

Investigating the structure-function relationships in insect cytokine-like stress-responsive
peptides

by

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Abstract

Insects employ a range of humoral and cellular responses to defend themselves against pathogen invasion. A family of peptides has been identified in many insects, including the tobacco hornworm, *Manduca sexta*, which may function as insect cytokines to regulate immune responses. They are activated at times of stress, such as pathogen invasion, wounding, and heat or cold shock. Previous structural studies reveal well-defined structures for two SRP homologs, consisting of two beta-strands and a beta-turn in the core region, with the relative stability and secondary structural features of this core region likely essential for their biological activity. The diverse roles played by the SRP family within the insect immune system make each of their structure-activity relationships intriguing. The structure of *M. sexta* SRP-3 (27AA) and SRP-6 (26AA), both stabilized by a disulfide bond, was investigated through 2D homo- and hetero-nuclear NMR spectroscopy. Nuclear Overhauser Effect (NOE) derived constraints were then used to calculate the lowest energy structures of SRP-3 and SRP-6.

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Chapter 1 – Review of Insect Cytokine-like Stress-Responsive Peptides

1.1. Introduction

Small signaling proteins called cytokines facilitate the communication between cells in the immune system. They trigger immunological responses in both vertebrates and invertebrates. Cytokines possess a significant role in almost all biological processes, such as embryonic development, stem cell differentiation, specific or non-specific immune responses, and aging (Vilcek and Feldmann, 2004). For the critical functions they play in the mammalian immune system, such as cell-to-cell interaction between macrophage, neutrophils, mast cells, T, and B-cells, as well as in immune cell activation, differentiation, and proliferation, a variety of most widely recognized cytokines, including interferons, interleukins, and chemokines, have been extensively studied (Stenken and Poschenrieder, 2015). Only a small number of cytokines have been recognized and investigated in any single insect species, compared to the hundreds detected and identified in mammals (Arango Duque and Descoteaux, 2014).

Insects deploy several humoral and cellular defenses, analogous to innate immunity in vertebrates, to protect themselves from the invasion of pathogens. Proteins or peptides, which are produced and emitted upon identification of abnormal tissues or pathogenic organisms, regulate and interact with the cellular and humoral responses of insects (Lemaitre and Hoffmann, 2007; Strand, 2008; Jiang *et al.*, 2010). Plasma proteins (such as serine proteases, serpins, and phenoloxidases) mostly control or directly engage in cellular responses while hemocytes within the open circulatory system carry out phagocytosis, nodulation, and encapsulation. Insects belonging to six different orders, which consist of Lepidoptera, Diptera, Hymenoptera, Coleoptera, Hemiptera, and Orthoptera, possess a variety of cytokine-like peptides identified

from their hemolymph (Matsumoto *et al.*, 2012; Duressa *et al.*, 2015). Their biological activities, such as hemocyte-chemotactic, differentiation, transient paralysis, growth inhibition, and wound healing, all significantly affect the immunological responses of insects. As implied by their names, PSPs promote plasmatocyte spreading (Clark *et al.*, 1997), GBPs slow the larvae's growth (Hayakawa, 1991), SRPs react to stress (Yamaguchi *et al.*, 2012), PPs elicit transient paralysis in larvae (Skinner *et al.*, 1991), HCP is hemocyte-chemotactic (Nakatogawa *et al.*, 2009), and CAP23 is cardio acceleratory (Furuya *et al.*, 1999). The evolutionarily primitive peptides might have developed additional physiological functions in addition to these partly intersecting immunity-related functions, such as head morphogenesis (Tsuzuki *et al.*, 2005), behavior balance (Kiyotake *et al.*, 2014), and hormonal control (Hayakawa, 1990). We located eight SRP genes in various regions of the *M. sexta* genome through a genome-wide search. Eleven cytokines were found in *M. sexta* along with uENF1, uENF2, and PP. The SRPs, uENFs, and PP all share certain similarities in their amino acid sequences and can control the expression of AMP or various innate immune pathways, despite their striking variations. In this review, we will provide an overview of the most recent findings on the structure-activity investigations of the multifunctional cytokine-like peptide family utilized by *Manduca sexta* to respond to a wide range of stresses. We will discuss the latest advances in the understanding of the ways by which these immune-regulating peptides for insects work.

To comprehend how certain peptides function in lepidopteran insects, their three-dimensional structures have been elucidated (Schrag *et al.*, 2019; Schrag *et al.*, 2017a; Aizawa *et al.*, 1999; Volkman *et al.*, 1999; Yu *et al.*, 1999; Miura *et al.*, 2002; Nakatogawa *et al.*, 2009). These cytokine-like peptides have a wide taxonomic range, which accounts for their diversity in structural and functional properties. These peptides are distinct from growth factors and peptide

hormones in how they regulate the physiological processes of SPRs, such as immunity versus growth, development, and reproduction. Pre-pro-peptides, larger precursor peptides with 60–150 primarily disordered residues, are shown to have SRPs at the C terminus (Matsumoto *et al.*, 2012; Hayakawa *et al.*, 1995; Wang *et al.*, 1999; Volkman *et al.*, 1999). Unique proteases identify the active peptide and cleave it between the pro-region and the first residue to release it before it can be activated and trigger a variety of immunological responses (Figure 1.1). SRPs thus remain non-functional when present as a part of pre-pro-peptides.

1.1.2. Insect Innate Immunity and Cytokine-like Peptides

Excluding marine habitats, insect species can be found in almost every known habitat and ecological niche. Insects are exposed to a variety of pathogenic and parasitic infectious agents due to their diversity, which causes immunological reactions that alter their physiology, development, and behavior. As a result, the disease only occurs in a limited proportion of these interactions. But it is undeniable that insects are incredibly effective infection-fighting organisms. While insects don't have an adaptive immune system, they do have a strong innate immune system that can combat infections (Vanha-aho *et al.*, 2016).

Innate immunity serves as the first line of defense for the host by triggering the immune response to pathogenic invaders. Innate immune responses are nonspecific, but since they are broadly dispersed throughout the body, they can be extremely important for maintaining homeostasis and preventing diseases and infections (Riera Romo and Pérez-Martínez 2016; Sun *et al.*, 2016). Behavioral adaptations and anatomical barriers such as the exoskeleton, tracheal and intestinal epithelia, and the gut peritrophic matrix are crucial for the first line of defense against pathogens (Erlandson *et al.*, 2019). Many insects move away or selectively lay their eggs free of contamination surfaces when they observe the presence of harmful microbes,

minimizing the likelihood of becoming affected. Additionally, insects intentionally consume low-quality, toxic food sources as a preventative effort to boost survival when parasitoids are present (Zelev et al., 2019).

Cellular and humoral responses make up the insect's innate immune system. The cellular mechanisms that insects can trigger comprise phagocytosis, cell aggregation, nodule formation, and encapsulation. These processes have analogs in other innate immune responses, including those of vertebrates. Insect hemocytes can be divided into many groups with different functions based on their morphological, histochemical, and functional properties as well as their molecular markers. For instance, there are three broad types of circulating functionally distinct hemocytes in the fruit fly *Drosophila melanogaster* can be divided (Letourneau et al., 2016): (a) A large number of circulating cells, or plasmatocytes, play a phagocytic role and have the ability to transdifferentiate into the other two cell types (Leitão and Sucena, 2015) (b) crystal cells, which are nonadhesive and produce enzymes of the phenoloxidase (PO) cascade and are involved in melanization; and (c) Lamellocytes, are flat, adhesive cells that are largely in charge of encapsulating large foreign objects, particularly parasitoid eggs (Cevik et al., 2019). In addition to functionally matching these cell types in other insect species, such as granulocytes that can stick to foreign surfaces identically to lamellocytes or oenocytoid cells which include cytoplasmic PO precursors, there are also hemocyte types that are absent in *D. melanogaster*, like as spherulocytes that may transport cuticular elements (Nunes et al., 2021). Hemocytes in insects regulate the cellular response, which targets pathogens by mechanisms like phagocytosis, superoxide generation, encapsulation, and enzyme release. Processes like melanization, clotting, and the secretion of antimicrobial peptides are all part of an insect's humoral response (Sheehan et al., 2018; Yang et al., 2018). Complex regulation along with the cooperation of

different immune responses amongst distinct tissues is crucial for more effective pathogen eradication. In insects, a collection of factors called cytokines performs regulatory functions (Figure 1.1). (Ishii *et al.*, 2015).

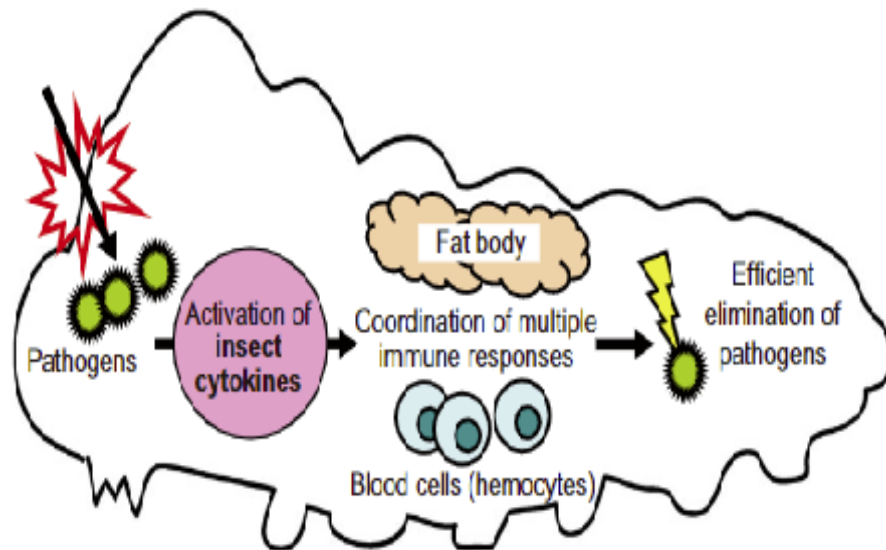


Figure 1.1. Schematic diagram of insect immunity and cytokines' response to pathogen invasion. The insect's innate immunity comprises humoral and cellular responses to infections, which consists of pathogen recognition via specific binding molecules that recognize microbial cell wall components. Plasma factors made in the fat body can induce melanization and antimicrobial peptide synthesis to assist in the efficient elimination of pathogens (Ishii *et al.*, 2015).

