

**Expression of Alpha-*N*-Acetylglucosaminidase Fused to the HIV-1 Protein
Transduction Domain and a Modified Protein Transduction Domain**

by

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ABSTRACT

The genetic disorder mucopolysaccharidosis IIIB, which primarily affects the central nervous system (CNS), is caused by a deficiency in the enzyme alpha-N-acetylglucosaminidase (Naglu). Recombinant Naglu is unable to enter cells or cross the blood-brain barrier (BBB), making potential enzyme replacement therapy infeasible. To enable Naglu to be endocytosed by cells and perhaps cross the BBB, two fusion proteins of Naglu with the HIV-1 Tat protein transduction domain (PTD) or a modified PTD were created. This project explored the use of a *Spodoptera frugiperda 9 (Sf9)* expression system utilizing the p2ZoptcxF vector to produce and purify active Naglu and active Naglu-PTD fusion proteins. It was found that the *Sf9* expression system produced active Naglu, that the addition of the PTD fusion moieties did not decrease its activity, and that it was possible to purify this protein to near homogeneity.

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LIST OF ABBREVIATIONS

μ- (micro-), **aa** (amino acids), **Abg** (beta-glucosidase), **APMSF** (4-amidino-phenylmethylsulfonyl fluoride), **BBB** (blood-brain barrier), **BSA** (bovine serum albumin), **CBD**, **CBD_{CEX}**, **CBD(IIA)** (modified cellulose-binding domain of xylanase 10A of *Cellulomonas fimi*), **cdNA** (complementary DNA; reverse transcribed from ribonucleic acid), **CHO** (Chinese Hamster Ovary), **CIP** (Calf Intestinal Phosphatase), **DMSO** (dimethylsulfoxide), **DNA** (deoxyribonucleic acid), ***E. coli*** (*Escherichia coli*), **EDTA** (ethylenediaminetetra-acetic acid), **eGFP** (enhanced green fluorescent protein), **ERT** (enzyme replacement therapy), **FACS** (fluorescence-assisted cell sorting), **Factor Xa** (activated Factor X), **FGF**, **FGF-1**, **FGF-2** (fibroblast growth factor; type 1 and 2), **FXa** (activated Factor X), **-g** (gram), **GAG**, **GAGs** (glycosaminoglycan(s)), **GFP** (green fluorescent protein), **GnRH** (gonadotropic releasing hormone), **GPI** (glycophosphatidyl-inositol), **HIV-1** (human immunodeficiency virus type 1), **HS** (heparan sulfate), **ie2** (immediate early 2 promoter of *Orgyia pseudotsugata* nucleopolyhedrosis virus), **kb or Kb** (kilobase or kilobases), **kDa** (kilodalton, equal to 1000 MW), **-l** (liter), **LSLB** (Low salt Luria-Bertani Medium), **m-** (milli-), **mRNA** (messenger ribonucleic acid), **M** (molar), **M-6-P** (mannose-6-phosphate), **MCC** (microcrystalline cellulose), **MDCK** (Madin-Darby canine kidney, an epithelial cell line), **MPS** (mucopolysaccharidosis), **MPS III** (mucopolysaccharidosis III), **MPS IIIB** (mucopolysaccharidosis III type B), **MW** (molecular weight, equal to the mass of 1/12 a C¹² atom), **n-** (nano-), **Naglu** (α -N-acetylglucosaminidase), **Naglu-PTD4** (α -N-acetylglucosaminidase-protein, transduction domain #4 fusion), **Naglu-tatPTD** (α -N-acetylglucosaminidase-tat

protein transduction domain fusion), **NMR** (nuclear magnetic resonance), **NPTD4** (α -*N*-acetylglucosaminidase-protein transduction domain #4 fusion), **Ntat** (α -*N*-acetylglucosaminidase-tat protein transduction domain fusion), **ON** (overnight), **p2Zop** (p2ZoptcxF), **p2Zop-Naglu** (α -*N*-acetylglucosaminidase-containing p2ZoptcxF vector), **p2Zop-NPTD4** (α -*N*-acetylglucosaminidase-protein transduction domain #4 fusion-containing p2ZoptcxF vector), **p2Zop-Ntat** (α -*N*-acetylglucosaminidase-tat protein transduction domain fusion-containing p2ZoptcxF vector), **p-Naglu** (α -*N*-acetylglucosaminidase-containing p2ZoptcxF vector), **p-NPTD4** (α -*N*-acetylglucosaminidase-protein transduction domain #4 fusion-containing p2ZoptcxF vector), **p-Ntat** (α -*N*-acetylglucosaminidase-tat protein transduction domain fusion-containing p2ZoptcxF vector), **pA** (poly-adenylation signal), **PCR** (polymerase chain reaction), **PMSF** (phenylmethylsulfonyl fluoride), **polyA** (poly-adenylation signal), **PTD** (protein transduction domain), **PTD4** (protein transduction domain #4 created by Ho *et al.* (2001)), **PVDF** (polyvinylidene fluoride), **RT** (room temperature), **-s** (seconds), **SA** (specific activity), **SDS** (sodium dodecyl sulfate), **SDS-PAGE** (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), **Sf9** (*Spodoptera frugiperda* 9), **TAE buffer** (Tris-Acetic acid-EDTA buffer), **TBS** (Tris-buffered saline), **TF** (transferrin), **TGF- β** (transforming growth factor- β), **TX100** (Triton X-100), **TTBS** (Tween 20-containing TBS), **U** (unit or units), **VEGF** (vascular endothelial growth factor).

For amino acids, standard one letter and three letter abbreviations are used.

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DEDICATION

This work is dedicated to my late grandmother, Mary “Mika” Bandsmer, someone who inspired me with her perseverance and hard work.

1 – Introduction

1.1 – Mucopolysaccharidosis III

1.1.1 – Heparan Sulfate Metabolism

Heparan sulfate is a common glycosaminoglycan (GAG), a side chain found on various proteoglycans. It consists of partially sulfated glucuronic acid and L-iduronic acid residues alternating with sulfated or acetylated glucosamine residues, and is often highly varied in organization (Cifonelli *et al.*, 1977; Fransson *et al.*, 1980; Linker *et al.*, 1975). Heparan sulfate (HS) is found in the extracellular matrix surrounding cells, on the surface of cells and in intracellular pools in various stages of anabolism and catabolism (Bienkowski *et al.*, 1984). HS has been implicated in many cellular functions as diverse as cell adhesion, motility, differentiation, and morphogenesis (Culp *et al.*, 1979; Endo *et al.*, 2003; Inatani *et al.*, 2003). HS is also known to interact with a large variety of growth factors, including fibroblast growth factors 1 and 2 (FGF-1, FGF-2), transforming growth factor beta (TGF- β), and vascular endothelial growth factor (VEGF) among others (Berry *et al.*, 2004; Folkman *et al.*, 1992; Segarini *et al.*, 1988; Tessler *et al.*, 1994).

HS is manufactured in the Golgi (Iozzo, 1987). It exists in a steady-state equilibrium between the extracellular matrix and intracellular pools (Bienkowski *et al.*, 1984). It is packaged onto proteoglycans while still in the Golgi apparatus; the proteoglycans are then transported to the cell surface via vesicles where they are exposed or released to the extracellular matrix (Iozzo, 1987). In hepatocytes, about two thirds of extracellular HS exists as loosely associated free chains, while the other

third can only be removed by trypsinization (Fedarko *et al.*, 1986; Kjellen *et al.*, 1980). After a specified amount of time dependent upon cell type, HS is recycled via vesicular intake and eventually lysosomal degradation (Brauker *et al.*, 1987; Iozzo, 1987). Any adsorbed growth factor or other molecule is taken into the cell along with the HS, for example, FGF (Chang *et al.*, 2000).

The enzymes involved in the breakdown of the various structures of HS GAGs include iduronate sulfatase, α -L-iduronidase, heparan *N*-sulfatase, acetyl-CoA: α -glucosaminide acetyltransferase, α -*N*-acetylglucosaminidase, glucuronate sulfatase, β -glucuronidase, and *N*-acetylglucosamine 6-sulfatase (Neufeld *et al.*, 2001).

1.1.2 – Cause and Manifestations of Mucopolysaccharidosis III

A deficiency in heparan *N*-sulfatase, α -*N*-acetylglucosaminidase, acetyl-CoA: α -glucosaminide acetyltransferase, or *N*-acetylglucosamine 6-sulfatase gives rise to the autosomal recessive genetic lysosomal storage disorder known as mucopolysaccharidosis III (MPS III), subtypes A, B, C and D respectively. The disorder is also known as Sanfilippo syndrome. MPS III has been observed in humans, mice, dogs and birds (Bhaumik *et al.*, 1999; Fischer *et al.*, 1998; Jones *et al.*, 1998). The incidence of MPS III, after correction for ascertainment probability, has been calculated to be approximately 1/24,000 births (Neufeld *et al.*, 2001).

Due to the inability of cells to completely catabolize HS, the products of incomplete HS degradation build up in vacuoles within cells. Build-up is especially noticeable in cells of the macrophage lineage in the mouse model of MPS IIIB (Li *et al.*, 1999). This storage has been observed to lead to a variety of cellular effects

including reduced neuraminidase activity and/or attenuation of fibroblast growth factor-receptor-1 mRNA production; both potentially interfere with neuroplasticity and neurogenesis (Li *et al.*, 1999; Li *et al.*, 2002). Secondary to HS product accumulation, it appears that some enzymes of ganglioside catabolism may be inhibited; gangliosides G_{M2} and G_{M3} accumulate in the brains of MPS III patients and mice (Baumkotter *et al.*, 1983; Constantopoulos *et al.*, 1978; Li *et al.*, 1999). This accumulation has been hypothesized to contribute to the deleterious effects MPS III has on the central nervous system (Neufeld *et al.*, 2001).

At the systemic level, the main effect of MPS III is central nervous system degeneration, with patients displaying severe cortical atrophy in the later stages of the disease (Zafeiriou *et al.*, 2001). On a symptomatic level, affected individuals show a deterioration of social/adaptive skills and cognitive functioning beginning a few years after birth. Individuals may also develop symptoms including aggressiveness and other severe behavior problems, sleep disturbances, hirsutism, and hearing loss, among others. Those affected with MPS III usually die in their second decade (Neufeld *et al.*, 2001).

1.1.3 – Treatment of MPS III

There is no cure for MPS III. Treatment is limited to symptom-dependent pharmacologic management or behavior modification for behavioral issues (Neufeld *et al.*, 2001). Bone marrow transplantation has been attempted, but there is no preservation of intellectual function and it carries a high risk of morbidity and mortality (Shapiro *et al.*, 1995).

Gene therapy for MPS III is still in the very early stages of development, and is subject to the shortfalls gene therapy presents as a whole: inefficient delivery and transient expression of the gene (Neufeld *et al.*, 2001; Schiffmann *et al.*, 2002).

A treatment that has proven effective for other lysosomal storage diseases, such as MPS I and Gaucher disease, is enzyme replacement therapy (ERT) (Kakkis *et al.*, 1996; Schiffmann *et al.*, 2002). Lysosomal storage diseases lend themselves readily to ERT because proper cell phenotype can be restored with only a small amount of normal activity of the enzyme in question (often less than 10%). As well, the recombinant enzymes can usually be produced in large quantities with an intact mannose-6-phosphate (M-6-P) marker, allowing uptake by cells via the M-6-P receptor-mediated pathway (Neufeld *et al.*, 2001; Schiffmann *et al.*, 2002). One downside to ERT for lysosomal storage diseases seems to be the rapid clearance of the enzyme from circulation and a corresponding slow infusion rate of the enzyme into the body (Kakkis *et al.*, 1996; Sands *et al.*, 1994).

1.1.4 – Alpha-N-Acetylglucosaminidase

Mucopolysaccharidosis IIIB (MPS IIIB), also known as Sanfilippo B, is the subtype of MPS III caused by a deficiency in the enzyme α -N-acetylglucosaminidase (Naglu). The gene encoding Naglu is comprised of 6 exons and 5 introns, spans 8.5 kilobases (kb) of DNA, and is localized to chromosome 17q21.1 (von Figura *et al.*, 1984; Zhao *et al.*, 1996). Most disease-causing mutations are private, meaning that there are few common mutations (Neufeld *et al.*, 2001). The protein is 743 amino acids in length, including a 23 amino acid signal sequence which is removed during co-translational processing (Weber *et al.*, 1996; Zhao *et al.*, 1996).

In normal human skin fibroblasts, the enzyme is produced as a precursor of approximately 87 KDa. Somewhat less than 30% of this precursor is secreted on route to the lysosome; this fraction can be taken up by other fibroblasts. Subsequent intracellular processing reduces the size of Naglu to 73 and 76 KDa; this protein remains stable for at least 4 days within the cell (von Figura *et al.*, 1984). All lysosomal enzymes, including Naglu, are housekeeping genes, and are therefore expressed in every cell type.

1.1.5 – Production and Characterization of alpha-N-acetylglucosaminidase

In view of potential ERT, two research groups independently produced and characterized human recombinant Naglu expressed in Chinese hamster ovary (CHO) cell lines. The recombinant protein was found to have properties nearly identical to Naglu secreted from human fibroblasts except that the recombinant enzyme was not well endocytosed by other cells in culture, unlike the native enzyme. The reduced endocytosis was hypothesized to be due to the limited M-6-P modification observed on the recombinant enzyme. This lack of cellular uptake led the two research groups to conclude that recombinant Naglu was an unlikely vector for ERT (Weber *et al.*, 2001; Zhao *et al.*, 2000).

However, studies of ERT utilizing a Naglu *-/-* knockout murine model unexpectedly displayed an improvement of some somatic symptoms in treated mice, primarily within the liver and spleen. The authors hypothesize that this is due to the fact that only a small amount of Naglu activity is necessary for restoration of proper HS metabolism, and that macrophages, the only cells to take up the enzyme, were able to correct the dysfunction of the entire organ. Unfortunately, the authors were

unable to show that this effect might not be due to native Naglu purified from the Chinese hamster ovary expression system, *i.e.* Naglu that would have had M-6-P modifications. They were also unable to show any evidence of Naglu within the central nervous system, and no uptake except in cells of macrophage lineage (Yu *et al.*, 2000).

Recently, both retroviral and a lentiviral vectors were developed to express Naglu. It was hoped that these different expression systems might give rise to Naglu that is phosphorylated in a manner similar to the native enzyme, allowing for some spread of active Naglu throughout an organism. However, both the retroviral and lentiviral systems also produced enzyme with little mannose-6-phosphorylation, and in turn, a correspondingly small amount of transduction of the enzyme between cells. For example, only 0.36% of the total Naglu secreted from retrovirally transduced MPS IIIB fibroblasts was endocytosed by non-transduced MPS IIIB cells (Villani *et al.*, 2002; Yogalingam *et al.*, 2000).

Thus, while it is possible to produce active recombinant Naglu, this enzyme is unlikely to enter most cell types due to the absence of mannose-6-phosphorylation when produced by either human or CHO cell lines.

1.2 – The Tat Protein Transduction Domain

1.2.1 – The Tat Protein and its Protein Transduction Domain

The tat protein is an essential viral protein responsible for the activation of genes expressed from the Human Immunodeficiency Virus Type 1 (HIV-1) long terminal repeat (Harrich *et al.*, 1997). In the 1980s, Frankel *et al.* (1988), and Green

et al. (1988) independently reported that purified tat protein had the surprising ability to be taken up by cells. Some researchers hypothesized that the tat protein is secreted from an infected cell to infiltrate and activate the latent HIV-1 genome within a neighboring cell (Ensoli *et al.*, 1993). Subsequent researchers used trial and error to find the portion of the tat protein which was responsible for the uptake, and narrowed it to a 9-12 amino acid sequence, YGRKKRRQRRRP, now known as the tat protein transduction domain (PTD) or cell penetrating peptide (Fawell *et al.*, 1994; Green *et al.*, 1988; Park *et al.*, 2002; Vives *et al.*, 1997).

