Cloning and Initial Expression Of Endopeptidase EC 3.4.24.16

Student: Martin Hall

Faculty Advisor: Professor Jeffrey Sigman

Background Information

Endopeptidase EC3.4.24.16, commonly referred to as neurolysin, is a metalloendopeptidase found within numerous mammalian species, including mice, rats, and humans. Metalloendopeptidases' primary function is cleaving protein peptide bonds, which modifies the shape and function of the original protein. Neurolysin is an important enzyme in many living organisms, including humans, because of its necessary role in the cleavage of messenger peptides that are involved with the cardiovascular, reproductive, and nervous systems.

Neurolysin is also very similar in structure to another metalloendopeptidase thimet oligopeptidase, known as the TOP enzyme, with which it shares a 60% identical protein structure. Neurolysin and thimet oligopeptidase both have a proposed induced-fit mechanism, in which the enzyme completely encloses the substrate protein like a clamshell in order to function.

The eventual goal of this line of research is to monitor the activity of neurolysin using a single fluorescent tag placed on the interior of neurolysin's active site. If the fluorescent readings vary greatly with the activity of the enzyme, the data will support proposed 'clamshell' mechanism. The desired fluorescent tag functions by attaching to all exposed cysteine groups. Therefore, it will be necessary to block all unwanted cysteine groups, because the fluorescence data will only be relevant and useable if the

only tagged cysteine is the desired target in the active site. Single-site mutagenesis will be used to change all excess cysteine groups into other amino acids of similar shape and polarity.

Because there will likely be many necessary mutations to prepare for the fluorescent tagging process, it is necessary to develop a procedure for efficient cloning and expression of neurolysin using Saint Mary's College's facilities. The project used mus musculus neurolysin, or neurolysin from the common mouse, due to its availability and its high similarity to the human strain.

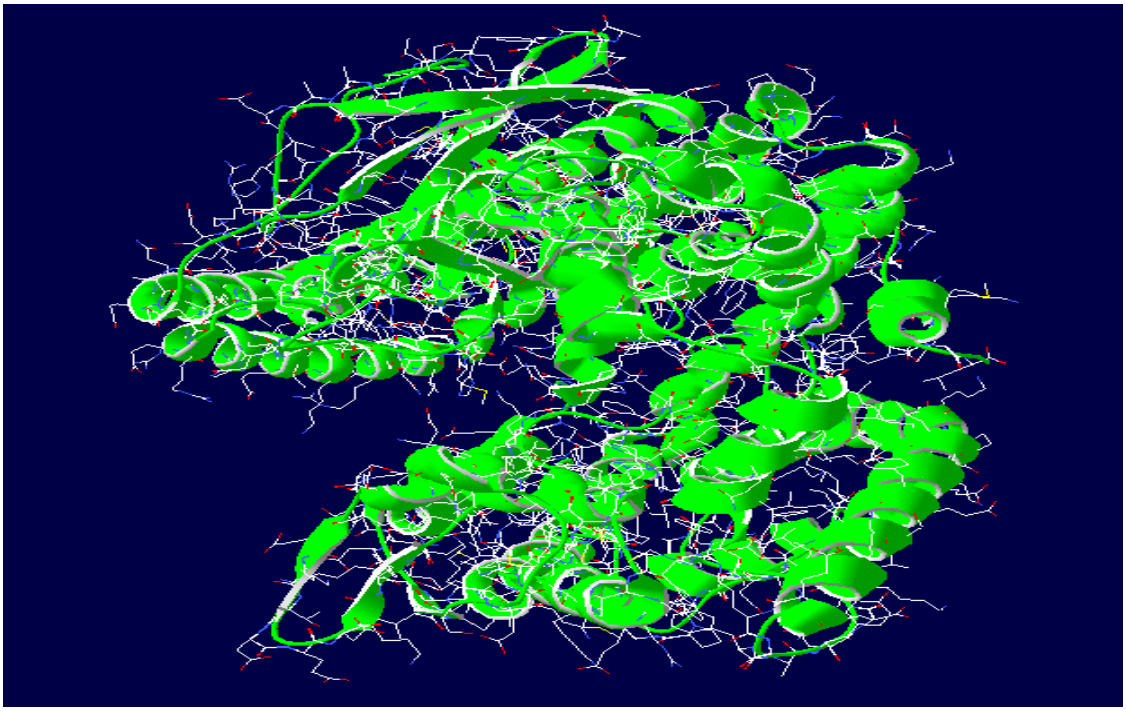
Because of the extensive similarity in protein structure between Neurolysin, TOP, and other metalloendopeptidases, there is a high probability that any new information discovered about one of these enzymes will also apply to other members of the enzyme family. Because TOP and neurolysin have been linked with a large number of bodily processes, including reproduction, pain perception, cardiovascular homeostasis, and more recently the onset of Alzheimer's disease, there is a chance that a better understanding of the function of these enzymes could have some level of medical significance.