1.1.3. Role of Insect Cytokine-like Peptides

The rapid onset of humoral immune responses is essential for the insect immune response. Any aspect of the immune response that is regulated by extracellular macromolecules is referred to as humoral immunity. Among the many different humoral responses seen in insects include the creation of antimicrobial peptides, the development of reactive oxygen and nitrogen intermediates, and intricate enzymatic cascades that control coagulation and hemolymph melanization (Lavine and Strand, 2002). Mammals require a group of signaling peptides known as cytokines to organize such a response. Cytokines are peptides that cause target cells to undergo cytokinesis in addition to pro-mitogenic responses. These humoral

responses in *Drosophila melanogaster* are organized by a variety of insect cytokines, including Spatzle, eiger, Vago, dawdle, decapentaplegic, and sex-peptide (Agaisse *et al.*, 2003; Weber *et al.*, 2003; Peng *et al.*, 2005; Deddouche *et al.*, 2008; Mabery and Schneider 2010; Clark *et al.*, 2011; Yamaguchi *et al.*, 2012). The stress-responsive peptide, a less well-known class of peptide that was first discovered in Lepidoptera, has crucial functions in organizing the humoral responses of insects. A class of short insect cytokine-like peptides known as stress-responsive peptides was initially discovered in the lepidopteran *Pseudaletia separata* (Hayakawa 1990). Following their discovery, 15 species of insects belonging to 6 orders—Lepidoptera, Diptera, Hymenoptera, Coleoptera, Hemiptera, and Orthoptera—and more than 21 stress-responsive peptides have been documented (Hayakawa 1990; Skinner *et al.*, 1991; Clark *et al.*, 1997; Matsumoto *et al.*, 2012; Yamaguchi *et al.*, 2012; Duressa *et al.*, 2015). Stress-responsive peptides don't take part in intracellular signaling in a direct fashion. Following production, either the hemocytes or the fat body secrete it into the hemolymph as a (pre-)pro-peptide (Clark *et al.*, 1998; Strand *et al.*, 2000; Nakatogawa *et al.*, 2009; Tsuzuki *et al.*, 2012). There are 60–150 total residues in the secreted (pre-)pro-peptides, which are inactive (Matsumoto *et al.*, 2012). Serine proteases must perform proteolytic cleavage for the mature stress-responsive peptide to be successfully activated. These serine proteases are activated when exposed to either abiotic stresses (heat shock and mechanical injury) or pathogen detection (Glucan Recognition Proteins or Peptidoglycan Recognition Proteins), subsequently leading to a serine protease conversion cascade (Ishii *et al.*, 2008; Nakatogawa *et al.*, 2009; Ishii *et al.*, 2010; Tsuzuki *et al.*, 2012; Ishii *et al.*, 2013). This ensuing serine protease cascade encourages the proteolytic conversion of a variety of secondary targets, such as Spatzle and stress-responsive peptides (Weber *et al.*, 2003; Tsuzuki *et al.*, 2012; Yamaguchi *et al.*, 2012). Among Lepidopterans, the probable location of

serine proteases' cleavage of stress-responsive peptides is localized to the peptide linkage when an Arginine or Lysine residue with the localizing sequence Glutamate (E)-Asparagine(N)-Phenylalanine(F) (Strand *et al.*, 2000). Yet investigations into other insect orders find greater sequence variability of this motif, suggesting that the presence of this cleavage localizing consensus sequence is restricted to Lepidopterans (Matsumoto *et al.*, 2003; Matsumoto *et al.*, 2012).

1.2. Stress-Responsive Peptides and Biological Importance

1.2.2. Discovery of Stress-Responsive Peptides

The very first one of these peptides, called growth blocking peptide (GBP) for its ability to prevent the development of host larva, was initially discovered in a wasp parasite lepidopteran species called *Mythimna separata* (Hayakawa, 1990). Later, these GBP peptides were discovered and researched in different lepidopteran species, and because of their various roles, they were given the names: growth blocking, paralytic, plasmatocyte spreading, and stress-responsive peptide (GBP/PP/PSP/SRP) (Clark *et al.*, 1997; Skinner *et al.*, 1991; Yamaguchi *et al.*, 2012). Despite being grouped together as a single group of peptides, their sequences, and functions can vary greatly. As this category of cytokines is triggered in response to various stress, we refer to them as stress-responsive peptides (SRPs).

In *Spodoptera litura*, the first cytokine designated as SRP was identified. Analog to PP, this *S. litura* SRP was found in moth larvae that were wasp-parasitized (Yamaguchi *et al.*, 2012). This study demonstrates that *S. litura* SRP carries out identical roles as PPs, such as inhibiting larval development and promoting hemocyte spreading. Parasitization, injury, and heat treatment may all trigger this SRP to express itself. The first SRP to be cloned was obtained from *Hyphantria cunea*. Various inducible genes were identified and cloned by injecting bacteria into

the larval hemocoel, one of them was called Hdd23, the homolog of *S. litura* SRP (Shin *et al.*, 1998). *Helicoverpa armigera* was also reported to exhibit a similar upregulation of SRP by bacterial infection. Injecting double-stranded RNA into *H. armigera* SRP would reduce nodule development and prophenoloxidase gene transcription while increasing *E. coli* survival rate in hemolymph (Qiao *et al.*, 2014). Although SRPs and PPs share certain functional similarities, SRPs have evolved independently of PPs having distinct sequence properties and are present across numerous Lepidopteran species.

1.2.3. Stress-Responsive Peptides in *M. sexta*

Eleven cytokines in total were identified in *M. sexta*. The SRPs contain a D-rich C-terminus that typically concludes with tyrosine. SRP-1 through SRP-5, -7 & -8 were placed together in the tree (Figure 1.3). For SRP-1, -2, and -3, the anticipated cutting site is between residues R and F in the sequences, and for SRP-6, it is between residues R and N (only F/N is shown in Figure 1.3). SRP-1 has properties more similar to SRP-1, -2, and -6 of *S. litura* when compared to other SRPs, and as a result, might possess comparable functions.

While SRP-6 already appears quite distinct from other sequences and may have specific uses, SRP-2 and SRP-3 have remarkably comparable sequences to SRP-1 and may also have similar functions.

1.2.4. Biological Importance of Stress-Responsive Peptides

Stress-responsive peptide molecules have been demonstrated to bind to extracellular receptors and have common biological functions that frequently correspond with mammalian cytokines. These biological functions involve stimulating plasmatocyte spreading, hemocyte proliferation, growth-retardation of the organism, promotion of nodulation or encapsulation, tissue differentiation of neuronal cells, involuntary muscle contraction (thanatosis), cardio

acceleratory function, and variation of antimicrobial peptide gene expression (Hayakawa 1990; Hayakawa, 1995; Skinner *et al.*, 1991; Clark *et al.*, 1997; Furuya *et al.*, 1999; Clark *et al.*, 2004; Ishii *et al.*, 2008; Nakatogawa *et al.*, 2009; Beschin and Müller 2004; Tsuzuki *et al.*, 2012; Dembic 2015). Antimicrobial peptide mRNA was recently found to be elevated in *D. melanogaster* due to a stress-responsive peptide, also known as a growth-blocking peptide. Immunodeficiency (*imd*), a key regulator of *relish*, is required for signal transduction in order for this action to occur (Tsuzuki *et al.*, 2012). As a result, it is possible that stress-responsive peptides co-opt a portion of the gram-negative cellular response for effective signal transduction activity.

1.3. Structural Features and Expression Profile of Stress-Responsive Peptides

The loose formula (R/K)-X_{1/15}-C-X_{7/9}-G-X_{1/2}-C-X_{1/15}, where R/K denotes the probable activation cleavage site and X denotes amino acids other than cysteine, describes the length of active SRPs, which range from 23 to 32 residues. Within their sequences, these peptides contain collections of conserved residues (Schrage *et al.*, 2017b; Matsumoto *et al.*, 2012) (Figure 1.2). Two cysteines that create a disulfide bond, are among one of the conserved residues, which provide structural constraint to the peptides inside their core (Matsumoto *et al.*, 2012). The sequence of this core region (C-X₍₂₎-G-X_(4,6)-G-X_(1,2)-C-[K/R]) appears more conserved than that of the N- and C-termini. The structure of the core region is distinct and stable (Schrage *et al.*, 2019; Schrage *et al.*, 2017b). It is anticipated that the alternating hydrophobic residues in this area might help the region bind to the SRP receptors (Clark *et al.*, 2001a; Clark *et al.*, 2001b). A considerable portion of SRPs' N-terminal region lacks structure (Aizawa *et al.*, 1999; Miura *et al.*, 2002; Volkman *et al.*, 1999; Umetsu *et al.*, 2009; Yu *et al.*, 1999) and appears to be linked to receptor-specific binding and proteolytic activation (Umetsu *et al.*, 2009; Strand *et*

al., 2000; Matsumoto *et al.*, 2012; Clark *et al.*, 1997). In contrast to the N-terminal region, the C-terminal region of SRPs exhibits a broad range of amino acid composition, structure, and mechanisms of regulation (Aizawa *et al.*, 1999; Aizawa *et al.*, 2001; Clark *et al.*, 2001b; Miura *et al.*, 2002; Zhang *et al.*, 2011).

The N-terminal extension, which appears prior to the first cysteine, is crucial for protease identification and activation. It additionally serves an essential role in the plasmatocyte spreading peptide's functionality. It is assumed that peptides have different functions for the C-terminal extension, which contains acidic residues and a hydrophobic tyrosine. The N-terminus could help stabilize the peptide structure and retain biological activity, as indicated by mutational investigations (Clark *et al.*, 2001a; 2001b; Schrag *et al.*, 2017b).

<i>MsSRP1</i>	FGV R VG T C PS G Y V R G T F C F DDDDY*
<i>MsSRP2</i>	FGV K D G K C PS G R V R LG I C V DDDDY*
<i>MsSRP3</i>	F L I K SS G C P K GY V R G T F C F DEDYDY*
<i>MsSRP4</i>	S L K R G T C P D G R V L F G R V C V EIDSLDY*
<i>MsSRP6</i>	N M I V V P P N C P P G Q M G S D G V C R V V F N *
<i>MsuENF1</i>	M I D V P L N H S A C S Q G T Y L D S S G V C R Q P W Q *

Figure 1.2. Aligned sequences of mature *M. sexta* SRP-1, -2, -3, -4, -6 and uENF1. Highlighted in color are residues that are conserved across the aligned sequences.

The precursor proteins which are ideally 60 to 150 residues long, contain a signal peptide (15 to 23 amino acids), a pro-region that lacks or has fewer cysteine residues, and a somewhat distinctive cleavage site like R/K or R/K-X1/2-R/K, as well as a functional peptide region, which is a characteristic structure of pre-pro-proteins. Pre-pro-SRPs' signal peptide suggests that these proteins are capable of being secreted outside of the cell. Pro-SRPs are released and preserved as active peptides in secretory cleavage by extracellular proteases or subsequently cleaved by intracellular processing enzymes (Hayakawa *et al.*, 1995; Nakatogawa *et al.*, 2009; Wang *et al.*, 1999).

1.3.2. Expression Profile of Stress-Responsive Peptides

PP, uENF1, uENF2, and SRPs are examples of peptides that are usually represented by insect cytokines. There were a total of eleven cytokines reported in *M. sexta*, including uENF1, uENF2, PP, and 8 SRPs. Tobacco hornworm *Manduca sexta* has a genome sequence that was published by Kanost *et al.*, (2004). They also used RNA-seq to examine the gene expression in various tissues at various developmental stages. According to RNA-seq data, the longer transcript, which codes for uENF1, uENF2, and PP, is only 10% as abundant as the shorter variant, which only codes for PP. The fat body is the main source of hemolymph proteins. After the pre-wandering stage, it gets highly transcribed and appears to be the only form in the midgut. Although it is uncertain how many uENF1, uENF2, and PP proteins were translated from this transcript, they are likely to have major significance in the midgut.

Nearly all libraries did not express SRP-5, -7, or -8. In most libraries, SRP-1 was less frequently expressed than SRP-2, -3, and -4. Astonishingly, SRP-6 developed a very high level of expression in the pre-wandering and wandering gut, suggesting that it may have influenced the midgut's behavior during these stages. Jiang *et al.* (unpublished data) used qRT-PCR to examine SRP expression during immunological challenges and in various tissues in order to examine their functions in immunity and to confirm the expression using RNA-seq data. Considering that they originate from the same mRNA, uENF1, and uENF2 showed similar expression patterns in various cDNA libraries. PP is primarily highly expressed in the fat body and has a considerably higher expression than uENF1 and uENF2. After the immunological challenge, PP's mRNA level dropped after 6 hours and was restored after 24 hours. SRP-2 was often expressed at higher levels than SRP-1 and SRP-3, and predominantly expressed at higher levels in hemocytes. However, SRP-2 did not activate after the immunological challenge.

However, after six hours, SRP-1 and SRP-3 can both be greatly induced, and SRP-1 even reaches a greater level than SRP-2 at that point. In contrast to SRP-1 and SRP-2, SRP-3 has substantially less expression. The expression of these cytokines was unaffected by heat treatment of 42 °C for an hour. Despite not observing its growth-blocking function, SRP-1 was most probably the ortholog of *S. litura* SRP.