1.2.2 – A Brief History of the Study of the Tat Protein Transduction Domain

It has taken many years of study to reach some consensus on the capabilities of the tat PTD, and how it is that the tat PTD is able to transduce cell membranes. Early research created much excitement. With attachment to the tat PTD, either by fusion or conjugation, varied moieties including proteins, peptides, nucleic acids, phages, adenoviruses, nanoparticles such as gold particles, and liposomes, were taken into almost every cell type tested, including non-dividing cells (Eguchi *et al.*, 2001; Fawell *et al.*, 1994; Gratton *et al.*, 2003; Levchenko *et al.*, 2003; Lewin *et al.*, 2000; Torchilin *et al.*, 2003; Torchilin *et al.*, 2001; Violini *et al.*, 2002). The only cells to date that have not facilitated tat PTD entry include MDCK renal epithelial cells and CaCo-2 colonic carcinoma cells, both of which are cell types forming tight junctions in monolayer cultures; it is currently unknown why these particular cells are impermeable (Violini *et al.*, 2002).

In another discovery, Schwarze *et al.* (1999) demonstrated that the 120 kilodalton (kDa) β -galactosidase, when fused to the tat PTD, was able to cross the

blood-brain barrier in mice. The blood-brain barrier is highly impermeable to most proteins, so the discovery of a small peptide that could not only transduce a large protein into nearly every cell type but also allow the protein to cross the blood-brain barrier was novel and exciting.

However, in further research, little consensus could be reached among researchers as to abilities and requirements of translocation. For example, some researchers showed that GAGs were necessary for translocation, while others demonstrated that they were not (Console *et al.*, 2003; Sandgren *et al.*, 2002; Sandgren *et al.*, 2004; Silhol *et al.*, 2002). Also, some researchers showed inhibition of uptake at 4°C, while others did not (Ferrari *et al.*, 2003; Vives *et al.*, 1997). Differences could not simply be accounted for by different cell types.

Conflicting results were found to be partially the result of misleading methods including methanol, acetone and paraformaldehyde fixation and flow cytometry. The above fixation methods were found to give erroneous results because of cell membrane disruption before the tat PTD was permanently fixed; flow cytometry was found to give misleading results because of its inability to distinguish between internalized fluorescent tagged-tat PTD and that bound to the surface (Leifert *et al.*, 2002; Lundberg *et al.*, 2003; Richard *et al.*, 2003; Vives, 2003).

1.2.3 – Tat PTD-Mediated Entry

Nuclear Magnetic Resonance (NMR) and liposome data have shown us that the tat PTD does not, as was once thought, cause a localized distortion enabling it to slide through the cell membrane, with the exception of giant unilamellar vesicles (Hakansson *et al.*, 2003; Kramer *et al.*, 2003; Thoren *et al.*, 2004). Rather, the

accepted hypothesis of internalization in a cellular system is that lysine and arginine residues of the tat PTD contribute to cell-surface binding via non-specific interactions with GAGs such as dextran sulfate and heparan sulfate; lysine and arginine residues of the tat PTD contribute to cell-surface binding (Console *et al.*, 2003; Sandgren *et al.*, 2002). Arginine, more so than lysine, has been shown to be absolutely necessary; it is thought that the guanidium group of arginine reacts well with hydrogen-bond acceptors such as phosphates, carboxylates and sulfurs, which are all abundant on the plasma membrane (Rothbard *et al.*, 2002; Vives, 2003). It is likely that arginine's ability to form a greater number of hydrogen bonds than lysine contributes to its greater ability to mediate non-specific binding to the cell surface. Because of its ability to bind non-specifically, entry of the tat PTD is M-6-P independent (Xia *et al.*, 2001).

Another well-established fact about tat PTD fusion proteins is that most internalization can be attributed to caveolae-mediated endocytosis. Caveolae are invaginations of the plasma membrane originating from lipid rafts and generally characterized by the presence of caveolin-1, an integral membrane protein (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003). Caveolae are involved in many endogenous processes such as cholesterol homeostasis, glycosphingolipid transport and GPI-anchored protein recycling, as well as growth factor signaling (Ikonen *et al.*, 2000; Simons *et al.*, 1997; Simons *et al.*, 2000). Caveolae have also been shown to recycle the HS side chains (*i.e.* GAGs) of the proteoglycan glypican-1 (Murakami *et al.*, 1999). Caveolae show different characteristics in different cell types; for example, CHO cells show fast and constitutive caveolae-mediated endocytosis, while HeLa

cells have caveolae which are largely immobile plasma membrane compartments which are not involved in constitutive endocytosis (Ferrari *et al.*, 2003). This difference in HS recycling may help to explain some of the different results received by different groups on the study of the tat PTD.

While it is known that caveolae are a major entry point for tat PTD fusion proteins, evidence suggests that the small tat PTD on its own is able to enter the cell through some other, nonendocytotic method (Silhol *et al.*, 2002; Thoren *et al.*, 2004; Thoren *et al.*, 2003). Nonetheless, all fusion protein data indicate that caveolae-mediated endocytosis is the primary, if not the only, method of entry of such fusions.

1.2.4 – Tat PTD and the Blood Brain Barrier

It is also an accepted fact that the tat PTD can confer upon some proteins the ability to cross the blood brain barrier (BBB). For example, fusion proteins of the tat PTD with the 120 KDa β -galactosidase protein, glial line-derived neurotrophic factor, and Bcl-xL, were all found localized in brain regions or affecting the brain in mice after intraperitoneal injection (Cao *et al.*, 2002; Elliger *et al.*, 2002; Kilic *et al.*, 2003; Schwarze *et al.*, 1999). What researchers do not know is how the tat PTD enables some proteins to cross the blood-brain barrier. Researchers are beginning to understand, however, how the *entire* tat protein can negatively affect the BBB; Andras *et al.* (2003) report that tat markedly affects both distribution and expression of specific tight junction proteins in brain endothelium, which could potentially lead to disturbances of BBB integrity. As well, the full tat protein has been found to induce oxidative inflammatory responses, and to recruit and induce monocytes to cross a BBB model (Toborek *et al.*, 2003; Weiss *et al.*, 1999). As of yet, however, it

is unknown if the smaller tat PTD or tat PTD fusion proteins cross the BBB by similar inductions and/or by other means.

1.2.5 – Tat PTD as a Secretion Signal

Another property of the tat PTD is that it serves as a secretion signal for some proteins. For example, β -glucuronidase was shown to be secreted 10-fold over the same protein expressed without C-terminal tat PTD (Elliger *et al.*, 2002). Herpes simplex virus type-1 thymidine kinase also showed significant translocating abilities when fused to tat PTD (Tasciotti *et al.*, 2003). On the other hand, this property of secretion has not held true for every protein; for example, NP₃₉₆ peptide and enhanced green fluorescent protein (eGFP) were not secreted more than they were without the tat PTD, nor is β -glucuronidase secreted more when the tat PTD is fused to its N-terminus (Elliger *et al.*, 2002; Leifert *et al.*, 2002). It currently remains unknown what properties of the protein and/or placement of the tat PTD allow one protein to be secreted while another is retained in the cell.

1.2.6 – Localization of the Internalized Tat PTD

While research into the internal trafficking pathway(s) of the tat PTD is still in its infancy, findings so far would seem to indicate that the exact subcellular localization of transduced protein may depend on cell type, the nature of the imported protein, and the delivery approach. Fischer *et al.* (2004) showed distinct cell-dependent differences in localization and trafficking patterns. Yang *et al.* (2002) showed that tat PTD-green fluorescent protein (GFP) fusion protein localized in either the cytosol or the nucleus/nucleolus depending on the delivery approach.

Tat PTD fusion proteins, the solitary tat PTD, and the full length tat protein have been visualized in endosomes, the cytoplasm, the nucleus and the nucleolus. Sandgren *et al.* showed that tat PTD-DNA or -GAG complexes localized to the endolysosomes, but that the tat PTD on its own migrated to the nucleus (Sandgren *et al.*, 2002). The data of Bonifaci *et al.* (1995) suggested that if proteins were to enter the nucleus, unfolding was required; the data of Ferrari *et al.* (2003) showed that vesicles with tat-GFP migrated in an actin skeleton-dependent fashion towards the nucleus, and if the protein entered the nucleus, unfolding was necessary, thus supporting this hypothesis. Eguchi *et al.* (2001) demonstrated that a tat PTD-lambda phage conjugate escaped the endosome via a non-toxic endosomal membrane destabilization. Excitingly, a tat PTD-GFP fusion protein with a mitochondrial signaling sequence localized to the mitochondria, indicating that it may be feasible to target tat PTD fusion proteins to specific intracellular locations (Del Gaizo *et al.*, 2003).

Data of Fischer *et al.* (2004) indicated that the tat PTD remained in the endosome of MC57 fibrosarcoma cells until acidification occurred. Once the pH dropped, the tat PTD exited into the cytoplasm via retrograde transport through the Golgi; this trafficking is similar to many plant and bacterial toxins. The integrity of the endosome was maintained throughout (Fischer *et al.*, 2004).

1.2.7 – A Modified Tat PTD

Ho *et al.* (2001) designed a series of peptides based on the tat PTD protein sequence in attempt to stabilize a predicted amphipathic helix structure. One construct, PTD4, amino acid sequence YARAAARQARA, showed 33X greater binding than

the tat PTD to Jurkat T cells *in vitro* and 5X greater binding to blood cells *in vivo* (it should be noted that the authors stated that they saw 33X and 5X better *transduction*, but since they did not trypsinize their cells, fluorescence-assisted cell sorting (FACS) would have detected bound PTDs as well as transduced PTDs). Aside from misleading methods, NMR data have recently been published indicating that the tat PTD as part of a fusion protein with IgG-binding domain of streptococcal protein G did *not* form a helical structure in solution (Hakansson *et al.*, 2003). Despite these detractions, it may still be possible that the PTD4 designed by Ho *et al.* (2001) is somehow capable of increased binding and/or transduction, since the 33X greater binding *in vitro* and 5X greater binding *in vivo* seems to be significant.

1.3 – Project Overview

1.3.1 – Methods

To date, no one has investigated the appropriateness of the insect cell expression system, more specifically, the *Spodoptera frugiperda* 9 (*Sf9*) system, for the production of active human recombinant Naglu. The *Sf9* system has already been shown to produce high levels of other active enzymes, including another human lysosomal enzyme, glucocerebrosidase (Martin *et al.*, 1988; Pfeifer *et al.*, 2001; Sinclair, 2001). The *Sf9* system, while producing proteins with post-translational modifications similar to those of the human system, does not produce mannose-6-phosphorylated proteins; this will allow future transduction studies of tat PTD and the modified tat PTD without the added variable of possible mannose-6-phosphorylation (Aeed *et al.*, 1994).

The vector p2ZoptcxF (a gift from Dr. Tom Pfeifer, University of British Columbia) was selected for the production of the three constructs. With this vector, transfected cells exhibit constitutive expression mediated by the *Orgyia pseudotsugata* nucleopolyhedrosis virus immediate-early 2 (ie2) promoter. It is an integrative plasmid, meaning it generates stably transformed insect cell lines; this avoids the potential problem of the disruption of the cells' protein processing machinery when using a baculovirus infection system (Hegedus *et al.*, 1998). To avoid time-consuming purification steps, the p2ZoptcxF plasmid includes the human transferrin secretion signal and the cellulose-binding domain from an exoglucanase of *Cellulomonas fimi*, CBD(IIA), also known as CBD_{CEX}, as a purification tag, allowing easy harvesting and purification of the proteins respectively. The vector is more fully described in Chapter 2, Materials and Methods.

A brief overview of the methods that were involved is as follows: three cDNA constructs were created, of Naglu, Naglu-tatPTD, and Naglu-PTD4, where PTD4 is the modified tat PTD constructed by Ho *et al.* (2001); these constructs were inserted into the p2ZoptcxF vector for *Sf9* insect cell expression and secretion; the three proteins were purified from insect cell medium using CBD(IIA) as an affinity tag; some characterization of the proteins in terms of physical properties and activity was performed.

1.3.2 – Goals and Hypothesis

This project aims to express Naglu and two fusion proteins, Naglu with the tat PTD, and Naglu and the modified tat PTD created by Ho *et al.* (2001). The eventual purpose of their creation is to test these purified proteins for transduction ability;

however, transduction studies will not be conducted within the scope of this study. The hope is that future studies will demonstrate that the fusion protein(s) of Naglu can enter cells in a mannose-6-phosphate independent manner, providing hope that ERT or gene therapy can one day be made possible by a Naglu that can enter cells and possibly be secreted from cells and/or cross the BBB.

The hypothesis of this author is that the *Spodoptera frugiperda* 9 (*Sf9*) expression system utilizing the p2ZoptxF vector is an appropriate system with which to produce and purify the three enzymes Naglu, Naglu-tatPTD, and Naglu-PTD4 in an active form.

Chapter 1 of this thesis addresses the backgrounds of MPS III and the tat PTD. Chapter 2 outlines the material and methods used in the research of this thesis. Chapter 3 presents the results achieved throughout the course of this research. Chapter 4 discusses the results obtained in light of the original hypothesis. The final chapter, Chapter 5, is a list of the publications or other information cited in this work.

2 – Materials and Methods

2.1 – Materials

2.1.1 – Chemicals and Reagents

The following were obtained from commercial sources: *Amersham Pharmacia Biotech, Piscataway, NJ*: ECL Plus Western Blotting Detection System, Hybond-P PVDF membrane; *Amicon, Inc., Beverly, MA*: Stirred Ultrafiltration Cell 8200, 50K DIAFLO® Ultrafiltration membrane, Microcon® YM-30 and YM-10 Centrifugal Filter Devices; *BioRad, Hercules, CA*: 40% acrylamide (37.5:1 acrylamide/bis), Gene Pulser® Cuvettes (0.1 cm), Bio-Rad Protein Assay Dye Reagent; *Eastman Kodak Company, Rochester, NY*: Kodak BioMax MR Film; *EMD Biosciences, San Diego, CA*: 4-methyl-umbelliferyl- α -*N*-acetylglucosaminide, Anti-CBD_{CEX}•Tag Antibody, colorpHast® pH strips, Factor X Cleavage/Capture Kit, 2 ml Spin Filters; *Invitrogen, Carlsbad, CA*: SF-900 II SFM, TOPO® TA Cloning Kit for Sequencing, *One Shot*® TOP10 Chemically Competent *E. coli*, 1 Kb DNA Ladder™, Zeocin™, deoxynucleotides; *Millipore Co., Bedford, MA*: Ultrafree®-4 Centrifugal Filter Device (Biomax 10K NMWL Membrane); *New England Biolabs, Beverly, MA*: all restriction enzymes, Prestained Protein Marker Broad Range (kDa ladder), Calf Intestinal Phosphatase (CIP), T4 DNA Ligase, 3X SDS Sample Buffer; *Pall, Ann Arbor, MI*: Nanosep® Centrifugal Devices, 30K; *Qiagen, Mississauga, ON*: Qiagen® Plasmid Midi Kit, QIAprep® Miniprep, QIAquick® Gel Extraction Kit, QIAquick® Nucleotide Removal Kit, QIAquick® PCR Purification Kit; *Qiagen Operon, Alameda, CA*: all primers; *Roche Molecular Biochemicals, Indianapolis, IN*:

FuGENE 6 Transfection Reagent; *Sigma-Aldrich, St. Louis, MO*: Sigmacell®
 Cellulose (Type 101 Highly purified Fibrous Cellulose); *Stressgen, Victoria, BC*:
 Goat Anti-Rabbit IgG:Horseradish Peroxidase (HRP) antibody; *TetraLink
 International, Buffalo, NY*: Ultra Therm DNA Polymerase.

