IN SEARCH OF A PAROTID SECRETORY PROTEIN PROTEASE: A FOCUS ON
GLANDULAR KALLIKREIN 22

By

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IN SEARCH OF A PAROTID SECRETORY PROTEIN PROTEASE: A FOCUS ON GLANDULAR KALLIKREIN 22

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Sjögren’s Syndrome is an autoimmune disorder that leads to a decrease in saliva and tear production by affecting the salivary and lacrimal gland functions. The NOD mouse model is an animal model that exhibits an autoimmune exocrinopathy similar to that of Sjögren’s Syndrome, making it an excellent model for the study of Sjögren’s Syndrome in humans.

Previous research has observed that the salivas of NOD mice exhibiting Sjögren’s Syndrome contain an unknown protease that aberrantly cleaves parotid secretory protein at a specific N-terminal point containing the NL-NL amino acid sequence. In an effort to identify the unknown PSP cleaving protease in the NOD saliva, earlier work determined, using purification and inhibition assays, the activity might be caused by an enzyme with strong homology to glandular kallikrein 22. The focus of the current research has been to test the idea that mGK22 is capable of cleaving PSP and to screen the cDNA library of
NOD mouse submandibular gland tissue for other kallikrein-like proteins that may be capable of cleaving PSP.

Screening of cDNA library provided evidence that many kallikrein and mutant genes known to have similar function and homology with mGK22 are expressed in the NOD mouse submandibular gland tissue. The homologies and functional similarity of these other kallikreins and mutant enzymes make them strong candidates as PSP cleaving enzymes. In addition it was possible to successfully clone mGK22 from NOD submandibular tissue and express it in a prokaryotic cell line. However, the crude extract of the prokaryotic cells expressing mGK22 showed no PSP protease activity, suggesting but not proving that GK22 may not be the PSP proteolytic entity. The lack of PSP proteolytic activity of mGK22 could be the result of expressing mGK22 in a bacterial, prokaryotic rather than an eukaryotic system. Further experimentation utilizing eukaryotic expression systems and enzyme purification directly from NOD saliva is probably necessary to further characterize mGK22 as well as identify if the PSP proteolytic activity is kallikrein mediated.
CHAPTER 1
INTRODUCTION

Sjögren’s Syndrome (SjS) is an autoimmune disorder that affects the salivary and lacrimal glands resulting in decreased saliva and tear production. SjS is also characterized by mononuclear lymphocytic infiltration of the lacrimal and salivary glands. Most SjS patients report the sensation of chronic dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). Moreover, SjS patients report dryness of other mucosal surfaces such as lungs, gastrointestinal tract, vagina, and skin. However, these symptoms are subjective and can be the result of causalities other than SjS such as prescription medication side effects. Therefore, several objective tests have been used to confirm SjS in patients (Delaleu et al., 2005). Although there are several tests for specific aspects of SjS, there are no widely accepted criteria for SjS classification. However, most classification models for SjS include the following manifestations: presence of ocular and oral sicca symptoms, measured decreased salivary and tear flow, lymphocytic infiltration of salivary glands, and presence of specific autoantibodies. The etiology of the disease remains to be elucidated. As a result, SjS in patients often goes under diagnosed and untreated.

Sjögren’s Syndrome

SjS is classified in two ways: primary and secondary SjS. The classifications are based upon clinical symptoms and the presence of other autoimmune disorders. SjS’s association with other autoimmune disorders and presence of autoantibodies implies that there are both local and systemic aspects of the disease. Primary SjS exists when the
lacrical and salivary glands are affected and no other connective tissue autoimmune
diseases are involved. Secondary SjS occurs when the typical SjS symptoms occur in
association with other autoimmune disorders such as rheumatoid arthritis, sclerodema, or
systemic lupus erythmatosus.

SjS displays a sexual dimorphism and age preference. Women are diagnosed with
SjS more often than men at a ratio greater than 9:1. This is one of the highest female to
male ratios when compared to other autoimmune rheumatic diseases. Moreover, in
women between the age of 40 and 60 years old the disease is even more prevalent.
Patients with SjS also have a high occurrence of lymphocytic malignancies especially in
B-cell lymphocytes (Voulgarelis et al., 2003).

Although the etiology of SjS remains unknown, many proposals of SjS
pathogenesis have been put forth. One explanation is that the characteristic decrease of
saliva and tear production in SjS is the result of glandular destruction caused by an
inflammatory autoimmune attack on the acinar cells of lacrimal and salivary glands.
Disruption of acinar cell apoptotic pathways is thought to play a critical role in the T-cell
mediated glandular destruction. Histopathological analyses confirms that the
lymphocytes infiltrating the minor salivary gland of SjS patients consist mostly of CD4+
T-cells and less of B-cells. Macrophages and dendritic cells have also been found in the
salivary gland infiltrates of SjS patients (Aziz et al., 1997; Zeher et al., 1991). A focal
score of 1 or greater, where a focus is defined as a cluster of >50 lymphocytes in a 4mm²
area, from lower lip biopsies are generally considered to be abnormal. The infiltrates are
positioned peri-ductal and peri-vascular to the lacrical and salivary glands (Cha et al.,
2002). Acinar epithelial atrophy and fibrosis have also been observed in histological
evaluations of salivary and lacrimal glands in SjS. It has been proposed that lymphocytic infiltrations cause SjS symptoms via disruption of apoptotic pathways. However, there have been many discrepancies between glandular destruction and hyposalivation in SjS patients observed by researchers (Delaleu et al., 2005). Histopathological evidence from labial salivary gland biopsies of lymphocytic infiltration is one of the key components of SjS diagnosis.

The manifestations of SjS extend far beyond the sicca symptoms of salivary and lacrimal glands. The extra glandular symptoms can affect musculoskeletal, pulmonary, vascular, gastrointestinal, hepatobiliary, hematological, dermatological, renal, and nervous systems. This suggests that SjS pathology has a systemic component affecting other tissues and organ systems.

Indeed one of the hallmarks and diagnostic criteria of SjS is the presence of organ specific and non-organ specific autoantibodies in SjS patient’s sera. Antibodies against nuclear proteins such as SS-A (Ro) and SS-B (La) have been commonly found in the sera of SjS patient’s. Patients with SjS commonly show high amounts of IgG and hypergammaglobulemia in serum analysis. Interestingly, rheumatoid factors (RF) such as IgM-RF and IgA-RF are found in the sera and saliva of SjS patients (Atkinson et al., 1989). In addition, antibodies reactive against the type3 muscarinic acetylcholine receptor (M3R) have been detected in the sera of SjS patients. Although the presence of autoantibodies is prevalent in SjS patients, the role of autoantibodies in the pathogenesis of SjS remains obscure.

Antibodies against M3R are thought to play a pivotal role in the pathogenesis of SjS by some researchers. Since secretion of water and electrolytes by acinar cells is
directly induced by acetylcholine and substance P, disruption of acetylcholine ligation with M3R would seem to affect saliva production (Baum et al., 1993). The importance of M3R in saliva production was confirmed via knockout mice experiments (Bymaster et al., 2003). Moreover, the infusion of monoclonal anti-M3R antibody from NOD mice or SjS patient’s sera into NOD and other mouse strains resulted in a loss of secretory function (Robinson et al., 1998). This suggests that the sicca symptoms associated with SjS could be the result of glandular dysfunction rather than acinar cell destruction. Thus, B-cell activation may be a critical point in SjS pathology indicating the importance of a humoral response in the pathology of SjS.

Although the presence of lymphocytes and acinar tissue destruction seem to point to glandular destruction as the main cause of the sicca symptoms in SjS, there are many documented discrepancies between lymphocytic infiltration of salivary tissue and hyposalivation. Another contributing explanation could be that the sicca symptoms of SjS are caused by an autoantibody mediated glandular dysfunction. The etiology of SjS remains to be elucidated in spite of the considerable data investigating the pathology of SjS. However, many hallmarks and characteristics of SjS have been identified. Moreover, research has identified many biomarkers that are used to help diagnose SjS in patients. Due to the fact that SjS symptoms occur in the late stages of the disease, the diagnosis of SjS in patients remains a major problem amongst clinicians and researchers.

**NOD Mouse Model**

In an effort to further investigate SjS pathology, many animal models have been used in SjS research. Limitations of using human tissue in SjS research range from ethical issues, environmental and dietary variance amongst subjects, and genetic diversity. The non-obese diabetic (NOD) mouse strain displays many symptoms with
considerable similarities to human SjS pathology. The NOD mouse develops type I diabetes via a unique H2^g^7 major histocompatibility (MHC) haplotype (Leiter and Atkinson, 1998). The expression of A^g^7 with no concomitant surface expression of an I-E molecule in the NOD mouse leads to a difference in binding affinity and affects antigen presentation. Also, the NOD mouse contains multiple mutations resulting in less potent IL-2, low expression of FcY receptor and high expression of prostaglandin synthase 2 in macrophages, lack of complement-dependent lysis, and functional NK1^+^ T-cells. The NOD mouse displays different incidence of diabetes rates between male and female in different colonies indicating many interactions of endocrine factors, environmental factors, and multiple genes that may confer protection from or susceptibility to the disease.