In the proteome peptidome analysis of the hemolymph of *M. sexta*, the mRNA level of PP is the highest, and among the 11 cytokines, it is the only one that was found (He *et al.*, 2016; Zhang *et al.*, 2014), demonstrating a greater protein content in the larval 5th instar hemolymph. This could possibly explain why PP was the first immune-related cytokine to be discovered and extensively studied. ProPP is stored in the hemolymph as extracellular precursors until activation by immunological challenges. Despite this, the upstream enzyme of ProPP remains unknown. With a comparable sequence and both being strongly induced following immunological challenge, SRP-1 is most similar to *S. litura* SRP. A similar function to PP has been identified for *S. litura* SRP. SRP-1, on the other hand, acts like AMPs, whose expression levels rise six hours after a challenge and fall after twenty-four hours. ProSRP-1 needs to accumulate in hemolymph over time, and by the time it does, PAP1 or PAP3, the activator of proSRP-1, may already be inactive. Surprisingly, six hours after the challenge, the PP expression decreases. ProSRP-1 might be kept in reserve for a future immunological response.

SRP-2 and SRP-1 both have the same PDDDY c-termini and are capable of being activated by PAP1 and PAP3. SRP-2 is expressed considerably more strongly in hemocytes than in fat bodies. SRP-2 expression rises from the wandering stage and peaks in the pupa for 1 to 3 days in the fat body, an indicator of immune-related genes (He *et al.*, 2015). Unknown mechanisms govern how SRP-1 and SRP-2 function differently to control immune response.

Lepidopteran species produce cytokines associated with SRP-6. In pre-wandering and wandering midguts, *M. sexta* SRP-6 becomes significantly expressed, and is probably a key regulator of midgut behavior during the wandering stage. The midgut expresses 131 different proteins. SRP-6 is typically not secreted to the hemocoel, and its exact mechanism is still understood. Investigating the activation and functioning processes of SRP-6, which might eventually be useful in pest management, will be fascinating.

The expression profiles of SRPs can be summarised as follows:

- After molting, SRP-1, -2, -3, -4, and -6 are expressed in the larval heads;
- SRP-2, PSP, and uENFs are expressed in the fat body; SRP-4 and -6 are presented in the early and late pupae, respectively;
- SRP-3 and PSP are expressed in the late embryo; uENFs, SRP-4 and SRP-6 are expressed in the entire larva;
- SRP-2, SRP-6, and PSP have a significant expression in the midgut; uENF expression levels range from pre-wandering to adult stages in the midgut;
- Some SRP-1 was found in Malpighian tubules.
- Expression in the testes and ovaries was modest, with the exception of SRP-3 and PSP
- Hemocytes and fat bodies both promoted the expression of SRP-1, -2, -4, uENFs, and PSP.
- SRP-5, -7, and -8 expression was hardly noticeable in the samples, although SRP-7 was induced in fat body.
- These findings imply that SRP-1, -2, -4, uENFs, and PSP could be contributing to immunity, whereas SRP-3, -2, -4, and -6 could be related to brain function. It's intriguing that SRP-2, -6, and PSP are highly expressed in the midgut.

1.3.3. Primary Structure

The sequence identity of the pro-segment is poor. Although it differs amongst insect orders, the mature SRP sequence is mainly conserved in insects of the same order (Hayakawa, 2021).

1.3.4. Conserved motif of orthologs and homologs

Different insect species include the gene that responds to stress in a cytokine-like fashion. This gene was discovered in *Spodoptera litura*, and it shared a small but significant amount of sequence similarity with a fall webworm immune-related gene called Hyphantria differentially-displayed clone 23 (Hdd23) (Shin *et al.*, 1998).

Both of the genes have a C-terminal peptide sequence with two cysteine residues identical to the ENF insect cytokine family, and they both encode an expected processing arginine residue in a comparable position. A contrast with a growth-blocking peptide from another insect revealed that both peptide sequences conserve numerous amino acid residues among the two cysteine residues (Strand *et al.*, 2000).

This resemblance led the researchers to propose that the C-terminal peptide acts as a growth-blocking peptide, and they verified that *S. litura*'s hemolymph contains it (Yamaguchi *et al.*, 2012). These 23-residue peptides, known as ENF peptides, were discovered to all start with Glu-Asn-Phe, and PSP was isolated from another hemolymph, *Pseudoplusia includens* (Strand *et al.*, 2000). A stable core domain, surrounded by unstructured N- and C-termini, defines three distinct domains in SRPs. According to studies on the structure, function, and binding of insects like *P. includens*' PSP and the GBP of *M. separata*, the C-terminus includes residues related to mitogenic activity, the N-terminus is essential for plasmatocyte spreading, and the core region is necessary for both activities and binding behavior (Wang *et al.*, 1999).

Lepidopteran PSPs/GBPs/PPs share characteristics with *M. sexta* SRPs in solution, the majority of which begin with Glu-Asn-Phe and are hence also known as ENF factors (Yu *et al.*, 1999). The structural characteristics of ENF factors are quite constant because these peptides are nearly identical (greater than 60% sequence identity in sequence), which corresponds to the initial amino acids of Glu-Asn-Phe for the 23-residue peptides as mentioned. According to numerous investigations using cDNA cloning, RNA-sequencing analysis, and genome sequencing, these ENF peptides are commonly present in lepidopteran insects. Certain hemolymph proteases have the ability to activate the ENF factor, which functions as a secreted precursor in stress-induced circumstances (Strand *et al.*, 2000). According to NMR studies, the ENF cytokine peptides GBP and PSP have flexible N and C termini and a structured core with a short antiparallel beta-sheet stabilized via a disulfide bridge (Aizawa *et al.*, 1999; Volkman *et al.*, 1999; Yu *et al.*, 1999). Investigations on GBP and PSP have revealed that two cysteine residues, along with other amino acid residues like tyrosine and arginine, are crucial for the biological functions of these proteins because they help to preserve the beta-hairpin structure at its core (Clark *et al.*, 2001a; Yoshida *et al.*, 2004). Many of these crucial amino acid residues are found in SRP at locations that are comparable to those of GBP and PSP, which could account for the cytokine-like activity of ENF. The N-terminal amino acid sequence of SRP, however, does not resemble ENF peptides much, indicating that SRP and ENF cytokines might stimulate various receptor types or activate the same kind of receptors with a broad range of specificity (Aizawa *et al.*, 2001; Clark *et al.*, 2001b).

1.4. Synthesis and Release

1.4.2. Gene and mRNA

In the host common cutworm *Spodoptera litura*, which had been parasitized by a single endoparasitoid wasp called *Microplitis manilae*, researchers investigated brain gene expression (Yamaguchi *et al.*, 2012). They discovered a new gene, the expression of which was greatly increased by parasitism. The gene encoded a pre-pro-peptide of a protein that resembled a cytokine and was mostly expressed in integuments, hemocytes, and neural tissues. With access to the *M. sexta* genome sequence, we have found ten open reading frames encoding PP homologs uENF1, uENF2, and SRP- 1 through -8 (Cao *et al.*, unpublished data). Although uENF1 and uENF2 are positioned upstream of PP, we will refer to the remaining eight stress-responsive peptides as such for the time being because biotic stress is a sort of stress that is more general than infection. Although we do not yet fully understand their physiological roles, we have been able to explain and compare their structures to those of known structures due to the variety of functions we have discovered in their homologs, which are spread across a wide range of taxonomic groups. A modified GBP consensus sequence was utilized to correlate with sequences that had a signal peptide at the N-terminus and a GBP-like sequence at the C-terminus in order to determine the characteristics of potential SRPs of *M. sexta*. Eleven sequences satisfied the requirements (Figure 1.2). The uENF1, uENF2, and PSP precursors are encoded by a single transcript with three open reading frames. A shorter transcript is also used to encode the 130-residue PSP precursor, which raises the possibility that it has a distinct promoter. SRP-1 through SRP-8 are eight more proteins with lengths varying from 72 to 144 residues (Figure 1.3) (Wang *et al.*, 1999; Schrag *et al.*, 2017b).

uENF1	...TKPPDNSAG K	MIDVPLNHSAC	SGQTYLDSSG V	CRQPWQ*	28	23	100
uENF2	...ENVQAGQ IIR	VPELE C PLGQRRDSLGN C RQRF*			22	22	72
PSP	...AETTTT K EG R	ENFAGG C ATGFLRTADG R CKPTF*			23	23	130
SRP1	...KPDVPSF ENR	FGVRVGT C PSGYVRR	GT F C FP DD Y*		25	16	113
SRP2	...THPIVT LED R	FGVKDG K PSGRVRR	LG I C VP DD Y*		25	16	113
SRP3	...QKTKVTF PNR	FLIKSSG C PKGYV KR	GT F C FP DE YDY*		27	20	107
SRP4	...NDDEKV RE RK	SLKRGT C PDGRVLF	GR V C V E I DSL D Y*		26	16	144
SRP5	...DPSTSQ R V KR	DI I KAP C PAGKV KV	MG K C T DD D P D Y GK *		27	20	74
SRP6	...AQPLPE VAL R	NMIVVPPN C PPG Q QMGSDGV	CR V FN*		26	15	77
SRP7	...PSSPLL R V KR	HIAGDP K CP K Q E K I	NG I C T E K S DD Y*		26	21	75
SRP8	...VGLPLA R V KR	ALT V VG I CP E G O KK I	NG I C S E T DS Y*		26	21	73

Figure 1.3. Sequence alignment of *M. sexta* ProSRPs (adapted from Schrag et al., 2017a). The active peptide, signal peptide, and entire pre-pro-peptide sizes are indicated by the three numbers at the end.

1.4.3. Distribution of mRNA

SRP-2 was used to analyze the mRNA levels of SRPs across different tissues and life stages in order to validate the expression patterns of SRPs in *M. sexta* (Cao et al., 2015). SRP-2 mRNA levels in the fat body and hemocytes were considerably higher than those in the comparable control samples, according to quantitative PCR analysis (Zhang et al., 2011). Hemocytes exhibited substantially higher mRNA levels than fat bodies. The enhanced expression in blood cells was confirmed when several tissues from day 3/5th instar larvae were examined. Very little mRNA is found in epithelial cells, the midgut, muscles, and salivary glands. Before the pupal-adult molt, there was an mRNA surge in the middle to the late pupal stage, and additionally, there was an increase in hemocytes during the wandering stage. Transcript levels in fat bodies were greater in day 0 larvae of the fourth and fifth instars compared to day 3 larvae of the same stages. The SRP-2 mRNA level in fat bodies peaked after the conclusion of the fifth instar feeding period (He et al., 2015; Schrag et al., 2017a). In different tissues of *M. sexta* and after various treatments, the expression of uENF1, uENF2, PP, SRP-1, SRP-2, and SRP-3 was investigated (Figure 1.4). This unpublished data was provided by Dr. Haobo Jiang's lab at Oklahoma State University.

The nerve expresses high levels of both uENF1 and uENF2, at similar levels. In a fat body, PP levels are significantly greater expressed. SRP-1 expression is elevated in the trachea and cuticle but is lower in the hemocytes, whereas SRP-2 and SRP-3 expression is high in the hemocytes and trachea, respectively.

