Duquesne University

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

Formula Grant Overview

The Duquesne University received $121,663 in formula funds for the grant award period January 1, 2010 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Implementation of an Asthma Program to Improve Asthma Identification and Education in Children - The purpose of the Asthma Program is to develop and implement a method to increase the identification of children with undiagnosed or uncontrolled asthma; to develop and evaluate educational interventions that teach caregivers and children with asthma how to properly control their disease state; and to encourage active healthy lives. The goal of the Asthma Program is to improve health outcomes and quality of life for children suffering from asthma.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

Project Overview

Research Aims

- To identify children with uncontrolled or undiagnosed asthma
- To determine the effectiveness of using the community-based screening method for recruiting and referring children to the Asthma Camps
- To identify relationships between lung function, hypertension, and obesity in children attending the community-based screenings
- To identify relationships between lung function, hypertension, obesity, asthma knowledge, and tobacco smoke exposure in children attending the community-based camps
- To evaluate the effectiveness of the asthma education interventions on child and caregiver knowledge of asthma
- To evaluate the longitudinal impact of the education interventions on clinical outcomes in children attending multiple camps

Research Design and Methods

The Asthma Program will consist of six screenings and three camps each year, for two

consecutive years. Screenings will be conducted in areas where populations are known to be at increased risk for asthma and its complications, such as inner-city, lower socioeconomic, African-American populations. Screenings will be conducted at churches or schools in these areas one month prior to each camp in an effort to identify children with undiagnosed or uncontrolled asthma and refer them to the camps. Camps will also be conducted in areas where populations are known to be at increased risk for asthma and its complications to improve ease of access. Asthma and smoking cessation education will be provided at each camp to children and caregivers. Baseline data will be collected at each camp and assessed. Longitudinal clinical outcomes will be assessed in children attending multiple camps.

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Other Participating Researchers

Nicole A. Marcotullio, PharmD - employed by Duquesne University

Expected Research Outcomes and Benefits

Screenings. It is expected that children with undiagnosed or uncontrolled asthma and related complications will be identified through the asthma screenings. Children will be screened for asthma control, obesity, and hypertension. It is expected that we will find a correlation between uncontrolled asthma and the presence of hypertension and obesity. We will refer children to a pediatrician if they screen positive for uncontrolled asthma or other related complications. Each child with a positive asthma screen will be referred to the asthma camps. It is expected that the screenings will increase recruitment and participation in the asthma camps.

Camps: Asthma education will be provided to caregivers and children attending each camp. It is expected that this intervention will improve asthma knowledge for both caregivers and children. Children attending the camps will be screened for asthma control, obesity, hypertension, asthma knowledge and tobacco smoke exposure. It is expected that we will find a correlation between uncontrolled asthma and the presence of hypertension and obesity. It is expected that we will find a correlation between tobacco smoke exposure and asthma control. In the cohort of children attending multiple camps, it is expected that participation in the asthma program will improve asthma control.

If proven effective, this Asthma Program strategy may become a national model for improving pediatric asthma identification, education, and outcomes.

Summary of Research Completed

Program Development

The first three months of this project were dedicated to Program Development. The collaborative research group between Duquesne University Mylan School of Pharmacy and Allegheny General Hospital Division of Allergy, Asthma and Immunology met several times from January 2010 through March 2010 developing the Asthma Program. Planning efforts focused on recruitment, training, and program design.

Recruitment encompassed recruiting children for each camp as well as demographically appropriate sites to conduct screenings prior to each camp. Recruitment efforts also focused on enrolling student pharmacists to participate in the screenings and camps. Fifty six student pharmacists and resident/fellows have been recruited to date.

Training efforts focused on training post graduate PharmD residents/fellows and student pharmacists to become proficient in conducting screenings and providing educational sessions developed by the physician and pharmacy faculty. Interested students and residents/fellows were required to attend a mandatory training session prior to involvement in the program.
A live training session was held on Tuesday, March 9, 2010. A blackboard site was also created to ensure all staff received adequate training on the screening equipment as well as the process and procedures of each event. A separate training session was conducted by the pediatric physician to ensure a select group of staff were adequately trained in performing spirometry testing and blood pressure. Students involved in the educational portion of the camp were required to read the Expert Panel Report 3 (ERP3): Guidelines for the Diagnosis and Management of Asthma, and develop a formal summary presentation to present to the pharmacy faculty. Faculty could then determine if the students were adequately prepared to participate in the educational component of the program.