The following were obtained as gifts: *Pfu* DNA polymerase was supplied by Dr. D. Levin (University of Victoria, Victoria, BC). CBD_{CEX} control protein was supplied by Dr. R.A.J. Warren (University of British Columbia, Vancouver, BC). Anti-Naglu primary antibody and the Naglu cDNA was supplied by Drs. Neufeld and Zhao (University of California, Los Angeles, CA). p2ZoptcxF vector was supplied by Dr. T. Pfeifer (University of British Columbia, Vancouver, BC).

2.1.2 – Media and Prepared Solutions

0.7% agarose gel: 0.7% (w/v) agarose in 1X TAE buffer (see below), 0.002% ethidium bromide. *10% methanol transfer buffer*: 10% methanol, 25mM Tris-HCl, 0.2M glycine. *4MU-Naglu substrate*: 0.2 mM 4-methyl-umbelliferyl- α -N-acetylglucosaminide in 0.1 M Na-acetate buffer, containing 0.5 mg/ml BSA, pH 4.3. *4X Upper Tris*: 0.5 M Tris base (pH 6.8), 0.4% (w/v) sodium dodecyl sulfate (SDS). *4X Lower Tris*: 1.5 M Tris base (pH 8.8), 0.4% (w/v) SDS. *40x TAE buffer*: 1.6 M Tris base (pH 7.2), 0.8 M sodium acetate•3H₂O, 40 mM ethylenediaminetetra-acetic acid(EDTA)•Na₂•2H₂O. *Blocking solution*: 7.5% w/v skim milk powder in TTBS (see below). *Cracking buffer*: 5 mM EDTA, 10% sucrose (w/v), 0.25% SDS, 100 mM NaOH, 60 mM KCl, bromophenol blue to deep color. *Gel Loading Buffer*: 50% glycerol in 1X TAE buffer, 1% bromophenol blue, 1% xylene cyanol. *Glycine-NaOH buffer*: 0.5 M glycine (pH 10.5). *Low salt Luria-Bertani Medium (LSLB)*; 1%

tryptone (w/v), 0.5% NaCl (w/v), 0.5% yeast extract (w/v), pH 7.5. *LSLB plates*: LSLB, agar (1.5% w/v). *SOC media*: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂•6H₂O, 10 mM MgSO₄•7H₂O. *Sodium phosphate buffer*: 6.84 mM NaHPO₄, 3.16 mM NaH₂PO₄, 150 mM NaCl, pH 7.2. *Tris-glycine electrode buffer*: 0.1% (w/v) SDS, 0.2 M glycine, 25 mM Tris base. *TTBS*: 20mM tris-HCl (pH 7.5), 0.1% Tween-20 (v/v), 500 mM NaCl. *Ultra Therm buffer*: 200 mM (NH₄)₂SO₄, 750 mM Tris-HCl (pH 8.8), 0.2% Tween-20 (v/v). *Ultra Therm PCR Mixture*: Ultra Therm DNA Polymerase diluted 1:10 in 100 mM KCl, 20 mM Tris (pH 8.0), 0.1 mM DTT, 0.5% Tween-20 (v/v), 0.5% NP40 (v/v), 50% glycerol (v/v).

2.1.3 – Equipment and Software

BioRad, Hercules, CA: Mini-protean II Electroblot Apparatus, Gene Pulser Xcell electroporator; *BTX, Hawthorne, NY*: ECM® 399 electroporator; Chromas v. 1.45, freeware by Connor McCarthy, Southport, Queensland; *DNASTAR, Inc., Madison, WI*: SeqMan™ II; *Ibis Therapeutics, Carlsbad, CA*: BioEdit; *Milton Roy, Pont-Saint-Pierre, France*: Spectronic® GeneSys™ spectrophotometer; *Owl Scientific, Woburn, MA*: Owl Polyacrylamide Protein Gel Electrophoresis Apparatus; *Perkin Elmer, Wellesley, MA*: GeneAmp PCR System 2400; *Stratagene, La Jolla, CA*: Eagle Eye® II apparatus and software; *Temptronic, Cherry Hill, NJ*: Thermolyne; *Turner Designs, Sunnyvale, CA*: Sequoia-Turner Model 450 Fluorometer.

2.1.4 – Bacterial and Cell lines

The *Escherichia coli* cell line *TOP10F'* used to create the recombinant vectors was obtained from Invitrogen (Carlsbad, CA). The Gibco™ *Spodoptera frugiperda* 9

(*Sf9*) cell line, a line derived from ovarian cells of the fall armyworm, was obtained from Invitrogen (Carlsbad, CA).

2.1.5 – p2ZoptcxF Vector

Attributes of the p2ZoptcxF vector include transcription of the transgene driven from the *Orgyia pseudotsugata* nucleopolyhedrovirus immediate early 2 (ie2) promoter (Hegedus *et al.*, 1998). This vector also contains a Zeocin™ resistance gene, also under the ie2 promoter, for the selection of recombinant plasmids in *Escherichia coli* and selection of stable genomic integrants in transformed *Sf9* cells. Furthermore, the vector encodes the human transferrin secretion signal followed by a modified CBD(IIA) prior to the insert.

The human transferrin secretion signal functions to secrete the recombinant protein; it is removed during processing (Funk *et al.*, 1990; von Heijne, 1983; Yoshiga *et al.*, 1997). The CBD(IIA), also known as carbohydrate-binding module 2a or CBD_{CEX}, is modified from the original cellulose-binding domain of xylanase 10A from *Cellulomonas fimi*; the three known glycosylation sites have been altered so that glycosylation does not occur, as glycosylation destroys its ability to bind cellulose (Boraston *et al.*, 2001). The CBD is followed by the DNA encoding a Ilu-Asp-Gly-Arg amino acid sequence, a cleavage site recognized by Factor X. Following this is a multiple cloning site allowing insertion of cDNA of interest. The polyA signal sequence from the immediate early 2 gene lies 3' to the insert (Hegedus *et al.*, 1998). Figure 2.1 shows a schema of p2ZoptcxF and the three constructs created by the author.

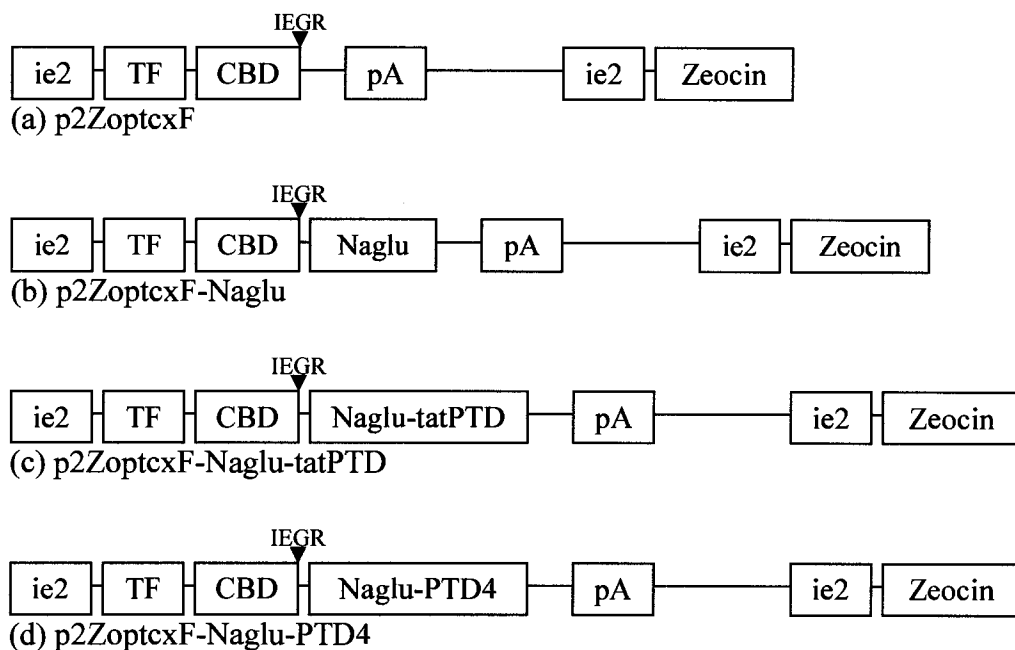


Figure 2.1. *Spodoptera frugiperda* 9 plasmid schemas. Abbreviations are as follows: ie2 (immediate early 2 promoter), TF (human transferrin secretion signal), CBD (modified cellulose-binding domain IIA), IEGR (Ile-Asp-Gly-Arg recognition site of Factor X), Naglu (α -N-acetylglucosaminidase), pA (poly A signal sequence from the ie2 gene), Zeocin (gene producing the ZeocinTM resistance protein for antibiotic selection), PTD (protein transduction domain).

2.2 – Methods

2.2.1 – Creation of Base Plasmid p2ZoptcxF-Naglu (Stop Codon Out-of-Frame)

To create the recombinant plasmid containing the Naglu insert, the Naglu cDNA from the plasmid pCMV-huNAGLU (a donation from Drs. E. Neufeld and K. Zhao, University of California, Los Angeles) was polymerase chain reaction (PCR)-amplified using the primer pair A/B (see Table 2.1). The amplified fragment, which contained 5' *EcoRI* and 3' *XbaI* cut sites, did not contain a start codon, the Naglu signal sequence, or a stop codon; start and stop codons were inherent in the p2ZoptcxF vector. The signal peptide was omitted to avoid complications in protein trafficking.

PCR was performed with the GeneAmp PCR System 2400 with conditions as follows: DNA template (1-2 ng), 0.25 mM deoxynucleotide mix, 10% v/v 10X Ultra Therm buffer, 1.5 mM MgCl₂, 0.6 μM each of forward and reverse primers, 8% dimethyl sulfoxide (DMSO), 1.3 M betaine, sterile deionized water to 49 μl. Betaine and DMSO were necessary to compensate for the extremely high GC content of the DNA at the beginning of the gene (85% over the first 200 bp, for example). After the reaction had reached 94°C, 1 μl of a 9:1 mixture of *Pfu* and Ultra Therm PCR-Mixture was added. The solution was held at 94°C for 10 minutes, followed by 30 cycles of 94°C (1.5 minutes) – 60°C (1 minute) – 72°C (1.75 minutes). This was followed by a 7 minute incubation at 72°C, followed by a 4°C incubation until the reaction was removed from the machine. PCR products were visualized by loading 2-10 μl on a 0.7% agarose gel and using the Stratagene Eagle Eye® II.

Primer	Sequence (5'-3')	Location	Orientation
A	TGAATTCGACGAGGCC GGGAG	<i>EcoRI</i> site, binds Naglu from amino acid 24 (bases 70-84)	sense
B	ATTCTAGATCCAAGAGC CGGCCACC	end of Naglu gene (bases 2214-2229), <i>XbaI</i> cut site including stop codon	anti-sense
C	GGCAGCCTGGGTGACCA	binds Naglu, bases 1413- 1429	sense
D	AATCTAGAATCGTCGAC GCTGACGTCGTTTCTTAC GACCGTATCCACCCCAA GAGCCGGC	end of Naglu gene (bases 2218-2229), DNA encoding tat PTD, <i>XbaI</i> site including stop codon	anti-sense
E	TATCTAGATTGCACGTG CCTGACGTGCTGCAGCTC GAGCGTATCCACCCCAA GAGCCGGC	end of Naglu gene (bases 2218-2229), DNA encoding PTD4, <i>XbaI</i> site including stop codon	anti-sense

Table 2.1. Primers used to construct α -N-acetylglucosaminidase plasmids. Bases in italics represent DNA encoding the tat protein transduction domain or PTD4.

The PCR fragment was gel purified using the QIAquick® Gel Purification Kit. This fragment and the p2ZoptxcF vector were then digested with *EcoRI* and *XbaI* in the following manner: 1-10 µg of DNA was incubated at 37°C overnight (ON) with 40 U *XbaI* in provided BSA and buffer. The next day, 30 U calf intestinal phosphatase (CIP) was added to the p2ZoptxcF digest for 2 hours while incubating at 37°C. Plasmid p2ZoptxcF was checked for linearization using 0.7% agarose gel electrophoresis. Subsequently, the insert and the p2ZoptxcF vector were purified using Qiagen's QIAquick® PCR Purification Kit. The entire sample of each of the two purified *XbaI*-cleaved DNA samples was then incubated with *EcoRI* in the manufacturer's provided buffer at 37°C for 6 hours, after which the fragments were purified using the QIAquick® PCR Purification Kit. Samples were stored at -20°C until use.

Ligation of insert and vector was performed in the following manner: 100 ng of vector was incubated for 20 minutes at room temperature (RT) with 3X and 6X the molarity of insert along with 400 U T4 DNA Ligase in supplied diluted buffer. The ligase was heat inactivated at 65°C for 11 minutes. 3 µl of this reaction was added to 40 µl ice-cold TOP 10 F' *E. coli* electrocompetent cells, which were prepared and electroporated according to the method of Sambrook *et al.* (2001). Ligation and cell mixtures were applied to 0.1 cm Gene Pulser® Cuvettes and electroporated with the BioRad Gene Pulser Xcell or the BTX ECM® 399 electroporator at the settings 25µF, 200 Ω, and 1.5-1.8 kV, giving a pulse of approximately 4 milliseconds. One ml of SOC media was added to the samples prior to a 60 minute incubation at 37°C.

Transformed cells (10 to 200 μ l) were spread on LSLB plates containing 25 μ g/ml ZeocinTM and incubated at 37°C overnight (ON). Apparent positive colonies were masterplated onto LSLB/ZeocinTM plates and again incubated overnight at 37°C. Colonies demonstrating growth on the masterplates were cracked to screen for size. To do this, a small aliquot of cells was removed from the surface of the plate using a flamed toothpick and swirled into a 0.5 ml Eppendorf containing 37°C Cracking Buffer. The Eppendorfs were placed in a 37°C waterbath for 5 minutes, followed by a 5 minute incubation on ice. The Eppendorfs were centrifuged at maximum speed (14,000 x g) on a tabletop centrifuge for 5 minutes, after which 35 μ l of the supernatant was loaded onto a 0.7% agarose gel for visualization. The few clones displaying plasmids of the correct size were selected for growth in an ON culture of LSLB containing 25 μ g/ml ZeocinTM by inoculation with a loop of colony. Cultures were grown and plasmids isolated using Qiaprep® Miniprep Kit. DNA was screened for concentration and purity by estimation with absorbance readings at 260 and 280 nm (A260, A280) using a Spectronic® GeneSys spectrophotometer. DNA sequencing was performed by the Centre for Biomedical Research DNA Sequencing Facility (University of Victoria). Sequences were screened by the author using BioEdit, Chromas, and/or SeqManTM II.

Unfortunately, the only plasmid p2ZoptxcF-Naglu created with error-free Naglu cDNA sequence contained an error after the coding sequence and before the *Xba*I cut site which put the stop codon out of frame; this plasmid was still useful in that it could be utilized to make the three correct plasmids in methods put forward in section 2.2.2. Briefly, a restriction enzyme cut site for *Bsr*GI within 800 bp of the end

of the coding sequence allowed the author to produce a smaller PCR product with less chance of error; these were created, subcloned for easy production of large amounts of insert, digested, and ligated to create correct plasmids. This plasmid with the stop codon out-of-frame is termed Base Plasmid p2ZoptxcF-Naglu.