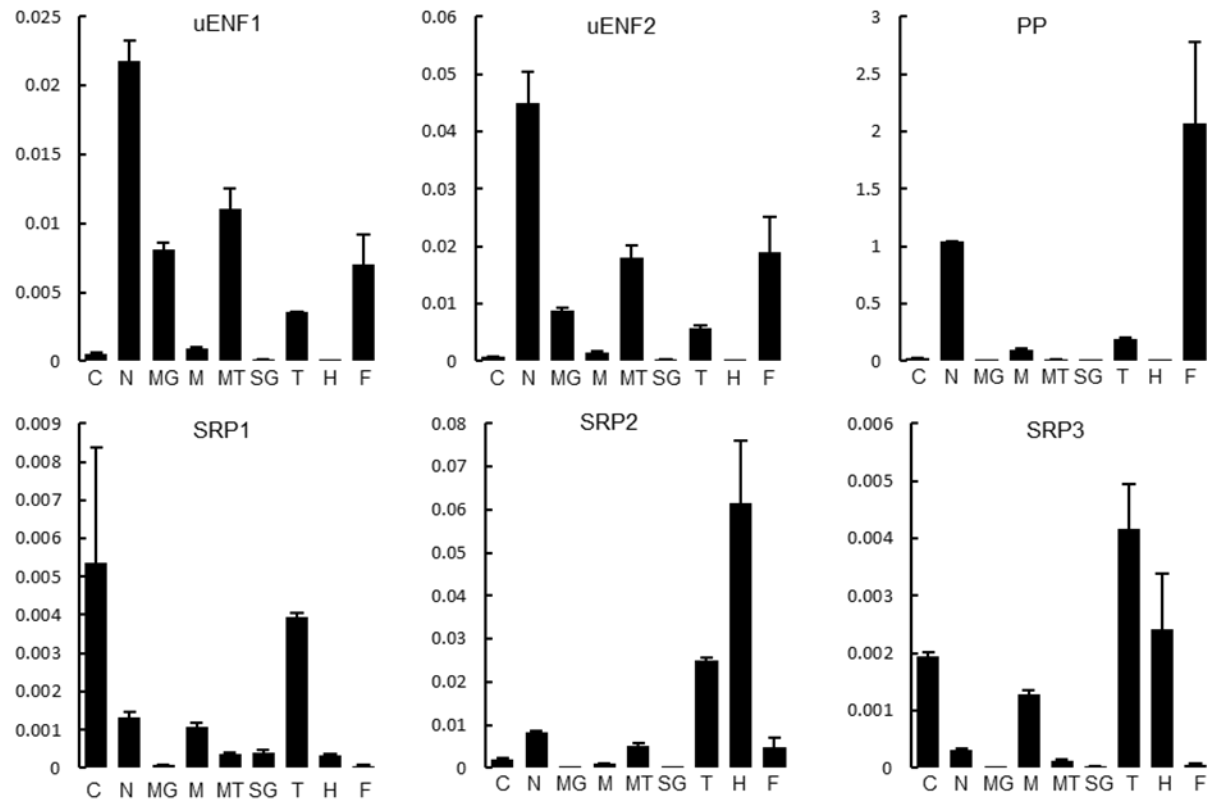


Figure 1.4. Expression of SRPs in different tissues. C, cuticle; N, nerve; MG, midgut; M, muscle; MT, Malpighian tubule; SG, salivary gland; T, trachea; H, hemocytes; F, fat body (Jiang *et al.*, unpublished data).

SRPs express themselves differently following various therapies. After a pathogenic injection, uENF and uENF2 expression rises after 24 hours, whereas SRP-1 and SRP-3 expression rises 6 hours after the injection. However, following an immunological challenge, PP drops. The expression of SRPs is not significantly influenced by heat as shown in Figure 1.5.

This unpublished data was provided by Dr. Haobo Jiang's lab at Oklahoma State University.

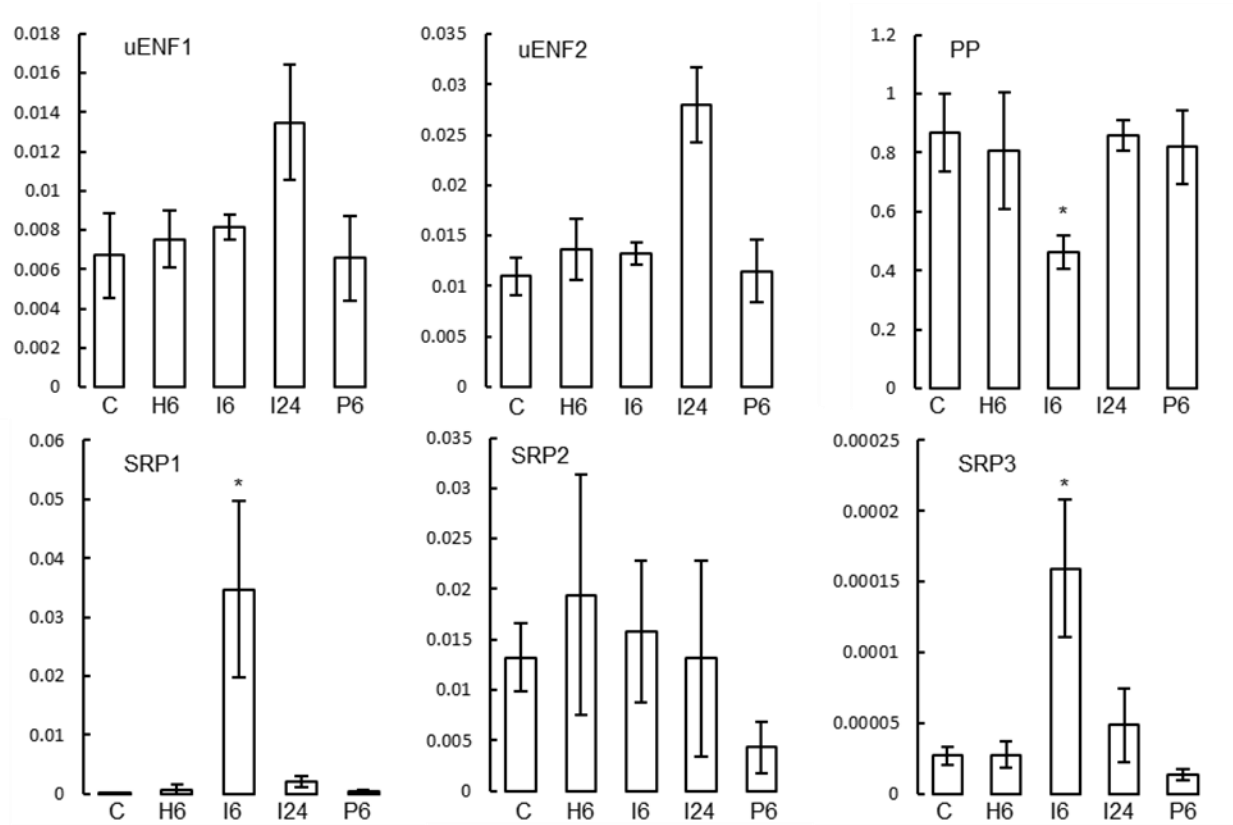


Figure 1.5. Expression of SRPs after different treatments. C, control; H6, 6 hours 42°C heat treatment; I6, 6 hours after immune challenge; I24, 24 hours after immune challenge; P6, 6 hours after injecting phosphate-buffered saline (PBS). *, $p < 0.05$ compared with P6 (Jiang *et al.*, unpublished data).

Under stressful circumstances, the gene was discovered to be more significantly expressed in *S. litura* larvae. After *M. manilae* had been parasitizing the host for 8 hours, the gene expression levels increased by about 4-fold. After eight hours of heat stress, SRP levels in the hemolymph were at least three times greater. The researchers came to the conclusion that reduced consumption and growth retardation in the host worms are inversely related to the gene's increased expression (Yamaguchi *et al.*, 2012).

1.5. Multifunctionality of Stress-Responsive Peptides and its Related Peptides

SRPs are a general term for insect peptides that govern the immune response. They perform a variety of functions. Their actions have a significant impact on the immune responses of insects, including the spreading of plasmatocytes, the proliferation of hemocytes, paralysis, wound healing, growth inhibition, and numerous other functions.

According to the literature so far, SRPs promote cell proliferation (i.e., the mitogenic effect of growth factors) amid cellular immune responses, trigger plasmatocyte spreading (a cytokine effect), and take part in the regulation of the development of the brain, integument, hematopoietic organ, and fat body. One can expect the roles of SRPs to go beyond immunity, growth, and development when numerous SRPs are detected in a single insect species (e.g., eleven in *M. sexta*). In this section, we concentrate on the function of SRPs in immunological control including their mitogenic impact on immune cells.

1.5.2. Plasmatocyte Spreading and Attachment

Cytokines which either stimulate or inhibit defensive cellular processes regulate immune responses in mammals and insects. Hemolymph and hemocyte functions are mostly the same as those of vertebrate blood, comprising carrying out immunological functions, retaining homeostasis, sealing wounds, and transporting hormones and metabolites. The expression of the cytokine receptor, cytokine receptor-like factor 3 (CRLF3), in the hemocytes of *Locusta migratoria*, has been established by Hahn *et al.*, (2019). The fat body, midgut, endocrine glands including the corpora allata or the corpora cardiaca, neurosecretory cells, and hemocytes, along with other organs produce and secrete hemolymph proteins into the circulation, which are engaged in immunological functions and adaptability to environmental stresses (Kodrík *et al.*, 2015). Many hemolymph proteins obstruct pathogen invasion directly (Yi *et al.*, 2014; Wu *et al.*,

2018) or induce hemocytes to neutralize pathogens through a variety of strategies, such as paralytic peptides, growth-inhibiting peptides, and plasmatocyte spreading peptides (Duressa *et al.*, 2015).

Many of SRPs, such as *M. separata* GBP, *P. includens* PSP, *M. sexta* PP1, *S. litura* SRP, and *L. migratoria* GBP, exhibit plasmatocyte spreading (Yamaguchi *et al.*, 2012; Duressa *et al.*, 2015). This reaction happens quickly after injury to promote wound healing and prevent hemolymph loss. A variety of cells are suspended in a liquid plasma that is rich in proteins and circulates across the cavity of the insect's body (Hillyer and Pass, 2020). Due to their open circulatory system, insects are extremely vulnerable to blood loss when wounded (Krautz *et al.*, 2014). Distributed plasmatocytes, combined with other hemocytes and plasma proteins, can produce a clot. It is probably due to plasmatocyte dissemination and adherence to tissues, the body wall, or invading pathogens when plasmatocytes suddenly disappear and then surprisingly reappear in *M. sexta* larvae after bacterial injection. When hemolymph proteins identify microorganisms, a serine protease system is activated, which could quickly result in the proteolytic activation of SRP precursors (Jiang *et al.*, 2010). In reaction to physiological stimulation, extracellular serine proteases and intracellular processing enzymes induce their proteolytic activation and/or release (Schrag *et al.*, 2017b). When SRP is introduced into armyworm larvae, MsGBP fails to boost SRP expression, showing that MsGBP cannot trigger SRP expression as long as SRP is present in the hemolymph beyond a certain threshold concentration (Matsumura *et al.*, 2018).

1.5.3. Paralysis

Paralytic peptide (PP), an insect cytokine, increases the expression of immune-related genes and aids in host defense in the silkworm *B. mori*. The results of this study showed that PP boosts nitric oxide (NO) production and NO synthase expression. A pharmacological NO synthase inhibitor inhibited the PP-dependent (i) stimulation of immune-related genes. (ii) induction of p38 mitogen-activated protein kinase, and (iii) delay in *Staphylococcus aureus* killing of silkworm larvae (Ishii *et al.*, 2013).

When active ENF factors are injected into the hemocoel of a lepidopteran insect, the larvae become rigidly paralyzed. In *B. mori*, it took several minutes for this change to become noticeable, and the length of it was observed to be dependent on the dose (Ishii *et al.*, 2015). PP might have caused a gradual muscular contraction that, in a way that is neuron-independent, suppresses insect movement. Although the PP therapy did cause a change in body length, there is insufficient proof that *B. mori* PP possessed a direct impact on muscular movements. (Ishii *et al.*, 2008). The hypothesis requires an evaluation of the electrophysiological response of the muscle. Additionally, it would be prudent to investigate any potential connections between hemocyte response and larval paralysis, an artifact brought on by injecting high amounts of the activated peptide or activating hemolymph from different larvae of the same species.

1.5.4. Wound Healing

Plasmatocyte aggregation to the gel surface in *P. includens* is stimulated by plasmatocyte spreading peptides added to plasmatocyte suspension in agarose-coated wells. In contrast, granulocytes spread evenly when the peptide is not present, but PSP inhibits their spreading in a concentration-dependent fashion (Strand *et al.*, 2000).