The NOD.B10.H2^b^ is the congenic partner strain to the NOD mouse whose MHC locus has been replaced with the non-diabetogenic MHC locus of C57BL/10. The NOD.B10.H2^b^ mouse displays an autoimmune exocrinopathy characterized by autoantibody generation, lymphocytic infiltration, and SjS-like sicca symptoms of the lacrimal and salivary glands. However, NOD.B10.H2^b^ displays SjS autoimmune exocrinopathy without developing type I diabetes, making it an excellent model for the study of primary SjS in humans (Carnaud et al., 1992; Robinson et al., 1998c). Additional studies on the C57BL/6 recombinant strain, which contained the insulin dependent diabetes (Idd) susceptibility loci Idd5 derived from chromosome 1 of the NOD mouse, displayed biochemical changes of autoimmune exocrinopathy without the loss of secretory function (Brayer et al., 2001). Thus, it appears that chromosome 1 of the NOD
mouse controls the biochemical events and other loci critical to immune infiltration and loss of secretory function.

Another SjS animal model is the NOD-scid mouse. The NOD-scid mouse was developed by breeding a homologous scid (severe combined immunodeficiency) locus into the NOD mouse background. NOD-scid mice are unable to produce B and T cells. As a result, the NOD-scid mouse has no functional immune system. The NOD-scid mouse has enabled researchers to explore the non-autoimmune component of SjS pathology. Indeed, the NOD-scid mouse shows no lymphocytic infiltration of salivary tissue and no decrease in saliva production. However, the NOD-scid mouse shows changes in protein composition and absent proteolytic activity in saliva when compared to the control mouse strain. This indicates that alteration of the NOD salivary gland occurs independently of lymphocytic infiltration. Moreover, salivary gland dysfunction may cause the immune response that leads to glandular destruction (Robinson et al., 1996).

**Temporal Changes in Salivary Composition of NOD Mouse Model**

Research on the NOD-scid mouse has led to the suggestion that SjS pathogenesis occurs in a two stages: an asymptomatic stage and a symptomatic stage (Cha et al., 2002). The asymptomatic stage precedes the symptomatic stage and is hallmarked by several biochemical changes in the glandular function of the NOD mouse. One of the biochemical changes of the NOD mouse is the presence of new and or aberrantly altered proteins in the NOD saliva. Specifically, the internal cleavage of parotid secretory protein (PSP) has been observed in the saliva of the NOD mouse and is considered a biomarker for the disease (Robinson et al., 1998).
PSP is a secretory glycoprotein found in abundance in the parotid gland of mouse and rat. It is also found in the lacrimal, sublingual, and submandibular glands at different ages. Although the function of PSP is not known, one study showed that it does bind to bacterial surfaces in a zinc-dependent manner suggesting that it may be capable of controlling bacterial growth (Robinson et al., 1998). Another study identified human PSP as having anti-bacterial function similar to bactericidal/permeability-increasing protein (Geetha et al., 2003).

Researchers also noted an age-dependent variance of the composition of salivary proteins in the NOD-scid mouse. Observations of the saliva composition of the NOD-scid mouse showed that its salivary protein profile and flow were similar to the BALB/C and prediabetic mice (Robinson et al., 1998). However, the observations also showed that the saliva composition of the NOD-scid mouse changed as the mouse aged. Changes in the salivary composition of the NOD-scid mouse include: increase in amylase expression, increase in proteolytic activity, and aberrant expression of PSP.

The temporal changes in the saliva profile of the NOD mouse were resolved by SDS-polyacrylamide gel electrophoresis. The gel analysis showed a disappearance of a 32kD band and the appearance of a 20kD band in the saliva of NOD-scid mouse over 15 weeks of age. Coincidentally, a 27kD band appeared in the saliva of the NOD-scid over 15 weeks of age as well. When the 32kD, 27kD, and 20kD bands were N-terminally sequences they were found to be homologous to the published murine-PSP sequence with interesting distinctions. The 32kD and 20kD bands were shown to have N-terminal sequences that were identical to the secreted form of PSP that is cleaved at the start sequence. The 27kD band was found to be identical to the PSP sequence that has been
internally cleaved at a position 27 amino acids downstream from the protein start sequence indicating the presence of an internally cleaved PSP isoform. The internal cleavage site was determined to be at a specific NL-NL amino acid sequence of PSP located 27 amino acids downstream from the protein start sequence. This specific NL-NL site appears to be an unusual cleavage site and does not serve as a substrate for any known proteases. Database searches of known proteases have been unable to find any proteases that cleave PSP at an NL-NL site (Day, 2002). Thus, the cleavage of PSP appears to be caused by an unknown unique protease in the saliva of the NOD mouse.

Western blot analysis using a polyclonal anti-murine PSP antibody confirmed the presence of aberrantly expressed PSP in mouse saliva of the NOD mouse. The western blots were used to compare saliva from 10-week-old NOD-scid, 20-week-old NOD-scid, 20-week-old BALB/c, and diabetic NOD mice. The saliva 32Kd and 20Kd band was found in both the 10 week old NOD-scid and 20 week old BALB/c. On the other hand, the 20-week-old NOD-scid and the diabetic NOD mouse contained the 27Kd and 20Kd isoforms of PSP. Moreover, the presence of the internally cleaved 27 kD PSP isoform is indicative of PSP cleavage that is present in the saliva of older NOD-scid and NOD mice and absent in the younger 10 week old NOD-scid and BALB/c mice.

PSP cleavage in the NOD mouse precedes the appearance of lymphocytic infiltrates of the salivary glands. Also, PSP cleavage in the NOD-scid mouse occurs at the time when lymphocytic infiltrates would begin to appear in the salivary glands of the NOD mouse. This sequential synchronization of proteolytic activity and lymphocytic infiltration of the NOD mouse and NOD-scid suggest that the changes in the exocrine glands are independent of lymphocytic infiltration and that the autoimmunity may be
caused by changes in the morphology of the exocrine glands. Thus, PSP cleavage could be one of the hallmark biochemical changes of the asymptomatic phase of SjS in the NOD mouse model.

PSP cleavage occurs in the NOD and many of its congenic strains, but not in the normal mice. Thus, the cleavage is present in the saliva of the diabetic NOD, NOD-scid, and NOD.B10.H2b mice. However, the PSP cleavage does not occur in the saliva of the 10-week old NOD-scid, 8-week old pre-diabetic NOD, C57BL/6 and BALB/c mice. Therefore, this PSP proteolytic activity could function as a biomarker for SjS.

In 2002, researchers developed a High Performance Liquid Chromatography (HPLC) assay to detect the cleavage fragments of a PSP-like peptide (Day, 2002). The PSP-like peptide employed by the HPLC assay is a 15 amino acid peptide that corresponds to amino acids 20 to 34 of the published sequence for mouse PSP. The PSP-like peptide also includes the unusual NL-NL cleavage site that serves as a substrate for the unknown PSP cleaving enzyme. In an effort to identify the protein responsible for PSP cleavage, purification methods were used to extract the PSP cleaving protease from NOD.B10.H2b saliva. The HPLC assay was used to detect the PSP cleaving proteases susceptibility to various specific protease inhibitors.

The protease inhibitor experiments conducted on the NOD.B10.H2b saliva revealed some interesting findings and candidate proteins that could be responsible for the PSP cleavage observed in the NOD.B10.H2b saliva. One interesting discovery from the experiments was total inhibition of the PSP cleavage by serine protease inhibitors. Conversely, the PSP cleavage was not affected by cysteine protease inhibitors and chelating agents designed to inhibit zinc-dependent metalloproteases as well as other
proteases stabilized by calcium (Day, 2002). The protease inhibitor assays suggest that the unknown PSP cleaving protease in the NOD mouse could be a serine protease.

The partial purification and protein analysis of NOD.B10.H2^b saliva also suggested candidate genes responsible for PSP cleavage. The NOD.B10.H2^b saliva was purified by Sephadex G-100 gel filtration column, which is used for batch separations of large peptides. The fractions that retained PSP cleaving activity were analyzed by SDS-PAGE and commassie blue staining then their protein profiles were compared to normal control mice. The protein profiles of the NOD mouse showed a unique protein band that was not present in the normal control mouse. When this protein band was N-terminally sequenced the following amino acid sequence was revealed: ILGXFKXEKDSQPXQ. This amino acid sequence was then searched against a database of known protein sequences and was shown to have strong sequence homology to mouse glandular kallikrein 22 (mGK22) (Day, 2002). Moreover, mGK22 is known to belong to a serine proteinase gene family whose inhibitor was shown to prevent PSP peptide cleavage. Although the N-terminal fragment showed near 100% homology with mGK22, it contained a single amino acid discrepancy with the published mgk22 amino acid sequence. Specifically, mGK22 contains asparagine residue at position 34 whereas the N-terminal fragment sequenced from previous research displayed an aspartic acid residue at that position. The research performed by Day on the NOD.B10.H2^b saliva strongly supports the hypothesis that the PSP cleaving enzyme in the NOD saliva is mGK22 or a protein with high homology to mGK22.

**Glandular Kallikrein**

The mouse glandular kallikreins (GK) are a group of biologically active peptides that function as highly specific esterases and are encoded by closely linked genes located
on chromosome 7. To date there are 28 GK genes in the mouse, 14 of which code for functional proteins. The kallikrein serine proteinase family was originally defined by their ability to release bioactive kinnin from high molecular mass precursors (Olsson and Lundwall, 2002). For example, a major GK found in kidney, pancreas, and salivary glands has been shown to cleave the precursor kininogen to release bradykinin which is a vasoactive peptide thought to play an important role in regulating blood flow (Schschter, 1980). GK’s are thought to be involved in a wide variety of peptide processing pathways and may represent potential regulatory steps in the conversion of inactive precursors into biologically active peptides (Evans et al., 1986).