2.2.2 – Creation of Plasmids p2ZoptxcF-Naglu, p2ZoptxcF-Naglu-tatPTD, p2ZoptxcF-Naglu-PTD4

Because of the difficulty in achieving full-length error-free sequences of the entire Naglu cDNA, and difficulties encountered with primer pairs A/D and A/E (Table 2.1), shorter fragments were PCR-amplified and inserted into the Base Plasmid p2ZoptxcF-Naglu plasmid using the *BsrGI* and the *XbaI* restriction enzyme cut sites which flanked from base pair 1413 of the Naglu cDNA to the end of the cDNA insert plus the stop codon. PCR reactions utilized primer pairs C/A, C/D and C/E to create the smaller fragments of Naglu, Naglu-tatPTD and Naglu-PTD4 respectively. PCR conditions were as above. Resulting fragments were subcloned into the TOPO® TA Cloning Kit for Sequencing vector as per the manufacturer's instructions and using the manufacturer's One Shot® TOP 10 Chemically Competent *Escherichia coli* cells. After ON incubation of 10-200 µl of the transformed cells at 37°C on LSLB plates with 100 µg ampicillin/ml, possible positive colonies were masterplated onto ampicillin-LSLB plates and again incubated at 37°C ON. Colonies which grew on the masterplate were cracked as above to screen for the size of plasmids. Some clones displaying plasmids of the correct size were selected to be cultured in 5 ml of LSLB media containing 100 µg/ml ampicillin at 37°C ON by using a flamed loop to swirl in a small amount of the colony. The plasmid DNA of

these clones was isolated using QIAprep® Miniprep as per the manufacturer's instructions. The appropriate sections of the insert were sequenced as above to check for an absence of mutations.

One error-free TOPO® clone each of Naglu, Naglu-tatPTD and Naglu-PTD4, as well as the p2ZoptcxF-Naglu plasmid were digested in the following manner: 1-10 µg of vector DNA was incubated with 40 U of *BsrGI* for 2 hours at 60°C; the temperature was dropped to 37°C, after which time 40 U of *XbaI* was added and incubated for 4 hours; 30U of CIP was added to the p2ZoptcxF digest 2 hours through the 37°C incubation. For the insert DNA, the digest was left to proceed ON. CIP was heat inactivated at 80°C for 20 minutes. The inserts and vector were purified using the QIAquick® Gel Extraction and Nucleotide Removal Kits respectively. DNA was estimated for concentration and purity using spectrophotometer A260/A280 readings.

Ligation of insert and vector was performed in the same manner as above. Transformed cells (10-200 µl) were spread on LSLB plates containing 25 µg/ml Zeocin™ and incubated at 37°C ON. Apparent positive colonies were masterplated onto LSLB/Zeocin™ plates and again incubated ON at 37°C. Colonies demonstrating growth on the masterplates were cracked as above to screen for size. Also as above, several clones displaying plasmids of the correct size were selected for growth in an ON culture of LSLB containing 25 µg/ml Zeocin™ by inoculation with a loop of colony. Cultures were grown and plasmids isolated using Qiaprep® Miniprep Kit, and screened for purity and concentration as above.

One clone each of p2ZoptcxF vectors containing Naglu, Naglu-tatPTD and Naglu-PTD4 inserts were selected for growth and isolation of plasmid with Qiagen®

Plasmid Midi Kit as above in order to attain concentrations of DNA appropriate for transfection.

2.2.3 – Creation of Stable *Sf9* Cell Lines Expressing Naglu, Naglu-tatPTD, Naglu-PTD4.

All *Sf9* cultures were maintained in SF-900 II SFM, a serum-free media for *Sf9* insect cultures. Adherent cultures were incubated in a non-humidified 26°C incubator, shaker cultures in a 27°C incubation room. 70 ml of shaker cultures were grown in 250 ml baffled flasks at 125 rpm. Shaker cultures were grown to stationary phase, that is, after cell number was no longer increasing and cell death was between 10 and 20%.

To prepare for transfection, *Sf9* cells were prepared in the following manner. 4.8×10^5 cells were seeded into each well of a 6 well tissue culture plate with 3 ml of SF-900 II SFM and grown to mid-log phase (50-70% confluency). Six μ l FuGENE 6 transfection reagent was mixed with 1 μ g of plasmid DNA and made up to 100 μ l with Sf900II SFM, incubated for 22 minutes and then added to the *Sf9* cells. For the selection of stable cell lines, Zeocin™ was added after 48-72 hours at a concentration of 0.5 mg/ml; the concentration of Zeocin™ was increased to 0.75 mg/ml after 2 passages. Selection with 0.75 mg/ml Zeocin™ was maintained for a total of at least 4 passages to obtain stable polyclonal cell lines.

2.2.4 – Activity and Protein Assays

The assay for α -*N*-acetylglucosaminidase activity present in culture media, on Sigmacell® pellets, and in purified fractions was performed following the protocol of Chow *et al.* (1981) and Zhao *et al.* (2000). Equal amounts (usually 25 μ l) of sample

and 4MU-Naglu substrate were incubated together at 37°C for up to 1 hour. The reaction was stopped by adding an excess of 0.5 M glycine-NaOH buffer (pH 10.5), and made up to 1.5 ml with the same for the purpose of measurement with the fluorometer. Fluorescence was assayed using a Sequoia-Turner Model 450 Fluorometer with a 360 nm narrow band excitation filter and a 415 nm sharp cut emission detection filter. Span dial was set completely counter-clockwise. Raw fluorescence readings were compared to a 4-methyl-umbelliferone (4MU) standard curve to calculate units of Naglu activity. One unit of Naglu is defined as the amount which releases 1 nmol of 4-methyl-umbelliferyl per hour (Zhao *et al.*, 2000).

Protein concentrations were calculated using the Bio-Rad Protein Assay Dye Reagent Concentrate as adapted from Bradford (*Bradford, 1976*). 5-100 µl of sample was made up to 800 µl with deionized water; Bio-Rad Protein Assay Dye Reagent Concentrate was added up to 1 ml, and readings were taken within 15 minutes with a Spectronic® GeneSys spectrophotometer. Absorbance readings at 595 nm (A_{595}) readings were compared to a standard curve made from known BSA concentrations.

2.2.5 – Concentration of Media

Because Naglu bound with greater efficiency to cellulose substrate when it was more concentrated, and because Factor X is reported to cleave more efficiently when its target protein is at a higher concentration, it was deemed necessary to concentrate the crude Sf-900 II SFM media harvested for efficient large-scale purification. A Stirred Ultrafiltration Cell 8200 apparatus and an inserted 50MW DIAFLO® Ultrafiltration Membrane were passivated by blocking ON at 4°C with 2% skim milk powder in deionized water. Prior to adding crude culture media, the

chamber was rinsed at least 5X with deionized water, and 10 ml of the same was passed through the device. Up to 120 ml of crude culture media was added and ultracentrifuged at room temperature (RT) for approximately 6 hours until an approximate volume of 3-4 mls was reached. This sample was pipetted off, and 2 ml of 10 mM sodium phosphate buffer was pipetted onto the membrane. After pipetting the buffer across the surface of the membrane numerous times, this wash was retained for further analysis/use. Samples were either frozen at -20°C, or stored at 4°C if they were to be used within 4 days.

Further concentration, if any, was performed using Ultrafree®-4 Centrifugal Devices, 10K. The concentrates from the ultrafiltration cell were centrifuged at 28°C at 7,500 x g until less than 500 µl of supernatant remained; a compact pellet containing much or nearly all of the total Naglu activity from the supernatant formed during this time; after supernatant removal, the pellet was resuspended in 600µl or more of 10 mM sodium phosphate buffer. Any protein which did not resuspend was removed by centrifugation. This sample was stored at -20°C or at 4°C if it was to be used further within 4 days.

2.2.6 – Protein Capture, Cleavage, and Purification

Crude media, concentrated or unconcentrated, was adjusted to pH 8.5-9.0 with 1 M NaOH; Triton X-100 was added to 0.1%. This was necessary to decrease non-specific binding of other media proteins to the cellulose substrate. After treatment, the media was placed on ice for a minimum of 20 minutes. Any protein or other precipitate that formed during this time was removed by centrifuging the sample at 1000 x g for 5 minutes. The supernatant was removed, and Sigmacell® was added.

To begin with, 5 mg of Sigmacell® was added to 8-10 ml unconcentrated media, 2 mg was added to any amount of media which had been concentrated more than 16-fold. The suspension was placed on ice or 4°C ON on a shaker to keep the cellulose suspended. The next day, the supernatant was assayed for activity; if much activity remained, either the pellet was removed by the methods as follows and more Sigmacell® was added, or more Sigmacell® was added to that already present and left to incubate ON again. To wash the Sigmacell® pellet, the supernatant was removed following by a brief, slow centrifugation (<1 minute). The pellet was washed 4X with 10 mM sodium phosphate buffer, twice with the buffer containing 0.1% Triton-X100 followed by twice without. The pellet was stored under 10 mM sodium phosphate buffer at 4°C or -20°C until further use.

To cleave the protein of interest from the cellulose pellet, the pellet was washed 2X with and resuspended in Factor Xa Cleavage/Capture Buffer. An excess of activated Factor X (Factor Xa), 2-20 U, was added to the pellet in a minimum volume of Capture/Cleavage Buffer (5-25 µl) to cleave the fusion proteins at the IEGR site between the CBD and Naglu, Naglu-tatPTD, and Naglu-PTD4. The pellet and Factor Xa were incubated at RT for a minimum of 48 to a maximum of 288 hours with gentle agitation to keep the pellet somewhat suspended.

Following this incubation, Factor Xa was removed using the Factor Xa Removal Kit following the manufacturer's instructions except that the Xarrest agarose was on occasion resuspended in less volume of Capture/Cleavage buffer in the final step to decrease the volume of eluate.

If the final eluted protein needed to be concentrated, either Nanosep® Centrifugal Devices, 30K or Microcon® YM-30 or YM-10 Centrifugal Filter Devices were used as per the manufacturer's instructions. If the spin column was spun to dryness, the sample was reconstituted with 10 mM sodium phosphate buffer. Sample was stored at 4°C or -20°C until use.

2.2.7 - Protein Visualization

Proteins were separated for visualization and analysis using tris-glycine, SDS-polyacrylamide gel electrophoresis (PAGE). Using the Owl Polyacrylamide Protein Gel Apparatus, 10% resolving and 4% stacking polyacrylamide gels were made with 4X Lower and Upper Tris respectively, deionized water, and the appropriate amounts of 40% acrylamide and electrophoresed at 15-25 mA. Samples were denatured with 3X SDS Sample Buffer in boiling water for 5 minutes, followed by a short high-speed centrifugation; if the sample was bound to cellulose, the pellet was resuspended in 3X SDS Sample Buffer in 10 mM sodium phosphate buffer, boiled for 5 minutes, briefly centrifuged, and the supernatant loaded. Chamber and reservoir of the gel apparatus were filled with tris-glycine electrode buffer during electrophoresis.

Proteins were visualized on SDS-PAGE gels by silver staining as follows. Gels were microwaved at maximum power for 90 seconds in fixative (50% methanol, 12% acetic acid, 0.1% formaldehyde) followed by a 90s microwave in 50% ethanol. The gels were then pretreated in 0.02% sodium thiosulfate pentahydrate for 90s in the microwave, washed in deionized water for 90s at room temperature, and stained with 2 mg/ml silver nitrate in 0.075% formaldehyde by microwaving twice for 40s with shaking in between microwave cycles. Bands were resolved in developer (60 mg/ml

sodium carbonate, 0.05% formaldehyde, 0.002% sodium thiosulfate pentahydrate) and reaction stopped in 50% methanol following a 90s room temperature deionized water wash.

For Western blotting, proteins were electroblotted from SDS-PAGE gels onto Hybond-P PVDF membrane ON at 10V in 10% methanol transfer buffer using the Mini-Protean II Electroblot Apparatus. Following the transfer, PVDF membranes were washed in TTBS for 5 minutes at room temperature followed by a one hour incubation in blocking solution. Primary antibody was then applied diluted in blocking solution. Primary antibodies included a rabbit monoclonal antibody raised against recombinant Naglu produced by *Lec1* CHO cells; this was diluted 1/7,500 in blocking solution. Anti-CBD antibody was a rabbit polyclonal, and was used 1/500 in blocking solution. Incubation with primary antibodies was carried out for 1 hour with agitation at RT. Following the incubation with the primary antibody, membranes were washed three times for 5 minutes in TTBS and incubated for 1 hour at RT in a goat anti-rabbit HRP-conjugated secondary antibody diluted 1/20,000 and 1/50,000 in blocking solution for the anti-Naglu and anti-CBD primary antibodies respectively. Membranes were washed 3 times for 5 minutes with TTBS and incubated with ECL Plus chemiluminescent reagent for 5 minutes, and visualized with autoradiography film for exposures ranging from 6 seconds to ON.

If the same blot was to be probed with a second antibody, the blot was stripped according to the ECL Plus kit instructions. Prior to incubation with second antibody, the blot was probed with ECL Plus and exposed to autoradiography film for 8 minutes to ensure that all signal had been stripped.

Protein sizes were estimated using pre-stained protein markers; however, according to New England Biolabs, this gives only an estimate, as the migration of the marker proteins through the gel is shifted due to dye-protein conjugation (NEB, 2004). Keeping this in mind, whenever possible (when the SDS-front had not exited the gel), the ratio of the distance traveled by the bands in comparison with the SDS-front (R_f) was calculated for each protein band and used to construct a logarithmic graph which was then used to extrapolate an estimated size of the sample in question; this number is reported as an apparent molecular weight. If the SDS-front had exited the gel, a crude logarithmic graph was constructed from the distance the protein standards migrated from the top of the gel, and samples were compared to this graph; sample size is reported as approximate molecular weight. If the protein bands of the gel were such that this was not feasible (*e.g.* if the migration was parabolic), protein size was estimated by visualization.

2.2.8 – Other Methods

Sf9 cell lysates were prepared by subjecting 1×10^7 cells suspended in 100 μ l sodium phosphate buffer to five repeated cycles of freeze-thaw in liquid nitrogen or dry ice in 100% ethanol to lyse the cells. Membrane and cytoplasmic preps were prepared according to the protocol of Sambrook *et al.* (2001). Briefly, lysates were centrifuged at 14,000 $\times g$ for 5 minutes; the supernatant was removed as the cytoplasmic fraction; the remaining pellet, the membrane fraction, was resuspended in 100 μ l of sodium phosphate buffer. Cell lysates were utilized directly or stored immediately at -80°C until use.