Modeling

As part of the research, two three-dimensional models were created. A basic model was created using iMolview, which was used to visualize and examine the three-

dimensional shape of Neurolysin. The main reason behind this model was to help understand how the unique shape of Neurolysin is involved with its induced fit mechanism. Neurolysin is shaped very similarly to a valley in that it has a hollow groove through its center. This groove, specifically the metal bonded to it, is believed to be the active site for the protein. When a substrate protein binds to Neurolysin, the groove encloses around the substrate, creating a micro-environment for peptide bond cleavage.

The second model was created using the computer program Swiss-PdbViewer. Mus musculus Neurolysin does not have a solved three-dimensional crystalline structure, so a homology model was created by taking the amino acid sequence of mus musculus Neurolysin and comparing it to an already solved crystalline structure with a similar sequence, in this case rat Neurolysin. The homology model is pictured here.



The Swiss-PdbViewer model will be used to examine in detail the location of all cysteine groups in Neurolysin. Because only surface-exposed groups need to be blocked for the fluorescent tag to work, any cysteine that can be determined to have a low enough exposure percentage will not need to be mutated.

Procedure

General Guidelines

Sterility was maintained whenever possible. Gloves were worn whenever handling cells or growths. Pipet tips, containers, and test tubes were sterilized using an autoclave, and then kept sealed to prevent contamination. Flame from a Bunsen burner was used to sterilize the metal loop prior to spreading cells on an agar plate. Additionally, appropriate doses of antibiotics were used to prevent unwanted contaminants during cell growths. Both ampicillin at a concentration of 100 ug/mL and kanamycin at a concentration of 30 ug/mL were used at different points of the research.

Following every cell growth in media, a 80% glycerol 20% water solution was used to make frozen stocks of the cells, by adding .1 mL of glycerol solution to .9 mL of cells and placing in the -80^{o}C freezer.

DNA sequencing, via the UC Berkeley DNA Sequencing Lab, was repeatedly used to verify the identity of our cells. DNA was extracted from the cells using a QIAprep spin