GKs, also known as tissue kallikreins, are a family of glycoproteins of varying molecular mass ranging from 25-40 kD. They are related to trypsin, chymotrypsin, and other serine proteases. The GKs all possess a histidine residue at amino acid position 41, an aspartate residue at amino acid position 96, and a serine residue at amino acid position 189. These three amino acids form what is known as the catalytic triad, a structure thought to be critical to the formation of the serine protease catalytic site (Young et al., 1978). Moreover, mouse GKs -22, -9, and –13 contain the aspartate residue at amino acid position 183 that is thought to be required for cleavage at basic amino acids (Kreiger et al., 1974). However, unlike trypsin, GK’s show a high degree of substrate specificity (Evans et al., 1986). In fact in amino acid sequence comparisons, the GK’s display a high degree of homology with each other except in regions that are thought to be important in determining substrate specificity. Thus the actions of GKs are highly specific suggesting that their role (if any) in bioprocessing is exclusive to certain pathways. The high degree of specificity and large multigene family that encodes GK’s supports the hypothesis that
GK’s have an integral role in the processing of a wide variety of hormone and growth factor precursors (Mason et al., 1983).

mGK22 is a 29 kD protein that is expressed in the salivary glands of mice in a pre-pro zymogen form. mGK22 is activated by cleavage of the zymogen peptide at an arginine residue located 24 amino acids downstream from the peptide start site. Although mGK22 has characteristics similar to trypsin, it lacks the trypsin calcium-binding loop and fails to form trypsin’s six disulfide bridges. In contrast, mGK22 has the characteristic kallikrein loop beginning at amino acid position 77 and forms five disulfide bridges (Blaber et al., 1987). mGK22, also known as epidermal growth factor binding protein (EGF-BP) type A, is one three GKs known to bind and cleave the mouse 9 kD epidermal growth factor (EGF) precursor at the carboxy terminus to produce the mature growth factor. Interestingly, EGF is a major protein produced by the salivary glands and secreted in saliva. Other kallikrein EGF-BPs are EGF-BP type B and type C coded by mGK13 and mGK9 genes respectively. Research has shown that mGK13 and mGK26 have a 99% homology leading researchers to conclude that they represent allelic variations of the same gene (Olsson and Lundwall, 2002). Although mGKs –9, -13, and -22 all bind and process pre-pro EGF, there are no identical residues between them other than any regions conserved between the majority of other kallikreins. Thus, there seems to be no obvious critical residues between the three EGF-BPs that would confirm EGF binding ability. Also, mGK22 and other EGF-BPs may play a crucial role in the regulation of mature growth factors (Blaber et al., 1987).

The importance of GKs in the progression of SjS remains to be shown in spite of the existence of intriguing data that shows GK’s involvement with inflammation and
immune responses. Interestingly, researchers have found mGK13, also known as EGF-BP type B, autoantibodies in the sera of another SjS mouse model (IQI/Jic mice). Moreover, mGK13 was shown to cause a proliferative response of splenic T-cells, in vitro (Takada et al., 2004). This data supports the hypothesis that mGK13 may act as an auto-antigen that increases the response of T-cells to organs that commonly express mGK13. This hypothesis, if true, may be further strengthened by the fact that mGKs –22, -9, -13 are expressed exclusively in the salivary glands, the target of the immune response associated with SjS (Drinkwater et al., 1987). Thus GKS may play an important role in the etiology of SjS in two ways: via lymphoproliferative activity and via autoantibody generation.

Previous data has suggested that mGK22 may be the enzyme required for PSP cleavage in the saliva of the NOD mouse model (Day, 2002). This hypothesis is supported by the fact that GKS are known to have proteolytic effects on various protein precursors and may be potential regulators of peptide activation in various specific pathways. However, it must first be shown that mGK22 is capable of cleaving PSP in isolation. Therefore in an attempt to further solidify mGK22’s candidacy as the enzyme responsible for PSP cleavage in the NOD mouse, mGK22 must be isolated and assessed for PSP cleaving activity. Since one of the most reliable and accurate methods of PSP cleavage assessment is via the aforementioned HPLC PSP assay, this method facilitates detection of PSP proteolysis.

The focus of the current study is to determine if mGK22 is expressed in the submandibular glands and if this GK is capable of cleaving PSP. At the same time, a NOD.B10.H2b submandibular tissue cDNA library will be screened by hybridization with
oligonucleotide probes complementary to the N-terminus of mGK22 to determine if other candidate genes for related proteins may be identified. In order to assess mGK22 PSP cleaving activity and find other candidates genes responsible for cleaving PSP the following 4 goals were established:

- Construct a cDNA library of NOD.B10.H2\(^b\) submandibular tissue and clone mGK22 into a suitable vector for expression.

- Probe the cDNA library of NOD.B10.H2\(^b\) submandibular tissue with primers complementary to the N-terminus of mGK22 for possible candidate genes and mutants capable of cleaving PSP. Express mGK22 from NOD.B10.H2\(^b\) submandibular tissue cDNA library.

- Detect PSP cleavage activity of expressed genes by HPLC-PSP assay.
CHAPTER 2
MATERIALS AND METHODS

Mouse Models

The animal mouse model used in this research was the NOD.B10.H2b mouse strain. All of the mice used in this research were approximately 14 weeks of age and purchased from the University of Florida Department of Pathology Mouse Colony. The mice were held under SPF conditions, provided food and water, and maintained on a 12-hour dark-light cycle until euthanized. Studies were carried out under IACUC-approved protocol CO17.

cDNA Library Construction

Total RNA was isolated and purified from the homogenized submandibular glands of 14-week old NOD.B10.H2b mice using the RNAeasy RNA extraction kit (Qiagen). RNA purity and concentration were confirmed by UV absorbance at 260/280 nm and formaldehyde gel electrophoresis. The cDNA first strand synthesis via reverse transcription polymerase chain reaction (RT-PCR) was performed on the purified total RNA from NOD.B10.H2b using the SMART (Switching Mechanism At 5' end of RNA transcript) cDNA Library Construction Kit (Clontech), as outlined in the manufacturer’s protocol. The SMART protocols (Clontech) employ a 5' SMART IV oligonucleotide and a 3' CDS III primer (modified oligo (dT) primer) in order to preferentially enrich for full-length cDNAs during first strand synthesis and subsequent PCR amplification. During first strand synthesis, the modified oligo (dT) is used to prime the initial reaction while reverse transcriptase’s terminal transferase activity adds extra nucleotides, primarily
deoxycytodine, to the 3' end of the cDNA. The SMART IV oligo has an oligo (G) stretch which base pairs with the deoxycytodine stretch of the cDNA creating an extended template. The result is a full-length cDNA that contains the complete 5' end of the mRNA and a complementary SMART IV oligo sequence that can serve as a universal priming site for the subsequent LD-PCR amplification. The first strand synthesis cDNA was amplified using the LD-PCR (Long Distance PCR) method according to the SMART cDNA Library Construction Kit manual (Clontech) and sub-cloned into pDNR-LIB plasmid (Clontech). The cDNA-containing pDNR-LIB plasmid was used to transform 25µL of “Electromax” Top10 electro-competent cells (Invitrogen) by electroporation according to the manufacturer’s instructions. The transformed cells were then added to 970 µL of LB broth and allowed to incubate for 1 hour at 37°C with shaking (225 rpm) to create the original, unamplified cDNA library. The titer of the original, unamplified cDNA library was calculated to be 6.6 x 10^5 colony forming units per milliliter (CFU/mL). The transformed bacterial cells were then plated at a concentration of 100 colonies per plate on 250 mm agar plates containing 1.5% agarose LB-media with chloramphenicol (30 µg/mL). The plates were then allowed to incubate at 37°C overnight.

**cDNA Library Screening for mGK22**

The transformed bacterial colonies were transferred to a positively charged nitrocellulose membrane and subjected to DNA hybridization analysis. The colonies were hybridized to 3’ tail labeled digoxigenin-11-dUTP/dATP DNA (dig-labeled) probes whose sequences were identical to the N-terminus of mGK22 (5’-ATACTTGAGGATTTAAATGTGAGAAGAATTCCCAACCCTGG-3’ corresponding to nucleotides 73-114). The hybridizations were performed according to the Genius
System Users Guide version 3.0 (Roche). Replica-plated nitrocellulose membrane colony lifts containing cDNA, were generated by placing the membranes on top of a cold agar plates for 1 minute. After the bacterial colonies were transferred to a positively charged nitrocellulose membranes, the membranes were placed in series of alkaline washes (0.5N NaOH, 1.5M NaCl and 0.5N NaOH, 1.5M NaCl, 0.1% SDS) to lyse the cells and a neutralization wash (1.0M Tris-HCl, 1.5M NaCl, pH 7.5). Next, the membranes were baked at a 120°C to fix the colony DNA to the membrane. The nitrocellulose membranes, with the fixed DNA, were pre-hybridized at 65°C overnight in hybridization buffer (5X SSC, 1.0% blocking reagent, 0.1%N-lauroylsarcosine, 0.02% SDS). After washing in 2X SSC, the membranes were incubated at 68°C with the dig-labeled oligonucleotide probes in hybridization solution (2.0 pmol/mL) for 1 hour. After hybridization, the membranes were washed in washing buffer (0.1M maleic acid, 0.15M NaCl, 0.3% Tween 20) to remove any non-hybridized probes. Next, the membranes were incubated in blocking buffer (0.1M maleic acid, 0.15M NaCl, 1% blocking reagent) for 30 minutes and then placed in blocking solution that contained anti-digoxigenin (150 mU/mL) antibody conjugated to alkaline phosphatase for 1 hour. To remove any unbound antibody the membranes were washed twice in washing buffer. Detection of the bound antibody was accomplished by colorimetric development of alkaline phosphatase. Colorimetric development was performed on the membranes by adding 10 mL color substrate solution, which contained nitroblue tetrazolium salt (0.3375 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.175 mg/mL) in 10mL of detection buffer (100mM Tris-HCl, 1.5M NaCl, 50mM MgCl₂, pH 9.5). Colorimetric development was allowed to continue
overnight (approximately 12 hours). To prevent over-development, the membranes were washed twice, in H₂O, and air-dried for storage.