3 – Results

3.1 – Creation of Plasmids p2ZoptcxF-Naglu, p2ZoptcxF-Naglu-tatPTD, p2ZoptcxF-Naglu-PTD4

The Base Plasmid p2ZoptcxF-Naglu (stop codon out-of-frame) was successfully created using PCR amplification and cloning the amplicon directly into the p2ZoptcxF vector. The constructed plasmid was successfully used to transform *TOP10F'* *E. coli* cells. While it was not the intention of the author to create this construct out-of-frame, the construct proved useful for ease of future work. Figure 3.1 shows the construct digested with *XbaI* and *EcoRI* after growth in a 5 ml culture and purification. Because the digest was not allowed to go to completion, the insert, the vector and the linearized the plasmid are all visible. Expected band sizes are 5.5 kilobases (kb) for the linearized plasmid, 3.2 kb for the vector p2ZoptcxF (p2Zop), and 2.3 kb for the insert; observed bands correspond to the predicted sizes. Sequencing data confirmed the lack of mutations within the coding region of Naglu cDNA, the lack of the native Naglu signal peptide (as designed), and displayed the out-of-frame stop codon.

p2Zop-Naglu, p2Zop-Ntat and p2Zop-NPTD4 plasmids were successfully created using PCR amplification, TOPO® cloning, digestion and ligation into the existing Base Plasmid p2ZoptcxF-Naglu (stop codon out-of-frame); bacterial transformation was successfully completed in *TOP10F'* cells. Figure 3.2 shows bands within a 0.7% agarose gel corresponding to true positive clones for these plasmids following growth in a 5 ml *TOP 10F'* culture and digestion of the produced, purified

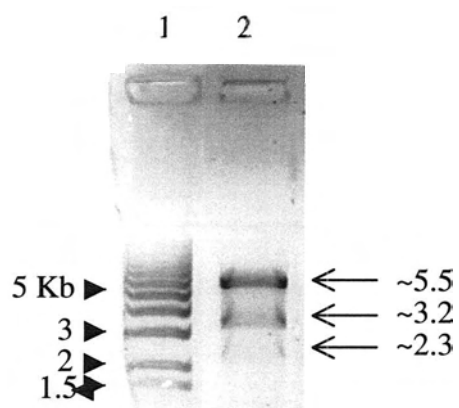


Figure 3.1. Insert size confirmation of base plasmid p2ZoptcxF-Naglu. 500 ng of purified vector from positive clone was digested overnight at 37°C with *EcoRI* and *XbaI* in buffer provided for *XbaI*. The entire sample was electrophoresed on a 0.7% agarose gel. Lanes: (1) 1 kb DNA ladder; (2) base plasmid p2ZoptcxF-Naglu digest.

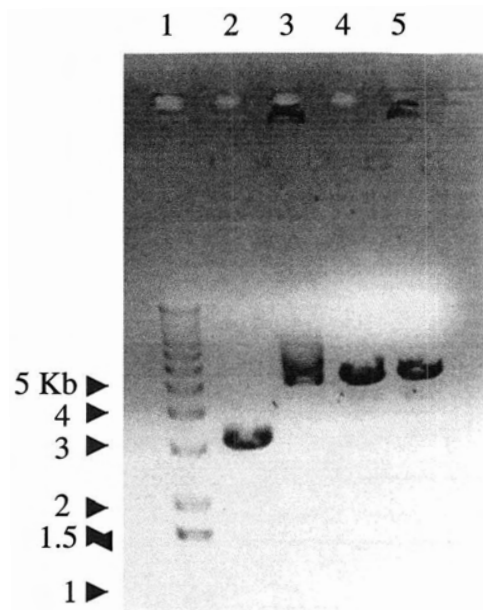


Figure 3.2. Size confirmation of vectors with insert. Two μg of plasmid DNA as produced by *TOP10-F'* *Escherichia coli* cells screened by overnight digestion with 20 units of *XbaI* and subsequent electrophoresis in a 0.7% agarose gel. Lanes: (1) 1 kb DNA ladder; (2) p2ZoptxcF only; (3) p2ZoptxcF-Naglu; (4); p2ZoptxcF-Naglu-tatPTD; (5) p2ZoptxcF-Naglu-PTD4.

plasmids with *XbaI*. Expected band sizes are 3.2 kb for p2ZoptexF without insert, and 5.5 kb for p2Zop-Naglu, p2Zop-Ntat and p2Zop-NPTD4; observed bands correspond to the predicted sizes. Sequencing results confirmed the absence of mutations from base pair 1400 to the stop codon while the inserts were within the TOPO® plasmid.

3.2 – Transfection of *Sf9* cells

Confirmed true positive plasmids were isolated from 25 ml large-scale cultures and successfully transfected into *Sf9* insect cells via transfection with FuGENE 6. Higher specific activities of Naglu were detected in the media of the polyclonal cell cultures transfected with either p2Zop-Naglu, p2Zop-Ntat, or p2Zop-NPTD4, as compared to *Sf9* cells alone or cells transfected with vector with no insert, confirming successful transfection as well as the production of active protein (Figure 3.3).

To assess the functioning of the transferrin secretion signal of p2Zop, *i.e.* that the recombinant protein was being secreted and not retained within the cell, *Sf9* cell lysates were probed with anti-Naglu and anti-CBD antibodies. The membrane preparation showed negligible binding of the anti-Naglu antibody (data not shown). No protein which bound both the Naglu and the CBD antibody was present within the cytoplasmic preparations (Figure 3.4). However, there were two proteins (or one processed protein) approximately 110 and 72 kDa which bound the anti-Naglu antibody. The smaller protein band was not equal in size to the secreted human recombinant protein, and the larger band appeared to be of a similar size although perhaps slightly larger. These protein(s) are found approximately equally in every

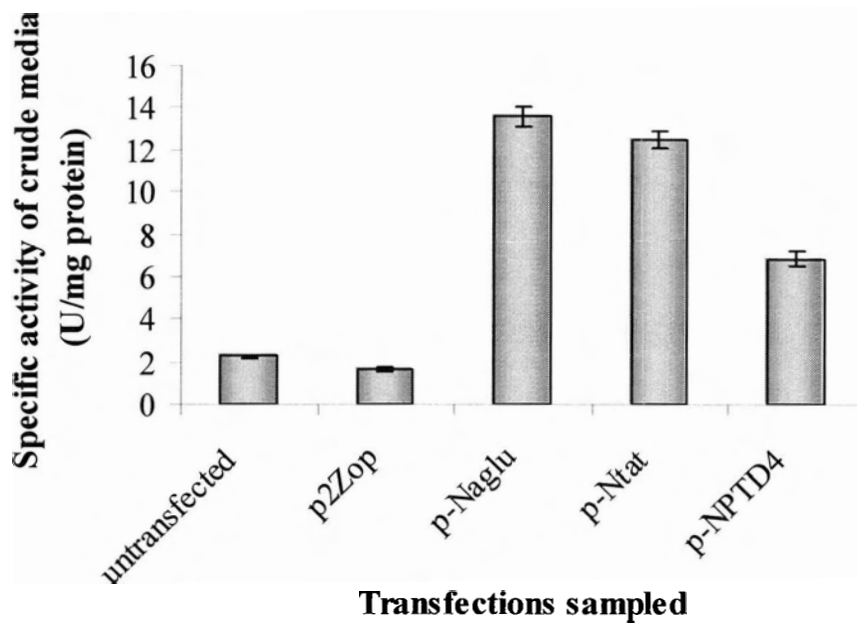


Figure 3.3. Specific activities of media from control and transfected cultures. Samples were taken from media of *Spodoptera frugiperda* cultures grown for 2-3 passes in 70 ml shaker flasks and assayed for specific activity of Naglu. All transfected cultures were selected in 0.75 mg/ml Zeocin™. Bars indicate standard deviation.

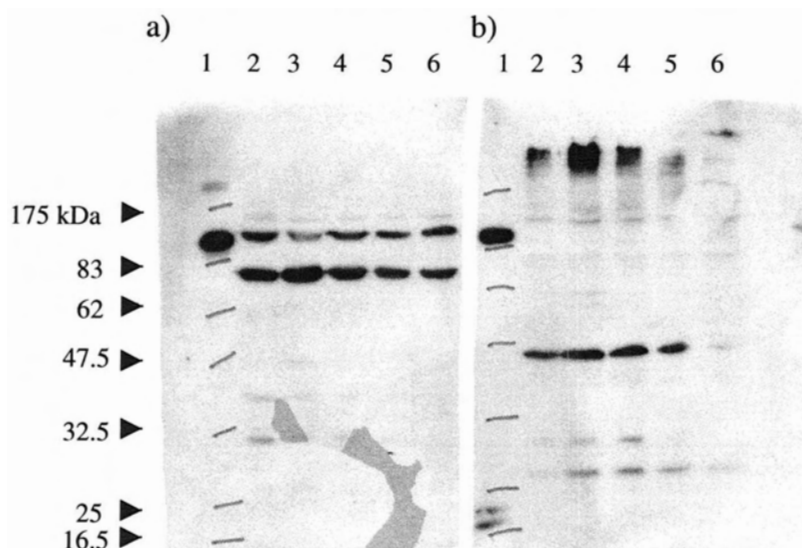


Figure 3.4. SDS-PAGE Western blot of cytoplasmic preparations of transfected and untransfected *Spodoptera frugiperda* 9 insect cells. 50 μ g of protein from freeze-fracture samples of adherent culture cells grown to confluency were loaded onto a 10% resolving gel. Immunodetection was performed with (a) an anti-Naglu antibody and (b) an anti-CBD antibody; both were exposed to film overnight. Lanes: (1) pre-stained protein marker *and* positive control (see below); (2) cells only; (3) vector only-transfected; (4) p2Zop-Naglu-transfected; (5) p2Zop-Naglu-tatPTD-transfected; (6) p2Zop-Naglu-NPTD4-transfected. Positive control is protein released from Sigmacell® pellet which had been bound overnight to contents of 3.5 ml unconcentrated Naglu-tatPTD-containing media.

sample, including untransfected *Sf9* cells. These same cytoplasmic protein(s) did not bind the anti-CBD antibody. The insect cytoplasmic preparations also displayed one protein of approximately 44 kDa which bound the CBD antibody relatively specifically.

3.3 – Protein Capture and Characterization

The p2ZoptcxF vector expressed a modified CBD(IIA), or CBD_{CEX}, as an N-terminal fusion to the constructed proteins in order to ease purification. To bind the three proteins of interest specifically to cellulose, it was found necessary to adjust binding conditions to minimize non-specific binding. Specifically, it was found that a pH between 8.4 and 9.0 and the addition of 0.1% Triton X-100 was necessary to reduce background binding to a level where the protein of interest could be visualized (Figure 3.5). In this figure, the protein of interest, the Naglu-tatPTD fusion protein (Ntat), is present just above the 83 kDa protein marker (this is confirmed later by Western blotting), apparent molecular weight 102 kDa. Being kept at pH 9.0 with 0.1% Triton X-100 at RT with agitation for up to one week did not negatively affect the activity of Naglu-tatPTD or Naglu-PTD4 (see Figure 3.6); no data were collected for Naglu.

After the treatment of media as above, the proteins which bound cellulose in the highest quantity were approximately 106 kDa and 165 kDa; the 106 kDa protein was not found in media from *Sf9* cells only, or cells which were transfected with p2Zop with no insert (Figure 3.7). This smaller protein also bound both the anti-Naglu and the anti-CBD antibody (Figure 3.8 (b) and (c)); this protein was not

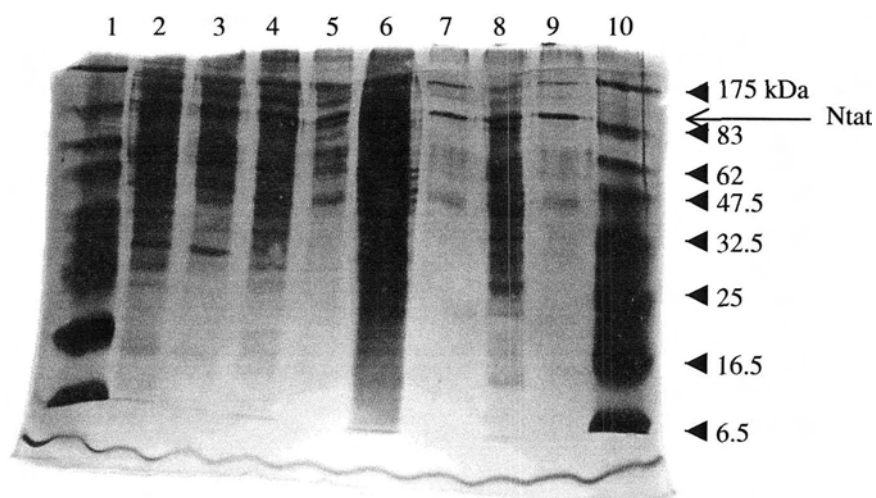


Figure 3.5. Increasing the binding specificity of media proteins to cellulose. Silver stained SDS-PAGE (10% resolving gel) of 5 ml of media containing Naglu-tatPTD treated in various ways to reduce non-specific binding of other media proteins. Samples were bound to 5 mg of Sigmacell® overnight. Lanes: (1) pre-stained protein marker; (2) untreated media (pH 6.2); (3) media (pH 6.2) with 0.1% TX100; (4) media (pH 7.1); (5) media (pH 7.4) with 0.1% TX100; (6) media (pH 8.5); (7) media (pH 8.4) with 0.1% TX100; (8) media (pH 9.0); (9) media (pH 9.0) with 0.1% TX100; (10) pre-stained protein marker. Arrow indicates Ntat protein.

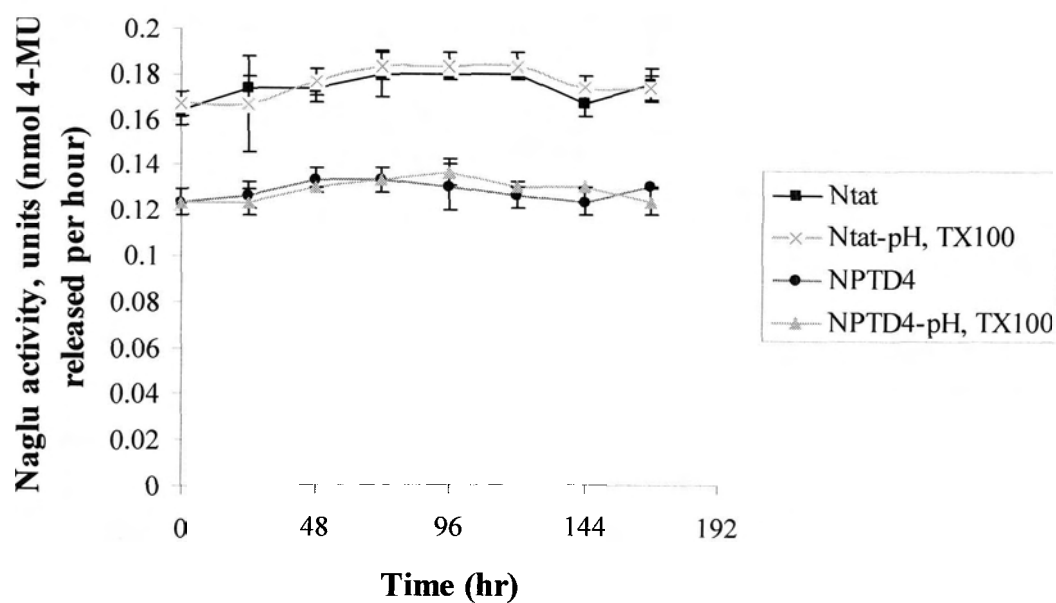


Figure 3.6. The effect of pH 9.0 and 0.1% Triton X-100 on Naglu activity. Graph compares activity present in 25 μ l samples taken from untreated Naglu-tatPTD and Naglu-PTD4 media, or from aliquots of the same media which had been adjusted to pH 9.0 and had Triton X-100 added to 0.1%. All samples were placed on a shaker at room temperature for the times indicated. Abbreviations are as follows: Naglu (α -N-acetylglucosaminidase), PTD (protein transduction domain). Legend abbreviations are as follows: Ntat (Naglu-tatPTD-containing media), Ntat-pH, TX100 (Naglu-tatPTD-containing media, pH 9.0, 0.1% Triton X-100), NPTD4 (Naglu-PTD4-containing media), NPTD4-pH, TX100 (Naglu-PTD4-containing media, pH 9.0, 0.1% Triton X-100). Bars indicate standard deviation.