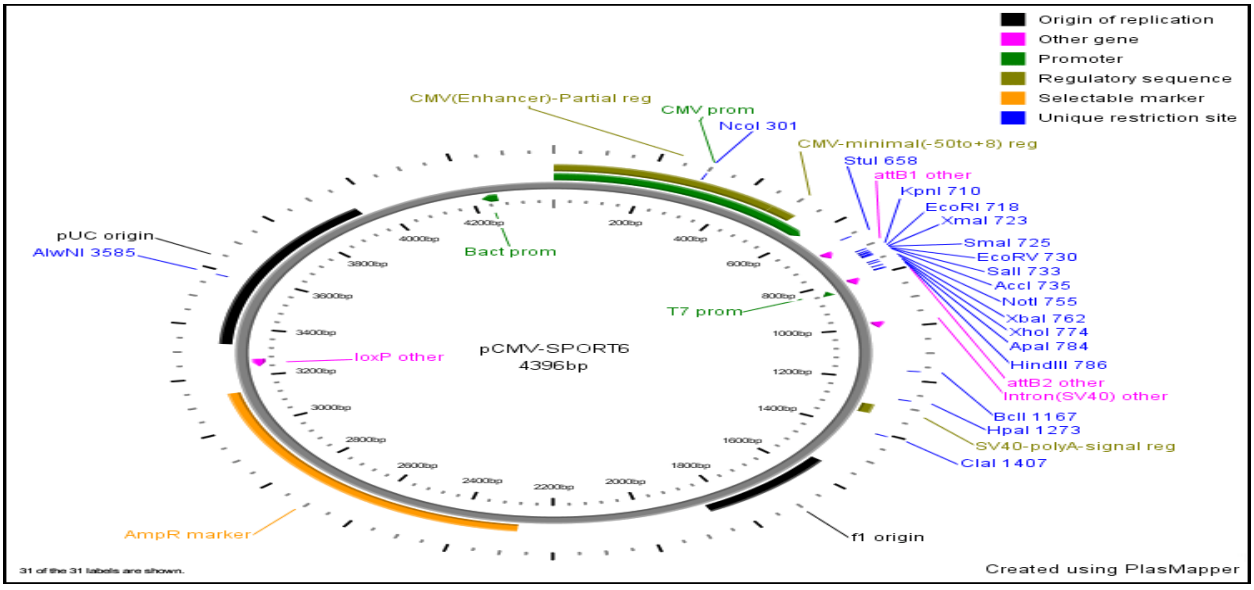
miniprep kit. The sequence then provided by the Berkeley staff was then compared to the expected result, generally determined using a modeling program such as SnapGene Viewer.

General Procedure

The gene for expression of Neurolysin, referred to as the NLN gene insert, was ligated into an appropriate expression vector. The DNA was then transformed into competent cells suited for expression. The cells were then grown in media, before initiating expression of the Neurolysin. Both the NLN gene insert and the expression vector had to be appropriately prepped for the ligation reaction.

Gene Insert Preparation

Cells with the pCMV-sport6 vector containing the mus musculus gene was purchased from Thermo Scientific. The pCMV vector is pictured here.



These cells were streaked with a sterilized metal loop on LB agar plates containing ampicillin and grown overnight at 37^{o}C. A single colony from the agar plate was grown overnight in 5 mL of Tsb broth in an incubator shaker at 37^{o}C and 180 rpm. This 5 mL growth was then used to inoculate a 50 mL growth. When the 50 mL growth finished, QIAprep spin miniprep kits were used to extract the DNA. Miniprep kits work by lysing the cell and breaking down it into its component parts, then filtering out everything other than the DNA. At the end, the cell DNA can be eluted out using water or another buffer.

After confirming the identity of the DNA via gel electrophoresis and DNA sequencing, about 3 ng of nln DNA was used for a polymerase chain reaction, referred to as a PCR. The PCR was carried out using a Phusion HF PCR kit from New England Biolabs. The PCR had an annealing temperature of 72^{o}C and an extension time of 45 seconds for 35 cycles, and was performed in a BioRad C1000 Thermal Cycler. Additionally, custom primers were ordered and used in this PCR. These custom primers were designed to extract and amplify the desired coding region of the NLN gene. Although the entire Neurolysin gene present in the pCMV vector was approximately 4000 base pairs long, the necessary coding region is only 2115 base pairs.

The PCR insert product consisted of the coding region as well as Xho1 and EcoR1 restriction sites on either end. This insert was then mixed in a restriction digest with Xho1 and EcoR1 restriction enzymes. The digest was incubated at 37^{o}C for 2-4 hours.

