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Protein Expression and Purification of Human Hormone Sensitive Lipase

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PROTEIN EXPRESSION AND PURIFICATION OF HUMAN HORMONE SENSITIVE LIPASE

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ABSTRACT

The purpose of this project was to develop a new product, purified human hormone sensitive lipase (HSL), for Blue Sky Biotech's product line. This was accomplished by cloning the HSL gene by gene synthesis, amplifying the annealed templates by PCR, cloning the amplicons into a plasmid in *E. coli*, packaging the gene into baculovirus, expressing the gene in cultured insect cells, and purifying the protein by affinity chromatography using histidine and biotin tags contained in the engineered vector. The results indicate that HSL has been purified and remains biologically active. Blue Sky Biotech will be able to sell this product to laboratories currently researching lipolysis pathways involved in diseases such as obesity, diabetes, and hyperlipidemia.

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ACKNOWLEDGEMENTS

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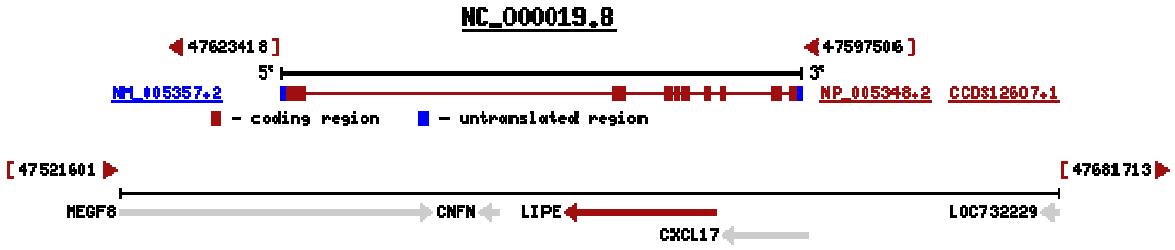
BACKGROUND

HORMONE SENSITIVE LIPASE (HSL)

Hormone sensitive lipase (HSL) is a key enzyme in fatty acid metabolism (Holm et al., 1988), and it also plays a role in steroidogenesis and overall energy homeostasis. It is an intracellular neutral lipase (Kraemer and Shen, 2002) that is capable of hydrolyzing cholesterol esters, monoacylglycerols, diacylglycerols, and triacylglycerols, and other water soluble substrates. The activity of HSL is regulated both post-transcriptionally (due to phosphorylation) and also by pre-translational mechanisms (Kraemer and Shen, 2002). It is expressed primarily in adipose and steroidogenic tissue; however, HSL is also expressed in cardiac muscle, skeletal muscle, macrophages, and islets. Its activity can be inhibited by insulin (Large et al., 1998), catecholamines (via α_2-adrenoceptors), prostaglandins, and adenosine. HSL can be activated by catecholamines via ß-adrenoceptors, which promote an increase of intracellular cAMP levels.

The human hormone sensitive lipase gene (LIPE) is located on chromosome 19, with a specific locus of 19q13.1-q13.2 (NCBI, 2008). Figure 1 illustrates the human HSL gene.

Figure 1: Diagram of the Human LIPE Gene.

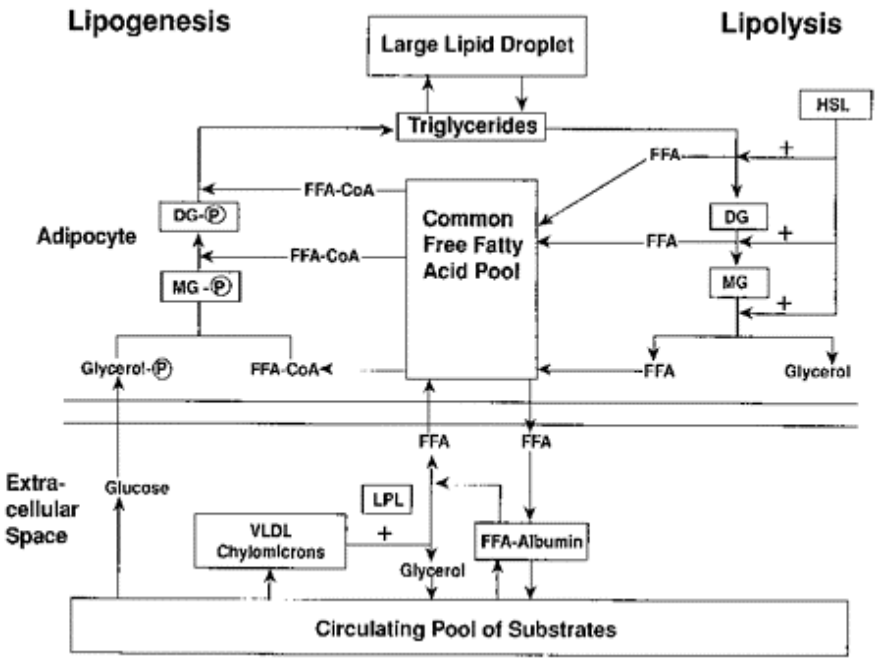


(NCBI, 2008)

The protein transcribed by the gene has two forms—long and short—which are produced through different translational start codons (NCBI, 2008). Steroidogenic tissues (testes, ovaries, adrenals, etc.) express the long form of the protein, which converts cholesterol esters to free cholesterol for the production of steroid hormones. Adipose tissue expresses the short form of the protein, which hydrolyzes triglycerides to fatty acids (NCBI, 2008). The short form of the protein catalyzes the rate limiting step in adipose tissue lipolysis. The protein exists in the cell cytosol, specifically on lipid droplets in adipocytes. The free fatty acids produced during this enzymatic reaction are circulated through the bloodstream, and are the primary source of energy when a deficiency in dietary substrates exists (Holm et al., 1988). The long form of HSL is involved in the hydrolysis of cholesterol esters in the adrenals, ovaries, testes, and macrophages. In general, human HSL is activated when energy is needed by the body. Because of this, human HSL has a positive response to glucagon and a negative response to insulin. HSL's dephosphorylation can be induced by insulin, which causes the hormone to have an antilipolytic effect (Holm at al., 1988).

Figure 2 shows the contribution of hormone sensitive lipase in lipolysis. Triglycerides from the large lipid droplets are hydrolyzed by HSL to free fatty acids and diglyerides. HSL then hydrolyzes the diglyerides to free fatty acids and monoglycerides, which are then hydrolyzed to free fatty acids and glycerol. At each hydrolysis the free fatty acids produced become a part of the common free fatty acid pool.

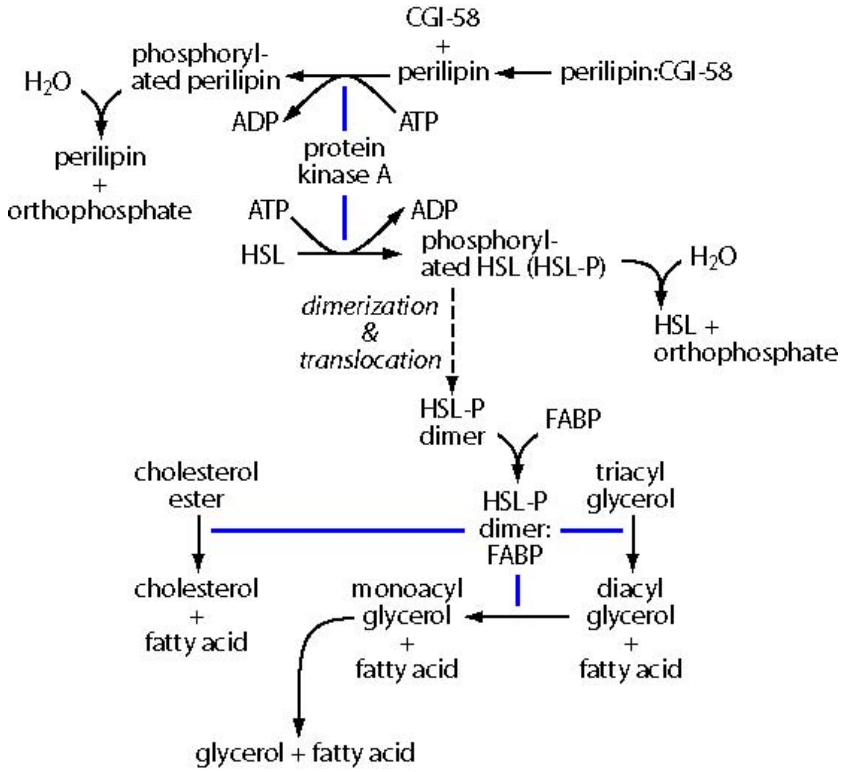
Figure 2: Diagram of Cellular Lipogenesis versus Lipolysis.



(Albright and Stern, 1998)

Figure 3 shows the activation and function of HSL within the cell. In mice, CGI-58: perilipin complexes exist at the cytosolic lipid droplet surface. These complexes need to be disrupted for HSL to be eventually phosphorylated. CGI-58: perilipin complexes have not been studied in detail in humans and therefore, the mechanism of action is assumed from the mouse model (D'Eustachio, 2005). When the complex dissociates, perilipin is phosphorylated by protein kinase A in the presence of ATP. The phosphorylated perilipin is re-organized on the lipid droplet surfaces, and protein kinase A is able to phosphorylate HSL at serine residue 563 in the presence of ATP. Phosphorylated HSL forms a dimer, and moves from the cytosol to the surface of lipid droplets. From here, it functions to hydrolyze triglycerides to free fatty acid and glycerol, and cholesterol esters to cholesterol and free fatty acid (D'Eustachio, 2005).

Figure 3: Diagram of the Activation of HSL.



(D'Eustachio, 2005)

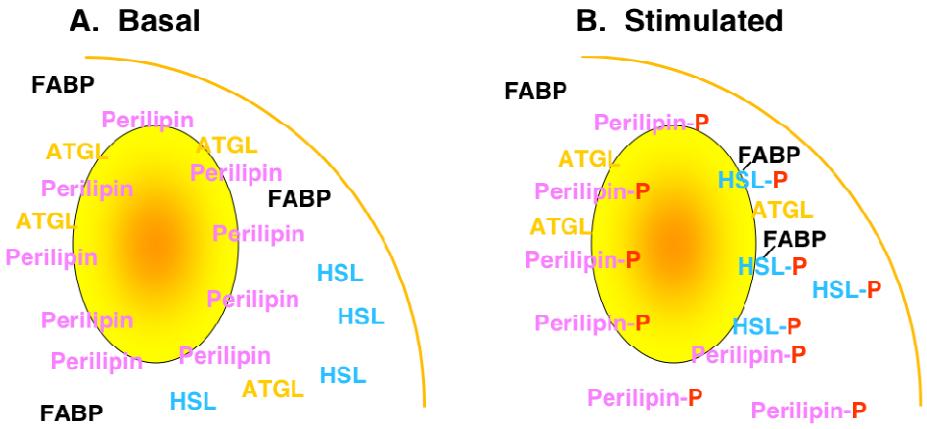
Understanding the function of HSL is important in finding a correlation between the lipase and several diseases. In order to find this correlation in humans, scientists are interested in analyzing the effect of HSL in mice.

HSL KNOCKOUTS AND TRANSGENIC MICE

Based on HSL's known role in lipolysis, researchers have been looking for a direct correlation between human hormone sensitive lipase and several diseases, including obesity, diabetes, and hyperlipidemia. A recent review, discussed below, from the Department of Medicine at Stanford University discussed the effects of HSL knockouts in mice (see below).

Figure 4 illustrates the localization of HSL and perilipin in lypolitic stimulation. In basal conditions, HSL and fatty acid binding protein (FABP) are localized in the cytosol, while perilipin and adipose triglyceride lipase (a droplet associated protein) are localized to the surface of lipid droplets within adipocytes. During lipolytic stimulation, protein kinase A is phosphorylated, which phosphorylates perilipin and HSL. HSL and FABP are then localized to the surface of lipid droplets (Kraemer and Shen, 2002).

Figure 4: Basal versus Stimulated Localization of HSL and Perilipin.



(Kraemer and Shen, 2002)

In mice where HSL has been inactivated by homologous recombination, physical appearance does not seem to be affected (non-obese). However, abnormalities in these KO mice are revealed when adipocytes are studied carefully (Kraemer and Shen, 2002). There are histological differences in both brown adipose tissue (BAT) and white adipose tissue (WAT). BAT has an increase in cell size, and WAT shows an increase in the differences in cell size (heterogeneity). Enzyme activity studies show an absence of neutral cholesterol ester hydrolase activity in both BAT and WAT, as well as in other tissues where HSL is normally present such as adrenal, testes, ovaries, and heart. Thus, HSL must be solely responsible for all neutral

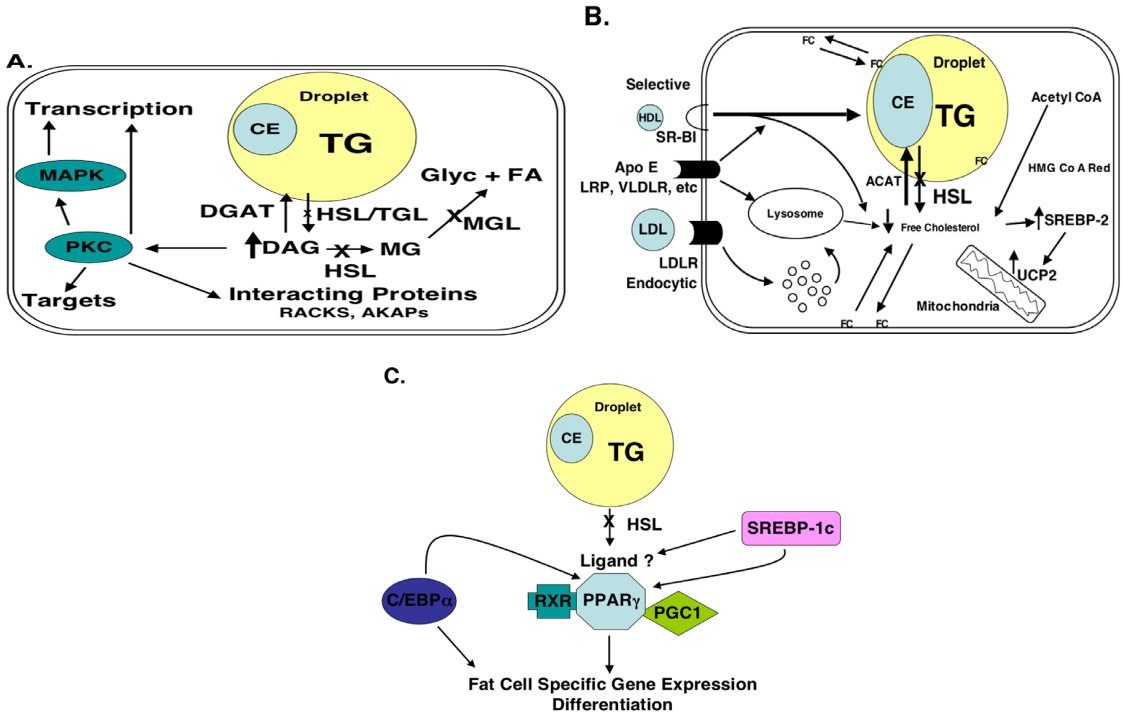
cholesterol ester hydrolase activity. Triacylglycerol lipase activity, on the other hand, was only decreased by 40-50% in these mice in WAT, and was similar to wild-type in BAT. It was concluded that triacylglycerol lipases other than HSL must be present in BAT and WAT (Kraemer and Shen, 2002). Researchers have discovered that ATGL (adipose triglyceride lipase) accounts for 60-70% of neutral triacylglycerol lipase activity in wild-type and HSL null mice (Kraemer and Shen, 2002).

