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Cloning and Expression of Bovine Alpha S1 Casein Peptides of Variable Size and Position of spPEP Inhibitor Sequence

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By

AVLEEN KAUR VISHRAM, B.Sc. in Biotechnology

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Cloning and Expression of Bovine Alpha S1 Casein Peptides of Variable Size and Position of spPEP Inhibitor Sequence

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AVLEEN VISHRAM, B.Sc. in Biotechnology

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ABSTRACT

Eurygaster integriceps Puton (Sunn pest) is the most damaging insect pest of wheat in areas of North Africa, Eastern Europe and Western and Central Asia. Sunn pest injects an enzyme characterized as a prolyl endylprotease (spPEP) into wheat grains that digests the gluten protein (Darkoh et al., 2010). Previous studies have shown that casein peptides generated by *Lactobacillus* species have inhibitory effects on human (hPEP) and bacterial prolyl endoproteases (bPEP) which makes casein a good natural candidate to inhibit spPEP. Developing biopesticides for food products from αS1 casein is complicated due to some allergenic amino acid sequences. Ruiter et al (2005) identified a non-allergenic peptide region on αS1 casein that includes a previously identified inhibitory sequence (LNENLLRFFVAPFPEVFG) to hPEP (Jeanneret, 2011). The Objective of this study was to produce 16 recombinant peptides derived from αS1casein of varying lengths and varying placement of the non-allergenic inhibitory sequence. The inhibitory region is positioned at 0, 10, 20 and 30 amino acids from either N' terminal or C-terminal end. Each of the peptides were cloned into pET15b for expression with a His tag for purification and thrombin site for removal of His-tag Peptides were incubated with either hPEP or spPEP in the presence of the

substrate GPpNA. We report the inhibition of both enzymes based on size and placement of the inhibitory sequence.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

PEP	**-** Prolyl endopeptidase
SpPEP	**-** Sunn pest prolyl endopeptidase
hPEP	**-** Human prolyl endopeptidase
bPEP	**-** Bacterial prolyl endopeptidase
BSA	**-**Bovine serum albumin
GPpNA	**-**Gly-Pro-p-nitroanalide
SDS-PAGE	**-**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
CEP	**-**Cell Envelope protein
PCR	**-**Polymerase Chain Reaction
IPTG	**-**Isopropyl β-D-1-thiogalactopyranoside
APS	**-**Ammonium Per Sulfate
TEMED	**-** Tetramethylethylenediamine

INTRODUCTION

Eurygaster integriceps Puton (Sunn pest)

Eurygaster integriceps Puton (Sunn pest; Heteroptera, Scutelleridae) is the most important insect pest of *Triticum aestivum L. subsp. aestivum* (bread wheat) and *T. turgidum L. Subsp. durum* (durum wheat) in West and Central Asia, Eastern Europe and North Africa (Kinaci *et al*., 2004, Rassipour *et al*., 1996). Sunn pest causes major damage in wheat crops by feeding on grains, leaves and stems. During this feeding process, amylases and proteases contained in the saliva are deposited into the grain. Only 3-5% of the wheat grains in a harvest may be contaminated which destroys the entire lot of wheat grain by making the dough sticky and having poor rising capability in bread. Previous studies indicate that one of these enzymes is a prolyl endoprotease (PEP) responsible for degrading gluten (Darkoh *et al*., 2010, Saadati and Bandani, 2011).

Prolyl endopeptidase (PEP)

PEPs are serine proteases that hydrolyze proline-containing peptides at the carboxylic ends of internal proline residues (Polgar, 2002). PEP has been

identified in many organisms including bacteria, archeabacteria, fungi, animals and plants. Most PEPs recognize comparatively short peptides 30 KDa or smaller as substrates. Sunn pest PEP (spPEP) is unique as it cleaves peptides and whole proteins greater than 30 KDa, the wheat gluten ranges from 30-130 KDa. Substrate specificity of PEP has been investigated primarily in mammalian PEP due to its medical implications and involvement in Alzheimer's disease, Parkinson's disease, depression, anorexia and other neurological disease states (Hannula, 2013). With 50% sequence similarity between the spPEP and mammalian PEPs, their catalytic functions are essentially the same except for the size of substrates. This size difference may provide a way to develop inhibitors specific for spPEP and not affect mammalian PEPs.

Gluten as a Target Substrate for spPEP

Wheat is a major source of protein, dietary fiber and energy. Out of the total protein content of wheat, 80-85% is gluten (Skerritt and Hill, 1991). Gluten (10-130 KDa) is a major storage visco-elastic protein. Based on solubility in alcohol and water, gluten can be classified into two types- gliadins and glutenins respectively (Koning, 2005). Further subdivisions of glutenins consist of 80-160 KDa high molecular weight (HMW) and 40-45 KDa low molecular weight (LMW) proteins. Gliadin (30-45 KDa) subdivisions are α-gliadins, β-gliadins, γ-gliadins and ω-

gliadins (Koning, 2005). Both gliadins and glutenins together are called prolamin due to being rich in glutamine (30-55%) and proline (15-30%) (Koning, 2005, Anjum et al., 2007). The high frequency of proline residues makes these prolamines a good substrate for the spPEP.

Need for Inhibitors of spPEP

The major control of Sunn pest is the use of pesticides but it is expensive, non-selective and the sunn pest develops resistant to the pesticides. Moreover, pesticides are often hazardous to human health and the environment (Critchley, 1998). Integrated Pest Management (IPM) is a combination of strategies to reduce pest damage such as rotation of crops, production of pest-resistant plants and use of environmentally friendly biopesticides such as parasitic fungi (Saadati and Bandani, 2011). Some small organic molecules such as pyrrolidine derivatives, alkaloids, heteroaryl ketones and peptide-like inhibitors are being used as inhibitors to mammalian PEP (hPEP). These inhibitors, however, are expensive and are not meant for general human consumption as they will inhibit mammalian PEP (Juillerat-Jeanneret, 2010). A more practical solution will be to identify natural environmentally friendly inhibitors that selectively inhibit spPEP and do not affect mammalian PEP (hPEP).

Peptides from casein generated by incubating casein with *Lactobacillus* have been shown to inhibit mammalian PEPs (Juillerat-Jeanneret, 2010). Similar casein digests have been shown to also inhibit the spPEP (Hargrove, 2013). *Lactobacillus* contains a cell envelope protein (CEP) that is expressed in the presence of casein which is responsible for the digestion of α and β caseins in milk. A recombinant source of casein peptide would provide a large amount of potential inhibitor and have lower production cost compared to chemical pesticides used to control Sunn pests. To make the casein inhibitors specific for spPEP and not inhibitory to mammalian PEPs, we hypothesized that if the length of the inhibitor is extended to greater than 30KD, then the inhibitors will be specific for the spPEP and not affect mammalian PEP.

Bovine Casein Proteins and Their Function

Bovine milk caseins are the predominant phosphoproteins existing as four polypeptides such as α_{S1} (39-46% of total caseins), α_{S2} (8-11% of total caseins), *β* (25-35% of total caseins) and *κ* (8-15% of total caseins) caseins (Stewart *et.al*, 1984, Srinivas *et al*, 2010, Pepe *et al*., 2013). In bovine milk, 80% of the total milk protein is casein and 20% is whey. α_{S1-} and *β-casein* are the most abundant proteins at 10-12 mg/mL and 10mg/mL respectively, α_{S2-} casein and *κ*- caseins are present at lower levels, 3.7 and 3.4 mg/mL respectively. The caseins are

encoded by single copy genes clustered in a region of about 200 kb on bovine chromosome 4 (Rijnkels, 1995). As per Griffiths *et al* (2013), *Lactobacillus* digested casein peptides have an inhibitory effect when incubated with mammalian PEP. Moreover, the casein derived inhibitory peptides were not harmful to human colon cells (Juillerat-Jeanneret *et al*. 2011) suggesting that guarding against any allergenic reactions, these natural inhibitors would be safe for human consumption as well as the environment. Besides acting as a transporter for calcium and phosphate in newborns, caseins provide numerous bioactive molecules. The bioactive molecules have been shown to play several physiological roles including defense against pathogenic bacteria, enhancement of immune function, antiviral and antioxidant activities (Pepe *et al*, 2013).

Use of αS1 Casein to inhibit spPEP

αS1 Casein consists of 214 amino acids. Within the casein sequences, a wide range of bioactive peptides are encoded of which some show more than one biological activity including inhibitory effects when digested and smaller inhibitory peptides are released. Previous studies have shown that casein peptides generated by *Lactobacillus species* have inhibitory effects on human (hPEP) and bacterial prolyl endopeptidases (bPEP) which makes casein a good natural candidate against spPEP. However, developing biopesticides for food products

from α_{S1} casein is complicated due to some people having allergies to selected caseins. Ruiter et al (2005) identified both allergenic and non-allergenic peptide regions in α_{S1} casein, one of the non-allergenic regions contained the 18-amino acid long peptide 'LNENLLRFFVAPFPEVFG' that also has been identified as an inhibitory sequence to hPEP ((Juillerat-Jeanneret *et al*. 2010). As a goal of this study, instead of digesting the α_{S1} casein by *Lactobacillus helveticus*, 16 potential inhibitory peptides were synthesized using bovine α_{S1} casein as a template (Fig.1). These 16 amplicons contained the common inhibitory sequence with additional amino acid sequences within the non-allergenic region to vary the length and position of the inhibitory sequence.

Even though the spPEP is unique in its substrate recognition, it has some features in common with post-proline cleaving enzymes in the S9 family of proteases such as a conserved catalytic triad consisting of Ser, Asp and His located between the two lobes of the protein (Laskar et al., 2012). The spPEP recognizes G-P-pNA substrate like other PEP's.

The natural substrate for spPEP is gluten (36-140KDa) but the natural substrate for hPEP is <30KDa (Polgar, 2002). By considering the difference in the substrate size affinity for both spPEP and hPEP, we expect to see differential inhibition between spPEP and hPEP based on substrate size among these 16 peptides. Long term studies will focus on the mechanism of spPEP inhibition and the substrate binding process.

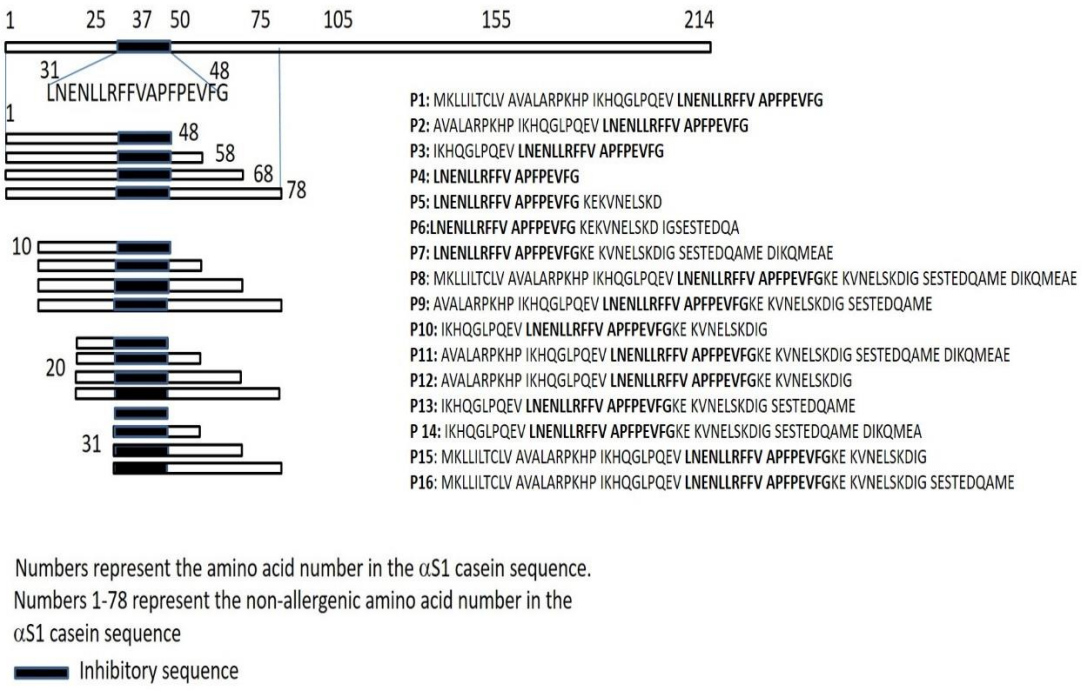


Figure 1: Diagrammatic representation of 16 amplicons with common inhibitory sequence. The black block in each insert represent the 18mer inhibitory sequence.