**Analysis of Plasmid DNA by Gel Electrophoresis and Enzyme Digestion**

The plasmids of the transformed bacterial cells from the cDNA library and mGK22 expression experiments were analyzed by gel electrophoresis and restriction enzyme digestion. Colonies from transformed cells were plated on antibiotic selective media. Next, they were used to inoculate 50ml cultures of LB-media with the appropriate selective antibiotic and allowed to incubate at 37°C overnight. The plasmids from the transformed cell cultures were isolated and purified using a Maxi-Prep plasmid purification kit (Qiagen). The protocol was performed as outlined in the Maxi-Prep plasmid purification kit manual (Qiagen). The purified plasmids were analyzed for UV absorbance at 260/280 nm using a spec 300 (Bio-Rad) to determine DNA concentration and purity. The plasmids were also loaded onto a 1% agarose gel containing 4.0% ethidium bromide and electrophoresed for 2 hours at 50V. The plasmids were then digested with restriction enzymes Xba I and Hind III (Promega), separately, to analyze the inserts they may contain. Approximately 10.0µg of purified plasmid DNA was combined with 10 units of Xba I (approximately 2.0µl) and incubated at 25°C for 1 hour. Separately, another sample of 10.0 µg of purified plasmid DNA was combined with 10.0 units of Hind III (approximately 2.0µl) and incubated at 37°C for 1 hour. Both digestions were prepared in 50 µl reactions with enzyme buffer (25.0 Tris acetate, 0.1 Potassium acetate, 10.0 mM Magnesium acetate, 1.0 DTT, pH 7.80, 0.1 mg/ml Acetylated BSA). After digestion incubation, both samples were incubated to 65°C for 15 minutes to deactivate the restriction enzymes. The samples were analyzed by gel electrophoresis for
their respective DNA fragment profiles. Finally, the identity and position of the inserts of the plasmids were confirmed by DNA sequencing.

**Expression of Recombinant mGK22**

The cDNA from the first strand synthesis was subjected to polymerase chain reaction (PCR) amplification using Proofstart high-fidelity DNA polymerase (Qiagen) and mGK22 specific primers (5′-CACCGCACCTCCTGTCCAGTCTCGAATAC-3′ corresponding to nucleotides 52-76 and 5′-TCAGGGGTTTTTGCCATAGTGTCTTTT-3′ complementary to nucleotides 753-780). To facilitate directional cloning, the forward primer contained 4 added nucleotides (CACC) as specified in the pET200 D-topo expression vector manual (Invitrogen). The PCR conditions were 94°C for 3 min followed by 30 cycles of 94°C for 45 sec, 65°C for 45 sec, 72°C for 2 min, followed by a final extension at 72°C for 7 min. PCR products of approximately 750 nucleotide base pairs (bp), which corresponds to the size of mGK22, were separated from other PCR fragments by gel electrophoresis. The purified cDNA, which contained the complete region of the mGK22 gene and stop codon, was sub-cloned into pET200 D-topo expression vector (Invitrogen). The orientation and sequence of the cDNA in the pET plasmid were confirmed by DNA sequencing. The ligated pET200 D-topo vector was used to transform *Escherichia coli* strain BL21 (DE3) cells (Invitrogen) for expression. The transformed cells were incubated at 37°C in 100 mL of LB media containing 50 µg/mL kanamycin until they reached an optical density of A600. The cells were harvested by centrifugation at 5000g for 10 min. The cell pellets were resuspended in 1 mL of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0). The cells were treated to freeze-thawing cycles and sonication (6 cycles of 10second bursts of sonication followed by 10 seconds cooling) to lyse the cells. To prevent overheating of the samples
and protect the expressed protein, the sonication of the cells was performed on ice. To separate insoluble cellular debris from the expressed recombinant mGK22 fusion protein, the crude cell lysates were centrifuged at 10,000g for 30 min. The presence of the fusion protein, which contains mGK22 residues and extra amino acids originating from the pET200 D-topo plasmid sequence at the N-terminus of mGK22, was detected in the lysates by western blot analysis with a 6X anti-histidine antibody. The crude cell lysates were analyzed for enzymatic activity using the HPLC-PSP assay.

**Protein Analysis**

**SDS-PAGE**

Crude cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate their constituent proteins by molecular weight. The crude cell lysates were combined with 2X laemmelli buffer (BioRad) in equal volumes (15µl) and incubated at 65 for 15 minutes. The entire sample, 15µl of crude cell lysates in 15.0µl of laemmelli buffer, was loaded onto a 15.0% Tris-HCl polyacrylamide gel (Bio-Rad). The gels were electrophoresed for 1 hour at 100V in Tris Glycine-SDS Buffer (Bio-Rad). After electrophoresis, the gels were washed twice in H2O in preparation for the next analysis.

**Commassie Blue Staining**

Visualization of the constituent proteins of the crude cell lysates was accomplished by commassie blue staining. The polyacrylamide gels from the SDS-PAGE of the crude cell lysates were placed in commassie blue staining solution (0.125% commassie brilliant blue R 250, 50.0% methanol, 10.0% acetic acid) and allowed to stain for 30 minutes. The polyacrylamide gels were then placed in destain solution (50.0% methanol, 10.0% acetic acid) for 15 minutes. Afterward, the destain solution was discarded and replaced by fresh
destain solution. The polyacrylamide gels were allowed to destain overnight. The polyacrylamide gels were then washed twice in H$_2$O and photographed.

**Membrane Blotting**

After separation of proteins, the proteins were transferred to a nitrocellulose membrane using a Semi-Dry Transfer Cell (Bio-Rad). The proteins from the polyacrylamide gel were transferred to the membrane at 20V for 30 minutes with membrane transfer buffer (80% Tris Glycine-SDS buffer and 20% methanol). Following the transfer, the membranes were washed twice in H$_2$O and used for western blot analysis with 6X anti-histidine antibody to detect the expressed protein.

**Western Blots**

The nitrocellulose membranes that contained the transferred proteins from the crude cell lysates were subjected to western blot analysis to detect the presence of a 6X histidine protein. The western blot analysis was performed as outlined in the Western Breeze western blot kit (Invitrogen) manual with the following specifications. All incubations were performed at room temperature. After the protein transfer, the membranes were washed twice with H$_2$O and incubated in blocking buffer (1.0% bovine serum albumin, 10mM Tris-HCl, 150mM NaCl, 0.05% Tween 20, pH 8.0) for 1 hour. The membranes were washed 3 times for 5 minutes in antibody wash (10mM Tris-HCl, 150mM NaCl, 0.05% Tween 20, pH 8.0). The membranes were then incubated with 6X anti-histidine primary antibody in blocking buffer at a concentration of 10 pmol/ml. Afterward, 3 washes of 5 minutes each in antibody wash were performed to remove unbound antibodies. Then the membranes were incubated with anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase (Sigma) at a dilution of 1:7500 in blocking buffer. The membranes were washed three times for 5 minutes in antibody wash.
to remove unbound antibodies. After pre washing in water twice, the membranes were

color developed by incubating the membranes in color substrate solution, which

contained nitroblue tetrazolium salt (0.33 mg/mL) substrate and 5-bromo-4-chloro-3-

indolyl phosphate (0.165 mg/mL) substrate in 10mL of alkaline phosphatase buffer

(100mM Tris-HCl, 100mM NaCl, 5.0mM MgCl₂, pH 9.5). To prevent over-development,

after 4 hours the membranes were washed twice for 5 minutes in H₂O. Membranes were

then air-dried for storage.

HPLC-PSP Assay

PSP Peptide Synthesis

The PSP peptide employed by the HPLC-PSP assay was a custom designed peptide

synthesized by the University of Florida ICBR Protein Chemistry Core Facility,

Gainesville. The PSP peptide was synthesized on an Applied Biosystems Peptide

Synthesizer model 432A using solid phase FMOC chemistry. The PSP peptide was

designed to mimic PSP, since it contains the amino acid residues of the PSP cleavage site

it can act as a substrate for the unknown PSP cleaving protease. The PSP peptide amino

acid sequence was N’-EAVPQNLLDVELLQ-C’. The PSP peptide amino acid sequence

was identical to the amino acids 20 through 34 of the entire published Mus musculus PSP

sequence (figure 2-1). Also, the PSP peptide contains the PSP protease specific NL-NL

cleavage site corresponding to the 26th and 27th amino acid positions of the Mus musculus

PSP sequence. The PSP peptide molecular weight and purity were confirmed by HPLC

(figure 2-2) and mass spectroscopy (figure 2-3). The University of Florida ICBR Protein

Chemistry Core Facility performed both analyses on the PSP peptide. For the purpose of

PSP cleavage detection by HPLC, 25 mg of the PSP peptide was dissolved in 10 mL of

PSP peptide buffer (10 mM Tris-HCl, pH 8.02). The concentration of PSP peptide in the
PSP peptide solution, used as the enzyme substrate in the HPLC-PSP assay, was 2.5 mg/ml.