Interestingly, HSL knockout (KO) mice are resistant to obesity, which makes HSL an important drug target. After 15 weeks of high fat diets, HSL null mice were 20-26% lower in weight than control mice. Fasting-induced weight loss and body temperatures in HSL KO mice were also observed (Kraemer and Shen, 2002). In addition, transcription factors were suppressed and mRNA expression of adipose differentiation markers including adiponectin, leptin, resistin, and adipsin were significantly decreased (60-90%). With the changes in gene expression because of the removal of HSL, lipolysis has been disrupted, and lipogenesis has been severely reduced. The alterations in mice resulting from the knockout of hormone sensitive lipase are concluded to be the following: lipase and lipid droplet-associated protein expression decreased, the amounts of WAT decreased with higher quantities of BAT, macrophages in WAT increased, thermogenesis and energy use increased causing resistance to obesity, and adipose differentiation genes (transcription factors, fatty acid and triglyceride synthesis enzymes, and alteration of the expression of adipocyte differentiation markers) (Kraemer and Shen, 2002).

The mechanisms for these alterations are not completely understood, but three possible mechanisms have been proposed (Figure 5). In part A, diacylglycerol increases in mice without HSL since HSL is the major diacylglycerol lipase in adipose tissue. Normal hydrolysis to free

fatty acids and glycerol does not occur. Instead, the build-up of diacylglycerol leads to activation of the protein kinase C family and targets such as MAPK, which affects cell proliferation, apoptosis, and differentiation. In part B of Figure 5, free cholesterol pools are decreased since HSL is the main cholesteryl ester hydrolase in adipose tissue. This leads to a greater amount of the transcription factor SREBP2 (sterol regulatory element binding protein 2) and increases in transcriptional targets like UCP2 (uncoupling protein 2). In part C of Figure 5, PPARγ ligands that are normally produced as a result of the release of fatty acids by HSL may be lacking in the HSL null mice. This lack of ligands could suppress activation of crucial adipocyte differentiation transcription factors, PPARγ and C/EBPα.

Figure 5: Potential Mechanisms of Altered Adipocyte Metabolism in HSL KO Mice.

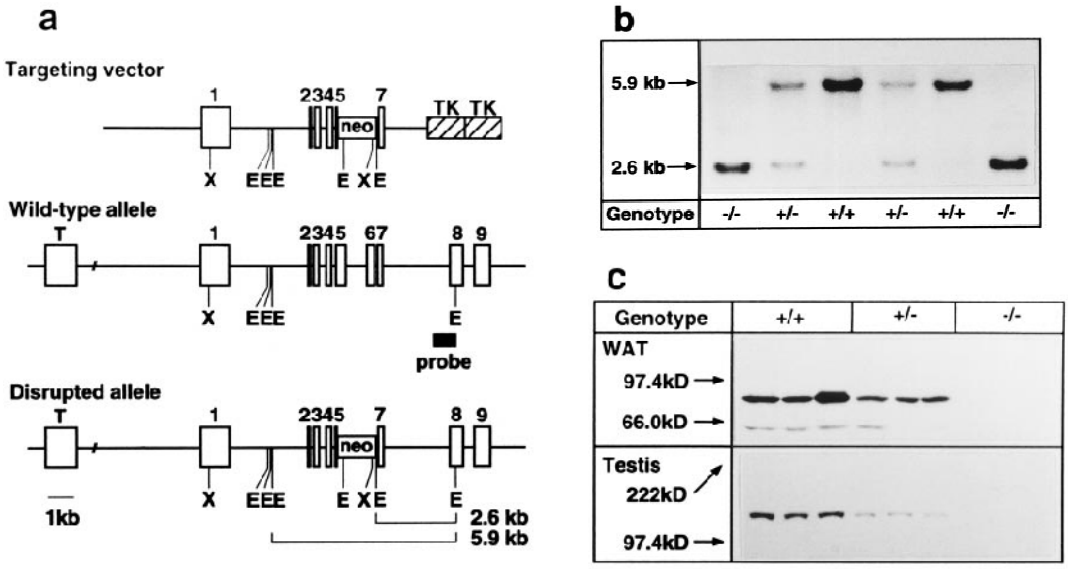


(Kraemer and Shen, 2002)

HSL AND MALE STERILITY

Scientists have also focused on the effect of HSL in the testes. Research from the University of Tokyo and Stanford University was also conducted on HSL knockout mice, focusing on both the hydrolysis of cholesterol esters and triacylglycerol. The HSL gene was disrupted by homologous recombination. Figure 6a shows the targeting vector, the wild-type HSL allele, and the disrupted allele (Osuga et al., 2000). Part of exon 5 and all of exon 6, which contain the catalytic domain, were deleted from the wild-type allele using the targeting vector to form the disrupted allele. The resulting HSL protein is inactive and lacks the C-terminal part. In Figure 6a, the rectangles represent exons, X and E are the restriction enzymes Xbal and EcoRI, respectively, and the shaded black box represents the probe used in the Southern blot in Figure 6b (Osuga et al., 2000). The Southern blot shows the size differences (5.9 kb wild-type and 2.6 kb mutated), with the genotypes shown below each lane. Figure 6c shows immunoblot analysis (Western) of WAT (84 kDa) and testes (130 kDa) without immuno-reactive HSL in KO -/- mice (Osuga et al., 2000).

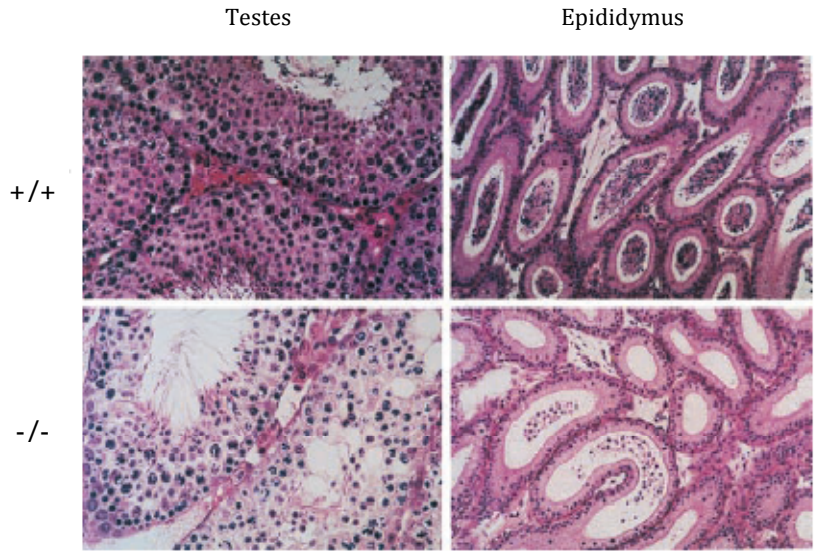
Figure 6: Homologous Recombination to Produce HSL -/- Mice.



(Osuga et al., 2000)

HSL -/- male mice were mated with female mice. Vaginal plugs were formed showing intercourse was normal, but none of the female mice became pregnant (Osuga et al., 2000). HSL -/- male mice had smaller testes than HSL +/+ male mice, shown by a lower weight: 86 ± 4 grams vs. 128 ± 6 grams, respectively. Epididymis preparations were measured for sperm counts. HSL -/- male mice had a sperm count of 94 ± 30 spermatazoa per epididymis, and HSL +/+ male mice had a sperm count of 7.7 ± 0.4 x 10^6 spermatazoa per epididymis, which is considerably higher for the KO mice, and may indicate a decreased release of sperm in those mice (Osuga et al, 2000). The sperm (60%) in HSL +/+ mice were motile, while none of the sperm in HSL -/- mice were motile. Figure 7 illustrates the difference in the thickness of epithelial layers in seminiferous tubules. The thickness was reduced from 12 layers in HSL +/+ mice, to 5-7 layers in HSL -/- mice. There was also a decrease in the number of mature spermatids from the HSL +/+ mice to the HSL -/- mice (Osuga et al., 2000).

Figure 7: Analysis of Testes and Epididymus in HSL -/- and HSL +/+ Mice.



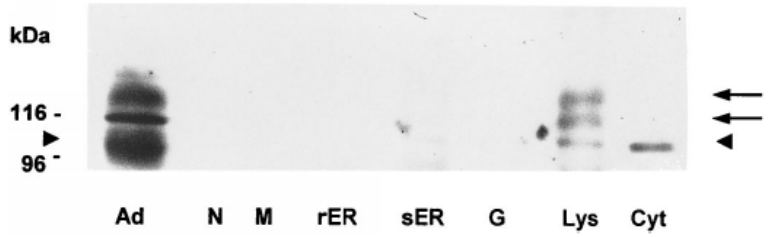
(Osuga et al., 2000)

Osuga et al also determined that in HSL -/- mice neutral cholesterol ester hydrolase (NCEH) activity was not present in the testes, which contained more cholesterol esters. NCEH activities in WAT and BAT were also absent in HSL -/- mice. Although there were phenotypic changes, such as enlarged BAT (5-fold) and WAT (2-fold), and BAT mass increase, HSL -/- were not obese. Triacylglycerol lipase activities (40% of wild-type) remained in WAT in HSL -/- mice. Thus, Osuga et al determined that HSL is not the only enzyme involved in triacylglycerol hydrolysis, and it is necessary for spermatogenesis.

A study was conducted on testicular HSL and its correlation with male fertility (Kabbaj et al., 2001). Testicular HSL was studied because of its importance with male fertility, as described in other studies where the KO mice were sterile rather than obese in some cases, proving HSL to be an important enzyme in the testis. More significantly, these studies suggest that HSL is important in the maintenance of the cholesterol content within the testicular cells. As seen in

Figure 8, there are several forms of HSL with varying molecular weights depending on its location: 104, 110, and 120 kDa. The smallest of the molecular weights were found in the seminiferous tubules (STf) total homogenate, interstitial tissue (ITf) and the cytosolic and lysosomal subcellular fractionations. As the testes developed, expression and activity increased. The larger of the molecular weights, 110 and 120 kDa were found only in the lysosomal subfraction of the STf homogenate. Although the detection of the testicular HSL in guinea pigs showed higher molecular weights than in other tissues, such as adipose tissue, the data does match that of studies conducted in other species. They concluded from their data that part of HSL may originate from germ cells, as seen in the HSL activity at varying molecular weights, which is then imported into Sertoli cells, which assist in the production of sperm in the male reproductive system. During spermatogenesis, spermatids attach to these cells in the seminiferous tubules in the testes.

Figure 8: HSL and Male Sterility in Guinea Pigs.
The figure shows HSL levels in various tissues and subcellular fractions.



(Kabbaj et al., 2001).

Although two isoforms of HSL in rats are currently known, HSLadi and HSLtes (84 kDa and 130 kDa respectively) and guinea pigs (104, 110 and 120 kDa) (Lindvall et al., 2003) research about HSL in humans, such as its functional importance and expression, must continue

(Large et al., 1998). Varying the amount of HSL could greatly impact the capacity in which adipose tissue hydrolyzes triacylglycerols. Increasing HSL mRNA levels two-fold, which was associated with plasma free fatty acid levels, was discovered in the adipose tissue of cancer patients. A decrease in HSL activity showed a decrease in lipolysis in fat cells of patients with combined hyperlipidemia and patients with hereditary obesity (Large et al., 1998).

HSL ACTIVITY

Contreras et al. (1998) expressed HSL in SF9 insect cells and purified the protein in two steps using anion-exchange chromatography on Q-Sepharose FF and hydrophobic-interaction chromatography on phenyl-Sepharose. They then conducted an HSL activity assay based on measuring the "[^{3}H] oleic acid released from either tri-[^{3}H]oleoylglycerol (TO), 1(3)-mono-[^{3}H]oleoyl-2-*O*-mono-oleoylglycerol (MOME, …), or cholesteryl [^{3}H]oleate (CO)" (Contreras et al., 1998). The HSL activity was measured in a crude extract, and then again after each purification step (Table 1).

Table 1: Human HSL Activity as Measured by Contreras et al.

Purification of Recombinant Human HSL

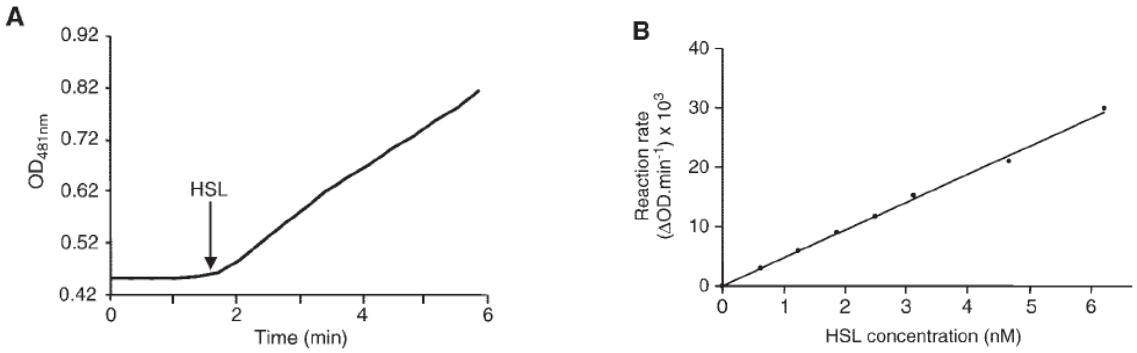
Purification step	Vol (ml)	Protein[a] (mg/ml)	HSL activity[b] (mU/ml)	Specific activity (mU/mg)	% Yield	Purification (fold)
Crude extract	300	13.1	89,880	6,861	100	(1)
Q-Sepharose	250	0.73	27,780	38,054	26	5.5
Phenyl–Sepharose	260	0.06	11,670	194,500[c]	11	28

[a] Protein concentration estimated by the method of Bradford (18) with BSA as standard.
[b] The diacylglycerol analogue (MOME) was used as substrate.
[c] This specific activity is based on determination of protein by the method of Bradford that underestimates the amount of human HSL protein. When a more accurate method was used for protein determination (17), a specific activity of 100 U/mg was obtained (see text).

(Contreras et al., 1998)

In a study conducted by Petry et al. (2005), they developed an assay using a monoacylglycerol (MAG) containing NBD (NBD-MAG). This was proven to be an efficient substrate for HSL, more specifically, human recombinant and rat adipocyte HSL. In their study, they recorded the kinetics of the reaction between NBD-MAG and HSL over a period of 6 minutes at OD 481nm (Figure 9A). The 180 µL reaction was incubated at 30°C, and HSL (at a concentration of 33 nM) was added after two minutes. Also, they plotted the reaction rate against the HSL concentration (in nM) (Figure 9B).

Figure 9: NDB-MAG Assay for Monitoring HSL Enzymatic Activity in Rat Adipocytes and Human Recombinant HSL.



(Petry et al., 2005)

BLUE SKY BIOTECH

Blue Sky Biotech (Worcester, MA) is a contract research organization in the pharmaceutical research and development market, providing both services and products. They specialize in gene cloning and expression, protein purification, and assay development. They offer services such as gene synthesis; subcloning and plasmid construction; vector construction; site-directed mutagenesis; protein expression scouting; scale-up of *E. coli*, yeast, mammalian and

insect cell expression; optimization of expression in bioreactors; and protein, tissue/organ and antibody purification. With respect to this project, Blue Sky Biotech is interested in producing purified HSL protein to add to their growing product line, which currently consists of 65 products such as Acetyl CoA Carboxylase (rat liver), human Lymphocyte-specific protein-tyrosine kinase, autotoxin (human), phosphodiesterases (human), and sirtuin T1 (human), just to name a few. HSL produced by Blue Sky Biotech can be used to facilitate research and drug discovery in the fields of obesity, diabetes, and hyperlipidemia.

PROJECT PURPOSE

The purpose of this project was to clone, express, and purify human hormone sensitive lipase (HSL) for Blue Sky Biotech's product line. Gene synthesis was chosen for the initial cloning for convenience and reliability. Expression in insect cells was chosen due to the high levels of expression of exogenous proteins. Protein purification by two separate epitopes (His and biotin tags) was chosen to increase product purity.