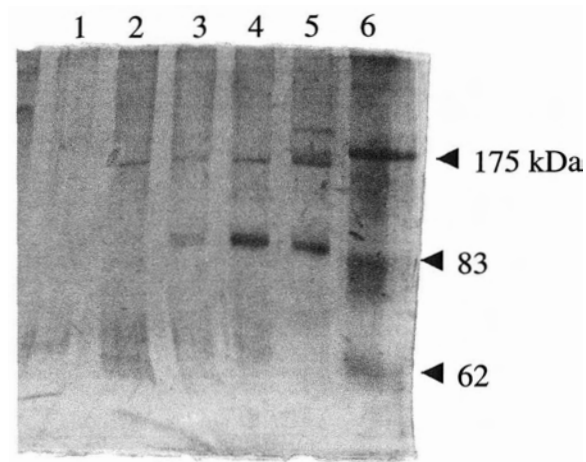


Figure 3.7. Cellulose-binding proteins in media of three constructs and controls. Silver-stained SDS-PAGE of proteins secreted from stationary phase *Spodoptera frugiperda* 9 cells and binding from unconcentrated media containing 4.3 U of Naglu activity (where applicable) to Sigmacell®. Protein from equal amounts of cellulose pellet were loaded onto each lane. Lanes: (1) cells only; (2) p2Zop only; (3) p2Zop-Naglu; (4) p2Ztop-Naglu-tatPTD; (5) p2Zop-Naglu-PTD4; (6) pre-stained protein marker.

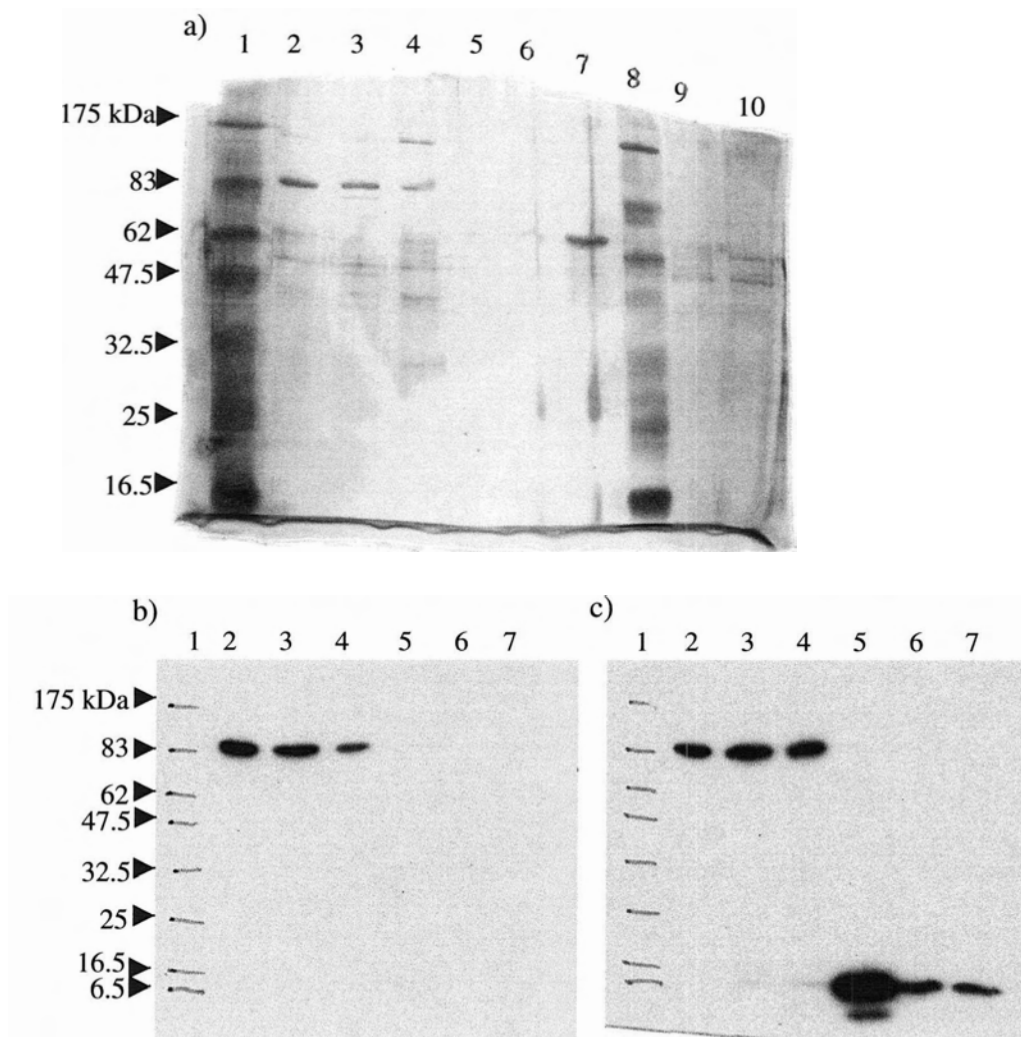


Figure 3.8. Protein identity, size, and amount. (a) Silver-stained SDS-PAGE, (b) anti-Naglu Western blot (stripped and re-probed with) (c) anti-CBD Western blot of proteins secreted from stationary phase *Spodoptera frugiperda* 9 cells and binding from unconcentrated media to cellulose. 10.3 U were loaded onto each silver-stain lane, 3.4 U per Western blot lane. Silver stain lanes: (1) pre-stained protein marker; (2) Naglu; (3) Naglu-tatPTD; (4) Naglu-PTD4; (5) BSA (50 ng); (6) BSA (100 ng); (7) BSA (200 ng); (8) pre-stained protein marker; (9-10) 2 lanes of 10 mM sodium phosphate buffer only. Western blots lanes: (1) pre-stained protein marker; (2) Naglu; (3) Naglu-tatPTD; (4) Naglu-PTD4, CBD (200 ng); (5) CBD (100 ng); (6) CBD (50 ng).

detected by either antibody in cellulose-bound media from *Sf9* cells or media from cells transfected with p2Zop with no insert. p2Zop-transfected cells did show expression of a small protein which specifically bound the anti-CBD antibody (data not shown); one cannot estimate the size of this protein accurately, other than that it is somewhat less than 16.5 kDa, as the 6.5 kDa marker and the protein of interest are still flush with the SDS front and have not separated yet.

There is the possibility that the addition of a tag can disrupt or decrease the activity of the protein of interest. To compare the sizes, activity, and relative amounts of the three proteins to each other and proteins of known concentration, amounts of Naglu, Ntat and Naglu-PTD4 (NPTD4), showing similar amounts of activity were loaded onto SDS-PAGE gels and silver-stained or Western blotted along with proteins of known concentration. Firstly, the author was unable to depict any significant difference in size between the three proteins with a 10% gel SDS-PAGE, no matter how far the proteins were electrophoresed (Figure 3.7). Band intensities (amount of protein) of the three constructs showed a general pattern: on both silver stains and Western blots, Naglu indicated slightly more protein than Ntat or NPTD4, with NPTD4 often displaying the least amount of protein (Figures 3.8, for example; also noticeable in other figures). This was not always detected when the Western blot was exposed for a long period of time; shorter film exposures however, often revealed this trend. Overall, 10.3 U was approximately 125 to 175 ng of protein (Figure 3.8 (a)), 3.4 U was approximately 125 ng (Figure 3.8 (c)). 1.7 U was less than 25 ng of protein (Figure 3.10). These rough comparisons indicate that 1 U corresponds to approximately 21 ng of protein; this estimate is semi-quantitative at best.

It was found that concentrating the media led to more efficient binding of Naglu activity from the media to microcrystalline cellulose (Figure 3.9). Figure 3.9 displays that cellulose substrate (Sigmacell®) bound NPTD4 more effectively when the initial media was more concentrated: 2 mg of cellulose was able to bind approximately 0.6 units of Naglu activity when applied to the 16-fold concentrated media, whereas 5 mg of cellulose was able to bind less than 0.2 units of activity when applied to the same media unconcentrated. When applied to unconcentrated Ntat media, 5 mg was able to bind less than 0.1 units of activity. This was not due to a lack of CBD on the remaining Ntat; if more cellulose substrate was applied to the unconcentrated media, more activity would leave the supernatant and become bound to the cellulose; for example, 35 mg of cellulose substrate was able to bind > 54% of the activity from the Ntat media pictured in Figure 3.9, a total of about 0.1 units. Application of even more cellulose led to a total of >80% capture of Ntat activity. The specificity of binding to cellulose did not increase with a greater concentration of Naglu; it was still necessary to add Triton X-100 and adjust the pH to 9.0 (data not shown).

During the course of the project, it was noted that some samples of Naglu, Ntat and NPTD4 showed hazy double-banding (Figure 3.10, Figure 3.11). This occurred in samples that had been concentrated with either or both of the Amicon 8200 Stirred Ultrafiltration Cell and/or the Millipore Ultrafree-4 Centrifugal Devices. The two bands in these samples were of apparent molecular weight 105 and 101 kDa

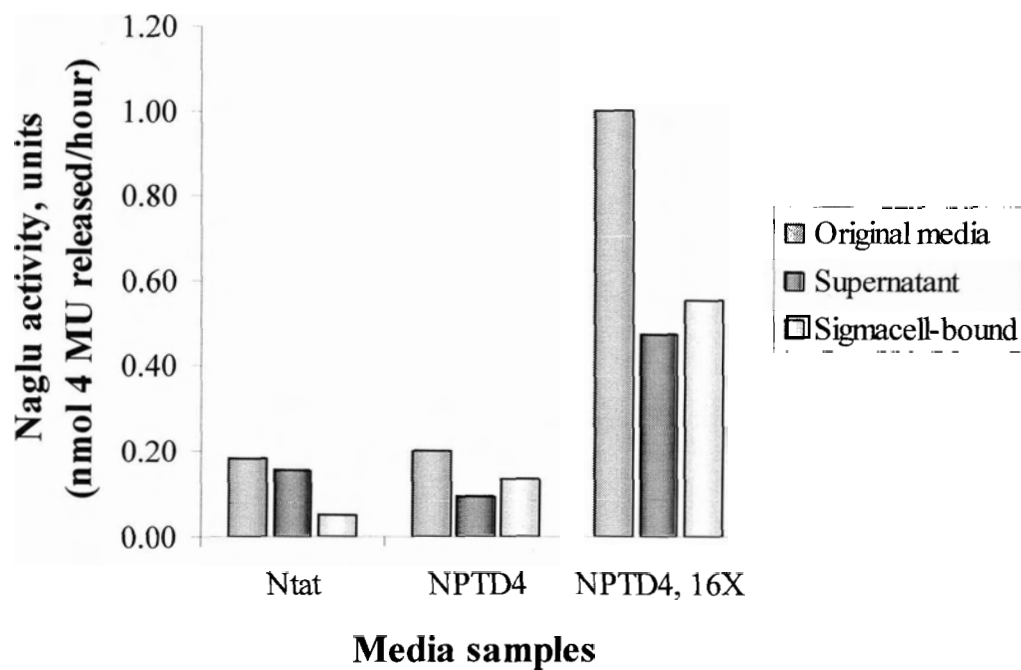


Figure 3.9. Comparison of binding efficiencies at different concentrations of starting media. Media containing varied concentrations of Naglu activity was bound overnight to 5 mg of Sigmacell® in the case of the unconcentrated samples, and to 2 mg of Sigmacell® in the case of the 16X concentrated sample; supernatant was assayed for activity the following day.

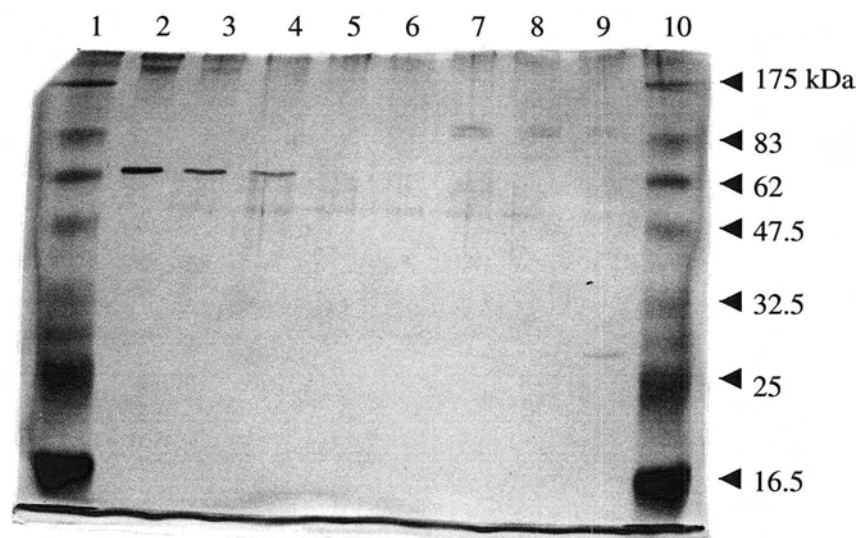


Figure 3.10. Double-banding of samples. Media from transfected samples was concentrated 240X in Amicon Stirred Ultrafiltration Cell 8200 and Millipore Ultrafree®-4 Centrifugal Devices and bound to 3 mg of Sigmacell® overnight; 1.7 U of Naglu activity (where applicable) was loaded onto an SDS-PAGE (10% resolving) and silver stained. Lanes: (1) pre-stained protein marker; (2) BSA (100 ng); (3) BSA (50 ng); (4) BSA (25 ng); (5) cells only; (6) vector only; (7) Naglu; (8) Ntat; (9) NPTD4; (10) pre-stained protein marker.

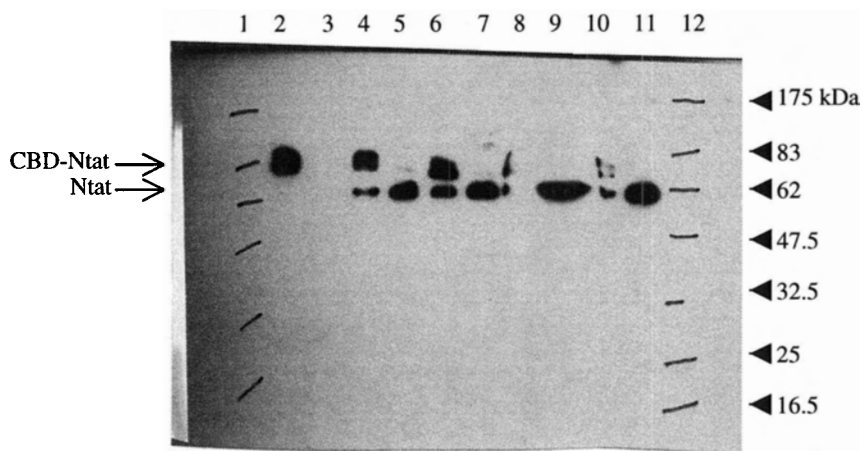


Figure 3.11. Double banding, and a Factor Xa time trial. 170X-concentrated medium of p-Ntat transfected samples concentrated in Amicon 8200 Stirred Ultrafiltration Cell and Millipore Ultrafree®-4 Centrifugal Device was bound to 2 mg of Sigmacell® overnight and split into 5 samples; each sample was incubated at RT for 2 to 12 days with 2 units of Factor Xa. SDS-PAGE (10% resolving) Western blot was immunodetected with anti-Naglu antibody; film was exposed for 20 seconds. Lanes: (1) pre-stained protein marker; (2) control pellet without FXa (12 days at RT); (3) washes from pellet without FXa (12 days at RT); (4) pellet (2 days); (5) washes from pellet (2 days); (6) pellet (4 days); (7) washes from pellet (4 days); (8) pellet (8 days); (9) washes from pellet (8 days); (10) pellet (12 days); (11) washes from pellet (12 days); (12) pre-stained protein marker.

in each of Naglu, Ntat, and NPTD4. Both of these bands were bound by the anti-Naglu antibody (Figure 3.11). No double-banding was noted in the final cleaved product, even from proteins which were double-banded before cleavage (Figure 3.11).