The three-step process of encapsulating foreign targets by granulocytes and plasmatocytes is most likely controlled by *P. includens* PSP. *B. mori* PP, *M. separata* GBP, HCP, and *M. sexta* PP1 all have been shown to increase plasmatocyte aggregation in a dose-dependent manner alongside plasmatocyte spreading (Nakatogawa *et al.*, 2009). It's fascinating to note that *M. separata* larval epidermis and hemocytes both express HCP. Upon wounding, the active peptide from ruptured epidermal cells brings hemocytes to the point of injury and, through a positive feedback mechanism, prompts the release of additional HCP from the assembled hemocytes. As HCP stimulates plasmatocyte aggregation and hemocyte deposition near the wound site, it is hypothesized that HCP accelerates chemotactic aggregation in reaction to tissue damage (Nakatogawa *et al.*, 2009).

1.5.5. Phagocytosis

Aseptic wounds rarely happen in a natural setting. With the help of phagocytosis, a crucial immunological function, insects are well-equipped to combat microbial infection. According to available data, SRPs aid in the cellular response that eliminates surface bacteria. Tetraspanin, a hemocyte surface protein responsible for encapsulation, is upregulated in expression by *B. mori* PP, according to quantitative PCR findings (Zhuang *et al.*, 2007). *B. mori* PP co-injection with *Staphylococcus aureus* significantly increased hemocyte phagocytosis of the bacterium (Ishii *et al.*, 2010). A metalloprotease from *S. marcescens* called serralyisin is thought to break down tetraspanin to interfere with hemocyte reactions. Serralyisin was injected into the hemocoel of *B. mori*, and this greatly reduced phagocytosis and plasmatocyte spreading (Ishii *et al.*, 2015).

1.5.6. Reactive Oxygen/Nitrogen Species and Nitric Oxide Synthase Production

With the help of their complex innate immune system, which is typically divided into hemocytic (phagocytosis, nodulation, and encapsulation) and humoral (the synthesis of anti-microbial peptides and prophenol oxidase activation) capabilities, insects protect themselves from pathogens and parasites. The major structures that provide immunity are hemocytes and the fat body of the larva.

At the point of injury or injection, bacteria are killed by (NO) and other reactive oxygen/nitrogen species. This is followed by extracellular serine proteases that produce active SRPs, Spätzle, and phenoloxidase being proteolytically activated. Larval death was delayed when PP and live *S. aureus* were administered together. These findings imply that nitric oxide is an SRP's downstream effector (Ishii *et al.*, 2013). ProGBP is quickly converted to active GBP by a serine protease under stressful conditions, and this is regarded to be a critical first step for insects to deal with stress-related negative effects through GBP-induced physiological changes. Prior research revealed that the hemolymph of *M. separata* larvae normally contains high quantities of proGBP and that the conversion of proGBP to active GBP happens right away after being exposed to heat stress (Matsumura *et al.*, 2018). In the fat body, which serves as the primary organ of proGBP synthesis, GBP mRNA levels are elevated 30 min to 1 hr after heat stress, compared to the quick processing (Matsumura *et al.*, 2018). When H₂O₂ was injected into larvae, an analogous trend of changes in GBP peptides and mRNA levels was seen, indicating that the heat stress-induced ROS increase first activates the proGBP processing enzyme before increasing the transcription level. The finding that heat stress raises the levels of ROS in tissues including the fat body, hemocytes, and hemolymph confirms this theory at least in part (Matsumura *et al.*, 2017). Nitric oxide is a tiny, membrane-permeable signal molecule that

mediates immunological and nervous system functions in both insects and vertebrates. Bacterial injection enhanced NO levels in the fat body and hemocytes of the larvae, and the *S. exigua* RNA interference (RNAi) NO synthase (NOS) gene reduced NO levels. The cellular and humoral immunological responses in insects are mediated by eicosanoids. For the cytokine plasmatocyte-spreading peptide (PSP) and two biogenic monoamines in *S. exigua*, eicosanoid immunological signals serve as a common downstream signal (Srikanth *et al.*, 2013). The rapid elevation of AMP transcript and protein levels following immunological exposure is regarded as induced AMP Synthesis and is a distinguishing feature of insect immunity (Buchon *et al.*, 2014). The rapid elevation of AMP transcript and protein levels following immunological exposure is regarded as induced AMP synthesis and is a distinguishing feature of insect immunity (Buchon *et al.*, 2014). Since the elimination of Spätzle or dorsal-dif did not affect GBP-induced Mtk expression, *D. melanogaster* GBP upregulated Metchnikowin (Mtk, an AMP), however not to the same extent as in *S. marcescens* infection (Tsuzuki *et al.*, 2012). The peptidoglycan recognition protein-LC or -LE (PGRP-LC or -LE) was RNA interference (i) by *Drosophila* immunity protein (Imd) or c-Jun N-terminal kinase (JNK) that resulted in the loss of MTK induction by GBP without affecting or even increasing MTK expression. In contrast to conventional relish-mediated upregulation of AMP genes, the signal of DAP-PG sensed by PGRP-LC and -LE and Imd does not seem to be sensed by PGRP-LC and -LE and Imd. SRPs have been demonstrated to have functions essential for organism survival. Under mechanical or thermal stress, red flour beetle *Tribolium castaneum*'s downregulated GBP genotype is significantly less stable than the upregulated genotype. The peptidoglycan recognition protein-LC or -LE (PGRP-LC or -LE) was RNA interference (i) by *Drosophila* immunity protein (Imd) or c-Jun N-terminal kinase (JNK) that resulted in the loss of MTK induction by GBP without

affecting or even increasing MTK expression. In contrast to conventional relish-mediated upregulation of AMP genes, the signal of DAP-PG sensed by PGRP-LC and -LE and Imd does not seem to be sensed by PGRP-LC and -LE and Imd. SRPs have been demonstrated to have functions essential for organism survival. Under mechanical or thermal stress, *T. castaneum*'s downregulated GBP genotype is significantly less stable than the upregulated genotype (Kiyotake *et al.*, 2014).

Growth-restraining activity SRPs, such as *M. separata* GBP, *P. includens* PSP, *D. melanogaster* GBP, *S. litura* SRP, *S. exigua*, and *L. migratoria* GBP, control the growth of insect larvae (Duressa *et al.*, 2015). In *M. separata*, GBP injection caused juvenile hormone esterase, a crucial enzyme involved in downregulating the hormone level in preparation for pupation, to exhibit a significant decrease in activity. Heat stress increases GBP mRNA levels in the fat body, which is the primary organ of proGBP synthesis, 30 minutes to an hour later (Matsumura *et al.*, 2018). When H₂O₂ was injected into larvae, an analogous trend of changes in GBP peptides and mRNA levels was seen, indicating that the heat stress-induced ROS increase first activates the proGBP processing enzyme before increasing the transcription level. The finding that heat stress raises the levels of ROS in tissues including the fat body, hemocytes and hemolymph confirms this theory to some degree (Matsumura *et al.*, 2017). GBP was used in HS-targeting delivery approach because of its high cell-penetrating ability and distinctive molecular interactions with cell surface HSPG (Fang *et al.*, 2013). In vivo, GBP targets HS-rich tissues namely mucosal and intestinal tissues and epithelial tumor tissues by interacting with negatively charged molecules on the cell surface, such as HS and lipids (Lien *et al.*, 2013; Chen *et al.*, 2015).

1.5.7. Mitogenic Activity

As a result of the initial findings about the mitogenic activity of *M. separata* GBP and its mutants, stress-response *L. cuprina* GBP boosts cell growth in a concentration-dependent approach (Tsuzuki *et al.*, 2012). Thymidine incorporation was boosted by ectopic injection of the fly's GBP into high five cells. Human keratinocytes and Sf9 cells both displayed a comparable incorporation response to *M. separata* GBP (Hayakawa and Ohnishi, 1998). Similar results were observed on day 0 as *M. separata* larvae of the final instar that were injected with 0.1 or 1 pMol of the peptide and grew greater in weight than the injection control. However, compared to the control, larvae given a 10 pMol GBP injection dramatically lost weight (Hayakawa and Ohnishi, 1998). These findings imply that modest concentrations of the stress-response GBP are more likely to exhibit mitogenic action than high amounts.

A number of analyses of SRP revealed biological actions that were comparable to those of GBP, including growth inhibition via decreased hunger and stimulation of hemocytes' cellular immunological responses. The active SRP peptide has 25-27 amino acids and two cysteine residues in its core region, which gives it some structural similarities to GBP. Active peptides are found at the C-termini of precursor polypeptides used in the synthesis of both GBP and SRP (Yamaguchi *et al.*, 2012).

1.6. Conclusions

Cytokines play a crucial role in immune system communication across both vertebrates and invertebrates, influencing various biological processes. While extensively studied in mammals, insect cytokines remain less explored. Insects deploy innate immune defenses, including humoral and cellular responses. SRPs in Lepidopteran insects, exemplified by *M. sexta*, serve diverse biological functions akin to mammalian cytokines. Structural studies reveal a conserved motif and distinct expression profiles across tissues and life stages. The identification of conserved motifs and diverse functions underlines the complexity of insect cytokine-like peptides. Their potential contribution to brain function opens avenues for exploring the broader physiological roles of these peptides beyond immune regulation—the diversity of SRPs, their activation mechanisms, and functional plasticity present opportunities for novel therapeutic interventions. Investigating the structural and functional aspects of these peptides can not only provide an understanding of immune dynamics but also offer insights into broader applications, including therapeutic applications such as against epidermal growth factor receptor (EGFR)-driven cancers.

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Chapter 2 – NMR Structural Studies of Insect Cytokines: *Manduca sexta* Stress-Responsive Peptide-3

2.1. Introduction

Stress-responsive peptides (SRPs), found in insects, are important for mitogenic and immunologic activities. SRPs are 19- to 32-residue peptides that have been identified in various insects that modulate the immune responses because of stress conditions such as wounding, and infections (Schrag *et al.*, 2016). They are cytokine-like peptides and exhibit a wide range of functions due to their diverse nature including plasmatocyte spreading, hemocyte proliferation, differentiation, paralysis, wound healing, growth blocking, and other activities (Schrag *et al.*, 2017b; Clark *et al.*, 1997; Duressa *et al.*, 2015; Hayakawa, 1990; Matsumoto *et al.*, 2012; Skinner *et al.*, 2016; Yamaguchi *et al.*, 2012). Despite over a hundred human cytokines having been defined, only a few insect cytokines and cytokine-like factors have been identified (Arango Duque and Descoteaux, 2014; Matsumura *et al.*, 2018). First isolated from lepidopteran species, they are classified as growth-blocking (GBP), paralytic (PP), plasmatocyte-spreading (PSP), or stress-responsive peptide (SRP). This family of bioactive peptides is now known to exist in at least six insect orders, exhibiting both structural and functional diversity. (Clark *et al.*, 1997; Duressa *et al.*, 2015; Hayakawa, 1990; Matsumoto *et al.*, 2012; Skinner *et al.*, 1991; Yamaguchi *et al.*, 2012). Nevertheless, the distinctions between them are often blurred as insect cytokines are implicated in the regulation of diverse innate immune responses (Beschlin and Müller, 2012; Dembic, 2015).