Hormone sensitive lipase affects lipolysis pathways in adipose tissue and the hydrolysis of cholesterol esters in steroidogenic tissue, such as the testes. Its activity is inhibited by the presence of insulin and activated by the presence of glucagon. Several diseases including obesity, diabetes, and hyperlipidemia are involved in varying the levels of HSL. The goal of this project is for Blue Sky Biotech to be able to sell this product to laboratories currently researching the role of HSL with these diseases.

METHODOLOGY

GENE SYNTHESIS

Gene synthesis was performed in three segments: P1, P2, and P3 to clone into plasmids pDONR-221-P1, pDONR-221-P2, and pDONR-221-P3 (Integrated DNA Technology). A total of 100 µL of molecular grade water was added to each of the wells to re-suspend each of the oligonucleotides. Once the oligos were entirely re-suspended, 10 µL from each well was then mixed together to anneal each of the individual segments. The annealed oligos were then used as template in PCR reactions. Primer mixes to be used in the PCR were made using the same primers from the gene synthesis plate; 10 µL of the first primer and 10 µL of the last primer for P1, P2, and P3. Refer to Appendix B for the complete list of primers.

GENE AMPLIFICATION AND CLONING INTO PFB-HBV3

GENE AMPLIFICATION AND ASSEMBLY

PCR was performed for P1, P2, and P3 in a total of 50 µL using primeSTAR HS DNA Polymerase from TaKaRa. The PCR products were then gel extracted and purified (Promega's Wizard SV Gel and PCR Clean-Up System). A Gateway (recombination) reaction was performed using the PCR product, pDONR vector, TE buffer, and the BP Clonase II enzyme. After one hour in 25°C, the three reactions were transformed into *E. coli* DH5α cells made competent by Blue Sky Biotech, and plated on LB+kanamycin plates. A PCR screen was done the following day to check for positive clones using a Taq 2X Mater Mix (New England BioLabs). The primers used for P1, P2, and P3 were M13R + HSL-P1-750F, M13R + HSL-P2-740F, and M13R + HSL-P3-735F, respectively. The annealing temperature for PCR was set to

52°C for P1, and 55°C for P2 and P3. The positive clones were then miniprepped (Fermentas GeneJET Plasmid Miniprep kit) and the samples were sent for sequence validation through SeqWright. The sequences were then analyzed using DNADynamo to obtain the positive clone numbers to use as the template for PCR assembly. PCR assembly was done in two parts: P1+P2, and P2+P3, then assembled into the full size HSL gene.

CLONING INTO PFB-HBV3

Once the PCR reactions were completed for P1, P2, and P3, the amplicons were gel extracted (Promega), digested using NotI and XhoI (New England BioLabs), and column purified (Qiagen QIAquick PCR Purification Kit). The elution (HSL insert) was then ligated to the pFB-HBv3 vector (see Appenix A) and transformed onto LB+ampicillin plates. Once positive results were achieved from the colony screens, the samples were miniprepped and digested to ensure that they contained the correct insert.

BACMID PREPARATION

Once a positive clone was obtained, the DNA was transformed into MAX Efficiency DH10 Bac Competent Cells (Invitrogen), which are used in the bac-to-bac baculovirus expression system. An expression bacmid with the gene of interest is produced from the combination of a donor plasmid, pFastBac, and a parent bacmid containing the *lacZa* from the DH10 bac *E. coli* strain. The *lacZa* gene enables blue and white selection of positive recombinants (Invitrogen, 2004). The samples were then prepped (Fermentas GeneJET Plasmid Miniprep kit), followed by a bacmid PCR.

BACULOVIRUS EXPRESSION OF HSL IN INSECT CELLS

PREPARING P1 VIRUS STOCK

A viral stock was prepared using SF9 cells diluted to $1.00x10^6$ cells/mL in 36 mL of SF900 II SFM media (Invitrogen) supplemented with 10 µg/mL of Gentimicin (Gibco) in a 125mL flask. A total of 10 µL of bacmid DNA was diluted into 750 µL of Opti-MEM (Gibco) media. Separately, 45 µL of Cell Fectin was diluted in 750 µL of Opti-MEM, then combined with the bacmid. The resulting 1.5 mL bacmid/cell fectin mixture was incubated at room temperature for 30 minutes and then added to 36 mL of SF9 cells. The P1 stock was harvested when the viability reached between 60-70%.

PREPARING SCOUT TEST SAMPLES

An experiment (SCOUT) was performed to determine the optimal host cell line and conditions that yielded the highest levels of HSL expression using the P1 Viral stock and two cell lines: SF9 and SF21. Twenty-four hours prior to the scout, the SF9 and SF21 cells were seeded using SF900 II SFM media (Invitrogen) and supplemented with 10 µg/mL of Gentamicin (Gibco). Cell and viability counts were performed on the Cedex (Innovatis) the following day. The P1 viral stock was then used to inoculate six, 50mL flasks containing one of the two cell lines and varying dilutions in SF900II SFM media: SF9 1:100, SF9 1:1000, SF9 1:10000, SF21 1:100, SF21 1:1000, and SF21 1:10000. The viable cell count, total cell count, percent viability, and diameter of the cells were recorded over 24-96 hours, after inoculation and samples from each of these time points were collected for the Western Blot analysis (amount calculated from $2.0x10^6$/total cell count). Refer to the SDS-PAGE Analysis and Immunoblotting section for sample preparation. Once the most advantageous conditions were found for expression of HSL, a

scale-up was performed under these conditions. When the cell viability decreased substantially, the 2L culture was harvested and the cell paste was used for the expression and purification of HSL.

CO-EXPRESSION OF HSL AND PBL

Due to the presence of the biotin acceptor domain on the protein, a co-infection of the HSL P1 viral stock and protein biotin ligase (PBL) was performed for further expression and purification. Four flasks containing 600mL SF21 cells were grown and split into four further flasks for use in the co-infection. Each flask contained 71mL of SF21 cells and 429mL of pre-warmed SF900 II SFM media (Invitrogen), containing 1ml/L Gentamicin (10 µg/mL) (Gibco) to reach a desired density of $7.5x10^5$ cells/mL. Each of the four flasks was infected with 500 µL of HSL P1/PBL viruses and mixed with 1 ml of 50 µM biotin. Once the cells grew for 48 hours, showing a large increase in diameter, they were harvested. For the harvest, all four flasks were combined and mixed into one flask (2L total), and spun at 4,000g for 15 minutes. A total of 25mL 1XPBS/L was added to each pellet and snap frozen for storage in -80°C.

PURIFICATION OF HSL

NACL CONCENTRATION TEST PURIFICATION

Three small-scale His purifications were performed using Ni-NTA (GE) with varying NaCl concentrations in the lysis buffer (20 mM Tris, 5 mM Imidazole, 1.25 mM Brij-35, 1 mM Triton X-100, 5 mM Tween 20, and either 150 mM, 300 mM, or 500 mM NaCl, pH 7.5) and elution buffer (20 mM Tris, 500 mM Imidazole, 1.25 mM Brij-35, 1 mM Triton X-100, 5 mM Tween 20, and either 150 mM, 300 mM, or 500 mM NaCl, pH 7.5). Half of a gram of cell paste from the 2L HSL expression harvest was resuspended in 5 ml of each of the three NaCl

concentrations. The resuspended cells were lysed by sonication and each lysate was centrifuged (20,000 rpm for 20 minutes) to separate the supernatant (soluble protein) from the pellet (insoluble fraction including the cell membrane). The protein in the supernatants was bound to washed Ni-NTA (GE) resin overnight at 4˚ with constant agitation. Each sample was poured into a column, and beads were washed with the appropriate lysis buffer, washed again with 25 mM Imidazole in the appropriate buffer, and eluted with 150 mM, 300 mM, and 500 mM Imidazole in 3 fractions each concentration. The fractions were analyzed by SDS-PAGE, Coomassie-stained and Western blotted, to determine the most effective NaCl concentration for binding.

NI-NTA PURIFICATION

A large scale His purification was performed using cold lysis (20 mM Tris, 5 mM Imidazole, 1.25 mM Brij-35, 1 mM Triton X-100, 5 mM Tween 20, and 300 mM NaCl, pH 7.5) and cold elution (20 mM Tris, 500 mM Imidazole, 1.25 mM Brij-35, 1 mM Triton X-100, 5 mM Tween 20, and 300 mM NaCl, pH 7.5) buffers. A 1L pellet from the HSL/pBL co-expression harvest was weighed and resuspended in an appropriate amount of lysis buffer (10 milliliters buffer per 1 gram cell paste). A protease inhibitor cocktail was added to the resuspension to reduce protein degradation. The resuspended cells were lysed by sonication and the lysate was centrifuged (20,000 rpm for 60 minutes at 4˚C). The supernatant was incubated with pre-equilibrated Ni-NTA (GE) resin overnight at 4˚ with constant agitation. A protease inhibitor cocktail was added to the resin and supernatant to reduce protein degradation. The resin and supernatant were poured into a column, and the beads were washed with lysis buffer, washed again with 25 mM Imidazole, and eluted with 150 mM, 300 mM, and 500 mM Imidazole in 3 fractions each. The flow through, washes, and fractions, as well as samples from each step before

the sample was added to the column, were analyzed by SDS-PAGE, Coomassie-stained and Western blotted to determine purity.

DIALYSIS

Protein samples were dialyzed to exchange buffers and eliminate imidazole from the samples. The pooled fractions from the His purification were added to 12 kD dialysis tubing (FisherBrand) and exchanged into 4L 1X PBS and 10% glycerol at pH 7.4.

BIOTIN PURIFICATION

The dialyzed biotinylated protein from the His purification was loaded onto washed and 1X PBS equilibrated SoftLink Avidin resin (Promega) in a column at 4˚C. The column was washed with 1X PBS and eluted using 5 mM biotin (dissolved in NaOH and diluted 1:20 in 1X PBS) into several fractions. The flow through, wash, and fractions were analyzed by SDS-PAGE, Coomassie-stained and Western blotted to determine purity.

BRADFORD AND NANODROP PROTEIN CONCENTRATION ESTIMATION ASSAYS

The Ni-NTA purified protein concentration was estimated using two different methods, a Bradford estimation and a NanoDrop estimation. In the Bradford method, a 1:2 serial dilution of Bovine Serum Albumin (BSA) was used as a standard concentration curve in a 95 well plate. A serial dilution of His purified protein was performed starting with 5 µl of undiluted protein, and diluting 1:2 with water serially. Bradford Reagent (195 µl) was added to each well containing either the BSA standard or the HSL protein. The samples were incubated at room temperature for 5 minutes and then analyzed using SoftMax Pro 5.2 software.

Protein estimation was done using the NanoDrop 1000 (Thermo Scientific) to verify the Bradford Assay. The apparatus was blanked with 2 µl of 1X PBS and then 2 µl of HSL protein was analyzed using NanoDrop 1000 V3.7.1.

SDS PAGE ANALYSIS AND IMMUNOBLOTTING

Samples from the test purification, His purification, and Biotin purification were prepped using 80 µl of SDS-PAGE loading dye and 20 µl of sample, boiled at 95°C for 5 minutes, and loaded onto either 7.5% or 4-20% SDS-PAGE gels to be analyzed using Coomassie-staining or Western blot analysis. Gels were run for one hour at 160 volts, and either Coomassie-stained or Western blotted. Gels for Western blot analysis were transferred to PVDF membranes for one hour at 100 volts with constant stirring. The membranes were blocked in milk for 20-30 minutes and left in primary antibody overnight. The membranes were put in the secondary antibodies for two hours before developing in Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega). The antibodies used included mouse anti-His mAb (primary) diluted 1:7,500 (Clontech) with a secondary of goat anti-mouse IgG-AP pAb diluted 1:10,000 (Jackson Immuno), rabbit anti-HSL (primary) diluted 1:1,000 (Cell Signal catalog number: 41075) with a secondary of goat anti-rabbit IgG-AP diluted 1:5,000 (Vector Labs catalog number: AP100), and Streptavidin-AP Conjugate diluted 1:25000 (Pierce) with no secondary antibody needed. No optimizations were attempted to increase the specificity of the HSL antibody, nor were other manufacturers' reagents tested.

PURITY DETERMINATION

The purity of HSL was determined using the 2100 Bioanalyzer with the Protein 230 Kit (Agilent Technologies). The kit contains a chip and reagents used for the analysis of proteins.

The chip has several micro-channels that separate proteins by size during electrophoresis. Gel-dye mix (12 µl), destaining solution (12 µl), protein samples (6 µl), and the ladder (6 µl) were loaded into marked wells on the chip. The chip was then placed into the 2100 Bioanalyzer and started immediately.

HSL ACTIVITY ASSAY

The activity of HSL was measured using the QuantiChrom™ Lipase Assay Kit (DLPS-100) for rapid colorimetric determination of lipase activity at 412nm (BioAssay Systems). A working solution was prepared using the kit reagents (5 mg Color reagent, 140 µl Assay Buffer, and 8 µl BALB Reagent per each well of reaction in a 96-well plate). Calibrator (150 µl) and 1x PBS (150 µl) were added to separate wells of the plate as controls. Protein samples from the Ni-NTA purification and the biotin purification were used undiluted and also diluted 1:10 and 1:100 for this assay. The undiluted, 1:10 diluted, and 1:100 diluted samples (10 µl each) were loaded into separate wells of the plate in triplicate from each purification. Working solution (140 µl) was added to each sample well, and the absorbance at 412 nanometers was taken every 30 seconds for a period of one hour. The activity was analyzed using SoftMax Pro 5.2 software and calculated using the following equation:

$$\text{Activity} = \frac{OD_{20min} - OD_{10min}}{OD_{Calibrator} - OD_{H2O}} \times 735 \quad (U/L)$$

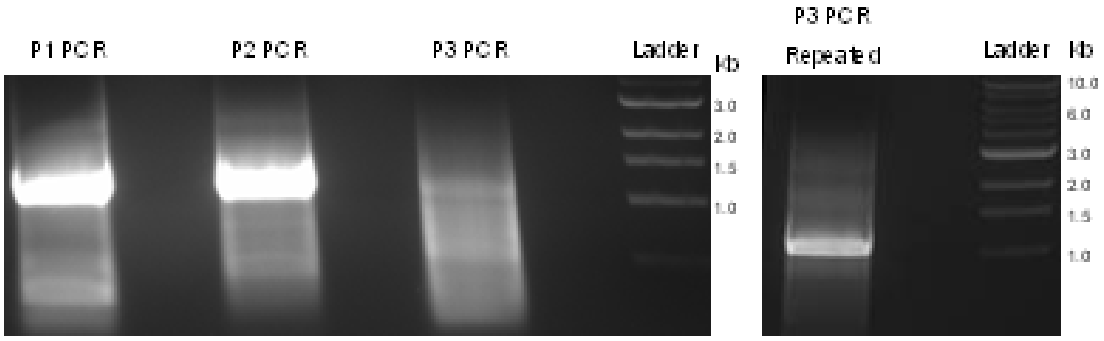
RESULTS

The following results were obtained from the HSL cloning, expression, and purification. The results indicate that HSL was successfully cloned into the pFB-HBv3 expression vector, transformed into DH10 bac cells (Invitrogen), transfected and expressed in SF21 insect cells co-infected with HSL P1 virus stock and protein biotin ligase (PBL), and purified using Ni-NTA resin followed by a Softlink avidin resin. It was also found that the purified HSL protein is biologically active as determined by an activity assay.