Program Progress

Screenings

Recruitment for each screening focused on identifying and building relationships with demographically appropriate sites to conduct screenings. A significant amount of time was spent developing contacts at sites that met the demographic criteria consistent with the project's anticipated population. As a result, four screenings were planned and completed to date. Sixty-one subjects have been enrolled in the study. Of the sixty-one subjects, 92% were African American and 5% were white. Thirty subjects were male and thirty-one subjects were female with a mean age of nine years. All four screenings took place at various inner-city after school programs in the following order: Wesley Center on April 12th; Hazelwood Library on April 14th; Blakely Center Girls Program on May 18; Blakely Center Boys Program on May 19. An April 16th screening at the Mifflin School was cancelled due to a power outage. The demographic characteristics of the subjects enrolled are consistent with the predicted sample; enabling us to work towards reaching our goal of addressing health disparities in inner-city, lower socioeconomic, African American populations.

Registration packets were delivered to sites approximately two weeks prior to each screening. These packets contained a description of the screening, IRB approved consent form, permission slip, and the Asthma Therapy Assessment Questionnaire (ATAQ). Parents were asked to fill out all forms and return with their child on the day of the screening. Six stations were set up at each screenings: Registration; Body Mass Index (BMI); spirometry; carbon monoxide breath test; blood pressure; and data entry. Participants rotated through each station. Staff individually assisted each child through the registration process. Registration consisted of explaining the assent form and administering the ATAQ as well as the Asthma Control Test (ACT) to each child. Staff created and supervised educational games and activities before and after the children rotated through each station. The games and activities focused on asthma, nutrition, and physical activity.

Camps

Two camps have been planned and completed to date, with the final camp of the year scheduled for August 7, 2010. Twenty-nine subjects have been enrolled in the camps. Of the twenty-nine subjects, 48% were African American, 38% white, 7% other, 3% Asian and 3% N/A. Eighteen subjects were male and eleven subjects were female with a mean age of ten years. Both camps took place at Duquesne University's A.J. Palumbo Center, located in Pittsburgh's inner-city. The demographic characteristics of the subjects enrolled are consistent with the predicted sample; enabling us to work towards reaching our goal of addressing health disparities in inner-city, lower socioeconomic, African American populations.

Recruitment focused on identifying children with asthma who would benefit from the various camp activities. The two screenings prior to each camp were developed in an effort to identify children in the community who suffer from uncontrolled or undiagnosed asthma and refer them to the camps. At this time, none of the children who attended the screenings attended either camp. Fliers were posted on bulletin boards throughout the city, in pediatric practice offices and mailed to past campers.

Registration packets were mailed to caregivers who registered via telephone approximately two weeks prior to each camp. These packets contained a description of the camp, IRB approved consent form and the Asthma Therapy Assessment Questionnaire (ATAQ). Caregivers could also elect to register onsite. Caregiver participation in the camps was strongly encouraged as ideal asthma management is a team effort. We therefore also incorporated adult screenings into the camp model. The following ten screening stations were set up at each camp: registration; adult BMI; child BMI; adult blood glucose/cholesterol testing; child spirometry testing; adult carbon monoxide breath test; child carbon monoxide breath test; adult blood pressure; child blood pressure; and data entry. Staff individually assisted each child and adult through the registration process. Registration consisted of explaining the assent form and administering the ATAQ as well as the Asthma Control Test (ACT) to each child. Caregivers and children were given pre-tests to assess their level of asthma knowledge prior to participation in the camp. Participants rotated through each screening station prior to rotating through the educational stations. Six education stations were set up focusing on the following topics: asthma pathophysiology; asthma symptoms; asthma triggers and avoidance strategies; medications used to control asthma; proper inhaler technique; and smoking cessation. A post- test was

administered to each caregiver and child after they rotated through all screening and educational stations.