Detection of PSP Peptide Cleavage by HPLC

To detect PSP cleavage activity in crude cell lysates, the PSP peptide was used as a substrate for the unknown PSP cleaving enzyme. 40 µl of crude cell lysates from the mGK22 expression experiments was added to 40 µl of the PSP peptide solution and incubated at 42 °C overnight, to ensure complete cleavage. As positive and negative control samples for PSP cleavage activity, saliva from NOD.B10.H2b and BALB/C mouse strains were analyzed for PSP cleavage activity by HPLC. The saliva control samples (NOD.B10.H2b and BALB/C) were separately incubated with PSP peptide. In each sample, 40 µl of the PSP peptide solution and 10 µl of saliva were combined along with 30 µl of PSP peptide buffer and incubated at 42°C overnight. All samples, both crude cell lysates and control saliva samples, were filtered through a 0.45 µm Regenerated Cellulose Micro-Spin filter tube (Alltech) by centrifugation at 5000g for 5 minutes prior to HPLC analyses.

The HPLC-PSP assay used to assess the PSP cleavage activity functions by detecting the cleavage fragments of the PSP peptide. The HPLC-PSP assay uses a reverse-phase 5µm 300Å Jupiter C18 column (phenomenex) to separate peptides and peptide fragments from each other and other compounds based on their binding affinities to the column. The HPLC-PSP assay uses a linear gradient elution of two buffers: buffer A (0.1 % Trifluoroacetic acid in Acetonitrile) and buffer B (0.1% Trifluoroacetic acid in HPLC grade H2O). The HPLC-PSP assay method used in this research specified for a linear gradient elution of 10% buffer A and 90% of buffer B to 90% buffer A and 10% of buffer B at a rate of 1.0 ml/min over a duration of 20 minutes for peptide separation. The
peptides were detected by an AD20 wavelength detector (Dionex), which distinguishes peptides by their respective ultraviolet (UV) absorbance at 214nm as they are eluted from the column. The elutions were profiled by documentation on a graph of retention time versus UV absorbance. Also, a linear regression of PSP peptide peak areas was constructed, enabling relative amounts of PSP peptide in samples to be determined.

Figure 2-1. Amino acid sequence of *Mus musculus* parotid secretory protein. The complete PSP is shown intact with the 20 amino acid leader sequence. The rectangular box outlines the synthetic PSP peptide used in the HPLC-PSP assay. The arrow labels the specific PSP protease NL-NL site.
CHAPTER 3
RESULTS

cDNA Library Screening for mGK22

A NOD.B10.H2<sup>b</sup> submandibular gland tissue cDNA library was screened with an (42-mer) oligonucleotide probe identical to the N-terminal sequence of the mGK22 gene. The oligonucleotide probe sequence was also identical to the back-translated sequence of the previously reported unique protein present in the PSP cleaving NOD.B10.H2<sup>b</sup> saliva (Day, 2002). The screening of 65 plates of the cDNA library at 100 colonies per plate, approximately 6500 clones, revealed 35 positive colonies. Since the initial colony titer was 6.6 X 10<sup>5</sup> CFU/mL, approximately 1% of the total library was screened. Thus, these 35 colonies probably represent abundant mRNA species. An example of a screened nitrocellulose membrane is shown in Figure 3-1.

The cloning region of each plasmid these positive colonies contained was directly sequenced to identify the cDNA inserts they contained. The sequencing revealed interesting gene identities of the positive clones including the kallikrein genes mGK9, mGK26, and mGK6. The alignments and comparisons of these kallikrein genes are shown in figures 3-2 thru 3-8. Other genes that were identified in the screening were: salivary protein1, salivary protein2, cytochrome P450, and PRL-inducible protein. An interesting result of the cDNA library screening was that mGK6 was found to be the most frequently expressed in the cDNA library. On the other hand, mGK22 was never identified in the cDNA library via screening with an mGK22 homologous probe.
However, mGK22 was identified and amplified from the cDNA library using mGK22 specific primers (Figure 3-9).

The products from two separate PCR reactions, using mGK22 specific primers and the cDNA library as a template, were sequenced. Each PCR reaction, when sequenced, showed that each reaction produced a different form of the mGK22 gene. The sequencing revealed that one PCR reaction produced a transcript identical to the published mGK22 sequence while the other PCR reaction produced a transcript with a single base pair deviation from the published mGK22 sequence. When translated, the single base pair deviation resulted in an aspartic acid for an asparagine amino acid substitution at position 34 in mGK22. This substitution was identical to the substitution found in the N-terminal sequencing of unique protein bands from purified fractions with PSP cleaving activity from previous research (Day, 2002). Thus, the PCR amplifications detected the presence of two different forms of the mGK22 gene present in the cDNA library of NOD.B10.H2^b submandibular gland tissue. Moreover, the different forms of mGK22 were produced by PCR in isolation and not as a mixture of both forms.

In addition to the cloned mGK genes showing 100.0% homology to the mGK published sequences, the screening revealed several genes in the cDNA library that contained minor base pair deviations from the published sequence. These point mutations, if translated, could result in amino-acid substitutions that deviate from the published amino acid sequences of the mGK proteins. For example, one positive colony whose sequence showed homology with mGK26 displayed a single nucleotide discrepancy resulting in a lysine to proline amino acid substitution at the 112\textsuperscript{th} amino-acid position (Figures 3-6 and 3-7). This nucleotide discrepancy was identified in
colonies obtained from two separate screenings (Table 1). Also, a positive colony with sequence homology to mGK6 that displayed another single nucleotide discrepancy resulting in a proline to lysine amino acid substitution at the 123rd amino-acid position was identified in colonies obtained from three separate screenings. Thus, the cDNA library screening of approximately 6500 clones did not identify any specific mGK22 colonies, but did show the presence of several other mGKs and mGK variants with significant frequency.

Analysis of cDNA Cloned mGK22 and pET200 D-topo Constructs

In order to express mGK22 from the NOD mouse, expression vectors for mGK22 were constructed. The expression of mGK22 was performed using the protocol as outlined by Matsui et al. (2000). Using NOD.B10.H2b submandibular tissue cDNA as a template, PCR amplification, with mGK22 specific primers produced a fragment of approximately 750 bp (Figure 3-9 lanes 2 and 3). A fragment of 750 bp is consistent with the published size of mGK22. Moreover, the predicted size of the PCR fragment generated from the mGK22 specific primers was 704 bp. Indeed the PCR fragment generated from mGK22 specific primers and mouse submandibular tissue cDNA displayed a slight shift below the 750 bp molecular weight marker (Figure 3-9 lane 1). Thus the mass of the nucleotide sequence generated from the PCR cloning of mGK22 was consistent with that of mGK22.

The fragment generated from the PCR reactions was ligated into pET200 D-topo vector for expression. The resulting plasmid-vector construct was designed to contain the entire coding region for mGK22 as well as a 6X histidine leader sequence. The plasmid-vector construct was used to transform *E. coli* (BL21).
The transformed *E. coli* (BL21) plasmids were purified and analyzed by gel electrophoresis to determine their size (Figure 3-10). If mGK22 has correctly ligated into the plasmid, the resulting plasmid’s approximate molecular weight is expected to be 6.5 Kbp. Lane 4 of figure 3-10 contains a plasmid with an approximate weight of 6.5 Kbp. Moreover, the negative control in lane 5 of figure 3-10 (an empty pET200 D-topo plasmid with a molecular weight of 5.6 Kbp) displayed its plasmid at a lower position than the plasmid in lane 4, thus indicating that the plasmid in lane 5 is of a lower molecular weight than the plasmid in lane 4. The negative control in lane 5 was determined to have an approximate molecular weight of 5.6 Kbps. Therefore, the molecular weight of the plasmid in lane 4 of figure 3-10 is consistent with expected molecular weight of the mGK22/pET200 D-topo construct.

The plasmids were analyzed by digestion with the restriction enzyme Hind III to yield predicted size fragments. Analysis of the nucleotide sequence of the vector construct showed that mGK22 contained a Hind III restriction enzyme cleaving site. Also, the pET200 D-topo plasmid, supplied by Invitrogen, has a Hind III restriction enzyme cleaving site. Therefore, if the mGK22 cDNA insert ligated into pET200 D-topo vector in the correct orientation, it should generate DNA fragments of approximately 803 bp and 5646 bp when digested with Hind III. On the other hand, the PCR positive control (lac Z) also contains a Hind III restriction enzyme cleaving site. However, the Hind III cleaving site in the PCR positive control (lac Z) is at a different position than the Hind III cleaving site in mGK22 causing it to produce a slightly larger fragment when digested with Hind III. The positive control lacZ/pET200 D-topo construct should generate DNA fragments of approximately 845 bp and 5646 bp, slightly larger than mGK22-pET200 D-
topo construct. Figure 3-11 displays the gel electrophoresis of the vector constructs digested with Hind III. Examination of figure 3-11 shows that a small DNA fragment in lane 4 (lacZ/pET200 D-topo positive control) was slightly larger than the small DNA fragment in lane 5 (mGK22). Moreover, the large DNA fragments of the 2 separate digests were relatively the same size, as expected. These DNA fragment profiles were consistent with the predicted DNA fragment profiles of mGK22/pET200 D-topo construct. The actual DNA sequence of the mGK22/pET200 D-topo construct was confirmed by DNA sequencing. The translation of the DNA sequence showed that the insert that was contained in the mGK22/pET200 D-topo construct is the complete pro-mGK22 gene transcript with extra sequences of amino acids on the N-terminus that were donated by the pET200 D-topo plasmid (Figure 3-8). Thus, the restriction enzyme digestion analysis and DNA sequencing of the mGK22/pET200 D-topo confirmed that the construct contained the PCR-cloned mGK22 gene, intact, in frame for expression, and in the correct orientation.