MATERIALS AND METHODS

Sequences of casein from NCBI

Bovine α_{S1} casein consists of 214 amino acids. The nucleotide sequences of bovine α_{S1} casein is shown in Figure 2 (a), (b) as deposited in the National Center for Biotechnology Information (NCBI).

Primers Design

The casein nucleotide and protein sequences were taken from NCBI database. α_{S1} Casein has 214 amino acids and the inhibitory region is 18 amino acid long (31-48 aa) shown as the bold-faced part of the protein (Figure 2b). Based on these sequences, four primer pairs were designed using primer-BLAST program, four forward primers with a *NdeI* site (CAATG) and four reverse primers with a *Hind III* site after a stop codon (TAG, TAA, TGA) as listed in table 1. Primers were ordered from Sigma Genosys (Sigma Aldrich, The Woodlands, TX). Figure 3 illustrates the positions of forward and reverse primers and expected amplicons with their respective base pair length.

(a) **>gi|31341348|ref|NM_181029.2| Bos taurus casein alpha s1 (CSN1S1), mRNA**

AGTAGGTTTAAATAGCTTGGAAGCAAAAGTCTGCCATCACCTTGATCATCAACCCAGCTTGCTGCTTCTTCCCAGTCT
TGGGTTCAAGATCTTGACAACC**ATG**AAACTTCTCATCCTTACCTGTCTTGTGGCTGTTGCTCTTGCTAGGCCTAAACA
TCCTATCAAGCACCAAGGACTCCCTCAAGAAGTCCTCAATGAAAATTTACTCAGGTTTTTTGTGGCACCTTTTCCAG
AAGTGTTTGGAAAGGAGAAGGTCAATGAACTGAGCAAGGATATTGGGAGTGAATCAACTGAGGATCAAGCCATGG
AAGATATTAAGCAAATGGAAGCTGAAAGCATTTCGTCAAGTGAGGAAATTGTTCCCAATAGTGTTGAGCAGAAGCA
CATTCAAAAGGAAGATGTGCCCTCTGAGCGTTACCTGGGTTATCTGGAACAGCTTCTCAGACTGAAAAAATACAAA
GTACCCCAGCTGGAAATTGTTCCCAATAGTGCTGAGGAACGACTTCACAGTATGAAAGAGGGAATCCATGCCCAA
CAGAAAGAACCTATGATAGGAGTGAATCAGGAACTGGCCTACTTCTACCCTGAGCTTTTCAGACAATTCTACCAGC
TGGATGCCTATCCATCTGGTGCCTGGTATTACGTTCCACTAGGCACACAATACACTGATGCCCCATCATTCTCTGAC
ATCCCTAATCCTATTGGCTCTGAGAACAGTGAAAAGACTACTATGCCACTGTGGTGAGGAGTCAAGTGAATTCTGA
GGGACTCCACAGTTATGGTCTTTGATGGTTCTGAAAATTCCATGCTCTACATGTCTTTTCATCTATCATGTCAAACCAT
TCTATCCAAAGGCTTCAACTGCTGTTTTAGAATAGGGCAATCTCAAATTGAAGGCACTCTTTCTTCTTGAGTTCTCTAC
TGTATTTTAGATAGTGTAACATCCTTAAGTGAAATTGTCCTAACAGCTTGTTACCTAAATTCCAGTAGTATCATGCTGGT
ATAAAGGCCACTGAGTCAAAGGGATTAAAGTCTTCATTAAATTTCTGTATGGAAAATGTTTTAAAAGCCTTTGAATCAC
TTCTCCTGTAAGTGCCATCATATCAAATAATTGTGTGCATTAACTGAGATTTTGTCTTTCTTCTTTTCAATAAATTACATT
TTAAGGCACT

(b) >gi|81673527|gb|AAI09619.1| Casein alpha s1 [Bos taurus]
MKLLILTCLVAVALARPKHPIKHQGLPQEV**LNENLLRFFVAPFPEVFG**KEKVNELSKDIGSESTEDQAME
DIKQMEAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKE
GIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTT
MPLW

Figure 2(a): The nucleotide sequences of α_{S1} casein taken from NCBI database**.** The bold part shown in the nucleotide sequence is the longest sequence of ORF starting with ATG and ending with a stop codon. **(b)** The amino acid sequence of α_{S1} casein. The bold part shows the inhibitory sequence.

Primers	Sequences
a_caseinf1	*5'- AGC**CATATG**AACTTCTCATCCTTACC -3'*
a_caseinf2	*5'- AGC**CATATG**GCTGTTGCTCTTG -3'*
a_caseinf3	*5'- AGC**CATATG**ATCAAGCACCAAGG -3'*
a_caseinf4	*5'- AGC**CATATG**CTCAATGAAAATTTACTCAG- 3'*
a_caseinr1	*5'-AC**AAGCTT**CATCCAAAC**ACT**TCTGG- 3'*
a_caseinr2	*5'- AC**AAGCTT**CAATCCTTGCTCAGTTC**ATT**G -3'*
a_caseinr3	*5'- AC**AAGCTT**CAGGCTTG**ATC**CTCAGTTG- 3'*
a_caseinr4	*5'- AC**AAGCTT**CAGCTTCAGCTTCC**ATT**G- 3'*

Table 1: Gene specific primers designed based on the DNA sequence available in NCBI for α_{S1} casein from bovine. The bold letters in red indicate the *NdeI* restriction site and the blue and green letters indicates *HindIII* restriction site and stop codon in the forward (f) primer sequences and the reverse (r) sequences respectively.

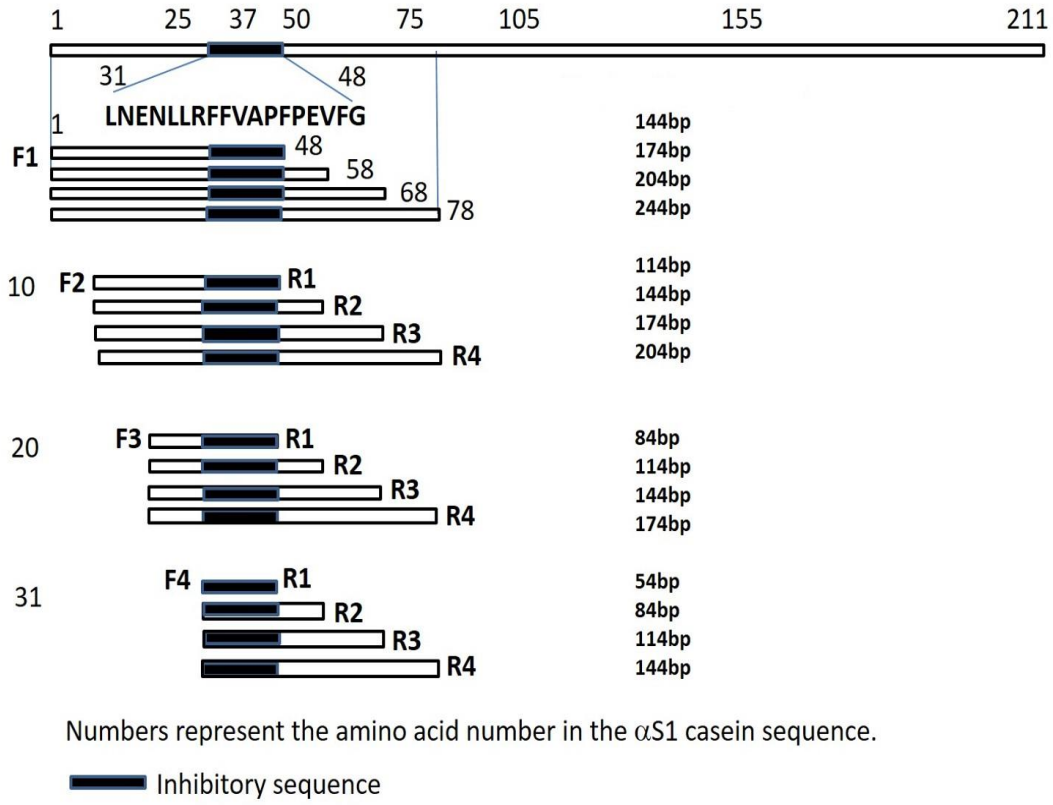


Figure 3: Diagrammatic representation of 16 amplicons with their primer pair and nucleotide number. The black block in each insert represent the 18mer inhibitory sequence

PCR Amplification

The PCR components and the PCR program used are listed in Table 2 and Table 3 respectively. The source of template was αS1 casein previously cloned by Basak (2013). Polymerase Chain Reaction (PCR) was performed using the gene specific primers and primer pairs respective to each amplicon is listed in Table 4. A High fidelity Taq polymerase, Accutaq LA DNA Polymerase (Sigma Aldrich) was used to amplify the sequences.

Agarose Gel Electrophoresis

After amplification, the PCR products were purified and analyzed on a 1.5% (w/v) agarose gel with 1X TAE as running buffer. Agarose gel electrophoresis was performed to verify the amplified product and to separate the expected band from any nonspecific bands (if present) for further gel purification, ethidium bromide was used to visualize the PCR products. For comparison of band size, 10 μL of DNA markers (High-low DNA Ladder, Bionexus Inc.) was added in one lane of each gel. The gel was run with 1X TAE buffer with an additional 5 μL of 5x bromophenol blue loading dye. Each gel was run at 100V for approximately 30 minutes. Ethidium bromide was used to visualize the bands on a Typhoon FLA 9500 (GE Life Sciences). The settings of gel analysis by the typhoon was 482 nm excitation and 605 nm emission.

Table 2: PCR reaction mix. Reaction volume was prepared at 50 µL total volume per tube. A reaction tube was prepared for each individual primer pair.

Components	Concentration (µL)
DNA Template	1 µL
Forward Primer	2.5 µL
Reverse Primer	2.5 µL
2X Reaction Buffer	5 µL
dNTps	2.5 µL
Taq Polymerase	0.5 µL
DI H_20	36 µL
Total Volume	50 µL

Table 3: PCR profile for amplification of 16 amplicons

PCR Steps	**Temp.**	**Time**
Initial Denaturation	95°C	30 sec
Denaturation	95°C	30 sec
Annealing	50°C	30 sec
Extension	68°C	1 min
Cycles (Step 2-4)	**34X**	
Final Extension	68°C	10 min
Store	4°C	∞

Table 4: Inserts with their primer pairs and respective sequences.