Once the digest was finished, the digested PCR DNA fragment was placed in a large well agar gel and subjected to gel electrophoresis. After conforming the DNA's identity using the gel, a purification step was taken, using a gel purification kit. Gel purification was used to remove any impurities left over from the digest.

Expression Vector Preparation

A pET-28b vector was purchased as the desired expression vector. This vector came as DNA, not cells, so it was necessary to create an adequate stock of the DNA. First, the vector was transformed into supercompetent XL1-Blue cells. These cells were spread on a kanamycin-containing agar plate and grown for approximately 16 hours. Afterwards, a single colony was grown in LB broth containing kanamycin in an incubator for 12-16 hours at 37^{o}C and 180 rpm. When the growth was finished, DNA was extracted from the cells using a miniprep kit in the same manner as before.

The vector DNA was then used in another restriction digest along with Xho1 and EcoR1 restriction enzymes for 2-4 hours at 37^{o}C. Again, the digest was followed by gel electrophoresis and gel purification to confirm the identity of the DNA as well as remove any unwanted impurities.

The two restriction digest steps, one for the vector and one for the insert, were necessary to prepare each for the ligation step. The restriction enzymes Xho1 and EcoR1 each cut the DNA at their respective sites, leaving behind a 'sticky' end. In the ligation

step, the Xho1 sticky ends from both the insert and vector will be able to join together, and the EcoR1 sticky ends will be able to do likewise, successfully placing the gene insert inside of the vector.

Ligation of Vector and Insert

For the ligation reaction, the digested insert and vector were mixed with a ligase buffer, DTT, ATP, and water. Once mixed, the ligation solution was incubated at 16^{o}C for 2-4 hours in a circulating water bath. During this incubation, the ends of the insert DNA combined with the ends of the vector DNA, to form a single, ligated piece of DNA consisting of the NLN gene coding region contained within the pET-28b vector.

Once incubation was finished, the ligated DNA was transformed into competent NovaBlue cells. Frozen NovaBlue cells were thawed on ice and placed in 1.5 mL polypropylene tubes. A sample of the ligated DNA was added to the tube, and then the tube was incubated on ice for five minutes. The tubes were then heat shocked in a 42^{o}C water bath for exactly 30 seconds. The heat shock provided the opportunity for the competent NovaBlue cells to uptake the available DNA. After the heat shock, the tubes were returned to ice for another 2 minutes, before the addition of room temperature SOC medium, which was followed by incubation at 37^{o}C and 250 rpm for one hour. When incubation had completed, the cells were spread on an agar plate to row overnight.

In addition to the DNA sample, two control transformations were performed to verify the success. A positive and a negative control were performed, with the positive control showing similar agar plate growth to the ligated DNA sample and with the negative control showing zero growth. For the positive control, a control DNA plasmid was used. This control verified the ability of the NovaBlue cells to properly uptake DNA. For the negative control, no DNA was mixed with the competent cells. This control verified that the NovaBlue cells had not already been contaminated with another source of DNA. For the negative control and the ligated DNA sample, agar plates containing kanamycin were used, while the positive control was grown on plates containing ampicillin.

After overnight growth, a colony from the ligated DNA plate was grown in25 mL of LB broth in an incubator at 32^{o}C and 180 rpm, and was then monitored using a UV-Vis system. Once the optical density had passed .6, a miniprep kit was used to extract the DNA from the cell growth. Part of this DNA sample was sent to the UC Berkeley DNA Sequencing Lab. However, before it could be sequenced, a PCR reaction with M13 primers was required for amplification. This was due to the low concentration of DNA in the sample. The results of the DNA sequencing matched the expected results obtained through SnapGene Viewer, confirming the successful cloning of the neurolysin gene into the pET-28b vector.