It was also noticed during the course of the project that samples which were kept for any significant amount of time at room temperature or higher, as in the case of samples concentrated with Millipore Ultrafree-4 Centrifugal Devices, showed a large amount of activity which did not bind to cellulose. For example, samples of each of the three proteins which had been concentrated about 240-fold with the Amicon 8200 and the Ultrafree devices (performed at RT and 28°C respectively) showed that 42-78% of the activity would not bind microcrystalline cellulose (data not shown); Naglu showed the greatest amount binding (42%); it was noted that this sample had been sitting at 4°C for the least amount of time after concentration before being frozen. A sample of the supernatant which contained 2000 U of the activity which would not bind cellulose was loaded onto an SDS-PAGE gel in attempts to visualize the size of this protein. Although the lanes were unusual in shape due to the large amount of protein loaded onto the supernatant lanes, three bands of proteins corresponding to the predicted sizes of cleaved Naglu, Ntat and NPTD4 were seen; this protein band bound the anti-Naglu antibody and did not bind the anti-CBD antibody (Figure 3.12). The bands at the tops of lanes 4, 6 and 8 are probably non-specific binding to large amounts of proteins or other substances remaining at the top of the lanes.

The major step involved in Naglu, Ntat and NPTD4 purification involved specific binding to cellulose. Binding to cellulose provided an increase in specific

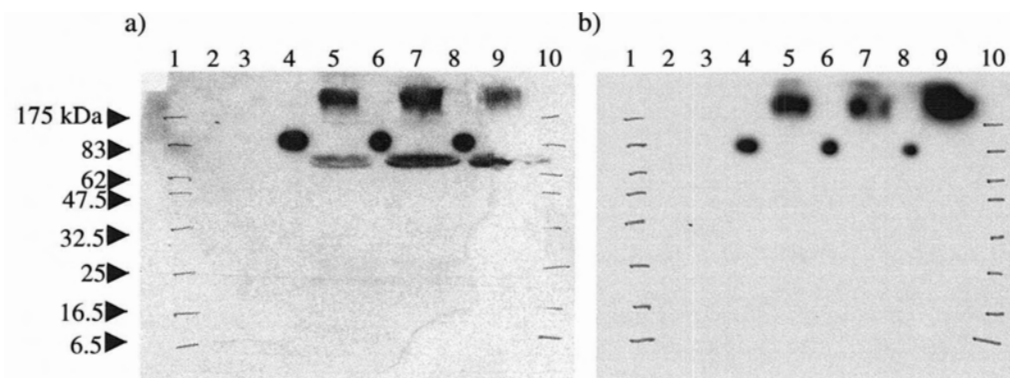


Figure 3.12. SDS-PAGE of media supernatant displaying activity which did not bind cellulose. The equivalent of 2000 U of Naglu activity was concentrated using a Microcon YM-10 centrifugal device, electrophoresed on a 10% resolving SDS-PAGE and Western blotted with a) anti-Naglu antibody, stripped and re-probed with b) anti-CBD antibody. Lanes: (1) pre-stained protein marker; (2) CBD (100 ng); (3) CBD (50 ng); (4) control Naglu pellet (2000 U); (5) Naglu-containing supernatant (2000 U); (6) control Ntat pellet (2000 U); (7) Ntat-containing supernatant (2000 U); (8) control NPTD4 pellet (2000 U); (9) NPTD4-containing supernatant (2000 U); (10) prestained protein marker. The control pellet consisted of protein bound from unconcentrated media onto cellulose.

activity of Ntat from 12.11 ± 0.10 U/mg protein (original media) to $29,409 \pm 4806$ U/mg protein, a 2400-fold increase. Similarly, the specific activity of NPTD4 increased from 10.06 ± 0.14 U/mg protein (original media) to $18,654 \pm 2996$ U/mg protein, a 1900-fold increase. Specific activity data were not available for Naglu, as the total amount of protein at this stage was so low that it was not possible to detect any protein to calculate the specific activity; however, the level of activity was similar to that of both Ntat and NPTD4. Yield at this stage was anywhere from 42-83%, dependant upon the amount of residual activity which would not bind cellulose. At no time did cellulose bind any noticeable amount of activity from media of *Sf9* untransfected cells or cells transfected with vector only (data not shown).

3.4 – Protein Cleavage

An 8-12 day incubation of Factor Xa with the cellulose-bound Ntat resulted in approximately 80% cleavage of the protein as evidenced by activity assay and Western blot (Figure 3.11; activity assay data not shown). A 38 day incubation resulted in approximately 99% recovery as evidenced by Western blot; there was no indication of non-specific cleavage of Naglu during this time (data not shown). Although it is reported that Factor Xa functions best with high concentrations of target protein (Novagen, 1998), it was found that increasing the amount of protein bound to cellulose did not decrease time required for cleavage of the fusion protein to any noticeable extent.

Naglu, Ntat and NPTD4 all evidenced cleavage; the final proteins are of apparent molecular weight 83, 84, and 87 kDa for Naglu, Ntat and NPTD4

respectively, down from the original apparent molecular weights of 104, 100, and 101 kDa (Figure 3.13 (a)). Therefore, there appears to be an average size difference of approximately 17 kDa between the cleaved and uncleaved proteins. The final cleaved protein binds the anti-Naglu antibody but does not bind the anti-CBD antibody (Figure 3.13 (b) and (c)).

Soluble Factor Xa was removed from the cleaved protein using the Factor Xa Cleavage/Capture Kit. Figure 3.14 displays a final protein showing only one strong band, the cleaved Ntat. Figure 3.13 (a) displays final cleaved proteins with a 30 kDa contaminant protein. Bovine Factor Xa is reported to show up as two bands of 34 and 29 kDa when reduced (Novagen, 1998); the contaminant band is most likely Factor Xa which has not been completely removed. It should be noted that the two bands which extend horizontally across the entire figure are protein contaminants from the sample buffer or running buffer, most likely keratin; many of the silver stain figures have these bands across the figures, although not so strongly as in Figure 3.13 (a). Even lanes 9 and 10 of Figure 3.8, buffer only, clearly show these two bands. These bands should therefore be ignored when looking at protein purity, other than an acknowledgment that these bands could be obscuring actual bands of protein.

If the cleaved protein needed to be concentrated before loading onto an SDS-PAGE gel, significant amounts of sample were lost when using Microcon YM-10 centrifugation devices (lanes 7 and 9 compared to 5 of Figure 3.13).

Final yields of the proteins ranged from about 7% to less than 0.5%. The longer a sample had been concentrated before binding to cellulose, the lower the yield, mainly because only 22-58% of activity would bind cellulose. As well, the removal of

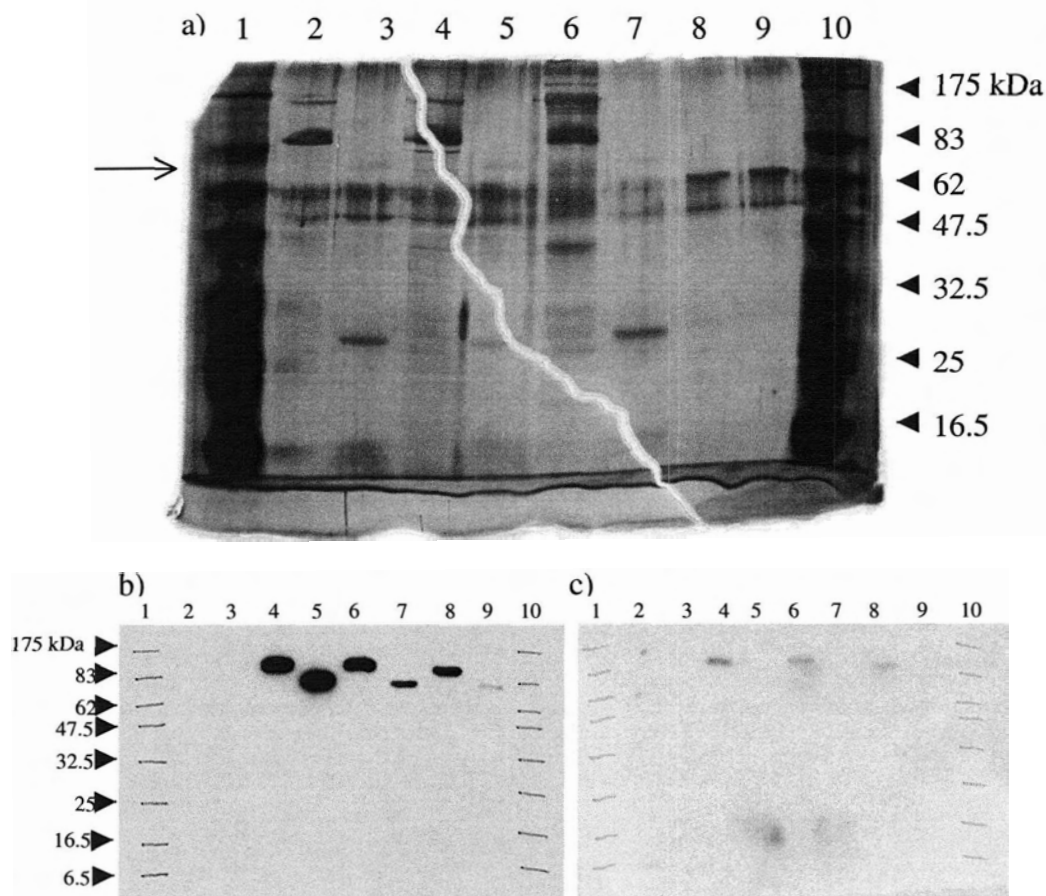


Figure 3.13. Cleaved Naglu, Ntat, and NPTD4. SDS-PAGE, 10% resolving, (a) silver stain and (b) anti-Naglu Western blot, stripped and re-probed with (c) anti-CBD. Protein from 240X media of stationary phase *Spodoptera frugiperda* 9 cells, concentrated with Amicon 8200 and Ultrafree-4 devices, was bound to cellulose, cleaved with 5 units of Factor Xa for 12 days, purified using Factor Xa Cleavage/Capture Kit, and concentrated using Microcon YM-10 centrifugal devices (except cleaved Naglu for the Western blot). Silver stain lanes: (1) pre-stained protein marker; (2) control Naglu (uncleaved); (3) cleaved Naglu; (4) control Ntat (uncleaved); (5) cleaved Ntat; (6) control NPTD4 (uncleaved); (7) cleaved NPTD4; (8) BSA (50 ng); (9) BSA (100 ng); (10) pre-stained protein marker. Western blot lanes: (1) pre-stained protein marker; (2) CBD (50 ng); (3) CBD (10 ng); (4) Naglu (300 U); (5) cleaved Naglu (300 U); (6) Ntat (300 U); (7) cleaved Ntat (300 U, YM-10 concentrated); (8) NPTD4 (300 U); (9) cleaved NPTD4 (300 U, YM-10 concentrated); (10) pre-stained protein marker. Arrow indicates cleaved proteins. Silver stain control were 6.9 units of Naglu activity bound to cellulose from unconcentrated media.



Figure 3.14. Step-wise purification of cleaved Ntat. Ntat protein from 9 ml unconcentrated media of stationary phase *Spodoptera frugiperda* 9 cells was bound to cellulose, cleaved with 10 units of Factor Xa for 12 days, purified using Factor Xa Cleavage/Capture Kit, and concentrated using Nanosep® centrifugal devices. Lanes: (1) sodium phosphate buffer; (2) pre-stained protein marker; (3) positive control of uncleaved protein (Ntat bound to cellulose); (4) original medium sample; (5) cellulose pellet after cleavage with FXa; (6) supernatant of pellet after 12 days with FXa; (7) unconcentrated final product; (8) concentrated final product; (9) pre-stained protein marker; (10) sodium phosphate buffer.

Factor Xa also seemed to remove much of the three proteins. For example, pellets containing 80, 41 and 33 U of Naglu, Ntat and NPTD4 activity respectively were cleaved for 12 days; the supernatants and washes were used with the Factor Xa removal kit, and the final yield (before further concentration) was 13.8, 4.4 and 4.6 U of activity. Assuming a 75% cleavage and recovery rate from Factor Xa cleavage, this is a loss of 80% of the protein applied to the Factor Xa removal kit.

4 – Discussion

It was found that the *Sf9* expression system using p2ZoptcxF was capable of producing secreted, active human Naglu of activity similar to that expressed in mammalian systems. Proteolytic activity on the CBD decreased yield, as did non-specific binding; the PTD-fusion proteins exhibited higher non-specific binding. Low yields indicate that greater optimization of the system should be attempted in order to produce enough protein for transduction studies.

4.1 – Protein Expression

The data indicate a range of expression levels between the three constructs, from 129 to 210 ng/ml (this estimation is obtained using the estimation that 48U of activity is approximately equal to 1 μ g – see Section 4.2.1). Such a range is not unexpected, and can be accounted for entirely by the method used to create stable expressing cell lines. Transfection with p2ZoptcxF is inherent to creating transfected cell lines of a polyclonal nature, with a range of genomic insert copy numbers at a variety of location in the genome. All of these factors can lead to a potential range of expression levels between transfections; the range of specific activities in the media of the three different clones illustrates this well.

The production of an average 175 ng/ml is 14- to 50-fold lower than the best producing clones reported by Pfeifer *et al.* (2001) in their use of the p2ZoptcxF vector to produce Factor X in *Sf9* cultures; they reported levels of 2.4-9.0 μ g/ml. This is likely due to more than one factor. Pfeifer *et al.* (2001) produced monoclonal cell lines, selecting only their highest expressing clones, which were what was reported. As well, Naglu is over twice as large as Factor X, and larger proteins often display

decreasing stability/expression of transcripts and proteins. Already being a large protein, the addition of the 24 and 118 amino acid (aa) transferrin and CBD sequences increases the size of Naglu from 720 aa (without its signal peptide) to 862 aa. For comparison sake, enhanced green fluorescent protein (eGFP), a 27 kDa protein (42 kDa with the CBD and transferrin signal), when produced in this same system under the same conditions, is produced at 1.2 µg/ml levels (Vaags, 2004); this is not dissimilar to the data of Pfeifer *et al.* (2001) considering that it is a non-optimized polyclonal cell line. This further confirms and strengthens the idea that levels between Pfeifer *et al.* (2001) and this author are comparable, and that size of the protein may correspond to expression levels.

To lend support to the hypothesis that lower levels of production than reported by Pfeifer *et al.* (2001) were due to non-optimization and/or larger size of Naglu, the author needed to rule out that some property of Naglu, Ntat and/or NPTD4 was not causing sequestration within the cell. The proteins of the cell lysate which bound either the anti-Naglu or the anti-CBD antibodies were mutually exclusive, and were found in all controls as well as the transfected samples; they therefore do not correspond to the proteins of interest. This indicates that secretion was either efficient, or that protein sequestration could not be detected within a 50 µg cell lysate protein sample. One can conclude that there is no data to indicate that transferrin signal peptide-mediated secretion is not occurring efficiently, supporting the hypothesis that large transcript/protein size may account for the lower levels of expression in comparison with Pfeifer *et al.* (2001).