Insert	Primer Pair	Primer sequences
Insert 1	**a_caseinf1** **a_caseinr1**	*5'- AGC**CATATG**AACTTCTCATCCTTACC -3'* *5'-ACAAGCTTCATCCAAAC**ACT**TCTGG- 3'*
Insert 2	**a_caseinf2** **a_caseinr1**	*5'- AGC**CATATG**GCTGTTGCTCTTG -3'* *5'-ACAAGCTTCATCCAAAC**ACT**TCTGG- 3'*
Insert 3	**a_caseinf3** **a_caseinr1**	*5'- AGC**CATATG**ATCAAGCACCAAGG -3'* *5'-ACAAGCTTCATCCAAAC**ACT**TCTGG- 3'*
Insert 4	**a_caseinf4** **a_caseinr1**	*5'- AGC**CATATG**CTCAATGAAAATTTACTCAG- 3'* *5'-ACAAGCTTCATCCAAAC**ACT**TCTGG- 3'*
Insert 5	**a_caseinf4** **a_caseinr2**	*5'- AGC**CATATG**CTCAATGAAAATTTACTCAG- 3'* *5'- ACAAGCTTCAATCCTTGCTCAGTTC**ATT**G -3'*
Insert 6	**a_caseinf4** **a_caseinr3**	*5'- AGC**CATATG**CTCAATGAAAATTTACTCAG- 3'* *5'- ACAAGCTTCAGGCTTG**ATC**CTCAGTTG- 3'*
Insert 7	**a_caseinf4** **a_caseinr4**	*5'- AGC**CATATG**CTCAATGAAAATTTACTCAG- 3'* *5'- ACAAGCTTCAGCTTCAGCTTCC**ATT**G- 3'*
Insert 8	**a_caseinf1** **a_caseinr4**	*5'- AGC**CATATG**AACTTCTCATCCTTACC -3'* *5'- ACAAGCTTCAGCTTCAGCTTCC**ATT**G- 3'*
Insert 9	**a_caseinf2** **a_caseinr3**	*5'- AGC**CATATG**GCTGTTGCTCTTG -3'* *5'- ACAAGCTTCAGGCTTG**ATC**CTCAGTTG- 3'*
Insert 10	**a_caseinf3** **a_caseinr2**	*5'- AGC**CATATG**ATCAAGCACCAAGG -3'* *5'- ACAAGCTTCAATCCTTGCTCAGTTC**ATT**G -3'*
Insert 11	**a_caseinf2** **a_caseinr4**	*5'- AGC**CATATG**GCTGTTGCTCTTG -3'* *5'- ACAAGCTTCAGCTTCAGCTTCC**ATT**G- 3'*
Insert 12	**a_caseinf2** **a_caseinr2**	*5'- AGC**CATATG**GCTGTTGCTCTTG -3'* *5'- ACAAGCTTCAATCCTTGCTCAGTTC**ATT**G -3'*
Insert 13	**a_caseinf3** **a_caseinr3**	*5'- AGC**CATATG**ATCAAGCACCAAGG -3'* *5'- ACAAGCTTCAGGCTTG**ATC**CTCAGTTG- 3'*
Insert 14	**a_caseinf3** **a_caseinr4**	*5'- AGC**CATATG**ATCAAGCACCAAGG -3'* *5'- ACAAGCTTCAGCTTCAGCTTCC**ATT**G- 3'*
Insert 15	**a_caseinf1** **a_caseinr2**	*5'- AGC**CATATG**AACTTCTCATCCTTACC -3'* *5'- ACAAGCTTCAATCCTTGCTCAGTTC**ATT**G -3'*
Insert 16	**a_caseinf1** **a_caseinr3**	*5'- AGC**CATATG**AACTTCTCATCCTTACC -3'* *5'- ACAAGCTTCAGGCTTG**ATC**CTCAGTTG- 3'*

Purification of PCR Products

The amplified PCR products were purified directly or by cutting out the band from the agarose gel using the Wizard SV Gel and PCR Clean-Up kit (Promega, Corp.). For processing PCR amplifications, an equal volume of membrane binding solution as provided by the kit was added to the PCR amplified products. For purification of fragments from the gel after electrophoresis, the DNA fragment of interest was excised from the gel using a clean scalpel with minimal volume of agarose in the sample. The weight of an empty 1.5 mL microfuge tube was recorded. The gel slice was transferred to the 1.5 mL microfuge tube and weighed. The weight of the empty tube was subtracted from the weight of the tube containing the gel to get the exact weight of the gel slice alone. Membrane binding solution provided with the kit (Wizard SV Gel and PCR clean-up system) was added to the gel slice at a ratio of 10 microliters per 10 mg of gel slice. The manufacturer's protocol was followed to purify the PCR products.

Measurement of DNA Concentration

After the purification of DNA samples, the concentration of DNA was measured using a Cary 50 Scan spectrophotometer (Agilent Inc., Santa Clara, CA). The instrument was zeroed using nuclease free water as a baseline and the absorbance of DNA was measured from 320 nm to 200 nm to obtain the 260 nm

and 280 nm values. The ratio [A_{260}/A_{280}] was used to check the purity of the DNA samples. For a pure DNA sample, the ratio must be between 1.8-2.0 (Carlos et al. 2001).

The formula used to calculate DNA concentration is as follows:

For a 1-cm path-length, the optical density at 260 nm (A_{260}) equals 1.0 for the following solutions:

a 50 µg /mL solution of dsDNA

1) dsDNA concentration = 50 µg /mL × A_{260} × dilution factor
2) Path length = 1cm, Hence Dilution factor = 10

Total yield was obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA yield (ng) = DNA concentration (ng/µL) × total sample volume (µL)

Cloning of Casein Fragments into the pGEM T-Easy Vector

The cloning procedure comprised ligation and transformation. pGEM T Easy vector (Promega Inc.) was chosen as it is a high copy number plasmid, contains a TA cloning site and contains M13F and M13R sites at the ends of the insert suitable for sequencing. The ligation reaction was performed in a 1.5 mL microcentrifuge tube. The concentration of reagents (insert DNA) was calculated per the technical manual provided with the pGEM-T Easy Vector (Promega Inc.). The recommended insert : vector molar ratio (3:1) was calculated using the following equation. The ligation reaction components were shown in Table 5.

2) $\frac{\text{ng of vector x kb size of insert}}{\text{Kb size of vector}}$ x insert: vector molar ratio = ng of insert

After mixing the reaction components, the ligation reaction was incubated at 4°C overnight.

Transformation

E.coli JM109 competent cells ($\geq$ 1 x 10^8 cfu/μg DNA) were provided with the pGEM-T Easy vector kit II (Promega, Inc.). The tubes containing the ligation reactions shown in the table were centrifuged to collect the contents at the bottom of the tube. Snap top tubes (15mL) were placed on ice with 2 μL of each ligation reaction added to the tubes. High efficiency *E.coli* JM109 competent cells were removed from the -80°C freezer and placed on ice until thawed. Once the cells were gently mixed, 50 μL of these cells were transferred to the tubes containing the 2 μL of ligation reaction. The tubes were then gently flipped to mix well and placed on ice for 15 minutes. The cells were heat shock at 42°C in a water bath without shaking for 45-50 seconds. The tubes were immediately placed back on ice for 10 minutes. In each tube 450 μL of chilled SOC medium (Sigma Aldrich) was added and incubated with shaking at 200 RPM in a New Brunswick 4300 shaker incubator for 1 hour at 37°C for recovery of the host cells. An aliquot

Reaction component	Volume
2X Ligation Buffer	5 µL
pGEM-T Easy Vector	1 µL (50ng/ µL)
PCR product	3 µL (20ng/ µL)
T4 DNA Ligase	1 µL (3 units/ µL)
Nuclease-free water to a final volume of 10 µL	

Table 5: Ligation Reaction Components per 10 µL Total Volume.

of 50 µL and 100 µL of the culture from the transformation mix was spread on individual LB/ ampicillin/IPTG/X-Gal (100 µg/µL ampicillin, 400 mM IPTG, 50 µg/µL X-Gal) plates in duplicate. The plates were then incubated overnight at 37°C. The successful cloning of an insert into pGEM-T Easy vector should result in white colonies on the plates due to disruption of β- Galacotsidase activity with X-Gal and IPTG. For each casein insert, one grid plate was prepared. White colonies were first transferred to the grid plates (figure 4) using a pipette tip. The remaining cells on the tip were used for preparing the overnights for miniprep DNA isolation by rinsing the remaining bacteria on the tip into 10 mL of LB/amp media (100 µg/µL) in a sterile polypropylene 15mL conical centrifuge tube and incubated overnight at 200 rpm with shaking. Eight colonies for each casein were individually grown in this way to obtain cultures for plasmid purification.

Plasmid Purification

From each 10mL overnight culture, the plasmid DNA was isolated using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI). The manufacturer's protocol was followed to purify the plasmid and the elution was done with 100 µL of nuclease-free water. The DNA was analyzed on a 1% agarose gel for confirmation of purity after which, DNA stored at -20°C.

Analytical Restriction Enzyme Digestion with *Hind III* and *NdeI*

First analytical restriction digestion was done with a small volume of 20 µL. Three digestion reactions including two single digestions with each enzyme *HindIII* (10 units/ µL) and *NdeI* (10 units/ µL) (Promega Inc.) and one double digestion with both enzymes were performed to confirm the presence of the insert into the plasmid. The pGEM-T Easy vector plus insert contains one *HindIII* and one *NdeI* restriction site at each 5' and 3' end of the insert respectively, thus the insert should be excised from the vector with double digestion.

Double digestion was performed to confirm the dropouts of inserts. Table 6 lists the components of the restriction digestion.

The samples were then centrifuged to collect all the liquid at the bottom of the tubes and then incubated in a water bath for overnight at 37°C. The digested and undigested samples with 5 µL of sample loading dye added to the reaction to make 25 µL total volume was loaded onto run on a 1.5% (w/v) agarose gel and electrophoresed at 100V until the bromophenol blue dye migrated at 2/3 the distance of the gel.

Preparative Restriction Enzyme Digestion of pGEM T Easy Containing Casein Constructs

After confirmation of the drop out of insert in the double digestion product, preparative restriction digestion was performed using the Multicore Reaction buffer [Promega Inc.], DNA (60 µL of each α_{S1} Casein insert), enzymes (*NdeI*/*HindIII*) and nuclease-free water per the reaction volume (100 µL) as listed in table 7.

All digestions were analyzed on a 1% (w/v) agarose gel. Two wells were taped together to accommodate the 100 µL volume sample. The band from the 100 µL double digestion reaction was excised from the gel and placed in a sterile 1.5 mL microfuge tube for further gel purification for each casein insert. Standard gel purification as described previously in section 'Purification of PCR products' was followed. The purified DNA was stored at -20°C for future ligation into pET15b linearized vector.