Children were then taught basketball skills by Duquesne University athletes. They were given inspirational talks by athletes about the importance of physical activity. Athletes shared personal stories about overcoming obstacles in pursuit of their dreams. This is done in an effort to inspire the children to remain physically active despite their diagnosis of asthma by applying what they learned about asthma control during the education portion of the camp.

Results

1. Spirometry Outcomes: Screening Data
    - Only three participants failed the CO exposure test. Thus, the test for associations with other factors was not performed.
    - No significant association was observed between the spirometry test outcome and blood pressure classification.
    - No significant association was observed between the spirometry outcome and weight classification with respect to BMI.
2. A strong association was observed between ACT outcome and weight classification ($P>0.002$). Less than half of all participants classified as "Overweight" or "Obese" passed the ACT, whereas no subjects classified as "Healthy weight" failed the test. (Figure 1)
3. A significant association was also observed between the ATAQ outcome and weight classification ($P> \sim 0.10$). A higher proportion of subjects classified as "Overweight" or "Obese" filed the ATAQ questionnaire as compared to subjects with a "Healthy weight" classification. (Figure 2)
4. The data collected from the Intervention Camps are not of sufficient size to test for associations with reasonable statistical power.

Summary

The community based screenings have proven to be an effective way of increasing the identification of children with undiagnosed or uncontrolled asthma. 33% of the children screened during the first three months of the study failed the asthma screen and are therefore referred for follow-up care. Results are mailed to the address given at time of registration. Recommendations for follow-up are made to all children who fail any of the screening measures.

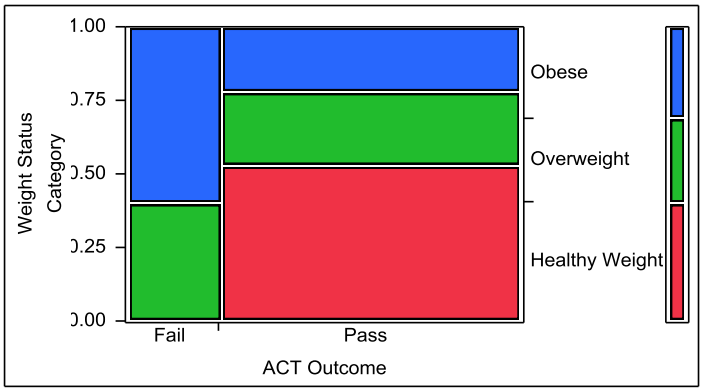
Six interactive educational sessions were developed and implemented at each camp addressing the following topics: asthma pathophysiology, asthma symptoms, asthma triggers and avoidance strategies; medications used to control asthma; proper inhaler technique; and smoking cessation. Additional interactive games were developed that focused on nutrition and physical activity.

Our group continues to work towards encouraging active healthy lives by providing basketball instruction at each camp as well as nutrition and physical activity education at all events.

Figure 1

Contingency Analysis of Weight Status Category By ACT Outcome

Mosaic Plot



Contingency Table

Weight Status Category

ACT Outcome	Healthy Weight	Overweight	Obese	
Count Total % Col % Row %				
Fail	0 0.00 0.00 0.00	4 9.52 33.33 40.00	6 14.29 46.15 60.00	10 23.81
Pass	17 40.48 100.00 53.13	8 19.05 66.67 25.00	7 16.67 53.85 21.88	32 76.19
	17 40.48	12 28.57	13 30.95	42

Tests

N	DF	-LogLike	RSquare (U)
42	2	6.4421403	0.1411

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	12.884	0.0016*
Pearson	9.490	0.0087*