**Analysis of mGK22 Expression**

The protein profiles of *E. coli* (BL21) transformed with mGK22/pET200 D-topo plasmid were examined by commassie blue staining (Figure 3-12) and western blot analysis (Figures 3-13 and 3-14) with an anti-6X histidine antibody. The protein profiles of *E. coli* (BL21) showed the appearance of a protein band with a 6X histidine tag following induction with IPTG. The recombinant protein from mGK22/pET200 D-topo should have an approximate molecular weight of 27.7 Kbp. The commassie blue staining and western blot analysis both showed the appearance of protein at an approximate molecular weight of 27 Kbp strongly supporting the conclusion that the *E. coli* (BL21) are expressing the recombinant protein from mGK22/pET200 D-topo plasmid.
**Commassie Blue Staining**

As stated above, the protein profiles of the *E. coli* (BL21) obtained from the commassie blue staining show the appearance of a protein band with the approximate weight of 27 kD. In figure 3-12, lanes 2 and 3 the appearance of a band just above the 25 kD molecular weight marker indicates the expression of the recombinant mGK22/pET200 D-topo protein. In lanes 1 and 11, the molecular weight markers that correspond to an approximate molecular weight of 35 kD and 25 kD are the 6th and 7th band down from the top, respectively. Moreover, the 27 kD protein band in lanes 4, 6, 8, and 10 of figure 3-12 are absent in lanes 2, 3, 5, 7, and 9 of the same figure. Lane 2 of figure 3-12 contains the crude lysate of the *E. coli* (BL21) prior to induction with IPTG (time point zero). Lanes 3, 5, 7, and 9 contain crude lysate of the *E. coli* (BL21) that was not induced with IPTG. Thus, the commassie blue staining shows the presence of an inducible protein in the crude lysate of *E. coli* (BL21) cells transformed with mGK22/pET200 D-topo plasmid.

**Western Blot Analysis**

To detect the expression of the recombinant mGK22 protein in the *E. coli* (BL21) transformed with mGK22/pET200 D-topo plasmid, a western blot analysis was performed on the crude lysates of the transformed *E. coli* (BL21). A western blot can be used to detect the presence of a 6X histidine tag via the employment of an anti-6X histidine antibody. The expression vector mGK22/pET200 D-topo has been designed to incorporate a 6X histidine tag on the N-terminus of mGK22. Thus the presence of the 6X histidine tag, at the approximate molecular weight of 27.7 kD, would further confirm the expression of recombinant mGK22 in the transformed *E. coli* (BL21).
In figure 3-13, lanes 3 and 4 show the presence of a 6X histidine tag at a position just above the parallel of the 25 kD molecular weight marker (Bio-Rad) located in lane 1. The position of the 6X histidine protein bands in lanes 3 and 4 of figure 3-7 corresponds to an approximate molecular weight of 28 kD. The molecular weight of the 6X histidine-tagged protein in the crude lysates is consistent with the production of the recombinant mGK22 protein from the mGK22/pET200 D-topo plasmid.

Figure 3-14 displays the western blot analysis of the crude lysates respective pellet and supernatant following centrifugation. Lanes 3 and 5, which contain the pellet component of the crude lysate at one and two hour time points, show the presence of a 6X histidine protein band with an approximate molecular weight of 28 kD. In contrast, there are no protein bands in lanes 4 and 6 which contain the supernatant component of the crude lysate at one and two hour time points. The 6X histidine protein present in the crude lysates appears to be in the insoluble, pellet of the crude lysates.

The western blots also revealed an increase in the concentration of a 6X histidine tagged protein associated with an increase in incubation time of the crude lysates of the transformed E. coli (BL21). The intensity of the 6X histidine protein bands increases from lane 3 to lane 4, which represents the time points 0.5 hours and 1.0 hour respectively. The time dependent increase of the protein in the crude lysates implies that it is an inducible protein whose concentration increases with time. Thus, there appears to be a 6X histidine-tagged inducible protein present in the crude lysates of the transformed E. coli (BL21). The western blot analysis performed on the crude lysates provides strong evidence of the presence of a 6X histidine-tagged inducible recombinant protein in the transformed E. coli (BL21).
**HPLC-PSP Analysis to Determine PSP Proteolytic Activity in Crude Lysates**

To assess the crude lysates of the transformed *E. coli* (BL21) for PSP proteolytic activity, they were subjected to analysis by the HPLC-PSP peptide assay (Day, 2003). The HPLC-PSP peptide assay uses a synthetic peptide as a substrate to detect the activity of a protease that cleaves PSP at a specific amino acid sequence. The 15 amino acid synthetic peptide that serves as the substrate in the assay is identical to the sequence of PSP from amino acids 20 to 34. The synthetic peptide’s sequence includes the leucine-asparagine (NL-NL) cleavage site located at amino acid positions 26 and 27 in PSP.

The peptide profiles can reveal PSP cleaving activity by detecting the presence of a single peak, representing the intact PSP-like peptide synthetic, or split peaks, representing the fragments of cleaved PSP-like synthetic peptide. The intact peptide and its cleavage fragments have their respective retention times for further identification.

To determine the retention time and peak area of the uncleaved, intact PSP peptide; it was subjected to the HPLC-PSP peptide assay. Exactly 40 µL of the PSP peptide solution was added to 40µL of lysis buffer and incubated at 42 °C overnight. The sample was then filtered and analyzed by the HPLC-PSP peptide assay. As shown in figure 3-15, this control sample displayed a single peak with a retention time of 13.46 min. The retention time of this peptide was used to compare the retention times of the uncleaved, intact PSP peptide in all future assays. The peptide-alone control sample enabled the relative peak area for a sample of PSP peptide, at a concentration of 1.25 mg/ml, to be determined. The PSP peptide-alone control sample was assayed to detect the effects of the components of the buffers used in the preparation of the crude lysates on the retention time and peak area of the PSP peptide during the HPLC-PSP peptide assay.
In an effort to determine the retention time and peak profiles of an enzymatically cleaved PSP peptide, the saliva of the NOD.B10.H2^b mouse was analyzed by the HPLC-PSP peptide assay. This sample, which includes NOD.B10.H2^b saliva, has been previously shown to cleave PSP peptide and serves as a positive control for the HPLC-PSP peptide assay. Figure 3-16 displays the HPLC chromatogram of sample containing 5 µL of NOD.B10.H2^b saliva combined with 40 µL of PSP peptide solution and 35 µL of lysis buffer. The sample was incubated at 42 °C overnight, filtered, and then subjected to the HPLC-PSP peptide assay. The two peaks at 9.16 minutes and 12.21 minutes represent PSP peptide fragments 1 and 2, respectively. This positive control was used to show that the enzymatic activity of the unknown PSP cleaving enzyme, present in the NOD.B10.H2^b saliva, is not affected by the components of the lysis buffer used in the crude lysate preparations.