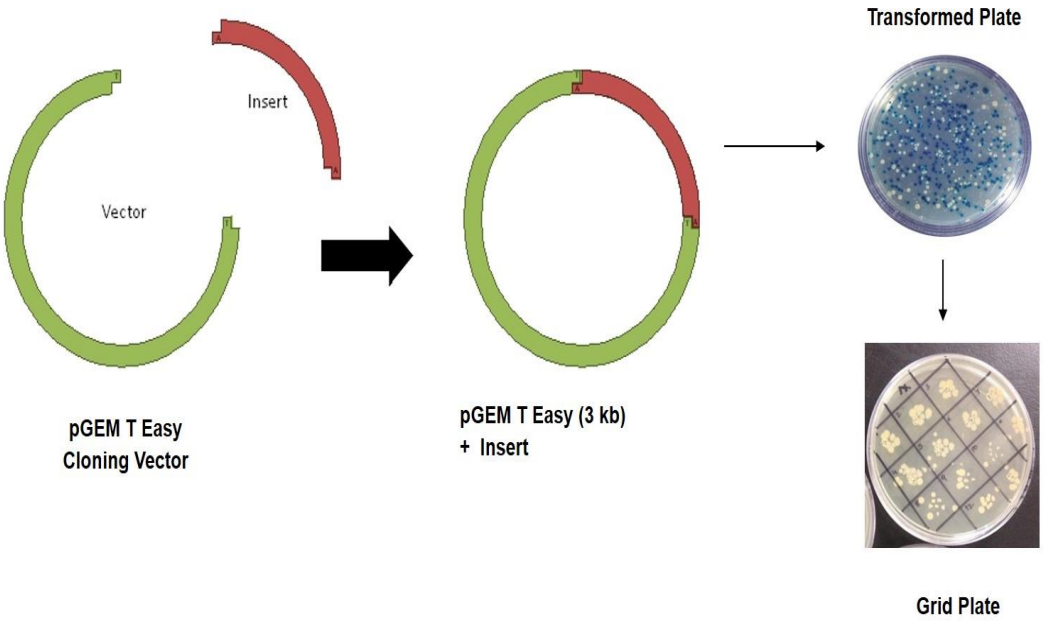


Figure 4: TA Cloning and transformation of casein inserts (with A overhang) into pGEM T Easy vector (with T overhang) and JM109 competent host cells.

Table 6: Analytical Restriction Digestion Reaction with *NdeI* and *HindIII*.

Reagents	**Single Digestion (NdeI)**	**Single Digestion (HindIII)**	**Double Digestion (NdeI & HindIII)**
Plasmid DNA	10 µL	10 µL	10 µL
5X Multicore Buffer	2 µL	2 µL	2 µL
NdeI	1 µL	0 µL	1 µL
Hind III	0 µL	1 µL	1 µL
BSA (2µg/µL)	1 µL	1 µL	1 µL
Nuclease free H_20	6 µL	6 µL	5 µL
Total volume	20 µL	20 µL	20 µL

Table 7: Preparative Restriction Digestion Reaction with *NdeI* and *HindIII*.

Reagents	**Double Digestion (*NdeI* & *HindIII*)**
Plasmid DNA	60 µL (20ng)
Multi-core Buffer	10 µL
NdeI	5 µL (50 units)
Hind III	5 µL (50 units)
BSA	5 µL (10 µg)
Nuclease free H_20	15 µL
Total volume	100 µL

Linearization of Expression Vector

The pET15b vector was linearized by digestion with *NdeI* and *HindIII* restriction endonucleases (Promega Inc.). A preparative digest reaction consisting of 20 µg of vector DNA was set up using the Multicore Reaction buffer (Promega Corp.), DNA, enzymes and nuclease-free water per the reaction volume as shown in Table 7 above. Uncut, single and double digestions were analyzed on a 1% (w/v) agarose gel for comparison. The linearized band from the 50 µL double digestion reaction was excised from the gel and placed in a sterile microfuge tube for further gel purification. Standard gel purification procedure as described in the previous section was followed. The purified DNA was stored at -20°C for further analysis.

Sub-Cloning of 16 casein amplicons into the pET 15b Expression Vector

For expression of the constructs, the pET15b vector (Novagen, Inc.) was used. It carries a N-terminal His tag sequence which facilitates the purification of the target protein, followed by a thrombin site and an inducible T7 promoter region. The ampicillin resistance gene is used for selection pressure. The *NdeI* and *HindIII* sites within the multiple cloning site were utilized for inserting each of the 16 casein inserts. Both *NdeI* and *HindIII* sites were unique in the vector. The ligation reaction was performed in a 1.5 mL microcentrifuge tube as described previously. The concentration of reagents and the PCR product (insert DNA) was calculated per

the technical manual provided with the pET15b Vector (Promega Inc.). The recommended insert: vector mole ratio (3:1) was calculated using the same equation used for the pGEM T Easy ligation. The ligation reaction components were shown in Table 8.

After mixing the reaction components, the ligation reaction was incubated at 4°C overnight as recommended by the manufacturer. Digestion and cloning of casein genes are shown in figure 5. Ligation reactions were transformed into *E.coli* JM109 competent cells ($\geq$ 1 x 10^8 cfu/μg DNA) (Promega, Corp.) using standard protocol as described previously in section 'Transformation'. An aliquot of 50 μL and 100 μL of the culture from the transformation mix was spread on LB/ ampicillin plates in duplicates (100 μg/μL ampicillin). The plates were then incubated overnight at 37°C. The successful cloning of an insert into pET 15b vector should result in white colonies on the plates.

Table 8: Ligation Reaction Components per 10 µL Total Volume.

Reaction component	Standard Reaction
2X Rapid Ligation Buffer	5 µL
pET 15b Vector	1 µL
Purified product	3 µL
T4 DNA Ligase	1 µL
Nuclease-free water to a final volume of 10 µL	0 µL

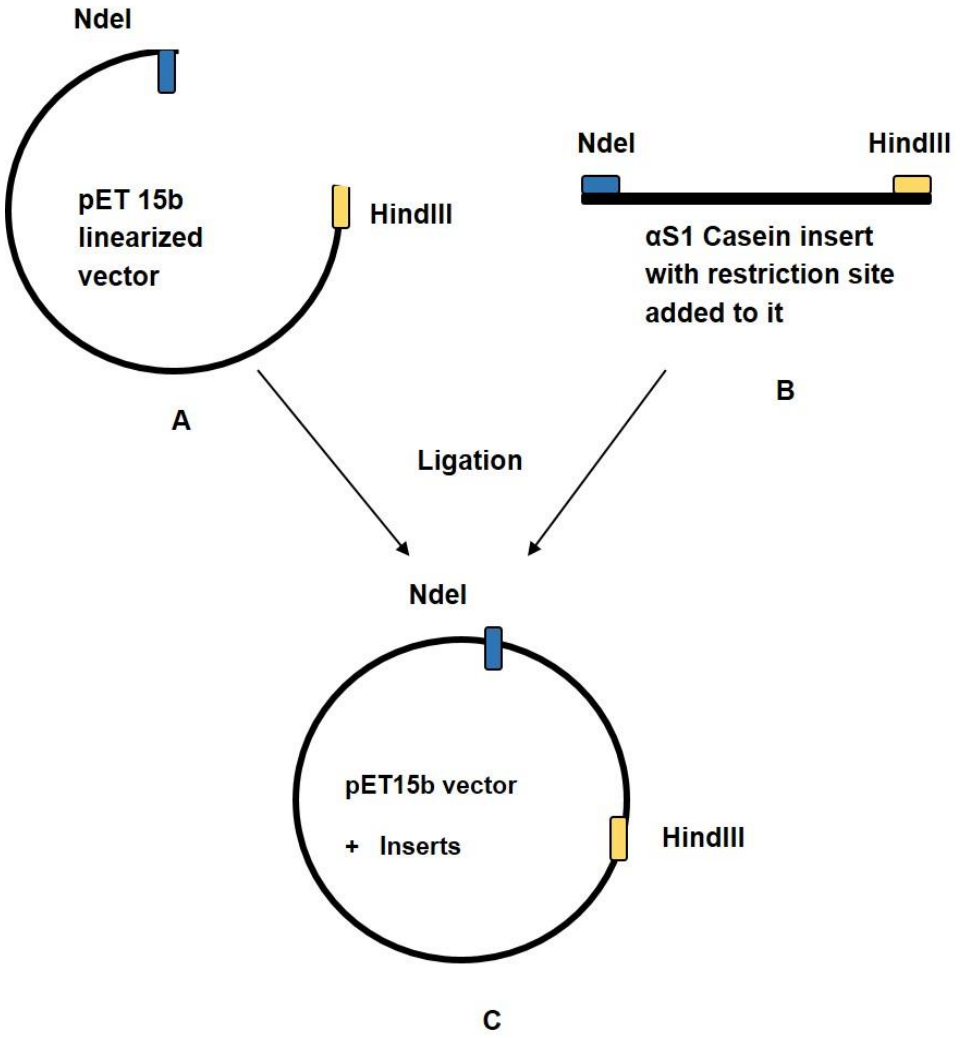


Figure 5: Digestion and cloning of casein genes into pET15b expression vector. [A] Restriction digestion of pET15b vector with *NdeI* and *HindIII* linearized the vector. [B] The primer pairs were redesigned with restriction sites *NdeI* and *HindIII* added to them. [C] The PCR amplified products were ligated to the pET15b vector and transformed into *E.coli* BL21(DE3) pLysS.

Grid Platting and PCR Screening (Colony PCR)

After transformation, the colonies containing insert DNA were verified by colony PCR where the cells were directly added as a template to the PCR reaction and lysed during the initial heating step. For each casein insert, one grid plate was prepared. Colonies chosen for the PCR were first transferred to the grid plates (figure 3) using a pipette tip. The remaining cells on the tip were used for the PCR reaction by rinsing the remaining bacteria on the tip into the PCR reaction buffer. The PCR reaction was set up as described in Table 2 and 3 above using the same program as used earlier for amplifying the DNA. The amplified products were separated on a 1% (w/v) agarose gel. Positive colonies showed an insert band consistent with the predicted band size of the corresponding casein insert.

Plasmid Purification

Ten milliters of LB/amp (100 µg/µL) media was prepared and aliquoted into 50 mL conical tubes. One white colony from each casein clone was inoculated into 10 mL of LB/amp media using a sterile pipette tips and incubated overnight at 200 rpm with shaking. Eight colonies for each construct were individually grown to obtain cultures for plasmid purification.