Initially discovered in *Drosophila*, GBP has shown the first evidence of an insect cytokine-like peptide. It has been isolated from the armyworm *Mythimna separata* after parasitization by the parasitoid wasp *Cotesia kariyai* (Hayakawa, 1990 and 1991). Soon after, the

paralytic peptide (PP) was identified in the tobacco hornworm *Manduca sexta* (Skinner *et al.*, 1991; Wang *et al.*, 1999). Later, SRPs were discovered in the hemolymph of *Spodoptera litura* larvae, whose development was also halted by parasitization with the parasitoid wasp *Microplitis manila* (Yamaguchi *et al.*, 2012). Since then, orthologous cytokine-like peptides have been identified in the hemolymph of six orders of insects: Lepidoptera, Diptera, Hymenoptera, Coleoptera, Hemiptera, and Orthoptera (Matsumoto *et al.*, 2012; Shears and Hayakawa, 2018). SRPs, a subclass of cytokine-like peptides, now identified in various insects, exert significant effects on insect immune responses (Schrag *et al.*, 2016). These peptides, which are usually 22-32 residues long and have a loose consensus sequence, affect the development of the brain, delay growth, cause muscular contraction, and change hemocyte activities. Although they do not strictly follow the conventional definition of cytokines, the term “stress-responsive peptides” is proposed to highlight their role in controlling the immune response of insects, acknowledging their involvement in various biotic and abiotic stress-related functions (Beschinn and Müller, 2012; Dembic, 2015; Schrag *et al.*, 2016).

2.1.2. Structural Features and Biological Functions

The core region of the SRP structure is stabilized by a combination of covalent disulfide bonds, hydrogen bonding with the beta-hairpin structure, and hydrophobic side-chain packing. This amphipathic plane is hypothesized to play a role in peptide binding to their receptors. Accordingly, mutation and amino acid replacement studies suggest that various residues within the core region alter biological activity in various ways (Clark *et al.*, 2001a; 2001b; Watanabe *et al.*, 2006). The peptides' structural integrity is maintained by a single disulfide loop, and appropriate folding is achieved via a loosely defined beta-hairpin sequence. (Schrag *et al.*, 2017b; Schrag *et al.*, 2019). The SRP structural core adopts an epidermal growth factor (EGF)-

like fold that is strikingly similar to the carboxyl-terminal region of the vertebrate EGF, despite the absence of sequence similarity (Yu *et al.*, 1999). Additionally, this structural similarity may imply that the interactions between the SRP receptor and its receptor are comparable to those between EGF and EGFR to initiate the biological activities of this stress response peptide. (Yu *et al.*, 1999; Schrag *et al.*, 2017b).

With the genome sequence of *Manduca sexta* made available by Kanost *et al.*, (2004) eleven open reading frames coding for SRP homologs have been identified (Cao *et al.*, unpublished data). The pre-pro-peptides that are associated with them are usually 60-150 residues long and are typically disordered and inactive. Therefore, when SRPs exist as part of the pre-pro-peptides, they are inactive. After being activated from the inactive proenzyme by numerous stresses such as parasitization, wounding, and environmental changes, the pro-SRP peptide is proteolyzed by the processing enzyme (a serine protease) (Schrag *et al.*, 2016; Hayakawa, 2021). Specific proteolytic cleavage by the extracellular proteases or the intracellular enzymes can release the C-terminal active peptide before storage by secretory vesicles for secretion and release into the extracellular environment (Nakatogawa *et al.*, 2009).

Eleven cytokines are reported in *M. sexta*, including uENF1, uENF2, PP, and 8 SRPs. Figure 2.1.1 shows the sequence alignment for SRP-1 through -6. Their C-termini are aspartic acid residue-rich and they typically end with a tyrosine (Cao *et al.*, unpublished data). While SRP-6 already appears quite distinct from other sequences and may have specific uses, SRP-2 has remarkably comparable sequences to SRP-1 and may also have similar functions.

MsSRP1 FGV**R**VG**T**C**P**SGYV**R**R GTF**C**F**P**DDDDY*
 MsSRP2 FGV**K**DG**K**C**P**SG**R**V**R**RLG I**C**V**P**DDDDY*
 MsSRP3 FLI**K**SSG**C****P****K**GYV**K**R GTF**C**F**P**DEDDYDY*
 MsSRP4 SL **K**RG**T**C**P**D**G**RVLF **G**RV**C**VEIDSLDY*
 MsSRP6 NMIVV**P**P**N**C**P**P**G**QMGSD**G**V**C**RVVFN*

Figure 2.1.1. Sequence alignment of mature *M. sexta* SRP-1, -2, -3, -4, and -6. Highlighted in color are residues that are conserved across the aligned sequences.

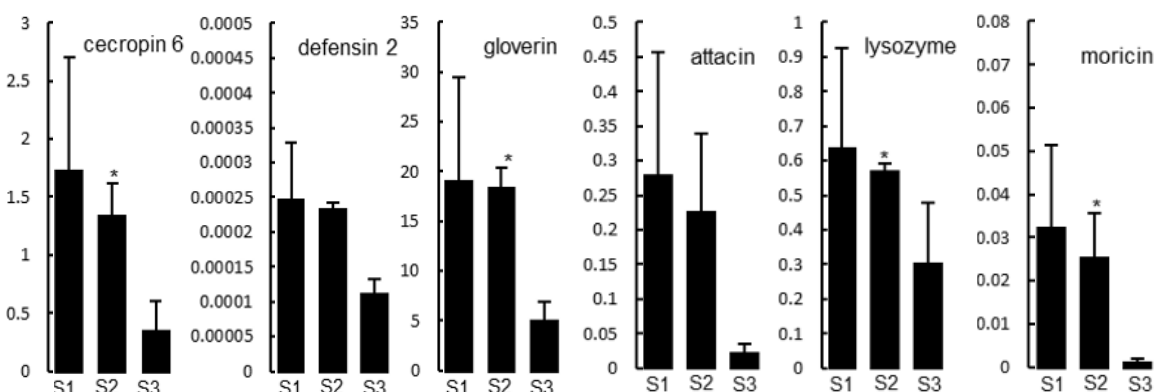


Figure 2.1.2. mRNA expression levels of AMPs by injection of SRP-1, -2, and -3 in *M. sexta* fat body (Jiang *et al.*, unpublished data). *M. sexta* larvae were exposed to active SRP-1, -2, and -3 (S1, S2, S3, respectively), and the fat body's antimicrobial gene expression was assayed. The results were normalized from base gene expression levels in larvae. Exploration of qPCR data demonstrated upregulation of known antimicrobial peptide gene expression within the fat body with regards to base expression after the introduction of SRP-1 and -2. However, injection of SRP-3 demonstrated little to no activity in antimicrobial expression (Jiang *et al.*, unpublished data).

In this chapter, the structure and function of *M. sexta* SRP-3 is described using NMR spectroscopy. Previous structural studies on *M. sexta* SRP-1 and SRP-2 suggest that both peptides display well-defined structures consisting of two beta-strands and a beta-turn in their core region necessary for their biological activity. The biological functions of different SRPs are diverse and we hypothesize that the relative stability and secondary structural features of the peptide core region regulate their function. In this chapter, structural studies using homo and hetero-nuclear multidimensional NMR spectroscopy show that *M. sexta* SRP-3 is a 27-residue

peptide (FLIKSSGCPKGYVKRGTFPDEDYDY), consisting of a disulfide bond between C8 and C19.

2.2. Materials and Methods for SRP-3's Structural Analysis

2.2.2. Peptide Synthesis

27-residue MsSRP-3 (FLIKSSGCPKGYVKRGTFPDEDYDY) was chemically synthesized (acquired from PepMic Ltd, Jiangsu, China) following protocols of step-wise solid phase synthesis using the standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The peptide was purified to >95% purity by reverse-phase HPLC on a C18 column. Its mass and purity were further confirmed by the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Applied Biosystems, Foster City, CA) as shown in Figure 2.2.1. SRP-3 was lyophilized and stored as a dry powder until used for NMR spectroscopy experiments.

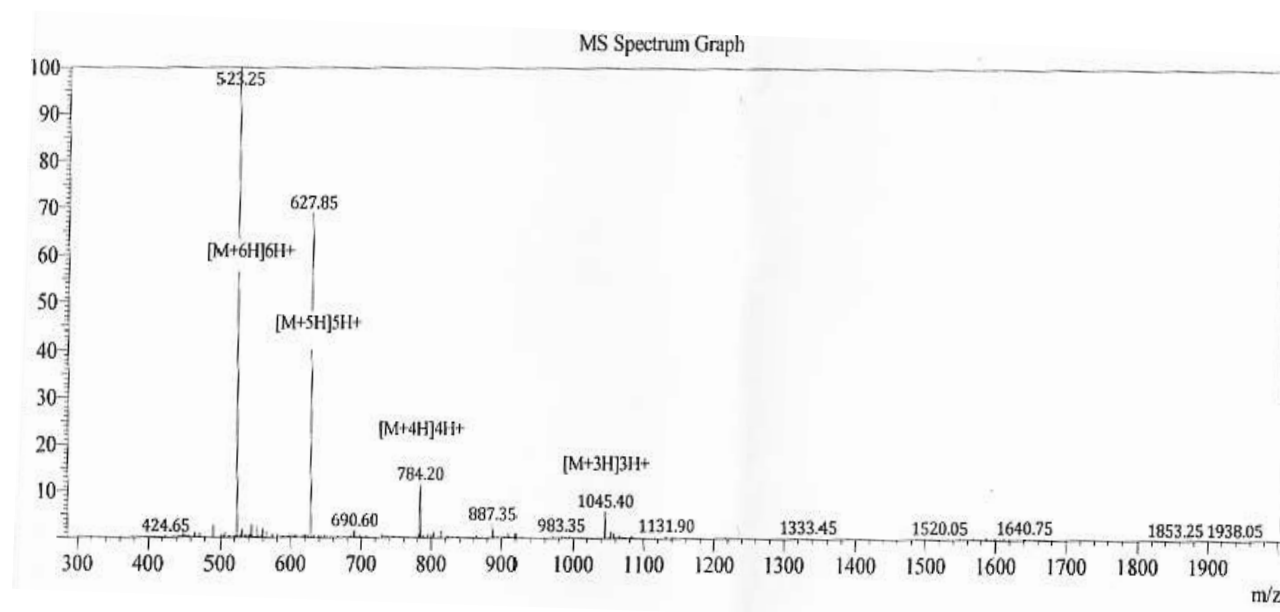


Figure 2.2.1. Mass spectrometry spectrum graph of SRP-3. The mass spectrometry analysis was performed on SRP-3 for its purity and molecular weight. SRP-3 shows a purity of >95% and the observed molecular weight of 3133.50 Da.

2.2.3. Circular Dichroism

Circular Dichroism (CD) analysis was performed with a Jasco-8155 spectropolarimeter (JASCO, Tokyo, Japan) using a 1mm path length cell over 190 to 250 nm range (Soulages *et al.*, 2001). The CD spectra data was acquired at 25°C every 1 nm, with a 2-second averaging time per point and a 1-nm band pass. SRP-3 samples were prepared in concentrations of 50 μ M in pure water with increasing volume/volume percentages (0, 10, 20, 30, 40, 50%) of 2,2,2-trifluoroethanol (TFE). The mean residue ellipticity was expressed as percent ellipticity.