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Correspondence Analysis

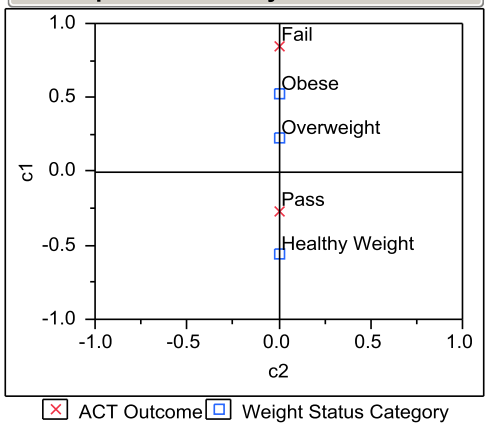
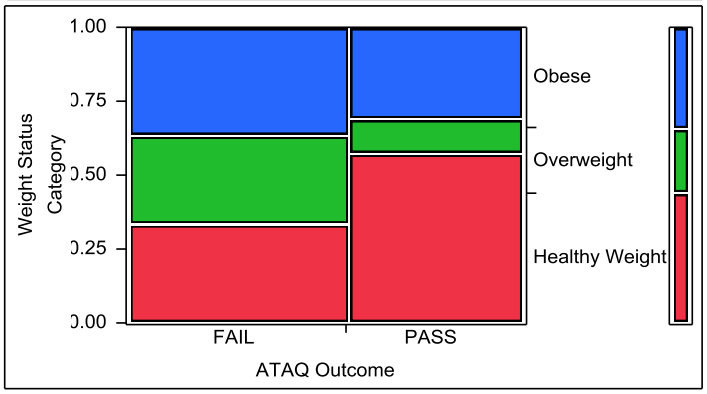


Figure 2

Contingency Analysis of Weight Status Category By ATAQ Outcome

Mosaic Plot



Contingency Table

Weight Status Category

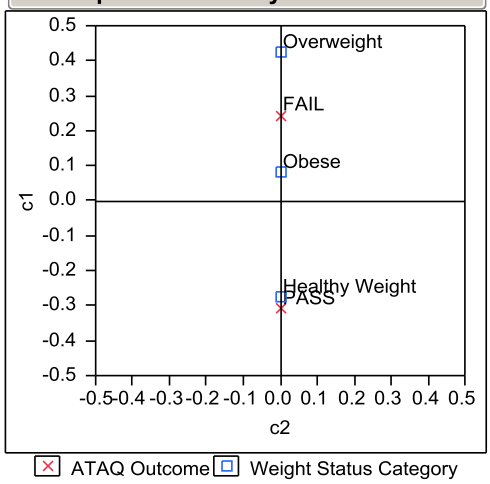
ATAQ Outcome	Count / Total % / Col % / Row %	Healthy Weight	Overweight	Obese	
	FAIL	11	10	12	33
		18.64	16.95	20.34	55.93
		42.31	76.92	60.00	
		33.33	30.30	36.36	
	PASS	15	3	8	26
		25.42	5.08	13.56	44.07
		57.69	23.08	40.00	
		57.69	11.54	30.77	
		26	13	20	59
		44.07	22.03	33.90	

Tests

N	DF	-LogLike	RSquare (U)
59	2	2.2836535	0.0365

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	4.567	0.1019
Pearson	4.416	0.1099

Correspondence Analysis



Research Project 2: Project Title and Purpose

Engineering Aβ-selective Enzymes for Treatment of Alzheimer's Disease - This project involves approaches aimed at engineering metalloendoproteinases to specifically cleave and clear Aβ peptide as a potential therapeutic approach for treatment of Alzheimer's Disease (AD). Dysfunctional metabolism leading to excess quantities of Aβ that is produced at normal levels throughout life may be the causative agent in many, if not most, cases of AD. Neprilysin, neprilysin-2 and insulin degrading enzyme have been shown to cleave Aβ *in vivo* or *in situ.* However, these enzymes are promiscuous with respect to substrate specificity and proteolyze other (neuro)peptides involved in critical physiological responses. The nano-engineering studies proposed here offer the opportunity to produce Aβ-selective enzymes that may be safely employed in subsequent gene transfer studies aimed at treating AD.