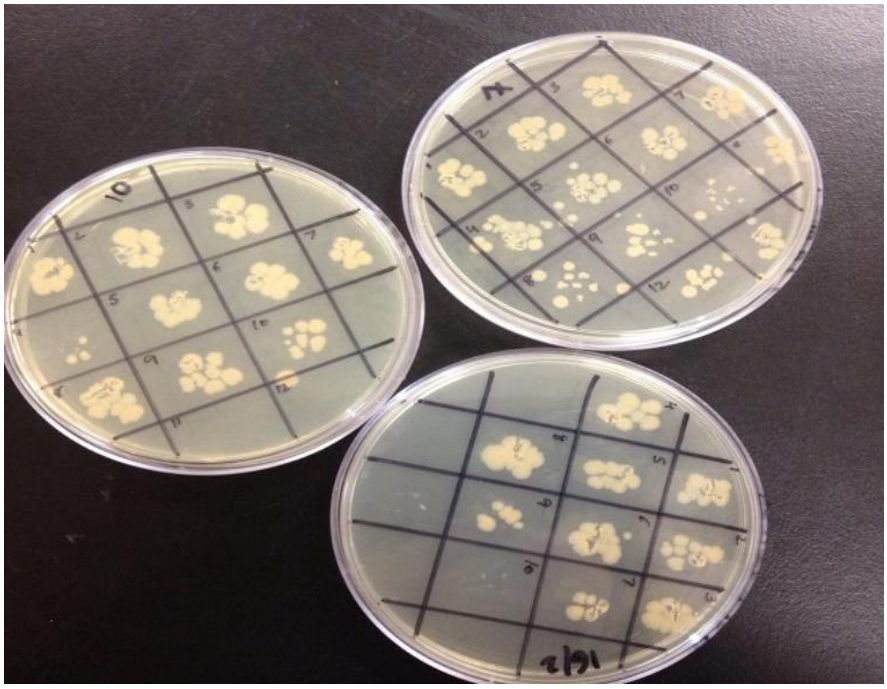


Figure 6: Grid Plates were prepared by drawing squares and numbering them. Each numbered square corresponds to an inoculation from a different colony in the original LB/amp plates.

From each 10mL overnight culture, the plasmid DNA was isolated using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI). The manufacturer's protocol was followed to purify the plasmid and the elution was done with 100 µL of nuclease-free water. The DNA was stored at -20°C.

Transformation into BL21(DE3) pLysS Expression Host

Final transformation of target genes was done using the BL21DE3 pLySs expression host cells ($\geq$ 1 x 10^8 cfu/µg DNA) (Promega, Corp.). The tubes containing the purified plasmids were centrifuged to collect the contents at the bottom of the tube. Sterile culture tubes were placed on ice and 1 µL of each purified plasmid was added to the tubes. High efficiency BL21(DE3) pLysS cells were removed from the -80°C freezer and placed on ice until thawed. Cells were mixed by gently flicking the tubes. Once the cells were mixed, 50 µL of these cells were transferred to the tubes containing ligation reaction. The tubes were then gently flicked to mix well and placed on ice for 15 min. The cells were then exposed to heat shock at 42°C in a water bath without shaking for 45-50 sec. The tubes were immediately placed back on ice for 10 minutes. In each tube 450 µL of chilled SOC medium (Sigma) was added and incubated for 1 hour at 37°C at 200 rpm. An aliquot of 50 µL and 100 µL of the culture from the transformation mix was spread on LB/ ampicillin in duplicates (100 µg/µL ampicillin). The plates were then

incubated overnight at 37°C. The successful cloning of an insert into pET 15b vector should result in white colonies on the plates.

Expression of 16 Casein Peptides

To determine which colonies expressed the recombinant peptides, the same colonies used for grid platting and PCR screening from each casein clone in the expression host was inoculated into 10 mL of LB/AMP media using a sterile pipette tip and incubated overnight at 200 RPM with shaking. The next day, 10mL of LB/amp (50 µg/µL) media was prepared and aliquoted into 50 mL conical tubes. Two tubes were inoculated with 100 µL overnight from one individual colony. After 5-6 hours of inoculation when the culture reached an OD 600, approximately at 0.6, one 50 mL conical from each overnight colony was induced with a final concentration of 0.4mM m IPTG (Basak, 2013), the other 50 mL conical was uninduced and incubated overnight in parallel to that of the induced culture shaking at 200 rpm with shaking. After overnight growth, each induced and uninduced culture was centrifuged at 6000 rpm for 15 mins. The pellets were collected and resuspended in 500 µL of 1X PBS by vortexing. Then 50 µL of Cell Lytic Express (Sigma-Aldrich) was added for 1 hour incubation on ice. After incubation, each lysate was sonicated with 3-4 pulses (0.20 Watts) and transferred into a sterile 1.5 mL microcentrifuge tubes. The lysate was centrifuged at 5,000 xg for 15 minutes,

the supernatant was transferred into fresh sterile tubes and stored at 4ºC along with the pellets.

SDS-PAGE Gel

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to observe the presence of expressed casein peptides and to confirm the molecular weights for all casein peptides. The protein sample loading dye (5µL) was added to each sample followed by denaturing at 90ºC for 2 min. Electrophoresis was performed at 100 V for 1 hour after which the gels were placed in Coomassie blue stain for overnight on rocker. Next day the SDS gels were destained using 40% Methanol, 10% HA and 50% H_2O. The gels were then stained using SYPRO Ruby (BIO-RAD, Corp.) overnight at room temperature on a rocker. The gels were destained first using 10% ethanol and then with nanopure H_2O followed by three consecutive washes in H_2O.

Tricine Gel Electrophoresis

Tricine-SDS-PAGE gels are generally separates protein ranging from 1-100KDa. It is the preferred electrophoretic system for the resolution of proteins smaller than 30KDa.

AB-3 stock solution: For the acrylamide-bisacrylamide (AB)-3 stock solution (49.5% T, 3% C mixture), dissolve 48 g of acrylamide and 1.5 g of bisacrylamide in 100 mL of water.

Overlay the polymerized resolving gel (16%/6 M urea) directly with a 4% sample (stacking) gel prepared as indicated in table 11. Sample loading dye (5μL) was added to each sample followed by denaturing at 90ºC for 2 min. Electrophoresis conditions used were an initial voltage of 30 V and maintain at this voltage until the sample has completely entered the stacking gel. The next appropriate voltage was 80 V until the samples approaches the end of the gel. The gels were then stained using SYPRO Ruby (BIO-RAD, Corp.) overnight at room temperature on a rocker. The gels were destained first using 10% ethanol and then with nanopure H_2O followed by three consecutive washes in H_2O.

Table 9: Running Buffers composition for Tricine-SDS-PAGE gel.

	Anode Buffer (10X)	**Cathode Buffer (10X)**	**Gel Buffer (3X)**
Tris (M)	1.0	1.0	3.0
Tricine (M)	-	1.0	-
12.2M HCl (M)	0.225	-	1.0
SDS (%)	-	1.0	0.3
Ph	8.9	8.25	8.45
Final Volume	1 L	1 L	250mL

Table 10: Resolving gel and stacking gel composition for Tricine-SDS-PAGE gel.

	4% Acrylamide (Stacking gel)	**16% Acrylamide (6M Urea) (Resolving gel)**
AB-6 (mL)	1mL	10mL
Gel Buffer (3X)	3mL	10mL
Urea (g)	-	10.8g
H_2O (mL) to final volume	8mL	-
Total Volume	12mL	30mL
polymerized by adding:		
APS (µL)	90µL	80µL
TEMED (µL)	10µL	10µL

Purification of 16 Casein Fragments with Ni Affinity Chromatography

For purification of the peptides, the cell lysates were centrifuged at 5000xg for 15 minutes to remove particulates. An equal volume of acetone was added to partially purify and concentrate the peptides by precipitating a large fraction of proteins out of solution. The mixture was incubated on ice for 15 minutes and then centrifuged at 5000xg for 30 minutes. The supernatant was lyophilized to dryness. Both supernatant pellet and the acetone pellet were resuspended in 5mL of 1X PBS. To further aid in the purification urea was added to final concentration of 8M to the resuspended acetone pellet to completely unfold the peptides as well as the His-tag for optimal binding to the Ni column. Tween-20 was added to a final percent of 0.1% (v/v). PBS equilibrated Ni resin (500µL) was added to the peptide mixture and allowed to mix by rocking for 10 minutes. Samples were then centrifuged to collect the Ni resin as a pellet. All but approximately 5 mL of supernatant was removed. A slurry was made of the Ni-peptide resin and transferred to an open column for washing. At least 5 column volumes of PBS were passed through the column to wash the resin free of unbound contaminants. Elution of the peptides was performed by the addition of increasing 0.05 mM imidazole concentrations added stepwise.

RESULTS

The focus of this project was to construct 16 recombinant peptides from amino terminus of α_{S1} casein containing the sequence known to inhibit human PEP and positioned at varying sites within the peptides which differed in length. The first objective of this project included PCR amplification of the 16-bovine αS1 casein inserts using gene specific primers. Then 16 amplicons underwent subsequent cloning and transformation into the final expression vector (pET15b) and *E.coli* BL21DE3 pLysS host cells. The second objective part of this project involved expression and partial if not total purification of the 16 α_{S1} casein peptides and then screening for percentage inhibition of spPEP.

PCR Amplification and Agarose Gel Analysis for 16 Casein Amplicons

Gene specific primers were designed and each casein insert was amplified using the α_{S1} casein as a template. The amplified products were purified and loaded on 1.5% agarose gel for quantification as shown in figure 7.

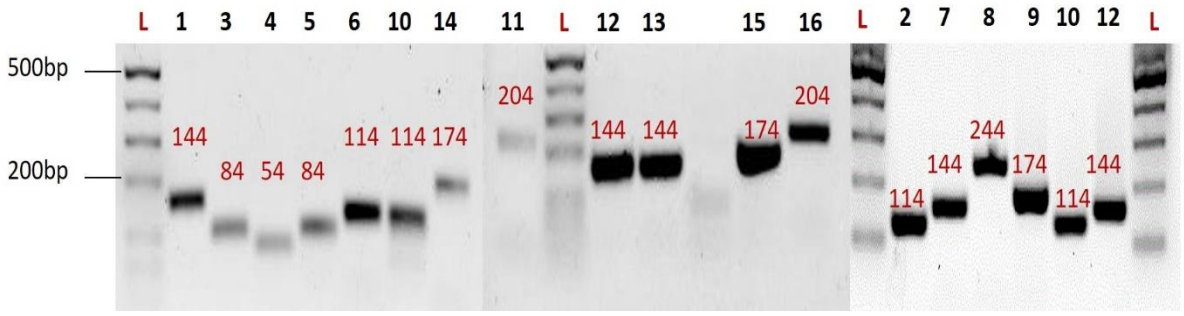


Figure 7. Shows 1.5% Agarose Gel Electrophoresis of all 16 amplicons of variable length. M; High Low DNA ladder, the digits 1-16 are representing each amplicon and the numbers on the top of each band indicates the base pair size of each amplicon.

Cloning into pGEM T Easy Vector , Confirmation with *NdeI and HindIII* Digestion

The 16 amplicons observed on the gel were successfully ligated into the pGEM T Easy vector (3 kb) and transformed into *E.coli* JM109 competent cells using a vector-:-insert ratio of 3 : 1 and 5 : 1. The transformed cells were spread on to LB/agar/amp/X-gal/IPTG plates and the presence of approximately 40% white colonies on the plate suggested the presence of insert. The vector : insert of 3 : 1 worked better for transformation.

Mole concentration of pGEM T Easy = 0.256 picomole/µL

From each transformed plate of each construct, 4 white colonies were chosen and inoculated in 10 mL of LB/amp media in sterile conditions. Plasmid DNA from overnight culture was purified and analytical restriction digestions with *NdeI and HindIII* restriction enzyme are shown in Figure 8 A-C.

Single, single and double digestion were performed with *NdeI* and *HindIII* enzymes as each insert carries a *NdeI* and *HindIII* restriction site on their either end. The double digestion confirmed the presence of insert into the vector as the insert dropped out from the vector. However, in the double digestion lane for each insert there were other smaller bands observed at size 50bp beside the expected drop out band. The expected sizes are in Table 12. Further observation revealed the presence of two bands with double digestion. This was obtained because *NdeI* cuts at two restriction sites (Figure 9): one in the pGEM T Easy Vector Multiple

Cloning site as shown in Figure 9 and one introduced in insert 5' from the PCR primer for downstream cloning into the pET15b expression vector.