The production of Naglu, Ntat and NPTD4 in this expression system was also comparable to that reported for the production of Naglu in two different CHO expression systems. Zhao *et al.* (2000) and Weber *et al.* (2001) report 0.82 and 1 $\mu\text{g}/\text{ml}$ expression, the latter using a microcarrier bead system. Zhao *et al.* (2000) report a specific activity of 20 U/mg protein. The non-optimized polyclonal lines created for the *Sf9* expression system used in this research produced levels only 4-5 fold lower than these mammalian systems, at specific activities slightly less than that of Zhao *et al.* (2000), indicating that the expression levels are somewhat comparable to the CHO mammalian expression systems in terms of protein levels.

Not only are protein expression levels comparable to that of other researchers working with recombinant Naglu, it also appears that the level of expression between Naglu and the Ntat are similar (Figure 3.3). It has been reported that tat fusion proteins are downregulated in terms of expression, but the data do not show any significant decrease between Naglu and Ntat expression levels (Cashman *et al.*, 2003).

4.2 – Protein Characterization

4.2.1 – Protein Activity

It also appears that the use of the *Sf9* system to generate Naglu, Ntat, and NPTD4 produces these proteins with activity similar to that reported for recombinant human Naglu produced in the CHO expression system of Zhao *et al.* (2000); data is not comparable to the activities of Weber *et al.* (2001) as they utilized a different activity assay. Zhao *et al.* (2000) report that their final purified protein showed a specific activity (SA) of 35 U/ μg of protein. In comparing Naglu, Ntat and NPTD4

protein amounts of known activity with proteins of known concentration (bovine serum albumin and CBD(IIA)), it was determined that 1 U corresponds to approximately 21 ng of protein, *i.e.* 48 U/ μ g protein; these figures are close enough to be comparable.

Data indicate that addition of the tat PTD and PTD4 did not disrupt Naglu activity. In most of the blots and silver stains which were loaded with the same amount of activity, intensities of the protein bands of Naglu, Ntat and NPTD4 showed a similar trend: in terms of protein, Naglu>Ntat>NPTD4. This would indicate one of three possibilities, that Naglu is less active than Ntat and NPTD4; that the addition of the moieties changes the pH optima of Naglu for the activity assay; or that Ntat and NPTD4 proteins are more likely to be lost in transfer or during the SDS-PAGE protocol. The latter seems the most likely explanation. It would be unusual for an addition to a protein to enhance its activity; however, it is possible that the PTD may enhance binding to the substrate 4-methyl-umbelliferyl- α -*N*-acetylglucosaminide, as the tat PTD is known to bind heparin and heparan sulfate (Console *et al.*, 2003; Rusnati *et al.*, 1997); perhaps Naglu would have an even greater affinity for the substrate with this addition. Secondly, it is also possible that the addition of the PTDs changes the pH optima of Naglu closer to that of the substrate buffer; native Naglus isolated from different sources have shown different pH optimas (Rohrborn *et al.*, 1978; Sasaki *et al.*, 1991; Vonfigura, 1977). A more likely explanation, however, is that the PTD fusion proteins are being lost in handling or during the SDS-PAGE protocols. Chico *et al.* (2003) report that the tat PTD binds polystyrene and borosilicate glass; at least 30% of their tatPTD-gonadotropic releasing hormone

(GnRH) fusion protein bound the sides of tissue culture wells and was removed only by washes with acid, base or 0.1% SDS. The binding was due to the cationic vector tag, not the GnRH. Chico *et al.* (2003) hypothesize that “it is possible that the unique structural properties that allow cationic vector peptides to penetrate cells might also be responsible for their unexpectedly high non-specific attachment to plastic and glass surfaces,” (p.8). However, in discussion with Dr. J. Ausio, it was mentioned that much non-specific binding could be due to amphipathic binding, rather than only cationic interactions (Ausio, 2004). The data lend support to this theory, as the modified tat PTD, which is *less* cationic and *more* amphipathic in nature, displays a greater loss during transfer due to non-specific binding. This hypothesis is further supported by the data of Ho *et al.* (2001) which show that NPTD4 has higher binding capabilities than the tat PTD (this protein showed the lowest amounts in many of the silver stains and Western blots).

One final observation about the activity of the three proteins was that none of them displayed any loss of activity upon binding to cellulose (Figure 3.9, for example).

4.2.2 – Protein Size

The size of the produced proteins corresponds well to reported sizes for both native and recombinant human Naglu. Reported sizes for native human Naglu range from 80-86 kDa when fully processed (Di Natale *et al.*, 1985; Salvatore *et al.*, 1982; Sasaki *et al.*, 1991; von Figura *et al.*, 1984). Recombinant human Naglu, as expressed by CHO *Lec1* cells, was reported to be 83 kDa when reduced and denatured; *Lec1* cells do not synthesize complex oligosaccharides (Zhao *et al.*, 2000). Weber *et al.*

(2001) report that their CHO expression system produced two functional Naglu proteins of 79 and 89 kDa. They hypothesize that the two sizes are due to different glycosylation patterns. The three final, cleaved proteins Naglu, Ntat and NPTD4 produced in the insect cell system are of apparent molecular weight 83-87 kDa. This corresponds well to reported sizes, indicating that the amount of glycosylation is likely similar to native and recombinant human Naglu. The type of glycosylation is most likely different from that produced in mammalian systems, although perhaps not the *Lec1* CHO system as neither produce complex oligosaccharides (Domingo *et al.*, 1988; Stanley *et al.*, 1985); there is most likely a high mannose complement and a lack of complex oligosaccharides (Martin *et al.*, 1988). This change in glycosylation has not seemed to affect activity of the enzyme (see above).

The author was unable to determine any significant size difference between the three proteins. SDS-PAGE gels are not optimal for discerning a 2% size difference between two proteins (Naglu is 720 aa, Ntat and NPTD4 are 735 aa). Bovine serum albumin, MW 66.4 kDa, was shown to be of apparent molecular weight 71 kDa in one silver stain for example, indicating a 7% or more discrepancy by determination with the pre-stained protein marker, making it unlikely that a 2% difference could be accurately determined.

The most probable explanations for the double-banding noticed in some of the samples is either that part of the CBD was being cleaved off by proteases or that the double-banding was an artifact of the specific electrophoresis. If part of Naglu were being degraded, or different glycosylation patterns are occurring, one would see a corresponding two sizes of product after removal of the CBD; this was not the case.

As well, one would perhaps see a small fragment which binds the anti-Naglu antibody. Neither of these data were present; only one band of final product was seen when the double band was cleaved, and there was no small anti-Naglu antibody-binding fragment (Figures 3.11). Therefore it is not Naglu that is being cleaved. It is also not the entire CBD which is being cleaved; if this were the case, the protein would not have bound cellulose. As well, the size difference would be greater than the approximate 4 kDa observed. Furthermore, samples showing double-banding one day, when electrophoresed two days later, migrated as a single band, indicating that the sample itself was not the cause of the double-banding. While not conclusive, the data suggest that the double-banding is either due to a small change within the CBD, or that it is an artifact of the gel/electrophoresis, most likely the latter.

4.3 – Protein Capture

To purify the protein by binding to microcrystalline cellulose (MCC), it was found necessary to adjust the pH of the sample between 8.4 and 9.0, and add the surfactant Triton X-100 to 0.1%. No other researchers have reported it necessary to modify any type of media used with any type of cell culture, including *Sf9* cells, before the addition of MCC (Assouline *et al.*, 1993; Greenwood *et al.*, 1992; Pfeifer *et al.*, 2001). Pfeifer *et al.* (2001) just added MCC directly to ESF-921 medium (Expression Systems, Woodland, CA) from *Sf9* cells to achieve specific binding. Concentration of the target protein is not likely to be the problem, as increasing the concentration of target proteins in this project, even though more protein would bind to a smaller amount of MCC, did not assist in specificity. Nor is the problem that the

CBD being used has been modified from the original to avoid glycosylation, as Pfeifer *et al.* (2001) used the same modified CBD. It may be a problem inherent with using SF-900 II SFM media. Another possibility is that Naglu interferes with the normal functioning of the CBD; this is not likely as glucocerebrosidase, being produced in the same lab as the author, is also showing similar tendencies (Vaags, 2004). Because the modification changes the pH and adds a surfactant, this would indicate that the problem is most likely due to non-specific binding via hydrogen binding, ionic interaction and/or electrostatic interactions. This idea is supported by data from a lab colleague who found that increasing the salt concentration of the media prior to addition of MCC also increased specificity, although not to the extent of changing the pH and adding Triton X-100 (Vaags, 2004).

In samples which had been left at room temperature or higher for long periods of time (7-19 hours), a significant percentage of activity was no longer able to bind cellulose (42-83%). This is most likely due to protease or other enzymatic activity, because frozen samples or samples stored at 4°C showed very little if any loss of binding. Greenwood *et al.* (1992) reported that much of their CBD(IIA)-beta-glucosidase (Abg) fusion protein failed to bind cellulose because of proteolytic degradation/cleavage of the CBD. The data from Figure 3.12 show that the Naglu remaining in the supernatant is approximately the same size as the final cleaved protein, indicating that the CBD has indeed been cleaved.

For all samples which showed some residual activity which would not bind, proteolytic cleavage is the likeliest explanation. However, another possibility could be that changing the pH of the media to 9.0 was sufficient to keep some CBD from

binding, as it is reported that CBD(IIA) is only stable up to pH 9.0, and that some fusion proteins with this CBD elute from MCC with buffers from 8.5-9.0 (Ong *et al.*, 1993; Tomme *et al.*, 1998). The hypothesis that the problem is due to proteolytic cleavage is more favorable, however, since the binding does not decrease when samples have been stored frozen or at 4°C despite the pH change. It is also possible that it could be a combination of both factors.

4.4 – Protein Cleavage

Factor Xa is reported to cleave target protein completely in 16 hours or less (Novagen, 1998). There are a few possible explanations as to why Factor Xa required a much longer time to cleave Naglu off of MCC, the first being that the target protein was not at an appropriate concentration; Factor Xa functions best when the target protein is at a concentration of 10 µg/µl or more (Novagen, 1998). These types of concentrations were not possible with this system. Despite that, it is the belief of this author that this was not the problem, as a substantial increase in the amount of Naglu, Ntat or NPTD4 bound to MCC did not result in faster cleavage. Also, a colleague achieved cleavage of a few hundred nanograms (in a volume of tens of µls) of eluted eGFP-CBD fusion protein with 0.03 U of Factor Xa with overnight incubation (Vaags, 2004). This would indicate that the slowness of cleavage has to do with Naglu, Ntat and NPTD4 being bound to cellulose. There have been no reports on the cleavage of a CBD-fusion protein while it is still attached to cellulose; Factor Xa functions when *it* is attached to cellulose, but perhaps it does not cleave other proteins well when the cleavage site is situated near to the cellulose substrate (Pfeifer *et al.*, 2001). A beta-

glucosidase from an *Agrobacterium* species showed less activity when bound to cellulose, indicating the possibility of steric or other interference in enzymatic reactions near the surface of the MCC (Ong *et al.*, 1991).

Samples bound to cellulose were purified to levels of specific activity slightly below the data reported by Zhao *et al.* (2000) of 35,000 U/mg for homogenous human recombinant Naglu; specific activities were 29,000 and 19,000 for Ntat and NPTD4 respectively, with protein levels of Naglu being undetectable. This indicates excellent purification in the first-step purification process using MCC. Yield at this stage was 22-83% (a large difference) depending upon the amount of probable proteolytic degradation/cleavage of the CBD domain. The excellent specific activity achieved in the first step was, of course, decreased in the concurrent step by the addition of Factor Xa. A further 80% cleavage (approximately) could be achieved by 8-12 day incubation with Factor Xa, with more being released after this time if incubation was continued. Despite this, final yields were below 10%. Removal of Factor Xa, which was not always complete, and subsequent concentration steps (if required) resulted in substantial loss of protein and in some cases a decrease in specific activity because of incomplete removal of Factor Xa. Loss in protein may be due to non-specific protein binding to concentrator columns and membranes (perhaps more so for Ntat and NPTD4 because of their binding properties), and perhaps capture of Naglu, Ntat and NPTD4 along with Factor Xa by the commercial kit.

4.5 – Future Directions

The purpose of the creation of these proteins is that they would be used in transduction studies to evaluate their ability to enter cells. However, in view of the fact that a transduction study may utilize $\mu\text{g}/\mu\text{l}$ concentrations (although amounts as small as 5 $\mu\text{g}/\text{ml}$ of tatPTD-eGFP fusion proteins have been visualized), it would be helpful to increase yield to obtain appropriate amounts of protein (Ferrari *et al.*, 2003; Lundberg *et al.*, 2003). Proteolytic activity could be decreased to avoid loss of the CBD by keeping all media on ice after collection or the addition of protease inhibitors. Greater yield of protein from cellulose pellets could be achieved with longer incubation times or the development of an elution strategy, the latter most likely resulting in faster Factor Xa cleavage and less Factor Xa to be removed. If Factor Xa removal with the commercial kit continues to be problematic, it is possible to inactivate Factor Xa with APMSF, a water soluble derivative of PMSF, a suicide substrate which effectively inactivates Factor Xa (Novagen, 1998); APMSF, however, could potentially inactivate Naglu and/or affect future transduction studies if not completely depleted. Also to increase yield, concentration of final samples should be avoided if at all possible. As well, avoiding concentration of the initial media, unless it is desirable to decrease the mass of the cellulose pellet used, would avoid protein loss due to protease activity and probable non-specific binding.

Secondly, future studies could also focus on increasing expression levels. Optimization by selecting and screening monoclonal cell lines, and/or fermentation (which can double the amount of protein produced with this system), could increase the amount of expression within the *Sf9/p2ZoptcxF* system (Pfeifer *et al.*, 2001). It may also be interesting and helpful to determine if Naglu can be produced in an

active form in a different expression system such as yeast; this would be relatively inexpensive, and the levels of expression from yeast systems (*Pichia pastoris*, for example) are higher than they are in any type of insect system.

Thirdly, it may also be helpful to add an eGFP tag to the Naglu being produced in order to avoid troubleshooting fluorescent labeling of small amounts of protein; however, this would make the protein larger, and perhaps decrease expression.

4.6 – Summary and Conclusion

The *Sf9* system expressed human recombinant Naglu, Ntat and NPTD4 in levels slightly less but comparable to reported mammalian systems. The three proteins were all active at levels comparable to published results from other researchers working with human recombinant Naglu. The Ntat and NPTD4 proteins were comparable in activity to the original Naglu protein, and were either more active at pH 4.3 or more likely to be lost in transfer, the latter seeming the more likely explanation.

The first steps of the purification process, binding to cellulose and cleavage of the protein of interest, provided excellent concentration and purification of Naglu, Ntat and NPTD4 with 8-12 day incubation periods of the proteins with Factor Xa, providing up to 64% recovery of pure sample/Factor Xa mixture. This process from crude media took 9-13 days. The latter steps of Factor Xa removal and concentration of final samples resulted in substantial loss of protein, giving final yields of less than 10%. Protease inhibition with low temperatures and specific inhibitors, avoiding

concentration of the initial media, as well as the use of APMSF to stop the action of Factor Xa instead of removal could be some responses to this low yield.

In terms of future directions, using this system to produce protein for transduction studies would require, in the opinion of the author, an optimization for better yields of protein and/or better expression of the proteins. It may also be helpful to create eGFP-Naglu and eGFP-Naglu-PTD fusion proteins.

In conclusion, although some caution is issued in using this system in attempt to produce relatively large amounts of protein without further optimization of yield and/or expression, the *S9* expression system utilizing the p2ZoptcxF vector is an appropriate system with which to produce and purify active forms of Naglu, Naglu-tatPTD and Naglu-PTD4. It is the hope of this author that future studies will be able to determine that the tat PTD or the PTD4 can enable cell entry of Naglu, thus providing hope for sufferers of MPS IIIB and their families.

5 – Bibliography

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