CLONING OF HSL

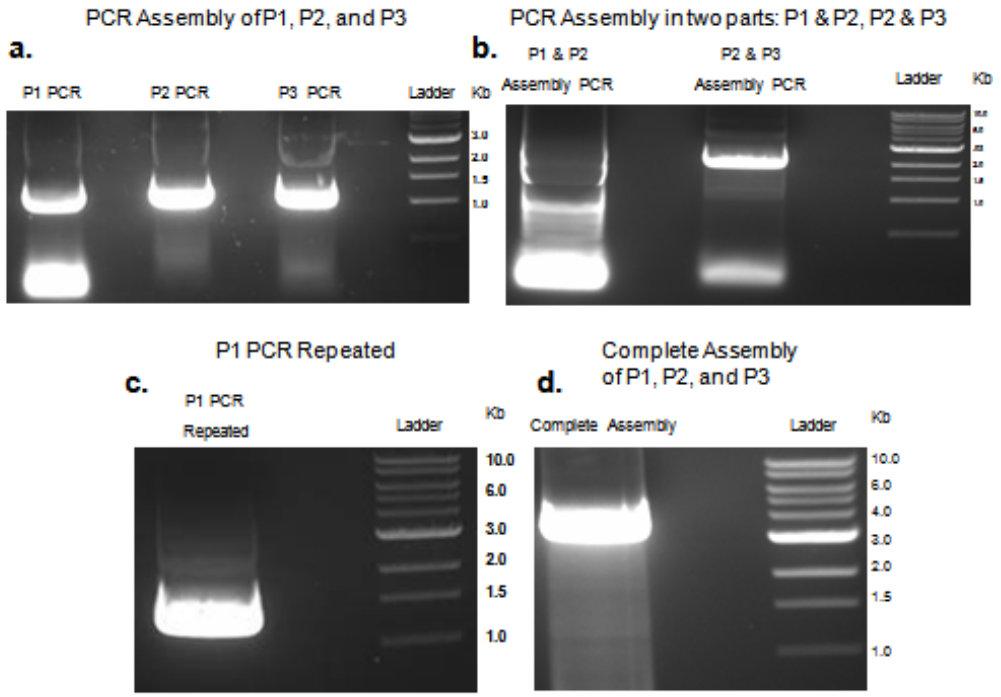
HSL was cloned in three segments: P1, P2, and P3, beginning with gene synthesis (synthetic primer annealing) followed by PCR. The first PCR was performed for P1, P2, and P3 (Figure 10); P3 was unsuccessful in the first PCR, but proved successful when repeated (Figure 10).

Figure 10: PCR of P1, P2, and P3 HSL Gene Fragments from Annealed Oligo DNA Templates.
Gels prepared with 1% Agarose (American Bioanalytical) in 1X TAE stained with EtBr.



Following gene assembly (first PCR reaction), PCR amplification was performed on P1, P2, and P3 (Figure 11a). The PCR products were purified from the gels, and despite attempts to assemble these three parts, complete assembly was unsuccessful. Assembly in two parts (Figure 11b) was then performed to determine where the problem was occurring. P1 was repeated after failing to assemble with P2 (Figure 11c). By using M13F as the forward primer and HSL P1 as the reverse at an annealing temperature of 55°C, all three HSL gene segments were successfully assembled together (Figure 11d).

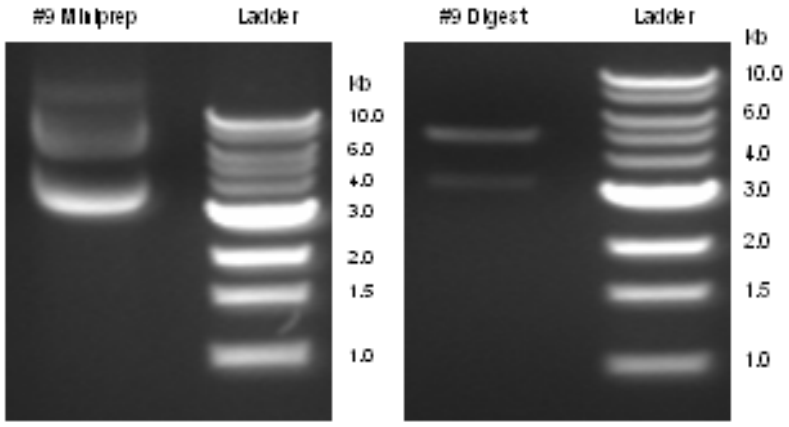
Figure 11: PCR Assembly of P1, P2, and P3.
Gels prepared with 1% Agarose (American Bioanalytical) in 1X TAE stained with EtBr.



The HSL insert (PCR full assembly) was ligated to the expression vector, pFB-HBv3, and transformed into *E. coli* DH5α cells. The cells were incubated overnight, followed by a colony (PCR) screen. One sample (HSL #9—a positive from the colony screen) was

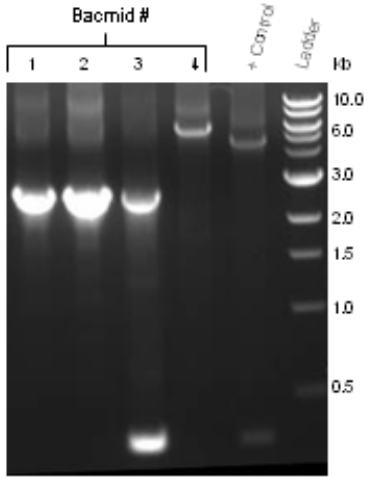
miniprepped and digested to ensure it contained the correct insert (Figure 12). This positive clone was then used for the transformation into DH10 Bac cells.

Figure 12: HSL Miniprepped and Digested with NotI and XhoI.
Gels prepared with 1% Agarose (American Bioanalytical) in 1X TAE stained with EtBr.



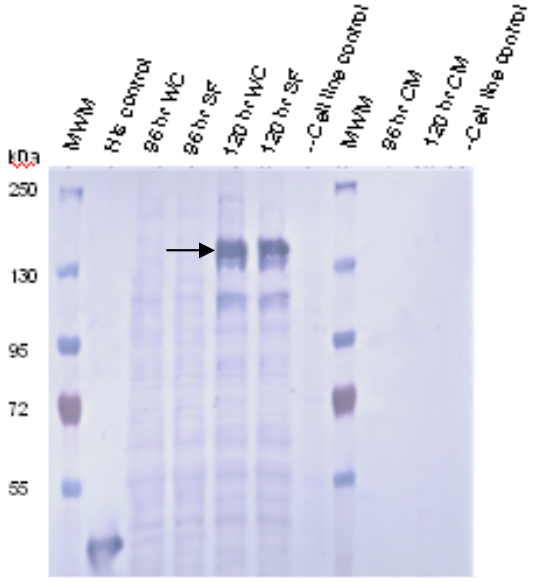
Several bacmids were prepared and screened by PCR to confirm identity. Of the bacmid samples prepped, one sample was determined to be a positive clone (HSL bacmid #4) (Figure 13, lane 4), which was then used to prepare the HSL virus stock used for expression and purification.

Figure 13: HSL Bacmid Quality Control.
Gels prepared with 1% Agarose (American Bioanalytical) in 1X TAE stained with EtBr.



A bacteriophage stock was prepared by combining SF9 insect cells with the bacmid DNA containing the HSL gene and cell fectin mix. This HSL P1 stock was harvested when the viability was between 60 and 70%. Figure 14 is an immunoblot for α-His-tagged HSL illustrating the expression of the HSL P1 virus stock after harvesting. The expression was strongest at 120 hours, and lane 6 shows that the HSL is soluble.

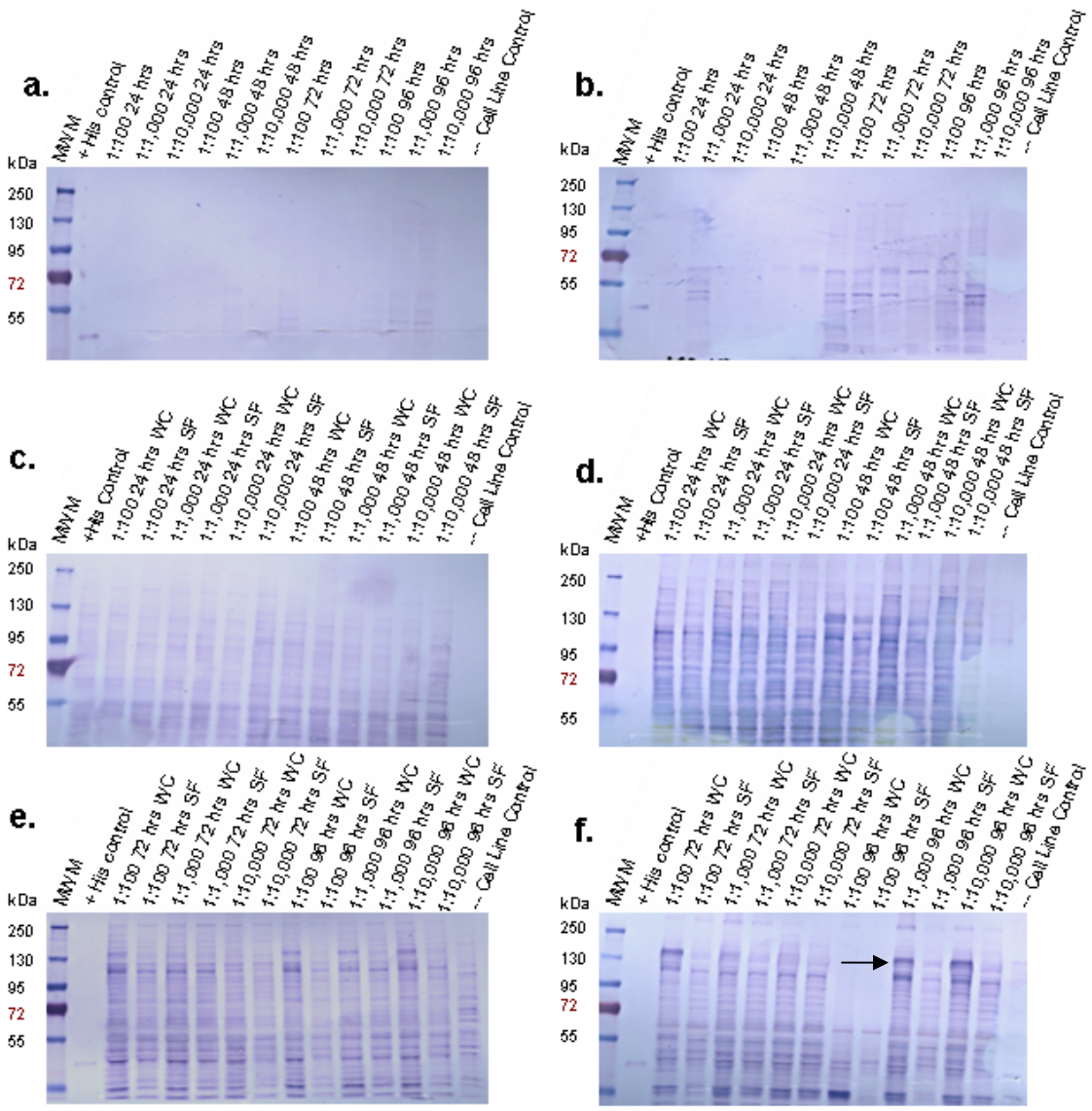
Figure 14: Analysis of HSL P1 Expression by Western Blot (α-His).
The arrow denotes the position of HSL protein.



After the P1 stock was prepared, an experiment (SCOUT) was conducted to determine the optimal host cell line and conditions that yielded the highest levels of HSL expression. The P1 virus stock was used to inoculate the two cell lines (SF9 and SF21) at three dilutions each. The cells were monitored every 24 hours until the viabilities were about 70%, and then the cells were harvested. Figure 15 illustrates Western blots (blotted with α-His) of the SCOUT. Panels a and b show the Western blots of the SF 9 and SF 21 insect cell line culture media, respectively,

of three dilutions (1:100, 1:1,000, and 1:10,000) at four different times (24 hrs, 48 hrs, 72 hrs, and 96 hrs). Panels c and d show the Western blots of the SF 9 and SF 21 whole cell lysates and soluble fractions, respectively, of three dilutions (1:100, 1:1,000, and 1:10,000) at two different times (24 hrs and 48 hrs). Panels e and f show the Western blots of the SF 9 and SF 21 whole cell lysates and soluble fractions, respectively, of three dilutions (1:100, 1:1,000, and 1:10,000) of two different times (72 hrs and 96 hrs). The description of the lanes loaded for each pair of panels is located to the right of the panels. The arrow in panel f indicates the conditions with the best expression of HSL. The SF 21 insect cell line at a dilution of 1:1,000 was used for large scale expression of HSL and cells were monitored over a period of 96 hours for viability.

Figure 15: SCOUT Experiment. Western Blots of HSL Expression Conditions in Insect Cells (α-His).

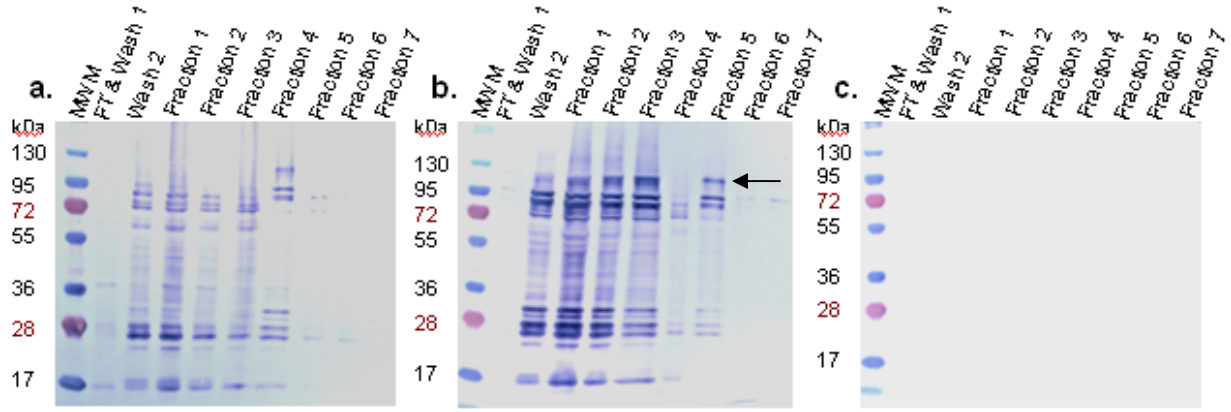


PROTEIN PURIFICATION

After the large scale (2L) expression of HSL in SF21 insect cells under optimal conditions, a test purification was conducted in order to determine the optimal NaCl concentration that produces the best His-tagged HSL binding to metal chelating resin and purity. Figure 16 illustrates analysis of the HSL test His purification. Panels a, b, and c show the

Western blots of the test His purification using lysis and elution buffer containing 150 mM NaCl, 300 mM NaCl, and 500 mM NaCl, respectively, all blotted with α-HSL. The arrow in panel b indicates the best binding of HSL to the Ni-NTA resin, and therefore the best purification of HSL. The lysis and elution buffers used in the large scale purification of HSL contained 300 mM NaCl. Because the protein specific antibody was used as the primary antibody, these membranes should have shown one band containing pure HSL. Some protein-specific antibodies, however, show high levels of cross reactivity. Due to the presence of multiple bands, it appears that HSL is very unstable and degrades considerably during purification.

