTGATAGAGGAAGTCTCAGACATGTTTGCATTCATC 2100
hPEP GGGGCGGGGAAGCCCACAGCCAAAGTGATAGAGGAAGTCTCAGACATGTTTGCGTTCATC 2100
Contig GCGCGGTGCCTGAACGTCGACTGGATTCCATAA 2134
hPEP GCGCGGTGCCTGAACGTCGACTGGATTCCATAA 2134

# VITA

After graduating from Oklahoma State University with a Bachelor of Science in Microbiology/Cell and Molecular Biology: Microbial Pathogenesis, Travis K. Moore enrolled at Stephen F. Austin State University and joined Dr. Clack's laboratory, from which he received the degree of Master of Science in Biotechnology in May, 2017.


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