2.2.4. Nuclear Magnetic Resonance Spectroscopy

SRP-3 was dissolved in 10% D₂O/ 90% H₂O with a final concentration of 2.2 mM. The sample was then placed in a 5 mm Wilmad 535 NMR sample tube for NMR experimentation. The NMR experimental data were all acquired at the Biomolecular NMR Core Facility in the Department of Biochemistry and Molecular Biophysics at Kansas State University. 2D ¹H-¹H NMR experiments were performed on a 14 Tesla Bruker AVANCE NEO (Bruker Biospin Corporation, Billerica, MA) NMR spectrometer, operating at 600MHz for proton frequency, using a 5mm Pulse Field Gradient inverse detection triple resonance HCN cryogenic probe. Data acquisition was carried out through standard 2D NMR methodologies: 2D ¹H-¹H Correlation NMR Spectroscopy (COSY), 2D ¹H-¹H Total Correlation NMR Spectroscopy (TOCSY), and a 2D ¹H-¹H Nuclear Overhauser Effect Spectroscopy (NOESY). A total of 512 increments of 2K data points were collected for these experiments. NOESY experiments were performed with 100, 200, 300, and 400 ms mixing times. 2D ¹H-¹H TOCSY spectra were recorded by using MLEV-17 pulse sequence for isotropic mixing for 80 ms at a B1 field strength of 8 KHz. Water peak suppression was obtained by low-power irradiation of the water peak during relaxation delay. 2D ¹H-¹⁵N Heteronuclear Single Quantum Coherence (HSQC) and ¹H-¹³C HSQC NMR spectra were

also recorded to obtain additional information on amide groups and proton-bearing carbons, respectively. The data sets were acquired at a temperature of 25°C. To collect information on hydrogen bonds, temperature gradient experiments were performed through 2D ^1H - ^1H TOCSY data at 5, 10, 15, 20, 25, 30, and 35°C. The processing of all data was performed on Bruker TopSpin 4.1.4. The Collaborative Computational Project for NMR (CCPN) 3.1 program (Skinner *et al.*, 2016) and the Computer Aided Resonance Assignment (CARA) 1.9 software (Keller, 2004) were used for pick peaking and assigning proton chemical shifts. The ^1H chemical shifts were referenced to the external standard 2,2-dimethyl-2-silapentane-5-sulphonic acid (DSS) chemical shift (0.0ppm) at 25°C. ^{15}N and ^{13}C chemical shifts were referenced indirectly from DSS.

2.2.5. Chemical Shifts Assignments and Structure Calculations

Bruker TopSpin 4.1.4 software was used to process the NMR spectra. The Collaborative Computational Project for NMR (CCPN) 3.1 program (Skinner *et al.*, 2005) and the Computer Aided Resonance Assignment (CARA) 1.9 software (Keller, 2004) were used for pick peaking and assigning proton chemical shifts. The Combined Assignment and Dynamics Algorithm for NMR Applications (CYANA) 2.1 program was used for structure calculations from the derived Nuclear Overhauser Effects (NOEs) constraints and chemical shift assignments (Güntert, 2004). The Visual Molecular Dynamics (VMD) 1.9 program was used to visualize and analyze the calculated structures (Humphrey *et al.*, 1996).

2.3. Results and Discussion for SRP-3

2.3.2. CD Data and Preliminary Structural Analysis

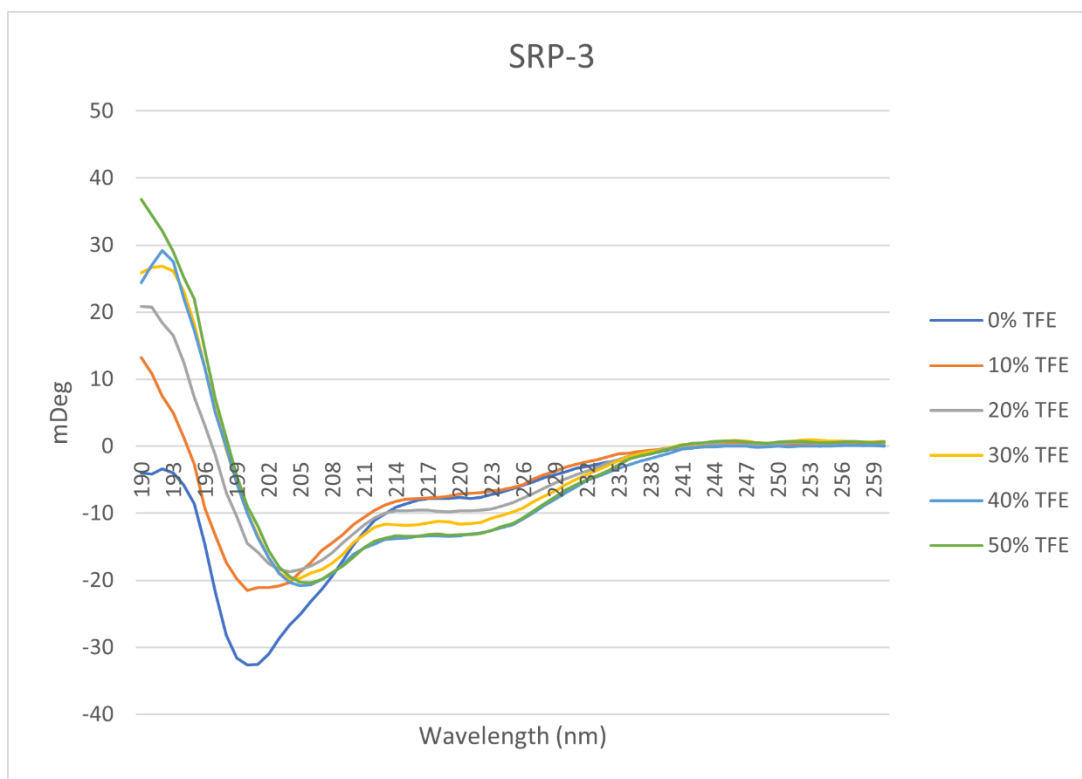


Figure 2.3.1. CD spectra of SRP-3 in v/v percent TFE concentration. SRP-3 samples were prepared to a concentration of 50 μ M in pure water with increasing volume/volume percentages (0, 10, 20, 30, 40, 50%) of 2,2,2-trifluoroethanol (TFE).

CD spectroscopy was performed to analyze the secondary structure and conformational changes of SRP-3. CD data was acquired for SRP-3 at increasing concentration of TFE in H₂O at 25°C as shown in Figure 2.3.1. TFE has the ability to induce and stabilize the secondary structure of the peptide in aqueous solutions. This stabilization occurs because the TFE molecules preferentially aggregate around the peptide. Water molecules around the peptide are displaced by TFE to produce a low dielectric environment that promotes the development of intrapeptide hydrogen bonds (Roccatano *et al.*, 2002). In pure water (0% TFE), SRP-3 did not show any considerable secondary structural characteristics. With increasing TFE concentration,

the structure of the peptide remains inconsistent. As a result, the CD spectrum indicated that SRP-3 was in the conformation of random coil in water.

2.3.3. NMR results and Chemical Shift Assignments

All the 1D and 2D homo- and hetero-nuclear NMR spectra were performed and processed on a Bruker 600 MHz NMR system using Bruker TOPSPIN 4.1.4 software. The amino acid resonances and their proton chemical shifts were assigned using the fingerprint region of 2D ^1H - ^1H TOCSY (Figure 2.3.2). Within the fingerprint region, the NH to side chain protons can be observed, with NH protons typically ranging between 6.5 and 10 ppm in the first dimension, and side chain protons falling within the 0 to 6 ppm range in the second dimension. All amino acid resonances, except for proline due to its lack of an amide proton, can be found within this region. To determine the chemical shifts of P9 and P21, the crosspeaks from 0 to 4.5 ppm in both dimensions of 2D ^1H - ^1H TOCSY were analyzed (Figure 2.3.3). This region encompassed the crosspeaks of aliphatic side chain protons of amino acids as well as of proline. This fingerprint region of COSY and NOESY spectra were analyzed for proton chemical shift assignments and identification of amino acid residues present in the peptide. The summary of the proton chemical shift assignments is summarized in Table 1.

To further elucidate the core region of SRP-3, an analysis of 2D ^1H - ^1H TOCSY temperature gradient experiments was conducted (Figure 2.3.4a). Amino acid resonances whose amide proton chemical shifts remained unchanged with different temperatures suggested that those amide protons were involved in hydrogen-bonded conformation. The analysis revealed that residues Gly11, Val13, Arg15, and Thr17 were engaged in hydrogen bonding, as evidenced by their consistency in proton chemical shifts across temperatures ranging from 5 to 35°C (Figure

2.3.4b). This analysis was done in conjunction with cross-referencing the lowest energy structure of SRP-3 to identify residues that are involved in hydrogen bonding as depicted in Figure 2.3.5.

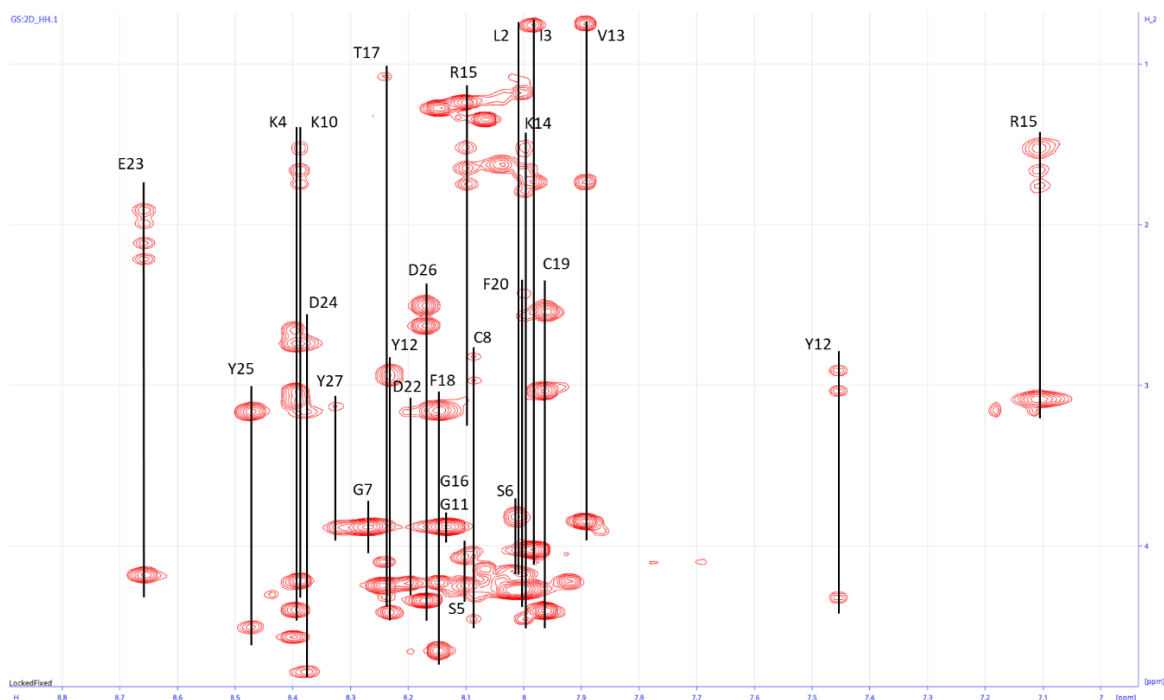


Figure 2.3.2. SRP-3 fingerprint region (NH-C α H/side chain protons) of 2D ^1H - ^1H TOCSY NMR spectrum. The vertical lines mark each individual amino acid spin systems of SRP-3.