Anticipated Duration of Project

1/1/2010 - 12/31/2011

Project Overview

Protein engineering offers the opportunity to design proteins with altered functions, via site-directed mutagenesis of regions targeted towards catalytic and/or substrate-binding sites as determined by atomic resolution structural studies. We propose two specific aims in order to engineer Aβ-selective enzymes for treatment of Alzheimer's Disease (AD):

Aim 1: Utilize peptidomic methods to profile substrates of neprilysin, neprilysin-2 and insulin degrading enzyme. These mass spectrometric methods will also be used in Aim 2 to similarly profile substrates of mutagenzied enzymes, and identify cleavage sites in Aβ.

Aim 2: Engineer a more selective form of these metalloendoproteinases with increased specificity solely towards Aβ to limit aberrant catabolism of other natural targets of the enzyme using phage display methods.

The nanoengineering of any of these three peptidases to safely express enzymatic activity will be a novel therapeutic tool in the treatment of AD by acting selectively to catabolize Aβ for requisite proof-of-concept data essential to integrate these studies in the *future* for preclinical trials. In addition, this systems biology approach using three similar, but unique, endoproteinases will provide data regarding enzyme design and basic information regarding targeted nanosculpting of protein activity for other aging disorders. This generalizable approach, wherein a protease is modified to more selectively, and specifically, degrade amyloidogenic substrates, may also be exploited to combat other amyloidoses, and provide a major tool for reducing plaque burden. *Overall, enzymes that cleave and clear* Aβ *can be protective, but these same enzymes also cleave other target peptides, and their use in treating this disease might have potentially lethal side effects.* Our successful implementation of nanoengineered enzymes will produce novel therapeutics that may be safe and efficacious agents for treating Alzheimer's Disease.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

Successful engineering of an Aβ-selective neprilysin, neprilysin2 and/or insulin-degrading enzyme would provide an important therapeutic tool in combating Alzheimer's Disease. Our ultimate aim is to develop an effective degradative enzyme that safely and specifically only targets neurotoxic Aβ to strategically combat AD. The approach pioneered in this project may have a value-added feature in that engineering of a more specific form of an endogenous enzyme with specificity for the pathological protein component in the amyloid deposit may prove to be of general use for other amyloidogenic diseases or protein misfolding diseases. Since the accumulation of amyloids appears to be a common pathological function in degenerative disease, the design and re-engineering of catabolic enzymes to specifically target a given amyloidogenic peptide/protein may have broad therapeutic utility.

Summary of Research Completed

We have initiated studies described in both Aims 1 and 2, and have focused our initial efforts on utilizing neprilysin as our first test protein to characterize and mutagenize. All of the work described in this progress report has been conducted under my direct supervision by Darrick Pope, a graduate research assistant and Tim Nacarelli, an undergraduate participant in the Duquesne summer research program.

All of the initial studies have been conducted using neprilysin mutants as the constructs were already in hand and these studies required Darrick and Tim to become familiar with all of the methods that will be re-utilized in examining other protease substrates. I have organized summation of the studies to date into the two respective Aims.

Aim 1: Utilizing peptidomic methods to profile substrates of neprilysin, neprilysin-2 and insulin degrading enzyme.

In focusing our initial studies on neprilysin, the first step in profiling its substrates is to purify a soluble form of this enzyme in a suitably inactivated form (the enzyme must be inactivated to examine its substrate-binding preferences as active enzyme would proteolyze its substrate instead of capturing it in high-throughput studies). This purified protein will serve as the bait in subsequent phage display and brain lysates-binding and mass spectrometry studies.

An expression plasmid (pGEX-2T), that encodes for the soluble domain of neprilysin (sNEP) containing its inactivated (E584Q sNEP) proteolytic domain fused to glutathione-S-transferase (GST) with an intervening thrombin cleavage site, was provided by our collaborator, Dr. M. Glucksman (see Fig. 1). The plasmid was used to transform host DH5α cells and the plasmid was harvested and purified from a large batch of clonal transformed host cells archived for use in subsequent studies to be conducted throughout the grant period.