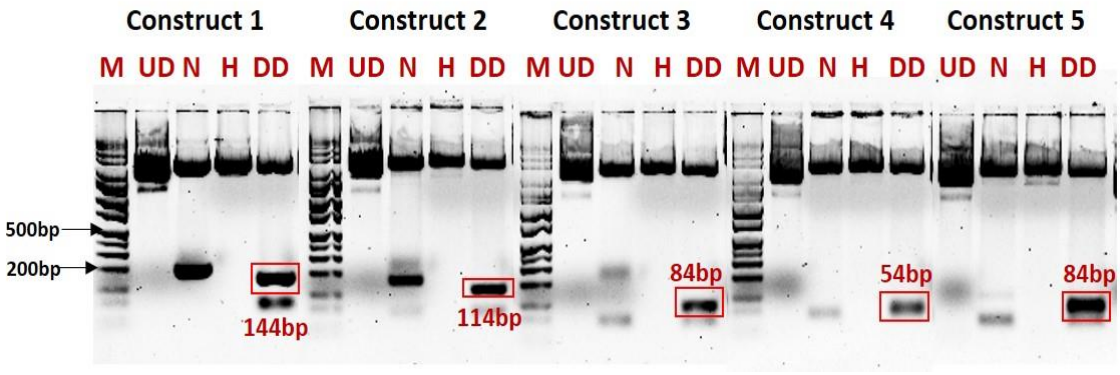
After the confirmation of the presence of each casein insert in the pGEM T Easy vector, preparative restriction digestion was performed and again confirmed with a 2% Agarose gel (w/v). The bands were cut out of the gel with sterile scalpel and purified to get the insert DNA. Figure 10 shows the insert bands that were purified and used for subsequent cloning into the pET15b.

Preparing DNA for Sequencing

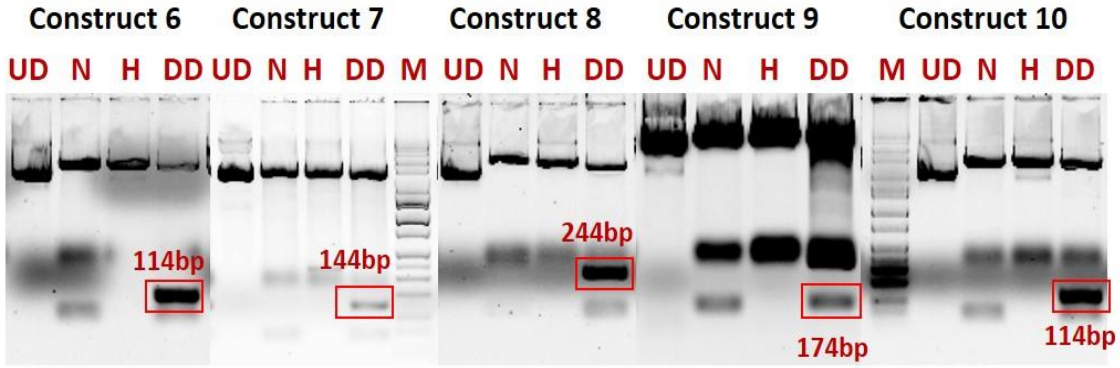
Upon receiving the confirmation of correct inserts in the pGEM T Easy vector DNA via enzyme restriction digestion. The positive colonies from each casein were sent for sequencing to Eurofins Genomics (Louisville, Kentucky). The pGEM T Easy vector have pUC/M13 Forward and Reverse Sequencing Primer binding site which will be used for sequencing of inserts in the plasmid vector (Table 13).

Overnight cultures of 100 mL of LB/amp media from each construct was grown. Midiprep was performed and the plasmid DNA obtained for each sample was 60 uL. All DNA samples showed to have an $A_{260/280}$ ratio of 1.8-2.0 suggesting the DNA was pure. The concentration and the purity of the midi prep purified DNA was determined and shown in Table 14 below. The results are pending.

(A)


Construct 1
Construct 2
Construct 3
Construct 4
Construct 5
M UD N H DD M UD N H DD M UD N H DD M UD N H DD UD N H DD
500bp
200bp
144bp
114bp
84bp
54bp
84bp

(B)


Construct 6
Construct 7
Construct 8
Construct 9
Construct 10
UD N H DD UD N H DD M UD N H DD UD N H DD M UD N H DD
114bp
144bp
244bp
174bp
114bp

(C)

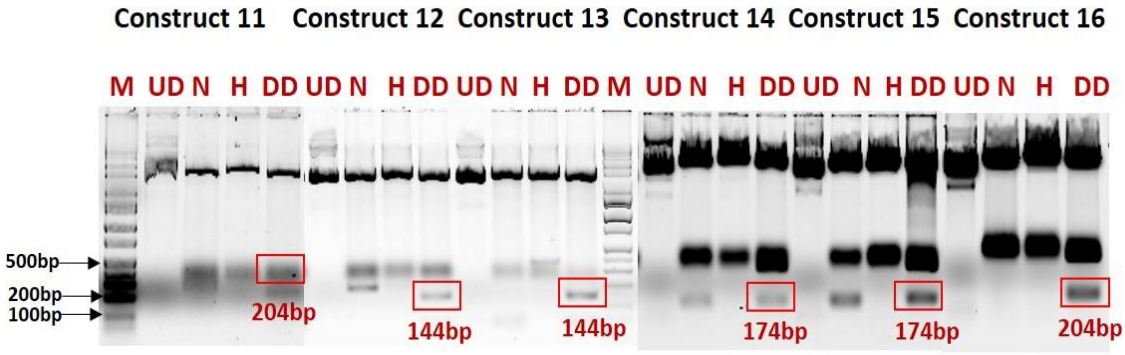

Construct 11
Construct 12
Construct 13
Construct 14
Construct 15
Construct 16
M UD N H DD UD N H DD UD N H DD M UD N H DD UD N H DD UD N H DD
500bp
200bp
100bp
204bp
144bp
144bp
174bp
174bp
204bp

Figure 8(A). Analytical Restriction digestion of each construct; C-1(144bp), C-2 (114bp), C-3 (84bp), C-4 (54bp), C-5 (84bp); **(B)** C-6(114bp), C-7 (144bp), C-8 (244bp), C-9 (174bp), C-10 (144bp); **(C)** C-11(204bp), C-12 (144bp), C-13 (144bp), C-14 (174bp), C-15 (174bp) and C-16 (204bp) in pGEM T-Easy with *NdeI* and *HindIII*. M; High Low DNA ladder, UC; Undigested, N; *NdeI* digest, H; *HindIII* digest and DC; Double digest with both *NdeI* and *HindIII*. The box represents the drop out band from double digestion for each construct with their base pair size.

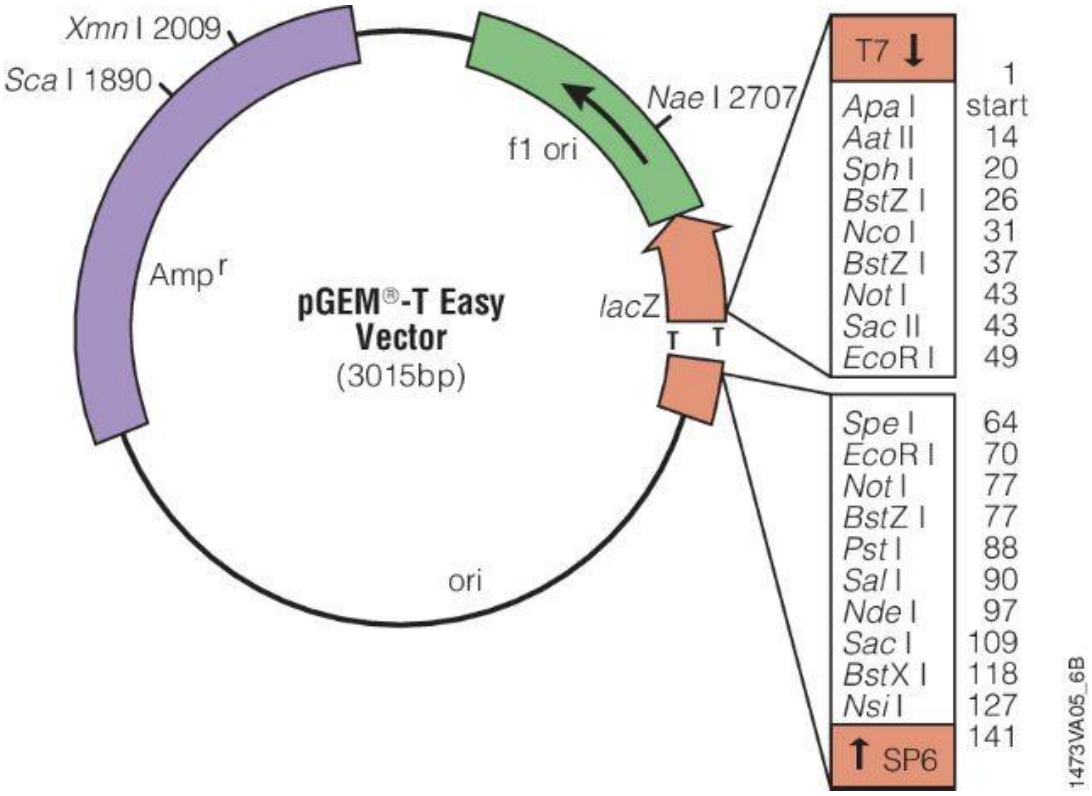


Figure 9. Shows the pGEM T Easy vector map with *NdeI* site at its multiple cloning site. Taken from Promega, Corp. Bulletin No. TM042.

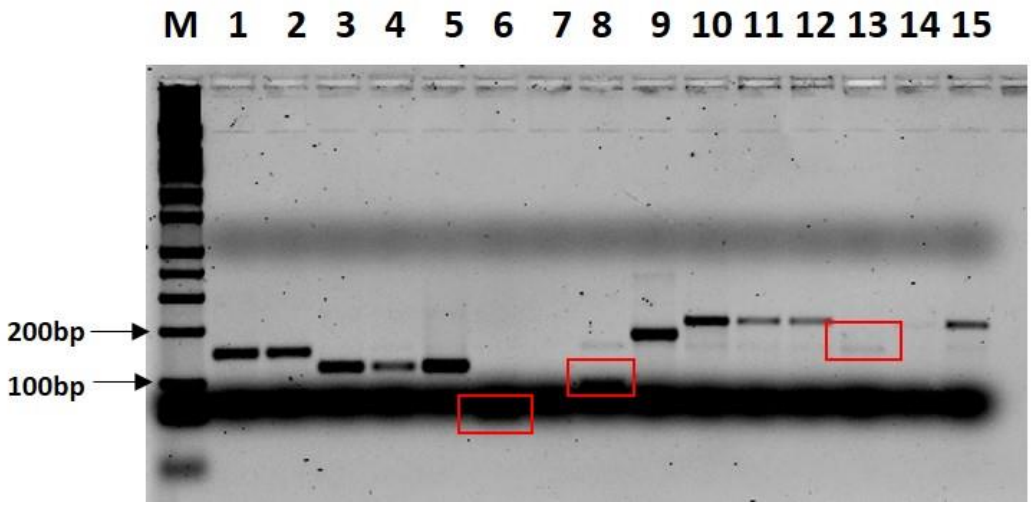


Figure 10. Gel purified 16 Casein inserts from double digests. Gel was 2% Agarose gel (w/v). The boxes indicate the bands overlapped with loading dye.