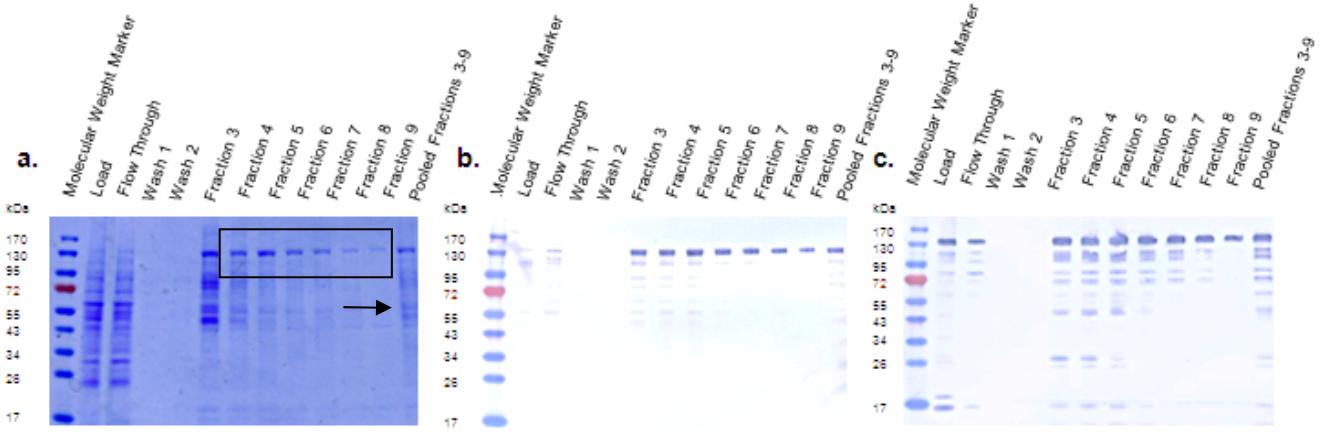
Figure 16: Analysis of HSL Test His Purification (α-HSL Western Blot).



Once the buffer containing 300 mM NaCl was chosen, a large scale His purification was performed on the co-HSL/PBL expressed insect cell pellets in order to purify HSL. Figure 17 illustrates analysis of the co-HSL/PBL His purification. Panel a shows the Coomassie-stained SDS-PAGE gel. Lanes 7-12 on the Coomassie-stained gel show pure HSL as indicated by the black box. However, because lane 6 was pooled with lanes 7-12, the pooled sample shown in lane 13 appears to contain degraded HSL, indicated by the black arrow. Panel b shows the SDS-

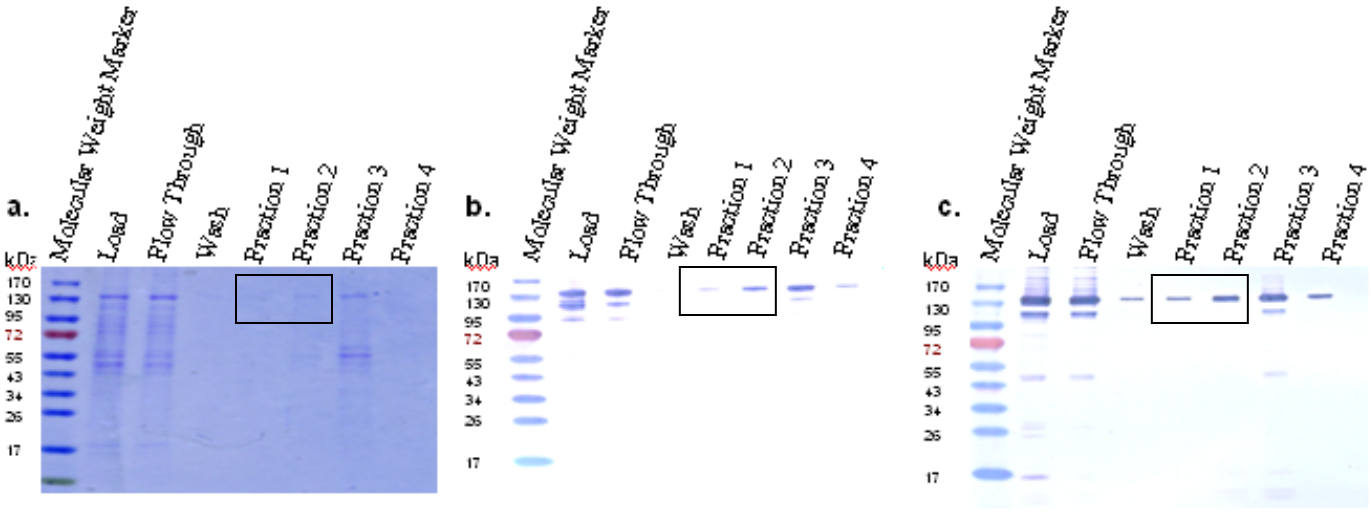
PAGE gel Western blotted with α-HSL. Panel c shows the SDS-PAGE gel Western blotted with α-His.

Figure 17: Analysis of co-HSL/PBL His Purification.
The black box denotes purified HSL fractions.



To further purify HSL, fractions containing HSL for the nickel-affinity purification were pooled and applied to SoftLink avidin. The biotinylated HSL binds to the mutant avidin on the resin, which increases the purity of the protein. Biotin binds avidin so tightly that mutant avidin is used in order to elute the protein. Figure 18 illustrates analysis of the co-HSL/PBL Biotin purification. Panel a shows the Coomassie-stained SDS-PAGE gel. Panel b shows the SDS-PAGE gel, transferred, and Western blotted with α-HSL. Panel c shows a Western blot using α-His as the primary antibody. The black boxes indicate the fractions (1 and 2) that appeared to be the most pure in all three panels. Fraction 2 contains the greatest concentration of pure HSL.

Figure 18: Analysis of co-HSL/PBL Biotin Purification.
The black box denotes the purest HSL fractions.



After the each purification, the concentration of the purified HSL was determined using NanoDrop Protein Estimation. A small amount (2 µl) of 1X PBS was added to the NanoDrop to blank the machine and then 2 µl of purified HSL was added for the concentration to be measured. The NanoDrop reported the purified HSL after the Ni-NTA purification to be at a concentration of 6.55 mg/ml (Figure 19) and 2.46 mg/ml (Figure 20) after the biotin purification. The Bradford protein assay reported purified HSL from the Ni-NTA purification to be at a concentration of approximately 8 mg/ml (Figure 21). The final volume after the biotin purification step was 6 ml, and therefore the final yield was 14.76 mg.

Figure 19: NanoDrop Estimation of Protein Concentration from HSL Purification 1.

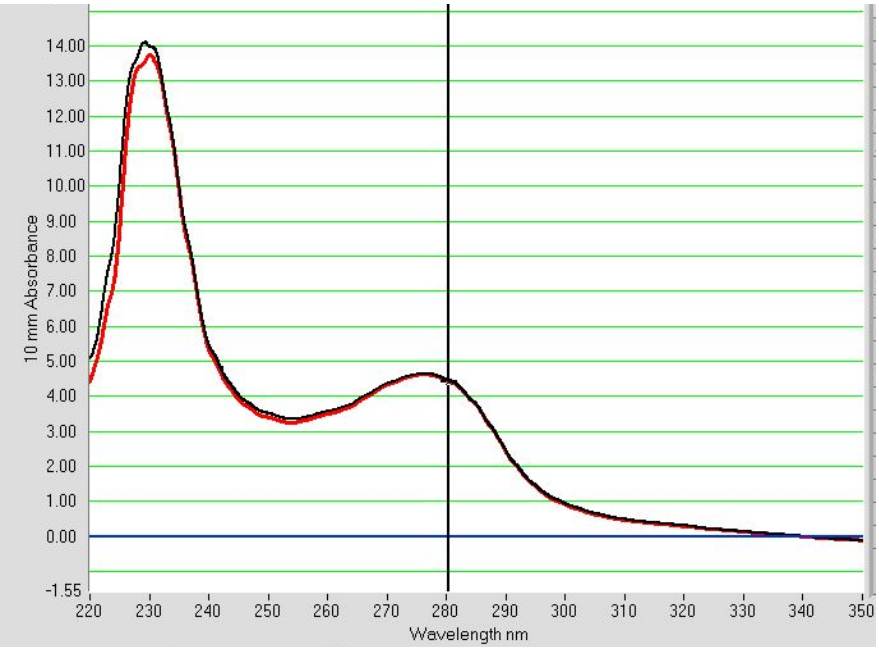


Figure 20: NanoDrop Estimation of Protein Concentration from HSL Purification 2.

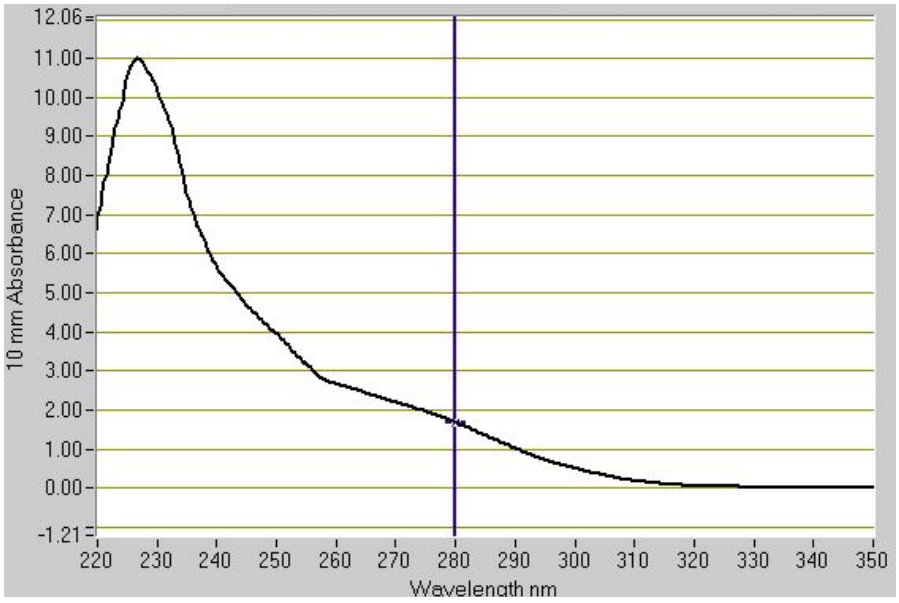
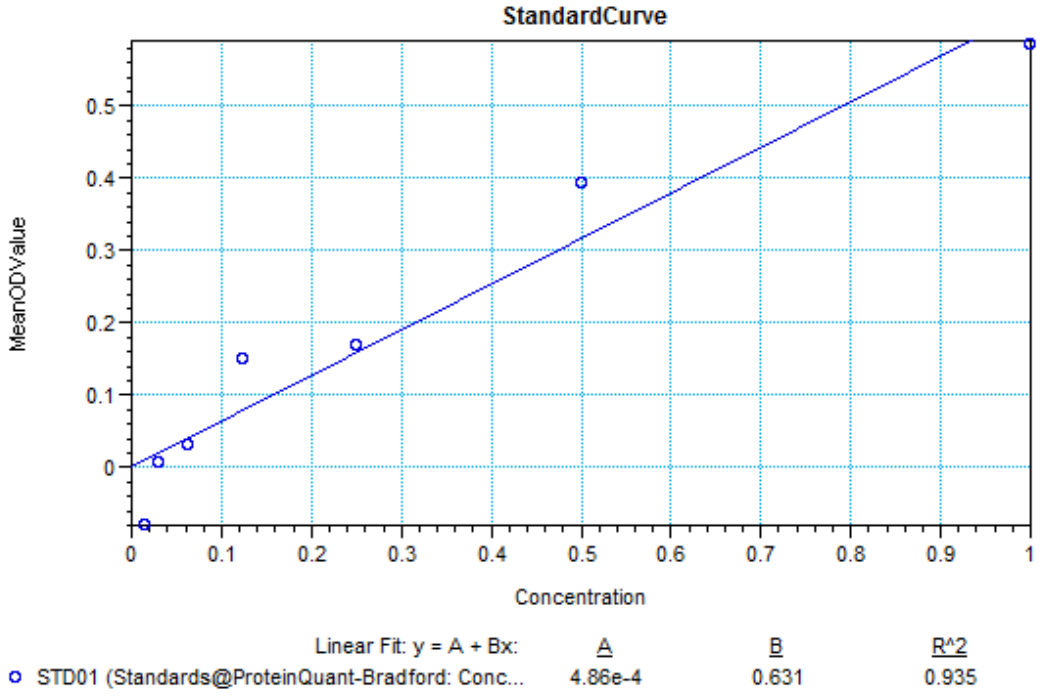


Figure 21: Bradford Assay Standard Curve Used to Estimate Protein Concentrations.



HSL ACTIVITY

The biological activity of the purified HSL was found using the QuantiChrom Lipase Assay Kit (DLPS-100) for rapid colorimetric determination of lipase activity at 412nm (BioAssay Systems). The purified HSL was determined to be biologically active. Table 2 shows the results of the activity assay performed on both the His-purified HSL (Purification 1) and the biotin-purified HSL (Purification 2). The protein from each purification step was assayed in triplicates under varying dilutions: undiluted, 1:10, and 1:100. These values were read every 30 seconds for a total of 60 minutes (Figures 22 and 23). As seen in Table 2, and Figures 22 and 23, the OD activity of the protein samples increased over time, and the undiluted samples tended to have a greater OD change over time than the diluted samples. The activity of HSL was calculated from the graphs using the following equation:

$$\text{Activity} = \frac{OD_{20min} - OD_{10min}}{OD_{Calibrator} - OD_{H2O}} \times 735 \quad (U/L)$$

(BioAssay Systems)

After concluding the protein to be biologically active, its purity was found using the 2100 Bioanalyzer (Agilent). After the first step of the purification, the purity of the protein was 19.97% and after the second step of purification, it reached 80.83% purity. After the second purification step, HSL appears to be more pure with fewer bands than the first purification step (data not shown).

Figure 22: HSL Activity Assay for HSL Purification 1.

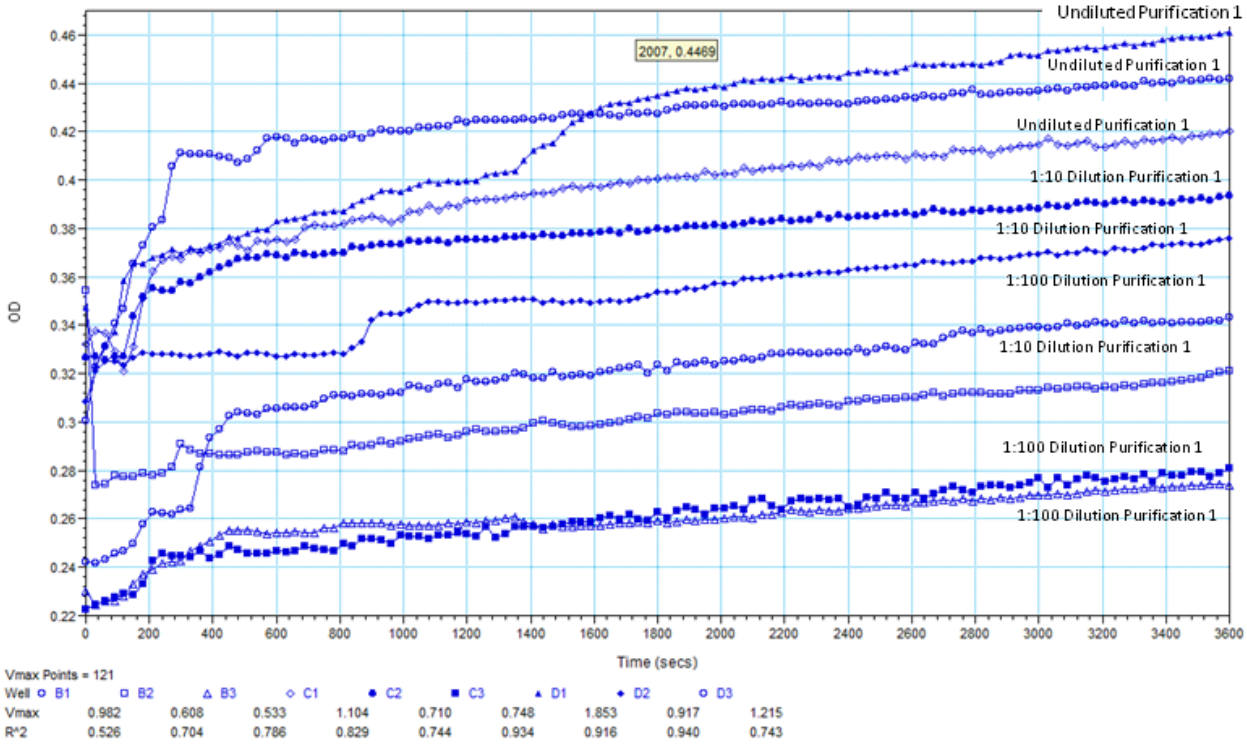


Figure 23: HSL Activity Assay for HSL Purification 2.