The plasmid was also used to overexpress GST-sNEP protein. The isolated plasmid was used to transform cells for the purification of E584Q sNEP. Following IPTG induction, cells were harvested and lysed. Soluble protein homogenates were purified by column chromatography on glutathione sepharose beads following an established protocol. After immobilization and extensive washing, purified sNEP was eluted by thrombin cleavage. An SDS-PAGE of a representative purification trial showed the evidence of a single protein band of sNEP at the expected MW_{app} (Fig. 2). This protein, both in soluble form and as a GST-fusion, was archived and is currently being used in subsequent studies. To identify novel and known peptides from brain lysates that are sNEP substrates, the GST-sNEP E584Q chimera will be immobilized on glutathione columns. Brain lysates will be added to the column, and selected peptides that bind to the immobilized sNEP will be identified by mass fingerprinting. Conversely, phage display studies identifying random 15mers that bind to immobilized GST-sNEP E584Q will be identified and sequenced. These studies are on-going.

Aim 2: Engineering a more selective form of metalloendoproteinases with increased specificity solely towards Aβ to limit aberrant catabolism of other natural targets using phage display methods.

In order to conduct the studies of this Aim, the cDNA of the inactivated form of soluble metalloendoproteinase must be inserted in phase to the appropriate site of the pIII coat protein of the M13 phage. Appropriate vectors, SAM and SAM33 (providing for fusion products in all or a single pIII coat protein, respectively), and *E. coli* host cells (XL-1 Blue F'tet) were provided by Dr. B. Kay, Univ. Illinois-Chicago. *A priori*, one cannot know if the phage will accommodate the insertion of a large protein fused to all three copies of its pIII coat protein, so initial studies were conducted trying both constructs. Initial trials utilize neprilysin as the protease, so the cDNA corresponding to the soluble form of inactivated E584Q sNEP was inserted in frame with the pIII gene in both vectors. SAM-E584QsNEP and SAM33-E584QsNEP vectors were constructed and are currently being confirmed by restriction analyses of isolated double stranded plasmids. As these vectors will be the template for all subsequent random mutagenesis studies, initial efforts were to produce recombinant virus in both single and double stranded forms for use in ongoing studies. These methods are new to our lab, so much time was spent mastering basic molecular biological techniques essential for conducting these and subsequent studies. The isolated SAM vectors containing the sNEP protein will be assessed for viability and then will be used as the template for random mutagenesis of selected discrete regions of the SNEP protein that are hypothesized to be part of the binding site for Aβ. Subsequent phage display studies assessing competitive binding studies of biotinylated Aβ and other substrates identified in Aim 1 will identify mutations in sNEP that result in selective Aβ binding.