Table 11: Sequencing primers in pGEM T Easy. The Tm's for each oligonucleotide determined by the base-stacking free-energy method are listed on Promega's technical resources.

Primer name	**Sequence**	**Catalog no.**	**Tm (°C)**
pUC/M13 Forward primer (17mer)	5′-d(GTTTTCCCAGTCACGAC)-3′	Q5391	50
pUC/M13 Reverse primer (17mer)	5′-d(CAGGAAACAGCTATGAC)-3′	Q5401	47

Table 12: Concentration of Midi-prep plasmid DNA for casein constructs in pGEM T-Easy isolated from *E.coli* JM109.

Sample	**A_{260} nm**	**A_{280} nm**	**A_{260}/ A_{280}**	**Concentration (ng/µl)**
Insert 1	0.268	0.146	1.835	134
Insert 2	0.483	0.258	1.872	241.5
Insert 3	0.216	0.119	1.815	108
Insert 4	0.314	0.166	1.891	157
Insert 5	0.493	0.266	1.853	246.5
Insert 6	0.352	0.186	1.892	176
Insert 7	0.373	0.200	1.865	186.5
Insert 8	0.342	0.194	1.762	171
Insert 9	0.326	0.175	1.862	163
Insert 10	0.475	0.253	1.877	237.5
Insert 11	0.591	0.315	1.876	295.5
Insert 12	0.404	0.218	1.853	202
Insert 13	0.371	0.199	1.864	185.5
Insert 14	0.110	0.067	1.641	55
Insert 15	0.387	0.208	1.860	193.5
Insert 16	0.464	0.254	1.826	232

Linearization of Expression Vector pET15b

In preparation of the vector for the ligation of the insert, an analytical digest was prepared with 5 µg of the vector and *NdeI* and *HindIII* enzyme which showed that the digest worked (Figure 11 a). The subsequent preparative digests of 20 µg of pET15b DNA for the double digest and the two single digestions showed a prominent dark band at approximately 5700 bp position from double digest which was excised from the gel and purified. Figure 11 (A) shows analytical restriction digestion of pET15b vector. Two bands were observed in the double digest (DD) lane, one at 5700bp (vector) and other close 200bp which was G5p. Figure 11 (B) showed the presence of linearized pET15b vector on 1.5% agarose (w/v) gel in lanes 1-4.

Screening for Vector pET15b + Insert Construct

After transformation, eight white colonies from each construct were randomly chosen from each plate. Colony PCR was done to amplify the inserts using their respective primer pair and the presence of the inserts was confirmed by running 2% Agarose gel. Figure 12 (A) shows PCR screening results for construct 1 and 2 into pET15b expression vector; all the transformed colonies on the grid plate was used. For construct 1, the expected size of inserts (144bp) was found in lane 4,9,12. For construct 2, the expected size of inserts (114bp) was consistent with all the lanes 1-16. Figure 12 (B) shows the PCR screening results

for all possible colonies for construct 3, 4 and 5 into pET15b expression vector. For construct 3 and 4, the expected size of inserts (84bp & 54bp) was consistent with all the lanes 1-6. For construct 5, the expected size of inserts (84bp) was consistent with all the lanes 1-14.

Screening for pET15b into BL21(DE3) pLysS Cells

After confirmation of inserts in pET15b vector JM109 *E.coli* host cells by colony PCR. The positive colonies were used to inoculate 10mL LB/amp media overnight cultures for miniprep plasmid purification. The concentration and purity of the DNA was measured and shown in Table 12 below. DNA samples showed to have an $A_{260/280}$ ratio 1.8-2.0 suggesting the DNA was pure.

As our aim was to produce the large-scale expression of 16 αS1 casein peptides and to investigate the if the peptides inhibited of spPEP. The next step was to move pET15b vector containing each of the casein sequences into *E.coli* BL21(DE3) pLysS cells by transformation.

The 16 casein inserts were transformed into the final expression host in batches. The first batch of transformed inserts included 1,2,4 and 11. From each transformed plate of each insert, 8 colonies were picked for PCR screening and confirmed with 2% agarose gel (figure 13). Overnight cultures of positive colonies were stored as glycerol stocks in -80°C for future growth.

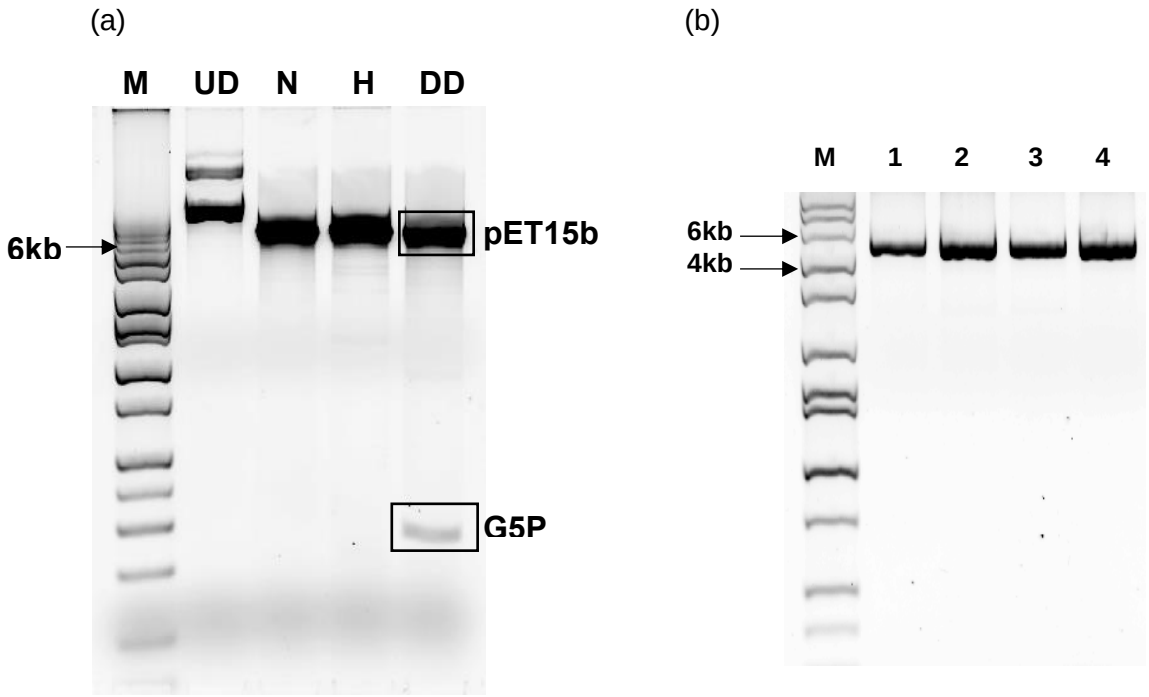
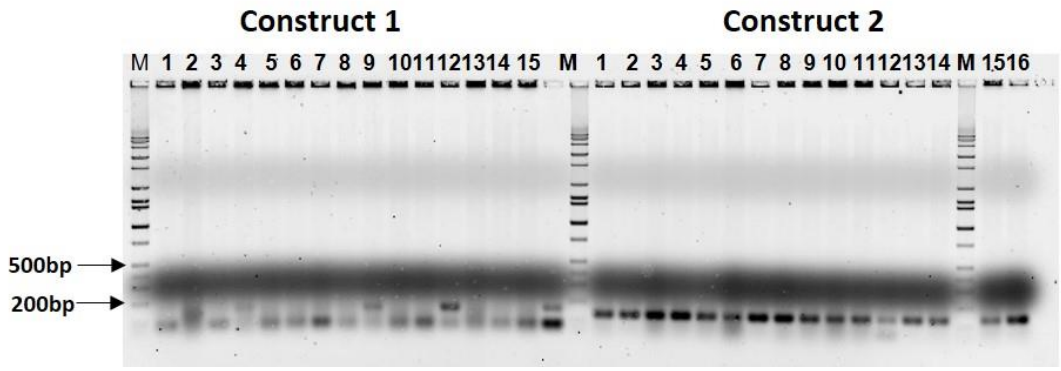


Figure 11(a). Restriction digestion of pET15b (5708 bp) containing G5P protein. Lane UD contains undigested vector. Lane N is the single digestion of vector with *NdeI* and Lane H is the single digestion of vector with *HindIII* restriction enzyme. Lane DD has the double digest of *NdeI* and *HindIII* which gave a darker band at about ~5700 bp and highlighted with square. Lane M is the Bionexus Inc. All purpose Hi-Lo DNA marker. **(b)** Purified digested linearized pET15b with correct size (Lane 1-4).

(a)



(b)

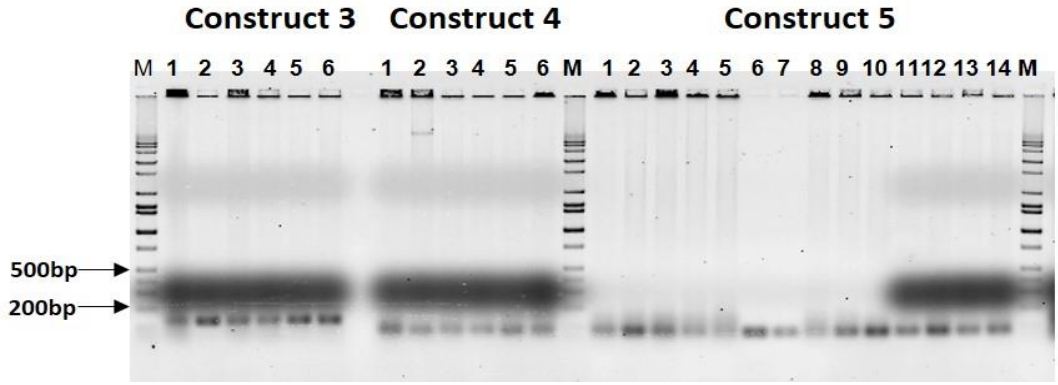


Figure 12(a). Colony PCR of the overnight cultures for the confirmation of the presence of insert 1(144bp), 2(114bp) and 3(84bp) into pET15b vector JM109 *E.coli.* host cells. **(b)** shows the presence of insert 4(54bp) and 5(84bp) into pET15b vector JM109 *E.coli.* host cells. All the colonies on the grid plate was used. Lane M is the Bionexus Inc. All purpose Hi-Lo DNA marker.

Table 13: Concentration and purity of Miniprep plasmid DNA for casein constructs in pET15b vector isolated from *E.coli* JM109.