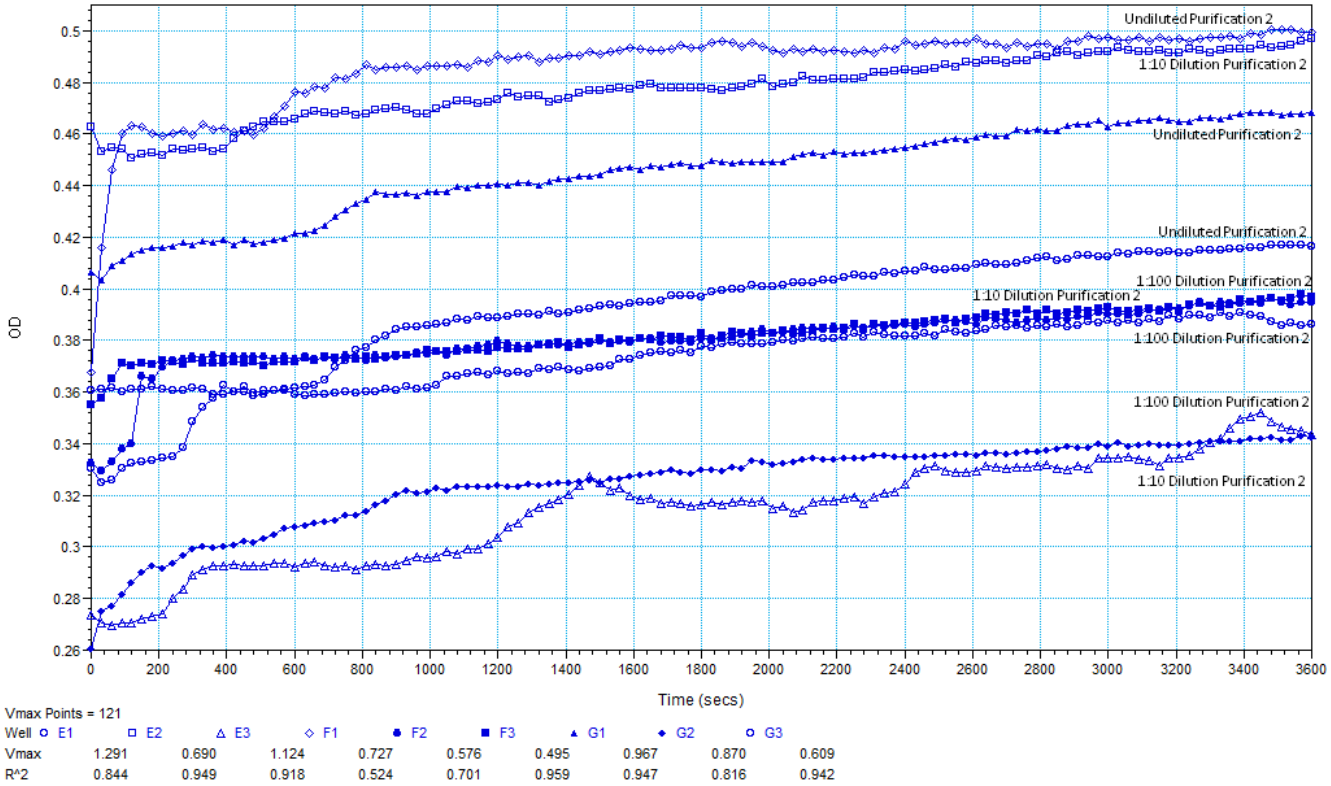


Table 2: Summary of HSL Activity Assay Results.

Purification 1			
Sample	**OD at 10 min**	**OD at 20 min**	**Activity (U/L)**
Undiluted	0.392	0.405	17.35
Diluted 1:10	0.329	0.339	13.34 X 10=133.4
Diluted 1:100	0.269	0.277	10.67 X 100=1,067
Purification 2			
Sample	**OD at 10 min**	**OD at 20 min**	**Activity (U/L)**
Undiluted	0.42	0.44	26.68
Diluted 1:10	0.382	0.392	13.34 X 10=133.4
Diluted 1:100	0.341	0.35	12.01 X 100=1,201

DISCUSSION

The purpose of this project was to develop a new product, purified human hormone sensitive lipase (HSL), for Blue Sky Biotech's product line. Blue Sky Biotech will be able to sell this product to laboratories currently researching lipolysis pathways involved in diseases such as obesity, diabetes, and hyperlipidemia. The results indicate that HSL was successfully cloned into the pFB-HBv3 expression vector, transformed into DH10 bac cells to create a bacmid, transfected and expressed in SF21 insect cells co-infected with HSL P1 virus stock and protein biotin ligase (PBL), and enriched using Ni-NTA resin followed by a Softlink avidin resin. It was also found that the purified HSL protein is biologically active as determined by a lipase activity assay (BioAssay Systems).

The objective of the initial molecular engineering work was to perform gene synthesis of three HSL gene fragments: HSL-P1, HSL-P2, and HSL-P3 and clone each into pDONR-221 individually. Gene synthesis was performed rather than traditional PCR because it is quicker, allows for codon optimization, and easier for storage if needed again. There are, however, cons to using gene synthesis: expensive, easy to make mistakes or introduce mutations, time consuming to put the list of primers together. By engineering a synthetic gene, it allows for the optimization of the nucleic acid sequence while still retaining the amino acid sequence. This was done in three segments due to the large size of the HSL gene, approximately 3.2kb. Thus, cloning into three vectors and then assembling them together enabled the full-length gene to be cloned into the expression vector, pFB-HBv3, and transformed into DH10 bacmid cells for use in the transfection. Gel electrophoresis was performed on the final PCR segments (P1, P2, and P3)

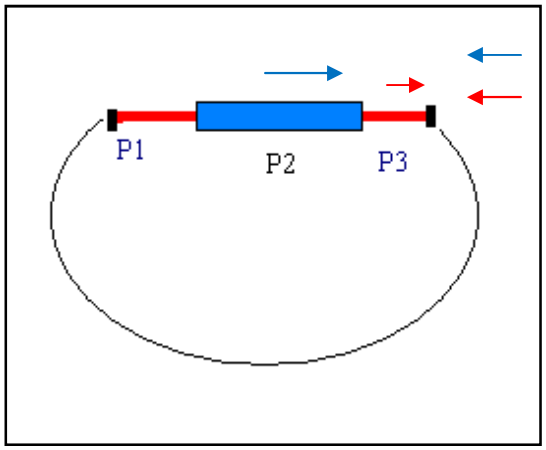
and it was shown that P1 and P2 had a band present at 1200 bp (Figure 10); however, P3 showed no band present (Figure 10) so a second PCR was repeated for P3 and proved successful (Figure 10); the only difference was that the primers were taken directly from the gene synthesis plate instead of the primer mix made previously.

When all three segments, P1, P2, and P3 were transformed individually, the colonies were screened for positive clones using primers specific to each segment. The results showed all negative clones, indicating self-ligation. This means that despite the growth of several colonies, none of the colonies contained the insert. Before repeating any of the previous steps, this theory was tested by setting the annealing temperature to 52°C instead of 55°C, which proved to be successful. The temperature for annealing is important because every primer has a temperature at which it will optimally anneal to its substrate. This is due to the hydrogen bonds between the complementary base pairs. Out of the many samples (12 for each) that were sent for sequence validation, only one from each was actually a positive clone; there were many mutations present in the other sequences, again pointing out a potential negative of synthetic cloning.

The next step involved the complete PCR assembly of P1, P2, and P3 (Figure 11). This should have produced a band at around 4.4kb. When this wasn't the case, P1 and P2 were run together through gel electrophoresis, and then P2 and P3 were run together. It was concluded that because the band for P1 and P2 was not the correct size (2-2.5kb), the primers for P1 were incorrect. The P1 PCR was repeated and the full gene was assembled via PCR, gel extraction, column purification, digestion, ligation to the expression vector (pFB-HBv3), transformation, and colony (PCR) screening for positive clones. Although positives were visible in the colony screen and DNA was present in the minipreps, the digest indicated that there were no positive

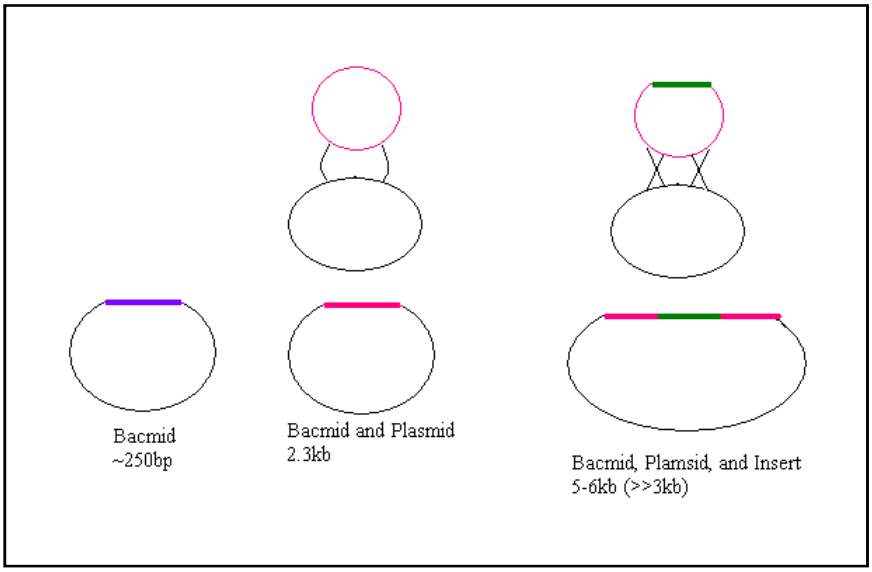
clones present. To ensure that this was in fact the case, the primers for the colony screen were changed to HSL-P2-740F and pFBacR. The predicted size, as seen in Figure 24 below, should now be ~1.3kb (indicated in blue) instead of the original design (indicated in red) that would only produce the gene at 500bp due to the location of the primers.

Figure 24: Primer Change to Produce Larger Size



The positive clone (#9) was miniprepped, digested (positive because the insert and vector were present on gel image), and then transformed into bacmid cells. The bacmid PCR, used to determine the positive sample, showed only one of the four samples to be positive (#4), as depicted in Figure 25 below. A bacmid without the plasmid or insert will be a very small size; however, when the bacmid, plasmid, and insert are present, the size will be substantially greater, as seen in the figure below. This bacmid sample was then used to make the P1 virus stock for use in the expression and purification of HSL.

Figure 25: Positive Bacmid Detection



After the HSL P1 stock was produced, a SCOUT was performed to determine the best conditions to express HSL in insect cells, as was described in the methods. Gel electrophoresis was performed on the SCOUT samples. At the time, it was not clear whether HSL was intracellular or secreted, so the control media was also run on SDS-PAGE with the SCOUT samples. The gels were transferred onto polyvinylidene difluoride (PVDF) membranes, and blotted with α-His. Blue Sky Biotech uses PVDF membranes because they tend to be easier to handle and to stain than nitrocellulose (Sigma-Aldrich). Figure 15f shows that the best HSL expression was seen in the lane that contains the SF21 insect cell line at 1:1,000 dilution after 96 hours. Because of this, the SF21 insect cell line at a dilution of 1:1,000 was used in the large scale expression in both the transfection of HSL and the co-infection of HSL/PBL. Although the expression of HSL was best after 96 hours, during the large scale expression of HSL the cells had to be harvested when the viability was between 60-70%, which occurred after 72 hours. During the large scale expression of the co-infection of HSL/PBL, the cells were harvested at 48 hours because the cell diameter was too large (~24 μm).

Following the expression of HSL in SF21 insect cells, a test purification using Ni-NTA resin was conducted to determine the conditions that produce the greatest amount and highest purity of HSL. Three different buffers containing three different NaCl concentrations (150 mM, 300 mM, and 500 mM) were used in the test purification. The protein concentration in a purification buffer affects the ability of the desired protein to bind to the resin. The buffer with the protein concentration producing the greatest amount of HSL in the eluted fractions is the most appropriate buffer to use in large scale purification. The Westerns were blotted with the protein specific antibody (α-HSL) to determine whether HSL was degrading during purification. Figure 16 shows that the buffer containing 300 mM NaCl produced a greater amount of HSL and therefore it is the concentration that was used in the large scale Ni-NTA purification. The numerous bands in Figure 16 show that HSL is very unstable at room temperature and degraded considerably during purification. Because this purification was performed at room temperature, it was determined that the large scale purification would be conducted at 4˚C with more protease inhibitor cocktail added to the cell lysate.

The large scale Ni-NTA purification of co-HSL/PBL was performed as described in the methods with the buffer containing 300 mM NaCl at 4˚C with more protease inhibitor cocktail. Gel electrophoresis was performed on the fractions from the large scale purification. One of the gels was stained with Coomassie blue dye, and two gels were transferred onto PVDF membranes, and blotted with α-HSL and α-His. The Coomassie-stained gel in Figure 17 shows that fractions 4 through 9 were fairly pure (130 kDa HSL band), with a few other faint bands. These bands are consistent with bands that are present in the α-HSL Western blot in Figure 17, which means that HSL degraded slightly during the large scale Ni-NTA purification. Because HSL is unstable, the fractions to pool were determined by a colorimetric test using Bradford

reagent. The fractions that contained the most protein (darkest blue) were pooled and frozen in liquid nitrogen to prevent further degradation; fractions 3 through 9 were pooled. As seen in the Coomassie stained gel in Figure17a, fraction 3 was impure and contained degraded HSL. Because this fraction was pooled with the pure fractions (4-9), the pooled fractions sample in lane 13 was impure. As a result, the large scale Ni-NTA purification was not successful in producing pure HSL and purification using SoftLink avidin resin was necessary.

The pooled fractions from the large scale Ni-NTA purification were applied to SoftLink avidin resin, washed, and bound protein was eluted with 5 mM biotin. Gel electrophoresis was performed on the fractions from this purification. One of the gels was stained with Coomassie blue dye and two gels were transferred onto PVDF membranes and blotted with α-HSL and α-His. Biotin binds tightly to avidin and the resin contains mutant avidin; biotinylated proteins can bind to the resin, but not so tightly that they cannot be eluted off. The biotinylated HSL in the applied sample adhered to the mutant avidin; however, HSL and other proteins that are not biotinylated flowed through the column. Lane 3 in Figure 18a is the flow through from the biotin purification, which shows a dark band at the 130 kDa molecular weight marker. This band represents HSL that was not biotinylated during expression. A considerable amount of HSL was lost due to this and could be prevented by adding more biotin to the co-infection to increase the biotinylation of HSL. Ten fractions were collected during the elution, but fractions 5 through 10 did not contain protein. In fractions 1 and 2, the undiluted elution buffer (100 mM biotin dissolved in 100 mM NaOH) was used accidentally. This mistake was corrected in fractions 3 through 10 by using the diluted elution buffer (100 mM biotin dissolved in 100 mM NaOH and diluted 1:20 in 1X PBS to produce 5 mM biotin). However, the undiluted buffer caused complete

elution of the protein in fractions 1 through 4 as seen in Figure 18. Fractions 1 and 2 appear to contain a considerable amount of pure HSL at the 130 kDa molecular weight marker.