To assess for PSP cleavage activity in *E. coli* (BL21) transformed with mGK22/pET200 D-topo plasmid, the whole cell lysate and the cleared supernatant of the crude lysate were subjected to the HPLC-PSP peptide assay. Briefly, 40 µL of the crude whole cell lysate and 40 µL of the cleared supernatant from the mGK22 expression experiments was added, separately, to 40 µL of the PSP peptide solution and incubated at 42 °C overnight, to allow sufficient time for complete cleavage. The samples were filtered and analyzed by the HPLC-PSP assay to determine the retention times and peak areas of their peptides. In figure 3-17, the crude whole cell lysate sample incubated with PSP generated a single peak of interest at a retention time of 13.58 minutes. Moreover, when the peak area was integrated and applied to a standard curve of a linear regression of PSP peptide, the peptide concentration in the sample was approximately 1.20 mg/ml,
indicating that PSP cleavage did not occur. Likewise, figure 3-18 shows that the cleared supernatant sample incubated with PSP peptide solution generated a single peak of interest at a retention time of 13.45 minutes, which is the approximate retention time for the uncleaved PSP peptide. Thus, there was no proteolysis of PSP by the cloned, expressed mGK22. These results may not be unexpected, since the assay to this point was carried out using the pro-mGK22 form. Moreover, Matsui et al have reported that the pro-mGK27 form was also inactive.
Figure 3-1. Photograph of replica-plated nitrocellulose membrane hybridized with oligonucleotide probe from cDNA library screening. Membranes contain replica-plated bacterial colonies transformed with plasmids containing cDNA library inserts from NOD.B10.H2b mouse submandibular tissue. The membranes were incubated with a Dig-labeled oligonucleotide probe, whose sequence was identical to N-terminal of mGK22, and detected with alkaline phosphatase conjugated antibody. Positive colonies appear as darkened spots on a heterogeneous background. Colonies were plated at a concentration of 100 CFU/ml.
<table>
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<tr>
<th>Gene</th>
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<th>Found mutant form in cDNA Library</th>
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<th>Frequency of Mutant in cDNA Library Screening</th>
<th>Amino Acid Substitution Result of Mutation</th>
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Table1. Summary of results from screening of cDNA library made from NOD.B10.H2b mouse submandibular tissue. NA= not applicable
Figure 3-2. Alignment of nucleotide sequences from cDNA library screening. The nucleotide sequences obtained from the cDNA library screening were aligned for comparison. The sequences donated by the pET200 D-topo plasmid are in red. The Pre-pro zymogen peptide nucleotide sequences are represented in blue. The nucleotide regions with high variability are represented in orange. The identities of the sequences are: mGK22/pET- PCR product using glandular kallikrein 22 specific primers and cDNA from NOD.B10.H2b submandibular tissue as a template ligated into pET200/D-topo plasmid, mGK22- *Mus musculus* glandular kallikrein 22(AAN78419.1), mGK6- *Mus musculus* glandular kallikrein 6(NP_034769.4), mGK9- *Mus musculus* glandular kallikrein 9(NP_034246.1), mGK26- *Mus musculus* glandular kallikrein 26(NP_034774.1).
Figure 3-2 Continued
Figure 3. Alignment of translated gene sequences from cDNA library Screening. The nucleotide sequences obtained from the cDNA library screening were translated into their corresponding amino acid sequences and aligned. The sequences donated by the pET200 D-topo plasmid are in red. The Pre-pro zymogen peptide sequences are represented in blue. The limits of the pre and pro regions are indicated by the arrows located below the N-terminal region of the sequences. The mature glandular kallikrein begins at amino acid position 25 (position 44 of mGK22/pET). The green sequences represent the N-terminal region of the kallikrein proteins with the greatest homology. The regions with high variability are represented in orange. The identities of the sequences are: mGK22/pET- PCR product using glandular kallikrein22 specific primers and cDNA from NOD.B10.H2b submandibular tissue as a template ligated into pET200/D-topo plasmid, mGK22- Mus musculus glandular kallikrein 22(AAN78419.1), mGK6- Mus musculus glandular kallikrein 6(NP_034769.4), mGK9- Mus musculus glandular kallikrein 9(NP_034246.1), mGK26- Mus musculus glandular kallikrein 26(NP_034774.1).

### Pre-pro regions

- **mGK22/pET**: MRGSIHIIHHGMASMTGQMGMRDLYDDDDDKPFTAPPVQSRILGGFKCEKN
- **mGK22**: MRFILLFLTSLGIDAAAPPVQSRILGGFKCEKN
- **mGK6**: MRFILLFLASLGIDAAAPPVRSIVFQGNEPA
- **mGK9**: MRFILLFLASLGIDAAAPPVHRSIVFQGNEPA
- **mGK26**: MRFILLFPASLGIDAAAPPVQSRIFVQGNEPA

### Amino acid sequences

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<th>Description</th>
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**Figure3-3.**
Figure 3-4. Alignment of nucleotide sequences of mGK6 from screening of NOD.B10.H2b submandibular tissue cDNA library and Mus musculus mGK6 (BC010754). The nucleotide sequence obtained from the cDNA library screening was aligned with Mus musculus mGK6 (BC010754) for comparison. The sequence identities are: mGK6/cDNA- the nucleotide sequence obtained from the cDNA library screening; mGK6- Mus musculus glandular kallikrein 6 (BC010754). The sequences are identical with the exception of a single nucleotide discrepancy, which is represented in red and indicated by an arrow.
Figure 3-5. Alignment of amino acid translation of the mGK6 nucleotide sequences from screening of NOD.B10.H2b submandibular tissue cDNA library and amino acid sequence of \textit{Mus musculus} mGK6 (BC010754). The nucleotide sequence obtained from the cDNA library screening was translated into the corresponding amino acid sequence and aligned with \textit{Mus musculus} mGK6 (BC010754) amino acid sequence for comparison. The sequence identities are: mGK6/cDNA- the amino acid translation of the nucleotide sequence obtained from the cDNA library screening; mGK6- \textit{Mus musculus} glandular kallikrein 6 (BC010754) amino acid sequence. The sequences are identical with the exception of a single amino acid discrepancy, which is represented in red and indicated by an arrow.
Figure 3-6. Alignment of nucleotide sequences of mGK26 from screening of NOD.B10.H2b submandibular tissue cDNA library and *Mus musculus* mGK26 (NM_010644). The nucleotide sequence obtained from the cDNA library screening was aligned with *Mus musculus* mGK26 (NM_010644) for comparison. The sequence identities are: mGK26/cDNA- the nucleotide sequence obtained from the cDNA library screening; mGK26-*Mus musculus* glandular kallikrein 26(NM_010644). The sequences are identical, with the exception of a single nucleotide discrepancy, which is represented in red and indicated by an arrow.
Figure 3-7. Alignment of amino acid translation of the mGK26 nucleotide sequences from screening of NOD.B10.H2b submandibular tissue cDNA library and amino acid sequence of *Mus musculus* mGK26 (NP_034774.1). The nucleotide sequence obtained from the cDNA library screening was translated into the corresponding amino acid sequence and aligned with *Mus musculus* mGK6 (NP_034774.1) amino acid sequence for comparison. The sequence identities are: mGK26/cDNA- the amino acid translation of the nucleotide sequence obtained from the cDNA library screening; mGK26- *Mus musculus* glandular kallikrein 26 (NP_034774.1) amino acid sequence. The sequences are identical with the exception of a single amino acid discrepancy, which is represented in red and indicated by an arrow.
Figure 3-8. Translated amino acid sequence mGK22/pET200 D-topo plasmid construct.

The figure displays the amino acid translation of the DNA sequence of the pET200 D-topo plasmid that contains mGK22 PCR product as an insert. The rectangular box outlines the leader sequence donated by the pET200 D-topo plasmid as indicated by the 6X histidine tag beginning at the 5th amino acid position. The mGK22 PCR product amino acid sequence begins at amino acid position 37. Mature mGK22 begins at amino acid position 44 which corresponds to amino acid position 24 of the published mGK22 amino acid sequence.
Figure 3-9. Results of gel electrophoresis analysis of PCR amplification using mGK22 specific primers. lane 1- 1Kb ladder molecular weight marker; lane2- mGK22 specific primers with first strand synthesis cDNA as a template; lane3- mGK22 specific primers with transformed cDNA as a template; lane4- mGK22 specific primers with no DNA(negative control); lane5- control primers and control template DNA (Invitrogen positive control).
Figure 3-10. Results of gel electrophoresis analysis of plasmid constructs. lane 1- 1Kb ladder molecular weight marker; lane 2- pET200 D-topo plasmid with mGK22 insert; lane 3- pET200 D-topo plasmid with PCR (lac Z) positive control; lane 4- empty pET200 D-topo plasmid.
Figure 3-11. Results of gel electrophoresis analysis of hindIII digested plasmid constructs. 
lane 1- 1Kb ladder molecular weight marker; lane 2- mGK22 without hindIII; lane 3- PCR (lac Z) positive control; lane 4- mGK22 with hind III; lane 5- empty pET200 D-topo plasmid with hind III.
Figure 3-12. Results of SDS-PAGE analysis and comassie blue staining of crude whole cell lysates induced with IPTG with uninduced crude whole cell lysates. lane1- SDS PAGE molecular weight standards; lane 2- 0.0 hours (pre-induction); lane 3- uninduced 1.0 hour; lane 4- induced 1.0 hour; lane 5- uninduced 2.0 hour; lane 6- induced 2.0 hour; lane 7- uninduced 3.0 hour; lane 8- induced 3.0 hour; lane 9- uninduced 5.0 hour; lane 10- induced 5.0 hour; lane11- SDS PAGE molecular weight standards.
Figure 3-13. Analysis of crude whole cell lysates induced with IPTG using western blot analysis with anti-6Xhistidine antibody. lane 1- 0.0 hours (pre-induction); lane 2- 1.0 hour post induction; lane 3- 2.0 hour post induction; lane 4- empty; lane 5- histidine tagged positive control.
Figure 3-14. Analysis of pellet and supernatant of crude lysates induced with IPTG using western Blot analysis with anti-6Xhistidine antibody. lane 1- 0.0 hour (pre-induction); lane 2-empty; lane 3- pellet 1.0 hour post-induction; lane 4- supernatant 1.0 hour post-induction; lane 5- pellet 2.0 hour post-induction; lane 6- supernatant 2.0 hour post-induction.
Figure 3-15. HPLC-PSP assay chromatogram of 40µL of PSP peptide incubated with 40µL of lysis buffer. The uncleaved, intact PSP peptide retention time was 13.46 minutes.
Figure 3-16. HPLC-PSP assay chromatogram of 40µL of PSP peptide incubated with 5 µL of NOD.B10.H2b and 35µL of lysis buffer. The retention times of the fragments of the cleaved PSP peptide were 9.16 minutes (peak 6) and 12.21 minutes (peak 7).
Figure 3-17. HPLC-PSP assay chromatogram of 40µL of PSP peptide incubated with 40 µL of crude whole cell lysates from the mGK22 expression. This assay was performed on whole cell lysates prior to centrifugation. The retention time of the uncleaved, intact PSP peptide was 13.58 minutes.
Figure 3-18. HPLC-PSP assay chromatogram of 40µL of PSP peptide incubated with 40 µL of supernatant of the crude cell lysates from the mGK22 expression. The retention time of the uncleaved, intact PSP peptide was 13.45 minutes.
The identity of the unknown PSP proteolytic enzyme present in the salivas of the NOD mice exhibiting SjS symptoms remains an elusive target. Although previous data have pointed to mGK22 as the PSP cleaving culprit in the NOD saliva, the data from the cDNA library screening of the NOD have revealed the presence of other candidate proteins with known functional similarity and genetic homology to mGK22 as present in the submandibular glands of the NOD mouse. A majority of these proteins identified in the cDNA library screening belong to the same kallikrein family of proteins that includes mGK22 such as: mGK6, mGK9, mGK26. However, there were a few non-kallikrein genes that were identified by the screening, e.g. salivary protein 1 and 2, PRL-inducible protein, cytochrome P450.