Sample	**A_{260} nm**	**A_{280} nm**	**A_{260}/A_{280}**	**Concentration (ng/µL)**
Insert 1	0.124	0.067	1.850	62
Insert 2	0.136	0.076	1.789	68
Insert 4	0.159	0.089	1.786	79.5
Insert 11	0.148	0.087	1.701	74

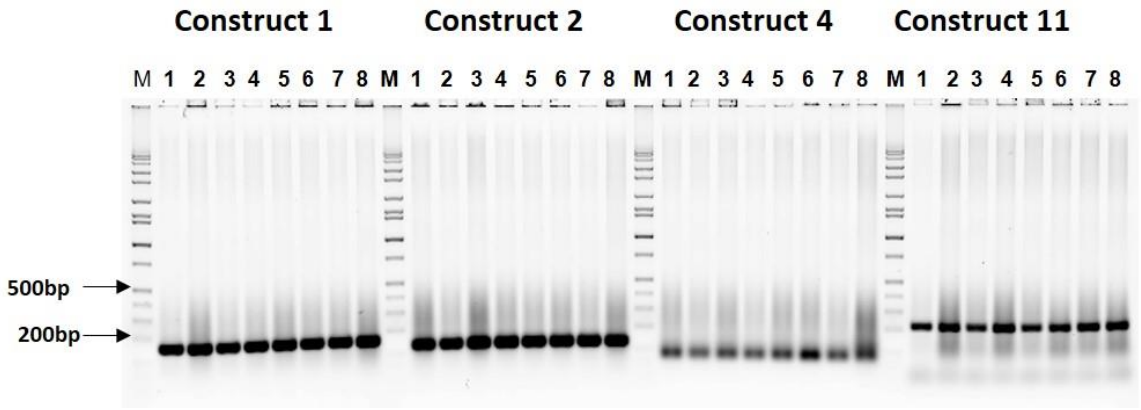


Figure 13. Colony PCR of the overnight cultures for the confirmation of the presence of insert 1(144bp), 2(114bp), 4(54bp) and 11(204bp) into pET15b vector BL21(DE3) pLysS host cells. Eight colonies were picked from each construct. Lane M is the Bionexus Inc. All purpose Hi-Lo DNA marker.

Optimization of Expression of Casein Peptides

For expression, two PCR confirmed colonies from construct 1,2,4 and 11 were grown. Cultures were grown for both induced and uninduced for comparison. The cells were harvested as a pellet and lysed. The cell lysates were loaded onto 4-20% SDS-PAGE for 1 hour at 200 volts. The gels were stained with Coomassie Blue R250 overnight and destained the next day. The image was captured using an Amersham Imager 600 (GE Lifesciences). After few unsuccessful attempts for expression, the conclusion was, that the peptides were too small (<10KDa) and were not resolved thoroughly with SDS-PAGE. Table 13 shows 16 peptides with their respective amino acid number and molecular weight.

After further investigation, some studies were found showing that different compositions of acrylamide and buffers provided results in better resolution of smaller peptides. Thus, the next batch of expressed peptides, were loaded on 16% Urea Tricine gels (Schagger et al, 2006). The smaller protein bands (<30KDa) were resolved better on the tricine gels. At first, the urea tricine gels were visualized with Coomassie Blue R250 stain but the bands were on visible not even the marker. It was evaluated that my peptides did not have the necessary amino acids (Arginine) that are stained by Coomassie Blue R250. So, Sypro Ruby was utilized for staining. Small bands were still very faint. The contrast settings of the Typhoon FLA 9500 were used to visualize the bands. Figure 14 (A) and (B) show the

comparison of the expression gel picture of peptide 11 (7.54 KDa) without and with contrast respectively.

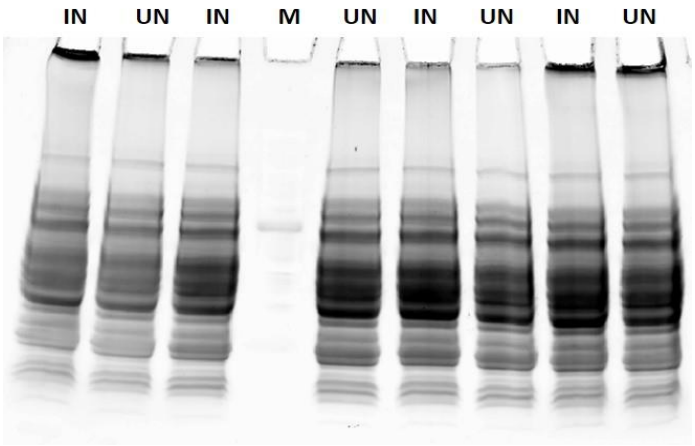
Purification of Casein Peptides

The cell lysate was purified with His-Spin Protein Miniprep™ (Zymo research). No peptides were found in Ni elute when using Ni spin columns. Therefore, an equal volume of acetone was added to purified lysate to partially purify the peptides by precipitating a large fraction of proteins out of solution. The peptides were expected to be found in supernatant however, P11 was found in the precipitate. Acetone precipitation helped with concentrating the peptide as shown in Figure 15 in the lane labeled AP pellet. No peptides were observed in supernatant (APsup) even with additional contrast of the image.

Table 14: shows 16 peptides with their respective amino acid number and molecular weight.

Peptides	Number of amino acids	Molecular weight (KDa)	Nucleotide size (bp)
Peptide 1	48mer	5.32	144
Peptide 2	38mer	4.21	114
Peptide 3	28mer	3.10	84
Peptide 4	18mer	2	54
Peptide 5	28mer	3.10	84
Peptide 6	38mer	4.21	114
Peptide 7	48mer	5.32	144
Peptide 8	78mer	9.02	244
Peptide 9	58mer	6.43	174
Peptide 10	38mer	4.21	114
Peptide 11	68mer	7.54	204
Peptide 12	38mer	4.21	114
Peptide 13	38mer	4.21	114
Peptide 14	58mer	6.43	174
Peptide 15	58mer	6.43	174
Peptide 16	68mer	7.54	204

(A)



(B)

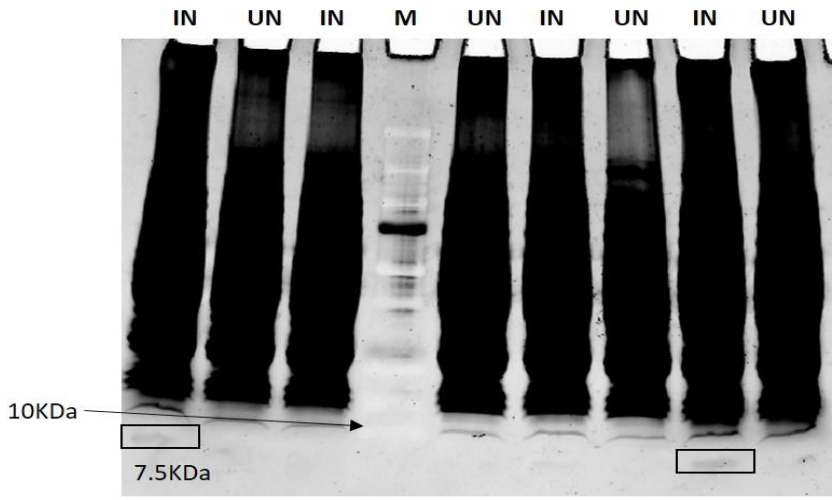


Figure 14(A). Shows the expression gel for peptide 11 (8.2KDa) without contrast, **(B)** with contrast. Lane M represents the Precision Plus Protein Marker. Lane IN represents the lysate from induced cultures from four colonies and Lane UN represents the lysate from the uninduced cultures. Sypro Ruby was used to stain the gels.

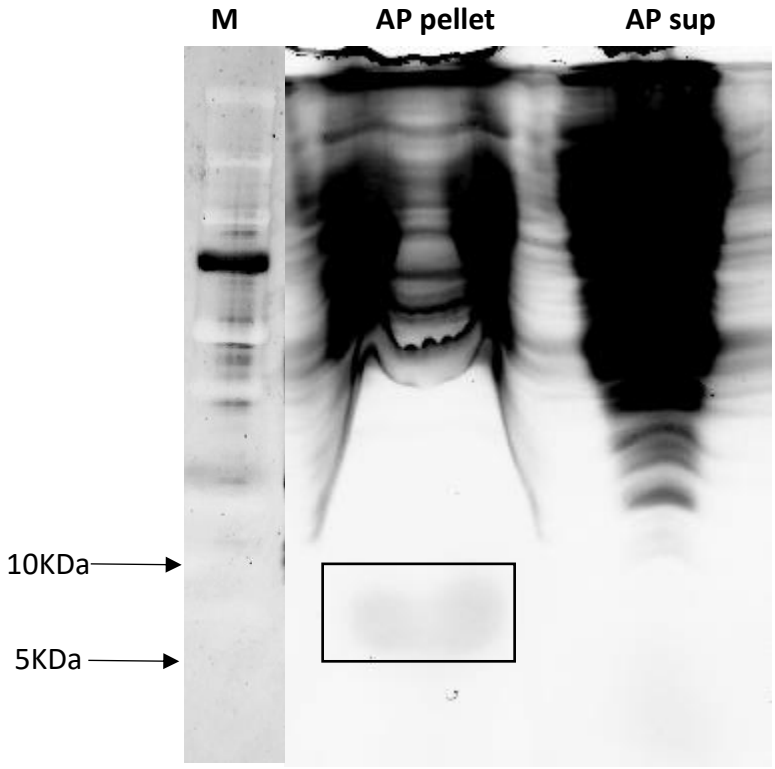


Figure 15. Lane M is Precision Plus protein marker. Lane AP pellet is acetone precipitated pellet shows expected size of the P-11 with 16% Urea Tricine gel. APsup is the supernatant of acetone precipitation.

FUTURE STUDIES

Additional colonies for each of the other 15 constructs needs to be grown and induced to screen for expression of the other 15 recombinant peptides. Once positives are identified, all of the recombinant peptides will be expressed on a large scale up to 1L in BL21(DE3) pLysS cells. The purification of the peptides will be optimized with acetone precipitation, the Ni-NTA column and the HPLC for purification. Each of the peptides will be assayed in presence of spPEP and GPpNA to determine the extent of inhibition before assaying the peptides (single or combination of peptides) in presence of gluten as a substrate. The outcome of these results will determine if one or a mixture of peptides will be used as an inhibitor of spPEP to prevent gluten hydrolysis in wheat.

DISCUSSION

The primary focus of this study consisted of cloning and expression of the 16-recombinant bovine αS1 casein peptides. The present project was started with designing four forward and four reverse gene specific primers that would result in amplification of 16 DNA fragments revolving around the inhibitory sequence of αS1 casein to have 16 DNA fragments of variable size and position as described in Figure 1.

After successful amplification, each amplicon was successfully cloned into pGEM T Easy vector and JM109 *E.coli* host cells (Promega, Corp.). The enzyme restriction digestion was performed and the products were analyzed on agarose gel which showed the same expected band sizes. After confirmation of each construct in pGEM T Easy vector, sub-cloning was successful into pET15b expression vector and BLD21(DE3) pLysS host cells. Upon IPTG induction of the cultures, only 1 construct expressed consistently the peptide expected is peptide 11.

Several experimental methods needed to be identified to provide resolution and visualization of the short peptides. Use of the Urea Tricine gels as described by Schagger (2006) provided resolution of peptides <10KDa as evidenced by

molecular size markers used BioRad and therefore will be used for future gel electrophoresis analysis of the remaining peptides. Sypro Ruby was successful at staining and providing visualization of smaller peptides in lieu of the normally used Coomassie Blue R250 stain used for larger peptides. What needs to be further optimized is the purification of the peptides with a standard procedure determined that will work for all of them.

The work presented in this thesis provides a foundation for the expression of the 16 αS1 casein peptides containing the sequence known to inhibit spPEP.

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VITA

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