An activity assay was performed on samples of HSL after each purification step. The absorbance measurements of undiluted, diluted 1:10, and diluted 1:100 purified HSL are shown in Figures 22 and 23. Contreras et al. (1998) measured HSL activity to be around 11,670 mU/ml after their final purification step. As seen in Table 2, the undiluted HSL after the last purification step was only about 26.7 U/L and appears to increase as the dilution factor increases. It is possible that the working solution from the activity assay did not contain enough substrate for the amount of HSL in the undiluted and somewhat diluted samples in order to determine an accurate measurement of the enzymatic activity of HSL. It is possible that all of the substrate in the working solution was already bound to enzyme before 10 minutes and only bound complex and unbound enzyme were left in the reaction wells. As a result, the change in optical density (OD) between 10 and 20 minutes was very small. Because the equation to determine the activity of the protein uses the change in ODs between 10 and 20 minutes, the activity calculations may not be accurate. If the samples had been diluted 1:1,000, the reaction would be slower since the enzyme and substrate would collide less frequently and a lower concentration of enzyme may allow for complete complex formation. It is possible that our activity measurement at a dilution of 1:1,000 would support that of Contreras et al. (1998). As the data currently is, however, it is difficult to report how active our purified HSL is.

The purity of HSL increased after the first purification step, reaching a total purity of 80.83%. After the first purification step, the purity was at 19.97%. By analyzing the graph (data not shown) of the data, it is evident that the protein became purer as seen by the number of other

bands on the gel. Though it appears that the concentration of HSL decreased slightly, the purity increased relative to the first purification step.

In the future, we recommend that Blue Sky Biotech add more biotin to the co-infection in order to produce more biotinylated HSL to increase the yield of product, and that they dilute our purified HSL 1:1,000 and 1:10,000 to determine a more accurate activity measurement. After an accurate activity measurement has been determined, Blue Sky Biotech will be able to produce purified HSL using our methodology. This purified HSL can be sold in order to further research into diseases such as obesity, diabetes, and hyperlipidemia.

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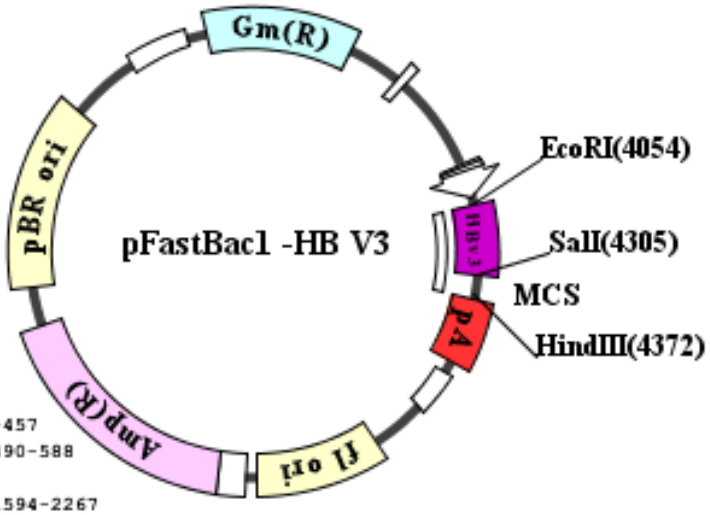
APPENDIX A

The following figure shows the expression vector, pFB-HBv3 used for the design of HSL.

```
Multiple Cloning Site - 4053 to 4392
            |EcoRI     |NcoI
4053        GGAATTCGCCACCATGGGAGGTTCTCACCACCATCATCATCATCACCACTCCGTCTTGCGTAGTCCTATGCCCGG
                        M  G  G  S  H  H  H  H  H  H  H  H  S  V  L  R  S  P  M  P  G

4128        TGTGGTCGTGGCCGTTTCTGTAAAGCCTGGTGACGCTGTAGCTGAGGGACAGGAAATCTGCGTGATTGAGGCTAT
              V  V  V  A  V  S  V  K  P  G  D  A  V  A  E  G  Q  E  I  C  V  I  E  A  M

4203        GAAGATGCAGAACTCCATGACCGCTGGAAAAACTGGTACTGTGAAGTCCGTGCACTGCCAAGCTGGCGATACCGT
              K  M  Q  N  S  M  T  A  G  K  T  G  T  V  K  S  V  H  C  Q  A  G  D  T  V
                                                |SpeI                |XbaI
                                       |SalI      |SacI      |NotI     |AsuII           |PstI
4278        GGGTGAGGGTGATCTGTTGGTGGAGCTGTCGACGAGCTCACTAGTCGCGGCCGCTTTCGAATCTAGAGCCTGCAG
              G  E  G  D  L  L  V  E  L  S  T  S  S  L  V  A  A  A  F  E  S  R  A  C  S
                           |KpnI
                        |Asp718I
            |XhoI      |SphI    |HindIII
4353        TCTCGAGGCATGCGGTACCAAGCTTGTCGAGAAGTACTAG
              L  E  A  C  G  T  K  L  V  E  K  Y  Z
```




```
rep_origin f1\origin 2-457
promoter bla\promoter 490-588
CDS Amp(R) 589-1449
rep_origin pUC\origin 1594-2267
misc_recomb Tn7R 2511-2735
CDS Gm(R) 2802-3335
promoter Pc\promoter 3524-3551
promoter PH\promoter 3904-4032
primer Polyhedrin\forward\primer 3920-3937
5' Tags HB tag 4053-4305
Multiple Cloning Site MCS 4053-4392
polyA_signal SV40\pA 4395-4635
misc_recomb Tn7L 4664-4829
```

APPENDIX B

The following are the protein sequences and list of primers used for P1 gene synthesis.

Protein sequence for synthesis: The red amino acids denote the signal sequence.

ENLYFQGEPGSKSVSRSDWQPEPHQRPITPLEPGPEKTPIAQPESKTLQGSNTQQKPASNQRPLTQQETPA
QHDAESQKEPRAQQKSASQEEFLAPQKPAPQQSPYIQRVLLTQQEAASQQGPGLGKESITQQEPALRQRHV
AQPGPGPGEPPPAQQEAESTPAAQAKPGAKREPSAPTESTSQETPEQSDKQTTPVQGAKSKQGSLTELGFL
TKLQELSIQRSALEWKALSEWVTDSESESDVGSSSDTDSPATMGGMVAQGVKLGFKGKSGYKVMSGYSGTS
PHEKTSARNHRHYQDTASRLIHNMDLRTMTQSLVTLAEDNIAFFSSQGPGETAQRLSGVFAGVREQALGLE
PALGRLLGVAHL

DNA sequence after addition of GATEWAY recombination sequences:

Gateway 5' end
GGGGACAAGTTTGTACAAAAAAGCAGGCT
Gateway 3' end
ACCCAGCTTTCTTGTACAAAGTGGTCCCC

1 GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGGCCGCTGAGAATCTGTACTTTCAGGGAG
61 AGCCCGGCTCCAAGTCCGTCTCGCGCTCGGATTGGCAACCAGAACCACACCAGCGTCCCA
121 TCACCCCGTTGGAACCCGGCCCAGAGAAAACGCCGATCGCTCAGCCGGAATCCAAAACCT
181 TGCAGGGCTCGAACACGCAGCAGAAACCAGCTTCGAATCAACGCCCGCTGACCCAGCAGG
241 AGACGCCAGCCCAGCACGACGCTGAAAGCCAAAAGGAACCGCGTGCCCAACAGAAATCCG
301 CTTCGCAAGAGGAATTTCTGGCTCCCCAGAAGCCAGCTCCGCAACAATCGCCATATATCC
361 AGCGCGTGTTGCTGACGCAGCAAGAAGCCGCCTCGCAGCAAGGACCAGGATTGGGCAAGG
421 AGTCCATTACGCAACAGGAGCCCGCTTTGCGCCAGCGCCACGTCGCTCAACCAGGTCCCG
481 GACCGGGCGAGCCACCGCCAGCTCAACAAGAGGCTGAGTCGACCCCCGCTGCTCAGGCTA
541 AACCAGGCGCTAAGCGTGAACCATCCGCCCCAACCGAGTCCACCTCCCAGGAAACCCCAG
601 AGCAGTCCGATAAACAAACGACGCCGGTCCAAGGAGCTAAGTCCAAACAGGGTAGCTTGA
661 CGGAACTGGGTTTTCTGACCAAACTGCAAGAACTGTCCATCCAACGCTCCGCCTTGGAAT
721 GGAAGGCCCTGTCGGAGTGGGTCACCGATAGCGAGAGCGAATCGGATGTCGGAAGCTCGT
781 CCGACACCGACTCGCCGGCTACGATGGGAGGAATGGTCGCCCAGGGAGTCAAACTGGGAT
841 TTAAGGGCAAGAGCGGTTACAAGGTCATGTCGGGCTATTCGGGCACGTCCCCGCATGAAA
901 AAACCTCGGCCCGTAATCACCGTCACTATCAAGACACGGCCTCGCGTCTGATCCACAACA
961 TGGATTTGCGTACCATGACGCAATCGTTGGTGACGTTGGCCGAAGATAACATCGCCTTTT
1021 TTTCCAGCCAGGGACCCGGTGAAACCGCCCAACGTTTGAGCGGAGTCTTTGCTGGAGTCC
1081 GTGAACAAGCCTTGGGTCTGGAACCGGCTTTGGGTCGCTTGTTGGGAGTGGCCCATCTGT
1141 AGACCCAGCTTTCTTGTACAAAGTGGTCCCC

Gene synthesis:

1 GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGGCCGCT 38
2 CGGGCTCTCCCTGAAAGTACAGATTCTCAGCGGCCGCAGC 40
3 CTTTCAGGGAGAGCCCGGCTCCAAGTCCGTCTCGCGCTCG 40
4 CGCTGGTGTGGTTCTGGTTGCCAATCCGAGCGCGAGACGG 40

5 CCAGAACCACACCAGCGTCCCATCACCCCGTTGGAACCCG 40
6 TGAGCGATCGGCGTTTTCTCTGGGCCGGGTTCCAACGGGG 40
7 AAAACGCCGATCGCTCAGCCGGAATCCAAAACCTTGCAGG 40
8 TTTCTGCTGCGTGTTCGAGCCCTGCAAGGTTTTGGATTCC 40
9 TCGAACACGCAGCAGAAACCAGCTTCGAATCAACGCCCGC 40
10 GGGCTGGCGTCTCCTGCTGGGTCAGCGGGCGTTGATTCGA 40
11 GGAGACGCCAGCCCAGCACGACGCTGAAAGCCAAAAGGAA 40
12 GGATTTCTGTTGGGCACGCGGTTCCTTTTGGCTTTCAGCG 40
13 CGTGCCCAACAGAAATCCGCTTCGCAAGAGGAATTTCTGG 40
14 GGAGCTGGCTTCTGGGGAGCCAGAAATTCCTCTTGCGAAG 40
15 CCCAGAAGCCAGCTCCGCAACAATCGCCATATATCCAGCG 40
16 CTTGCTGCGTCAGCAACACGCGCTGGATATATGGCGATTG 40
17 GTTGCTGACGCAGCAAGAAGCCGCCTCGCAGCAAGGACCA 40
18 CGTAATGGACTCCTTGCCCAATCCTGGTCCTTGCTGCGAG 40
19 GGGCAAGGAGTCCATTACGCAACAGGAGCCCGCTTTGCGC 40
20 GGGACCTGGTTGAGCGACGTGGCGCTGGCGCAAAGCGGGC 40
21 CGCTCAACCAGGTCCCGGACCGGGCGAGCCACCGCCAGCT 40
22 AGCGGGGGTCGACTCAGCCTCTTGTTGAGCTGGCGGTGGC 40
23 AGTCGACCCCCGCTGCTCAGGCTAAACCAGGCGCTAAGCG 40
24 GGACTCGGTTGGGGCGGATGGTTCACGCTTAGCGCCTGGT 40
25 GCCCCAACCGAGTCCACCTCCCAGGAAACCCCAGAGCAGT 40
26 GACCGGCGTCGTTTGTTTATCGGACTGCTCTGGGGTTTCC 40
27 ACAAACGACGCCGGTCCAAGGAGCTAAGTCCAAACAGGGT 40
28 AAACCCAGTTCCGTCAAGCTACCCTGTTTGGACTTAGCTC 40
29 GCTTGACGGAACTGGGTTTTCTGACCAAACTGCAAGAACT 40
30 GGCGGAGCGTTGGATGGACAGTTCTTGCAGTTTGGTCAGA 40
31 ATCCAACGCTCCGCCTTGGAATGGAAGGCCCTGTCGGAGT 40
32 ATTCGCTCTCGCTATCGGTGACCCACTCCGACAGGGCCTT 40
33 CCGATAGCGAGAGCGAATCGGATGTCGGAAGCTCGTCCGA 40
34 CCCATCGTAGCCGGCGAGTCGGTGTCGGACGAGCTTCCGA 40
35 GCCGGCTACGATGGGAGGAATGGTCGCCCAGGGAGTCAAA 40
36 CCGCTCTTGCCCTTAAATCCCAGTTTGACTCCCTGGGCGA 40
37 GATTTAAGGGCAAGAGCGGTTACAAGGTCATGTCGGGCTA 40
38 TTCATGCGGGGACGTGCCCGAATAGCCCGACATGACCTTG 40
39 CACGTCCCCGCATGAAAAAACCTCGGCCCGTAATCACCGT 40
40 ACGCGAGGCCGTGTCTTGATAGTGACGGTGATTACGGGCC 40
41 CACGGCCTCGCGTCTGATCCACAACATGGATTTGCGTACC 40
42 TCACCAACGATTGCGTCATGGTACGCAAATCCATGTTGTG 40
43 TGACGCAATCGTTGGTGACGTTGGCCGAAGATAACATCGC 40
44 GGTCCCTGGCTGGAAAAAAAGGCGATGTTATCTTCGGCCA 40
45 TTTTCCAGCCAGGGACCCGGTGAAACCGCCCAACGTTTGA 40
46 ACGGACTCCAGCAAAGACTCCGCTCAAACGTTGGGCGGTT 40
47 GTCTTTGCTGGAGTCCGTGAACAAGCCTTGGGTCTGGAAC 40
48 TCCCAACAAGCGACCCAAAGCCGGTTCCAGACCCAAGGCT 40
49 GGGTCGCTTGTTGGGAGTGGCCCATCTGTAGACCCAGCTT 40
50 GGGGACCACTTTGTACAAGAAAGCTGGGTCTACAGATGG 39

The following are the protein sequences and list of primers used for P2 gene synthesis.