The cDNA library screening failed to detect mGK22 in the cDNA library. However that could be the result of a sample screening whose size was too small to be representative of the complete cDNA library. Calculations of the screening confirm that approximately 6500 colonies of the 6.6 X 10^5 independent clones generated in the cDNA library were screened or about 1% of the library. Possibly, the abundance of mGK22 in the cDNA library was not great enough for it to be detected by the screening. Thus, the small fraction of the cDNA library that was screened could have been the reason for the failure to detect mGK22. Further screening may detect the presence of a gene with a low copy number such as mGK22.
Although the cDNA library screening failed to detect mGK22, the PCR amplification, using mGK22 specific primers, did detect mGK22 in the cDNA library. Moreover, the PCR amplification showed the presence of two different forms of mGK22 in the cDNA library. One of the PCR reactions produced a sequence that was identical to the published mGK22 sequence while another, separate PCR reaction, produced a sequence that contained a one base pair deviation from the published mGK22 sequence. When the sequence was translated to its corresponding amino acid sequence, the base pair substitution resulted in an aspartic acid for asparagine amino acid substitution. This amino acid substitution is consistent with the N-terminal amino acid sequence identified from a unique protein band of a purified saliva fraction shown to have PSP cleaving activity from previous research (Day, 2002). The two forms could represent alleles of the mGK22 gene or the existence of an unidentified glandular kallikrein gene. Errors generated during the PCR process are unlikely, since the protein encoded by this allele was identified in mice (Day, 2002). Further sequencing of genomic DNA in the NOD mouse should be performed to detect the presence of both forms of the mGK22 gene. Genomic DNA sequencing of the NOD mouse would help to understand the nature of the mGK22 discrepancies that have been found.

The cDNA library screening showed the presence of mGK6, mGK9, and mGK26 in the NOD saliva (Table 1). The N-terminal amino acid sequences of mGK6, mGK9, and mGK26 share a high degree of homology with mGK22 (figure 3-2 and 3-3). This homology between these GKs and mGK22 may explain why they were detected with an oligonucleotide probe that was complementary to the back-translated sequence of the N-terminus of mGK22.
The functional similarity and homology of mGK9, mGK22, and mGK26 strongly supports their candidacy as the enzyme responsible for PSP cleavage in the NOD saliva from previous research. Considering that mGK9, mGK22, and mGK26 share functional similarities by recognizing, binding, and processing pre-EGF, it is probable that all three kallikreins could similarly bind and cleave PSP, if indeed mGK22 possesses that ability. However, it should be noted that mGK22’s PSP cleaving activity, at least in the pro- mGK22 form, has yet to be proven. Therefore, future research should be broadly directed towards the isolation and assessment of mGK9, mGK22, and mGK26 for PSP cleaving activity should the mGK22 form not show activity.

The cDNA library screening also revealed the presence of some interesting base pair polymorphisms of mGK6 (figures 3-4 and 3-5) and mGK26 (figures 3-6 and 3-7) that make them highly suspect as PSP cleaving enzymes. The mutations were identified in several different screenings further supporting the conclusion that these nucleotide substitutions are indeed mutations and not random base pair substitutions due to transcribing or PCR replication errors. Sequencing of genomic DNA of the NOD mouse could be used to characterize the base pair polymorphisms of mGK6 and mGK26 found in the cDNA library screening as mutations. These mutations could result in a gain of function, resulting in PSP cleaving activity for mGK6 and mGK26. The mutations of mGK6 and mGK26, identified by the cDNA library screening, resulted in amino acid substitutions that involved a proline residue, when translated. The cyclic orientation and nature of a proline residue could be indicative of a beta turn or some other great structural deviation from the wild type isoform of the protein. Moreover, the mutations could confer a three-dimensional conformational change in mGK6 and mGK26 such that a PSP
cleaving function is gained. Further characterization by isolation or purification is required to ascertain the changes in activity, if any, caused by these mutations.

The cDNA library screening results generated from this thesis showed several candidate genes that may be responsible for the cleaving PSP in the NOD saliva. The proof of PSP cleaving ability and further binding characterization by these genes or mutations could help to confirm that one or more of them encode the protein responsible for PSP cleavage in the NOD saliva observed by researchers.

The data of the expression of mGK22 from this thesis shows that an inducible 6Xhistidine-tagged recombinant protein with a molecular mass equal to the mass of mGK22 was produced. The crude extract of the cells that were transformed to produce mGK22 were assessed for PSP cleavage activity after they were shown to produce an inducible protein with a 6X histidine tag.

Although the crude extract had no PSP cleaving activity, mGK22 may still be the PSP protease. The inability of the crude extract, which contained mGK22, to cleave PSP may be the result of interference from some of the transformed bacterial cell constituents. Further purification could be performed to remove cell constituents that may be interfering with mGK22 PSP cleaving activity. Another technique would be to analyze the NOD saliva for PSP cleaving activity while in the presence of mGK22 cell extract that has been previously shown to be inactive. This technique would show if the secreted form of mGK22 was affected by some other component present in the cell extract resulting in inactivity of the PSP cleaving protein. Furthermore, the cell extracts ability to inhibit PSP cleaving activity in the NOD saliva could serve as a possible explanation for the crude extracts inability to cleave PSP.
Another possible explanation for the crude extracts inability to cleave PSP could be that mGK22 was expressed in an inactive form due to the presence of the 7 amino acid pro sequence on the N-terminus of mGK22. The pro-sequence on the N-terminus of the mGK22 that was expressed in the transformed bacterial cells could be the cause of the inactivity of mGK22. Future experiments could be designed to express mGK22 in the mature form, without the 7 amino acids that comprise the pro-sequence on the N-terminus of mGK22.

Alternatively, the 6X histidine tag could have interfered with mGK22’s activity. Activation by enzymatic cleavage of the recombinant protein expressed in this research could confer PSP cleaving activity in the crude extract. Trypsin cleavage at amino acid position 24 is thought to be important and necessary step in the activation of GKs (Matsui et al., 2000). Perhaps activation via cleavage with trypsin could confer PSP cleaving activity in the crude lysates.

Moreover, the reformation of the disulfide bridges present in mGK22 could have been inhibited by the prokaryotic expression system thus preventing correct refolding and preventing the formation of required three-dimensional structures. Inappropriate refolding and incorrect three-dimensional conformation of the protein could induce the formation of inclusion bodies. The inclusion bodies are insoluble aggregates of proteins in E. coli. The analysis of the insoluble pellet and soluble supernatant of the crude lysates showed that the protein expressed was present mostly in the pellet of the crude lysate (figure 3-14). This suggests that although mGK22 was expressed in the transformed bacterial cells, much of it was probably in an insoluble form, which could result in expression of an inactive protein.
Also, post-translational modifications may be required for PSP cleaving activity in mGK22. Post-translational modifications such as glycosylation, which may be necessary for PSP cleaving activity, do not occur in a prokaryotic expression system. The expression of mGK22 in a eukaryotic expression system would produce a complete mGK22 with great similarity to the secreted form of mGK22 produced in the NOD saliva.

The data generated by this research has shed some light into the identity of a PSP cleaving protease present in the saliva of the NOD mouse. Although mGK22 cannot be ruled out completely, it is less likely that it is the PSP protease, which suggests that the other kallikrein genes and possibly kallikrein mutants that have been identified to be present in the NOD cDNA library may be the PSP protease. Further isolation, purification, and characterization of these other genes and mutants, as well as mGK22, are required to assess their ability to cleave PSP. However, confirmation of PSP cleavage by the candidate genes named in this thesis does not prove that they are solely responsible for the PSP cleavage in NOD saliva previously observed. The possibility that another unknown protein is responsible for the observed PSP cleavage in the NOD saliva cannot be ruled out yet. Purification and isolation, via ammonium sulfate precipitation for example, directly from the saliva of the NOD mouse could identify the protease responsible for PSP cleavage in the NOD saliva. Finally, once identified molecular techniques such as site-directed mutagenesis and gene knockout mice could be developed to further characterize the nature of the PSP cleavage reaction and its possible role in SjS pathogenesis.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

My name is Javier Brian Alvarado and I am a 36 year old graduate student living in Gainesville, Florida. I am the only child of Javier and Kathy Alvarado. I attended the University of South Florida in Tampa, Florida, where I earned a bachelor’s degree in biology in 1995.

After graduating, I expanded my work experience through employment in private industry. I have had work experience as a laboratory analyst, researcher, teacher and athletic coach over a period of 7 years.

In the future, I plan to reside in Gainesville, Florida, where I will raise my family and endeavor to expand my career as a molecular biologist.