Initial Expression

The remaining DNA from the NovaBlue media growth was transformed into competent BL-21 cells. Bl-21 cells are used for expression of proteins and enzymes. The transformation was performed in the same manner as the NovaBlue transformation, including the use of positive and negative controls. Once grown on a kanamycin-containing agar plate, a single colony was grown in 5 mL of LB broth in an incubator at 30^{o}C and 150 rpm for approximately 12 hours. 250 uL of this cell growth was used to inoculate 4 additional 250 mL LB broth growths. These 4 samples were kept in an incubator shaker to grow. The optical density of the growths was monitored using a UV-Vis system. The 250 mL samples were allowed to grow in the incubator at 30^{o}C and 150 rpm until their optical density at 550nm reached .4, at which point the temperature was decreased to 20^{o}C. When the optical density reached .6-.7, 250 uL of 100 mM IPTG solution was added to each growth, resulting in a final concentration of .1 mM. The IPTG initiated the expression of protein. The growth was then continued for another 14-16 hours. Afterwards, centrifugation at 5000 rpm and 4^{o}C for 10 minutes was used to collect the cells, with the cell pellet then being stored at -70^{o}C.

A number of imidazole buffers, each containing 50 mM NaH_2PO_4 and 500 mM NaCl, were made. The buffers ranged in concentration of imidazole from 10 mM to 500 mM. Each buffer was also balanced to a pH of 8.0 using a pH meter, HCl, and KOH. While on ice, the cell pellet was then resuspended in approximately 20 mL of the 10 mM

imidazole buffer. Enough lysozyme was added to reach 1 mg/mL concentration, and then the mixture was left on ice for 45 minutes. At this point, 2 mL of 0.2% Triton X-100 as well as RNase and DNase were added. RNase and DNase were added until a final concentration of approximately 5 ug/mL. Following another incubation period on ice of 30 minutes, the solution was centrifuged at 13,000 rpm for 30 minutes, at which point the supernatant was recovered.

The recovered supernatant was then applied to a HisTag purification column. Ideally, the Neurolysin protein would have bound to this column, allowing other contaminants to be washed off with the imidazole buffers. Eventually, at a high enough concentration of imidazole, the imidazole would have had a higher affinity to the column, and the protein would have been washed out and collected. Towards this end, a gradient ranging from 10 mM to 500 mM imidazole was used, while the absorbance of the wash was observed. There should have been a peak at the point when the protein washed off, which would have then been collected in a fraction. However, when imaged on a protein gel, the fraction matching the peak did not contain a band at the correct location. While the gel did contain multiple bands in the columns in which the fractions were loaded, none of these bands matched the band from the 2.2 kb TOP standard.

Conclusions

The successful cloning of the mus musculus Neurolysin gene into the pET-28b vector was confirmed via both gel electrophoresis analysis and DNA sequencing. However, the negative result of the protein gel following HisTag column purification suggests that the expression procedure utilized is either not working or is not efficient enough for confirmable results. One possible reason for the failure of the expression process is that the Neurolysin protein does not bind strongly enough to the HisTag column to not be immediately washed off by the imidazole. Another suspicion is that during the expression process, the protein is breaking down due to the presence of the lysozyme, which was intended to break down any impurities.

Future Research

The next step in the research project will be troubleshooting the expression procedure until a working one can be found. The current plan is to consult multiple articles and papers dealing with the expression of Neurolysin protein and then compare their approaches. Once a working expression procedure has been found and tested, the activity of the protein will be monitored and then single-site mutagenesis will be used to begin mutating out troublesome cysteine groups.

All cysteine groups over a yet to be determined level of surface exposure will need to be mutated into another amino acid. After each mutation, the protein will be expressed again to ensure that the activity of the protein was not negatively affected. Because of this, exposed cysteine groups will be intentionally mutated into other amino acids that match the shape and polarity of cysteine.

Once all necessary cysteine groups have been replaced, a new cysteine group will be mutated into the structure of Neurolysin, placed as close to the active site as possible without affecting the activity. This will be the cysteine that will be fluorescently tagged for the final step of the project, which will be monitoring the fluorescent readings when Neurolysin is active and inactive.

Works Cited

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