Protein sequence for synthesis:

FDLDPETPANGYRSLVHTARCCLAHLLHKSRYVASNRRSIFFRTSHNLAELEAYLAALTQLRALVYYAQRLL
VTNRPGVLFFEGDEGLTADFLREYVTLHKGCFYGRCLGFQFTPAIRPFLQTISIGLVSFGEHYKRNETGLSVA
ASSLFTSGRFAIDPELRGAEFERITQNLDVHFWKAFWNITEMEVLSSLANMASATVRVSRLLSLPPEAFEMP
LTADPTLTVTISPPLAHTGPGPVLVRLISYDLREGQDSEELSSLIKSNGQRSLELWPRPQQAPRSRSLIVHFHG
GGFVAQTSRSHEPYLKSWAQELGAPIISIDYSLAPEAPFPRALEECFFAYCWAIKHCALLGSTGERICLAG

DNA sequence after addition of GATEWAY recombination sequences:

Gateway 5' end
GGGGACAAGTTTGTACAAAAAAGCAGGCT
Gateway 3' end
ACCCAGCTTTCTTGTACAAAGTGGTCCCC

1 GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTTCGACCTGGACCCGGAGACGCCCGCTA
61 ATGGATACCGTTCCCTGGTGCATACGGCCCGTTGTTGTCTGGCTCACCTGCTGCACAAAA
121 GCCGTTACGTCGCTTCGAATCGCCGTTCGATTTTCTTCCGCACCAGCCACAATCTGGCCG
181 AGTTGGAAGCCTACTTGGCTGCCTTGACGCAGTTGCGCGCCTTGGTCTACTACGCCCAAC
241 GCCTGCTGGTCACGAACCGTCCGGGAGTCTTGTTCTTTGAAGGCGATGAAGGATTGACCG
301 CTGATTTTTTGCGTGAGTACGTCACGCTGCATAAGGGCTGCTTCTATGGACGTTGCTTGG
361 GCTTTCAATTCACGCCAGCCATCCGTCCCTTTCTGCAAACCATCAGCATCGGATTGGTCT
421 CCTTCGGTGAGCACTACAAGCGCAATGAGACCGGTTTGTCCGTCGCCGCTTCCTCGTTGT
481 TCACCAGCGGCCGTTTTGCCATTGACCCAGAGTTGCGTGGCGCTGAATTTGAGCGCATTA
541 CCCAGAATTTGGACGTGCACTTTTGGAAGGCTTTCTGGAACATCACGGAGATGGAGGTGC
601 TGTCGTCGCTGGCTAACATGGCTTCGGCTACGGTCCGCGTGAGCCGTCTGTTGTCCCTGC
661 CACCCGAGGCCTTTGAGATGCCGCTGACGGCTGATCCCACGCTGACCGTCACGATCTCGC
721 CACCGTTGGCTCATACCGGACCAGGACCCGTCTTGGTCCGTTTGATTAGCTATGACCTGC
781 GCGAGGGTCAAGACAGCGAAGAATTGTCCAGCTTGATTAAGAGCAATGGCCAGCGTAGCC
841 TGGAGTTGTGGCCGCGTCCACAGCAAGCTCCGCGTTCCCGCTCCCTGATCGTCCACTTTC
901 ATGGTGGAGGATTTGTCGCCCAGACGTCGCGCTCGCATGAACCCTATTTGAAGAGCTGGG
961 CTCAGGAGTTGGGAGCCCCGATCATCTCGATTGATTACTCCTTGGCTCCGGAAGCTCCAT
1021 TCCCGCGTGCTTTGGAGGAATGCTTCTTTGCTTATTGCTGGGCCATCAAGCACTGCGCCC
1081 TGTTGGGATCGACGGGTGAACGCATTTGTTTGGCCGGATAGACCCAGCTTTCTTGTACAA
1141 AGTGGTCCCC

Gene synthesis:

1 GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTTCGA 37
2 GCGTCTCCGGGTCCAGGTCGAACATAGCCTGCTTTTTTGT 40
3 TGGACCCGGAGACGCCCGCTAATGGATACCGTTCCCTGGT 40
4 ACAACAACGGGCCGTATGCACCAGGGAACGGTATCCATTA 40
5 CATACGGCCCGTTGTTGTCTGGCTCACCTGCTGCACAAAA 40
6 GATTCGAAGCGACGTAACGGCTTTTGTGCAGCAGGTGAGC 40
7 CCGTTACGTCGCTTCGAATCGCCGTTCGATTTTCTTCCGC 40
8 CTCGGCCAGATTGTGGCTGGTGCGGAAGAAAATCGAACGG 40

9 GCCACAATCTGGCCGAGTTGGAAGCCTACTTGGCTGCCTT 40
10 GACCAAGGCGCGCAACTGCGTCAAGGCAGCCAAGTAGGCT 40
11 TTGCGCGCCTTGGTCTACTACGCCCAACGCCTGCTGGTCA 40
12 AGAACAAGACTCCCGGACGGTTCGTGACCAGCAGGCGTTG 40
13 CGTCCGGGAGTCTTGTTCTTTGAAGGCGATGAAGGATTGA 40
14 TCACGCAAAAAATCAGCGGTCAATCCTTCATCGCCTTCAA 40
15 CCGCTGATTTTTTGCGTGAGTACGTCACGCTGCATAAGGG 40
16 CAAGCAACGTCCATAGAAGCAGCCCTTATGCAGCGTGACG 40
17 TGCTTCTATGGACGTTGCTTGGGCTTTCAATTCACGCCAG 40
18 TTTGCAGAAAGGGACGGATGGCTGGCGTGAATTGAAAGCC 40
19 CATCCGTCCCTTTCTGCAAACCATCAGCATCGGATTGGTC 40
20 TTGTAGTGCTCACCGAAGGAGACCAATCCGATGCTGATGG 40
21 TCCTTCGGTGAGCACTACAAGCGCAATGAGACCGGTTTGT 40
22 ACAACGAGGAAGCGGCGACGGACAAACCGGTCTCATTGCG 40
23 GCCGCTTCCTCGTTGTTCACCAGCGGCCGTTTTGCCATTG 40
24 TTCAGCGCCACGCAACTCTGGGTCAATGGCAAAACGGCCG 40
25 TTGCGTGGCGCTGAATTTGAGCGCATTACCCAGAATTTGG 40
26 GCCTTCCAAAAGTGCACGTCCAAATTCTGGGTAATGCGCT 40
27 CGTGCACTTTTGGAAGGCTTTCTGGAACATCACGGAGATG 40
28 CCAGCGACGACAGCACCTCCATCTCCGTGATGTTCCAGAA 40
29 GTGCTGTCGTCGCTGGCTAACATGGCTTCGGCTACGGTCC 40
30 GCAGGGACAACAGACGGCTCACGCGGACCGTAGCCGAAGC 40
31 CCGTCTGTTGTCCCTGCCACCCGAGGCCTTTGAGATGCCG 40
32 GTCAGCGTGGGATCAGCCGTCAGCGGCATCTCAAAGGCCT 40
33 GCTGATCCCACGCTGACCGTCACGATCTCGCCACCGTTGG 40
34 GACGGGTCCTGGTCCGGTATGAGCCAACGGTGGCGAGATC 40
35 CGGACCAGGACCCGTCTTGGTCCGTTTGATTAGCTATGAC 40
36 CTTGACCCTCGCGCAGGTCATAGCTAATCAAACGGACCAA 40
37 CTGCGCGAGGGTCAAGACAGCGAAGAATTGTCCAGCTTGA 40
38 CGCTGGCCATTGCTCTTAATCAAGCTGGACAATTCTTCGC 40
39 AAGAGCAATGGCCAGCGTAGCCTGGAGTTGTGGCCGCGTC 40
40 AGCGGGAACGCGGAGCTTGCTGTGGACGCGGCCACAACTC 40
41 TCCGCGTTCCCGCTCCCTGATCGTCCACTTTCATGGTGGA 40
42 ACGTCTGGGCGACAAATCCTCCACCATGAAAGTGGACGAT 40
43 GATTTGTCGCCCAGACGTCGCGCTCGCATGAACCCTATTT 40
44 ACTCCTGAGCCCAGCTCTTCAAATAGGGTTCATGCGAGCG 40
45 GAGCTGGGCTCAGGAGTTGGGAGCCCCGATCATCTCGATT 40
46 TTCCGGAGCCAAGGAGTAATCAATCGAGATGATCGGGGCT 40
47 ACTCCTTGGCTCCGGAAGCTCCATTCCCGCGTGCTTTGGA 40
48 CCAGCAATAAGCAAAGAAGCATTCCTCCAAAGCACGCGGG 40
49 AATGCTTCTTTGCTTATTGCTGGGCCATCAAGCACTGCGC 40
50 CGTTCACCCGTCGATCCCAACAGGGCGCAGTGCTTGATGG 40
51 GGATCGACGGGTGAACGCATTTGTTTGGCCGGATAGACCC 40
52 GGGGACCACTTTGTACAAGAAAGCTGGGTCTATCCGGCCAAAC 43

The following are the protein sequences and list of primers used for P3 gene synthesis.

Protein sequence for synthesis:

DSAGGNLCFTVALRAAAYGVRVPDGIMAAYPATMLQPAASPSRLLSLMDPLLPLSVLSKCVSAYAGAKTED
HSNSDQKALGMMGLVRRDTALLLRDFRLGASSWLNSFLELSGRKSQKMSEPIAEPMRRSVSEAALAQPQG
PLGTDSLKNLTLRDLSLRGNSETSSDTPEMSLSAETLSPSTPSDVNFLLPPEDAGEEAEAKNELSPMDRGLG
VRAAFPEGFHPRRSSQGATQMPLYSSPIVKNPFMSPLLAPDSMLKSLPPVHIVACALDPMLDDSVMLARRL
RNLGQPVTLRVVEDLPHGFLTLAALCRETRQAAELCVERIRLVLTPPAGAGPSGETGAAGVDGGCGGRH

DNA sequence after addition of GATEWAY recombination sequences:

Gateway 5' end
GGGGACAAGTTTGTACAAAAAAGCAGGCT
Gateway 3' end
ACCCAGCTTTCTTGTACAAAGTGGTCCCC

1 GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGATTCCGCCGGTGGTAACTTGTGCTTTA
61 CGGTCGCTTTGCGTGCTGCTGCTTACGGCGTCCGTGTCCCGGATGGCATTATGGCTGCCT
121 ATCCCGCCACGATGCTGCAACCAGCTGCCTCCCCATCCCGTTTGTTGAGCCTGATGGACC
181 CGCTGTTGCCCTTGTCCGTCTTGTCCAAGTGCGTCTCCGCTTACGCCGGAGCCAAGACCG
241 AAGACCACTCCAATTCCGACCAAAAAGCTCTGGGCATGATGGGATTGGTGCGTCGCGATA
301 CGGCTTTGTTGTTGCGCGACTTTCGTTTGGGCGCCTCCTCCTGGTTGAACAGCTTCCTGG
361 AGTTGAGCGGTCGTAAATCCCAGAAAATGTCCGAACCAATTGCCGAGCCCATGCGCCGCT
421 CGGTCAGCGAAGCTGCCCTGGCCCAACCACAAGGTCCCCTGGGTACCGACAGCCTGAAAA
481 ACTTGACCTTGCGTGATTTGTCGCTGCGCGGTAATTCGGAAACGTCCTCGGACACCCCGG
541 AGATGTCGTTGTCCGCCGAAACCCTGAGCCCCTCGACGCCATCGGACGTCAACTTTTTGT
601 TGCCACCGGAAGATGCTGGTGAAGAGGCTGAGGCTAAAAACGAACTGTCGCCGATGGATC
661 GCGGTCTGGGAGTGCGTGCCGCCTTTCCAGAAGGATTTCATCCACGTCGTAGCTCCCAAG
721 GAGCCACCCAGATGCCATTGTACAGCTCGCCAATTGTCAAAAATCCATTCATGTCCCCCC
781 TGCTGGCCCCAGACAGCATGTTGAAGTCCCTGCCACCAGTCCATATTGTGGCTTGCGCTC
841 TGGACCCCATGCTGGACGACTCGGTGATGTTGGCCCGTCGTCTGCGCAATTTGGGACAGC
901 CCGTCACGCTGCGTGTGGTCGAGGACTTGCCGCATGGCTTTCTGACGTTGGCTGCTTTGT
961 GTCGCGAAACGCGTCAGGCCGCTGAGTTGTGTGTGGAACGCATCCGCTTGGTCTTGACGC
1021 CGCCAGCCGGTGCCGGACCGTCGGGTGAGACGGGAGCCGCTGGCGTGGACGGTGGTTGCG
1081 GCGGTCGCCATTAGCTCGAGTCTAGAACCCAGCTTTCTTGTACAAAGTGGTCCCC

Gene synthesis:

1 GGGGACAAGTTTGTACAAAAAAGCAGGCTAT 31
2 CCACCGGCGGAATCCATAGCCTGCTTTTTTGTACAAACTT 40
3 GGATTCCGCCGGTGGTAACTTGTGCTTTACGGTCGCTTTG 40
4 GACGCCGTAAGCAGCAGCACGCAAAGCGACCGTAAAGCAC 40
5 CTGCTGCTTACGGCGTCCGTGTCCCGGATGGCATTATGGC 40
6 CAGCATCGTGGCGGGATAGGCAGCCATAATGCCATCCGGG 40
7 CCCGCCACGATGCTGCAACCAGCTGCCTCCCCATCCCGTT 40
8 ACAGCGGGTCCATCAGGCTCAACAAACGGGATGGGGAGGC 40
9 CCTGATGGACCCGCTGTTGCCCTTGTCCGTCTTGTCCAAG 40

10 CCGGCGTAAGCGGAGACGCACTTGGACAAGACGGACAAGG 40
11 CTCCGCTTACGCCGGAGCCAAGACCGAAGACCACTCCAAT 40
12 CCAGAGCTTTTTGGTCGGAATTGGAGTGGTCTTCGGTCTT 40
13 TCCGACCAAAAAGCTCTGGGCATGATGGGATTGGTGCGTC 40
14 GCAACAACAAAGCCGTATCGCGACGCACCAATCCCATCAT 40
15 CGATACGGCTTTGTTGTTGCGCGACTTTCGTTTGGGCGCC 40
16 AGGAAGCTGTTCAACCAGGAGGAGGCGCCCAAACGAAAGT 40
17 TCCTGGTTGAACAGCTTCCTGGAGTTGAGCGGTCGTAAAT 40
18 TGGTTCGGACATTTTCTGGGATTTACGACCGCTCAACTCC 40
19 CCCAGAAAATGTCCGAACCAATTGCCGAGCCCATGCGCCG 40
20 GGGCCAGGGCAGCTTCGCTGACCGAGCGGCGCATGGGCTC 40
21 AGCTGCCCTGGCCCAACCACAAGGTCCCCTGGGTACCGAC 40
22 CGCAAGGTCAAGTTTTTCAGGCTGTCGGTACCCAGGGGAC 40
23 CCTGAAAAACTTGACCTTGCGTGATTTGTCGCTGCGCGGT 40
24 CGGGGTGTCCGAGGACGTTTCCGAATTACCGCGCAGCGAC 40
25 TCCTCGGACACCCCGGAGATGTCGTTGTCCGCCGAAACCC 40
26 GTCCGATGGCGTCGAGGGGCTCAGGGTTTCGGCGGACAAC 40
27 CTCGACGCCATCGGACGTCAACTTTTTGTTGCCACCGGAA 40
28 CAGCCTCTTCACCAGCATCTTCCGGTGGCAACAAAAAGTT 40
29 GATGCTGGTGAAGAGGCTGAGGCTAAAAACGAACTGTCGC 40
30 CCCAGACCGCGATCCATCGGCGACAGTTCGTTTTTAGCCT 40
31 TGGATCGCGGTCTGGGAGTGCGTGCCGCCTTTCCAGAAGG 40
32 GGGAGCTACGACGTGGATGAAATCCTTCTGGAAAGGCGGC 40
33 TCCACGTCGTAGCTCCCAAGGAGCCACCCAGATGCCATTG 40
34 TTTTGACAATTGGCGAGCTGTACAATGGCATCTGGGTGGC 40
35 ACAGCTCGCCAATTGTCAAAAATCCATTCATGTCCCCCCT 40
36 AACATGCTGTCTGGGGCCAGCAGGGGGGACATGAATGGAT 40
37 GCCCCAGACAGCATGTTGAAGTCCCTGCCACCAGTCCATA 40
38 GGGTCCAGAGCGCAAGCCACAATATGGACTGGTGGCAGGG 40
39 CTTGCGCTCTGGACCCCATGCTGGACGACTCGGTGATGTT 40
40 AAATTGCGCAGACGACGGGCCAACATCACCGAGTCGTCCA 40
41 CCGTCGTCTGCGCAATTTGGGACAGCCCGTCACGCTGCGT 40
42 AAGCCATGCGGCAAGTCCTCGACCACACGCAGCGTGACGG 40
43 GACTTGCCGCATGGCTTTCTGACGTTGGCTGCTTTGTGTC 40
44 CAGCGGCCTGACGCGTTTCGCGACACAAAGCAGCCAACGT 40
45 GCGTCAGGCCGCTGAGTTGTGTGTGGAACGCATCCGCTTG 40
46 GCACCGGCTGGCGGCGTCAAGACCAAGCGGATGCGTTCCA 40
47 CGCCAGCCGGTGCCGGACCGTCGGGTGAGACGGGAGCCGC 40
48 ACCGCCGCAACCACCGTCCACGCCAGCGGCTCCCGTCTCA 40
49 GTGGTTGCGGCGGTCGCCATTAGCTCGAGTCTAGAACCCA 40
50 GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGACTCGAGCTAA 45