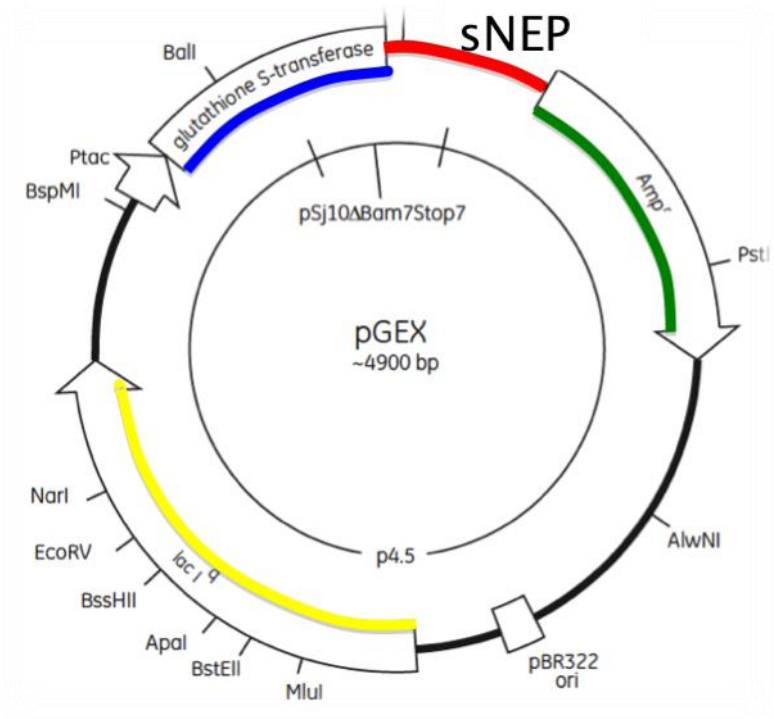

Figure 1. Map of pGEX-2T expression vector containing E584Q sNEP

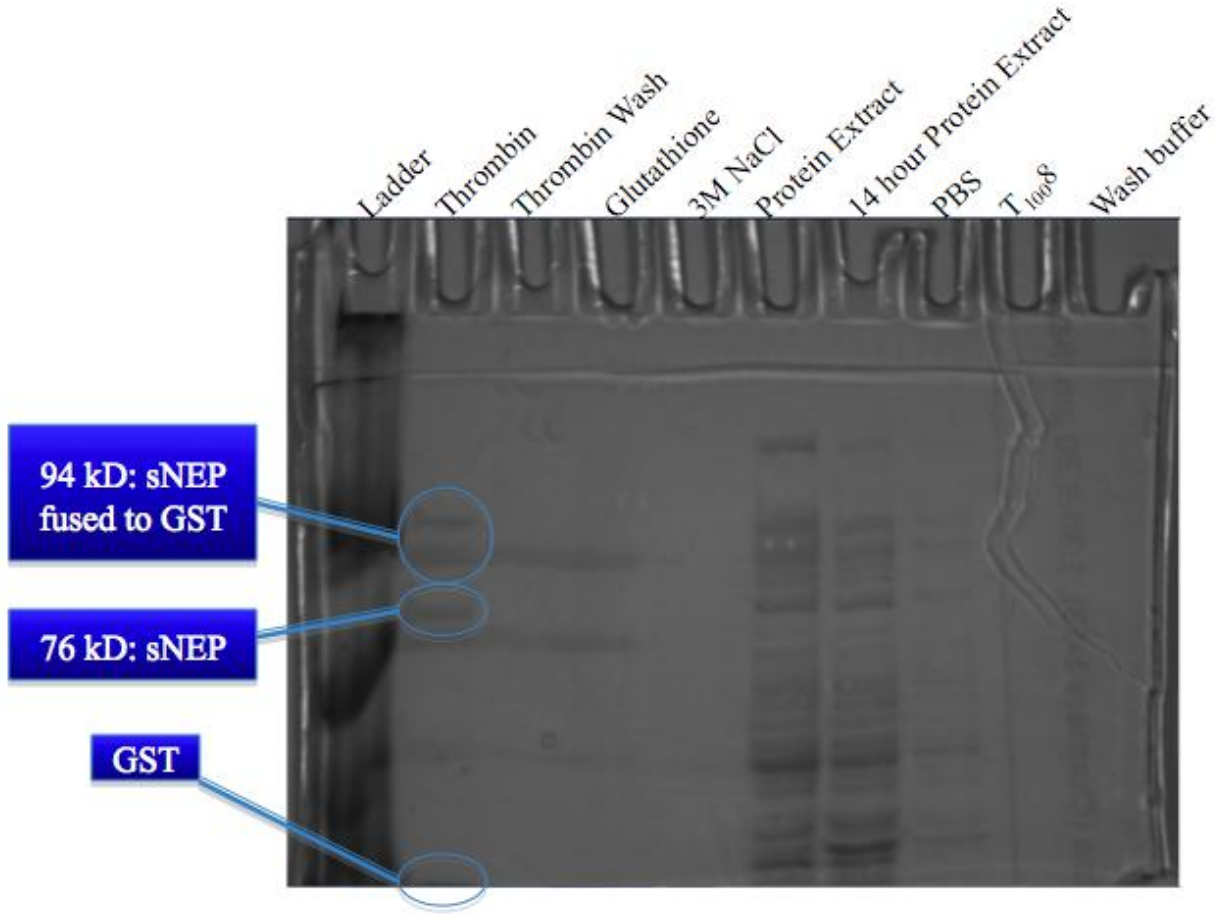

Figure 2. SDS-PAGE of sNEP purification.