Table 1. SRP-3 proton chemical shift (ppm) assignments

Residue	HN (ppm)	HA (ppm)	HB (ppm)	HD and others (ppm)
1PHE	-	4.397	3.03	Hδ 7.223 Hε 7.105
2LEU	7.975	4.075	1.708, 1.753	Hδ 0.738, 1.033 Hγ 1.313
3ILE	7.884	3.849	1.74	Hδ 0.753 Hγ 1.045, 1.289
4LYS	8.095	3.886	1.731	Hδ 1.627 Hγ 1.544 Hε 3.092
5SER	8.127	4.253	3.878	-
6SER	7.996	4.43	3.881	-
7GLY	8.277	3.881	-	-
8CYS	8.142	4.632	2.738, 3.134	-
9PRO	-	4.407	2.221	Hδ 3.626, 3.75 Hγ 1.951
10LYS	8.384	4.232	1.66, 1.738	Hδ 1.634 Hγ 1.52 Hε 3.127
11GLY	8.235	4.099, 4.243	-	-
12TYR	8.228	4.414	2.906, 2.954	Hδ 7.013 Hε 6.707
13VAL	8.017	4.144	1.221	Hγ 1.081, 1.074
14LYS	7.992	4.241	1.669, 1.804	Hδ 1.632 Hγ 1.517 Hε 3.075
15ARG	8.094	4.236	1.645, 1.724	Hδ 3.081 Hγ 1.513 Hε 7.102
16GLY	8.008	3.821	-	-
17THR	8.056	4.129	3.85	Hγ 1.343
18PHE	8.165	4.628	2.881, 2.971	Hδ 7.1
19CYS	8.384	4.543	2.659, 2.736	-
20PHE	8.368	4.778	2.735, 3.151	-
21PRO	-	4.427	1.957, 2.22	Hδ 3.627, 3.749 Hγ 1.833
22ASP	8.385	4.396	3.033, 3.094	-
23GLU	8.65	4.174	1.906, 2.016	Hγ 2.113, 2.209
24ASP	8.096	4.066	2.919, 2.956	-
25TYR	8.081	4.445	2.849, 2.962	-
26ASP	7.961	4.395	2.541, 3.031	-
27TYR	8.164	4.256	2.499, 2.632	-

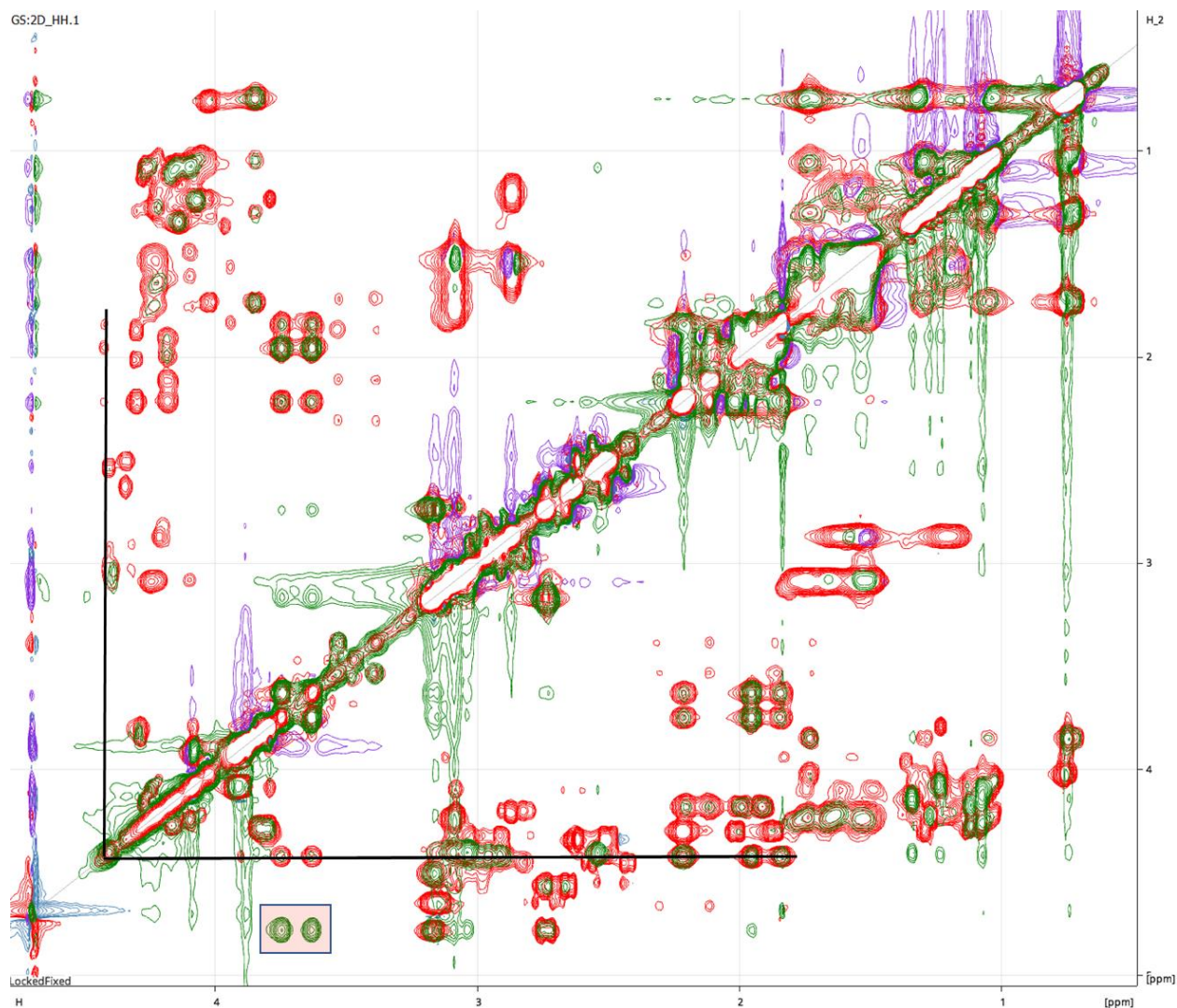


Figure 2.3.3. SRP-3 ^1H - ^1H TOCSY side chain region (red) overlaid with NOESY (green). Shown in purple are the negative contours of the ^1H - ^1H NOESY spectrum. The lines indicate the spin system of Pro21. The shaded area displays the NOE connectivity between the alpha protons ($\text{C}\alpha\text{H}$) of Phe20 and delta protons ($\text{C}\delta\text{H}$) of Pro21. This strong NOE connectivity also indicates that Pro21 is in a trans conformation with Phe20.

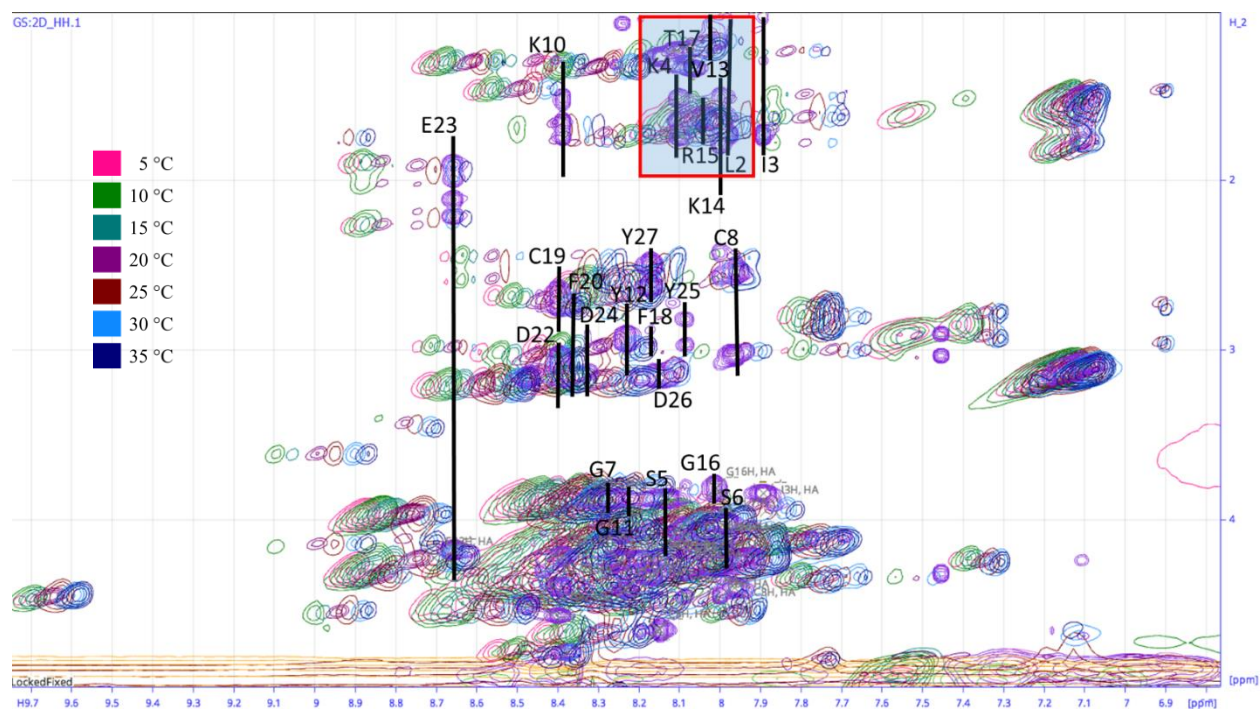


Figure 2.3.4a. 2D ^1H - ^1H TOCSY temperature gradient experiment. ^1H - ^1H TOCSY was collected at 5°C, 10°C, 15°C, 20°C, 25°C, 30°C and 35°C to determine exchanging amide protons. The shaded area indicates the region where amino acid resonances whose chemical shifts did not fluctuate much between different temperatures, which is zoomed in Figure 2.3.4b.

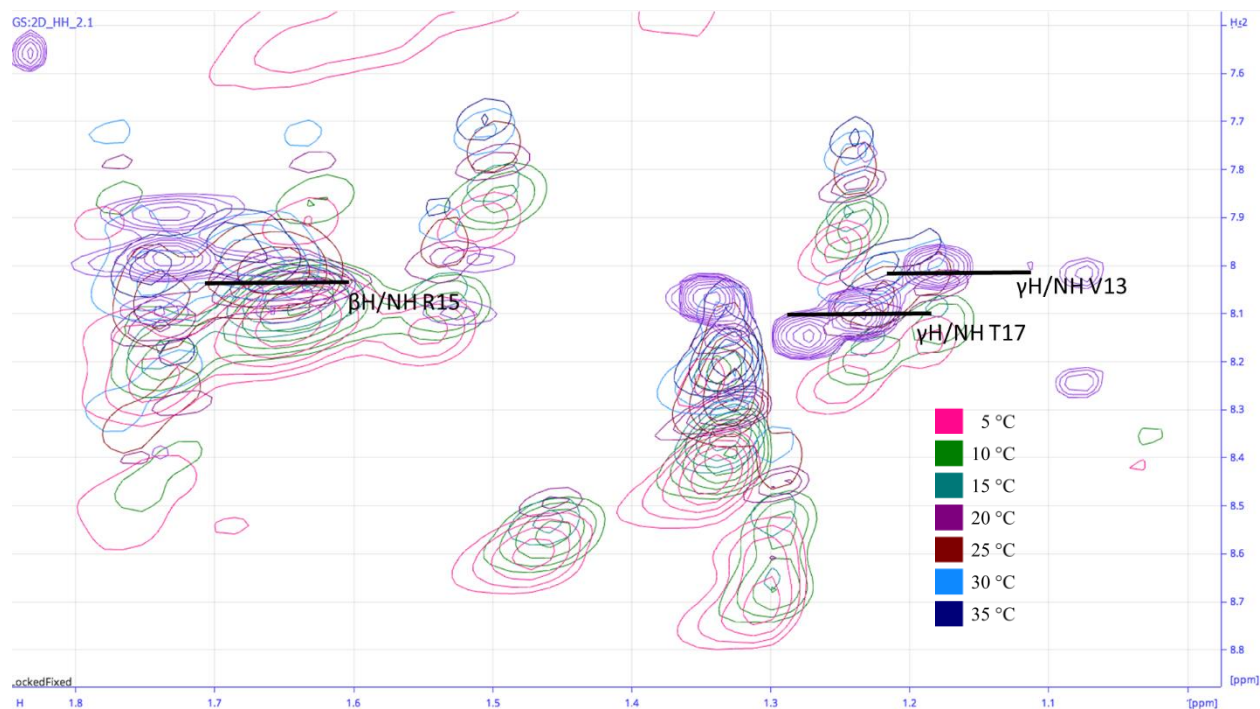


Figure 2.3.4b. 2D ^1H - ^1H TOCSY temperature gradient experiment. A zoomed in view of the shaded area from Figure 2.3.4a, represents the amino acid resonances whose proton chemical shifts did not alter as much. The residues not involved in amide proton exchanging are Gly11 (not displayed here in this figure), Val13, Arg15, and Thr17. Temperature-dependent NMR experiments offer a view of which amino acids are involved in hydrogen bonding as residues involved would not be affected by temperature changes. Thus, hydrogen bond constraints for Gly11, Val13, Arg15, and Thr17 were included in structure calculations as depicted in Figure 2.3.5.

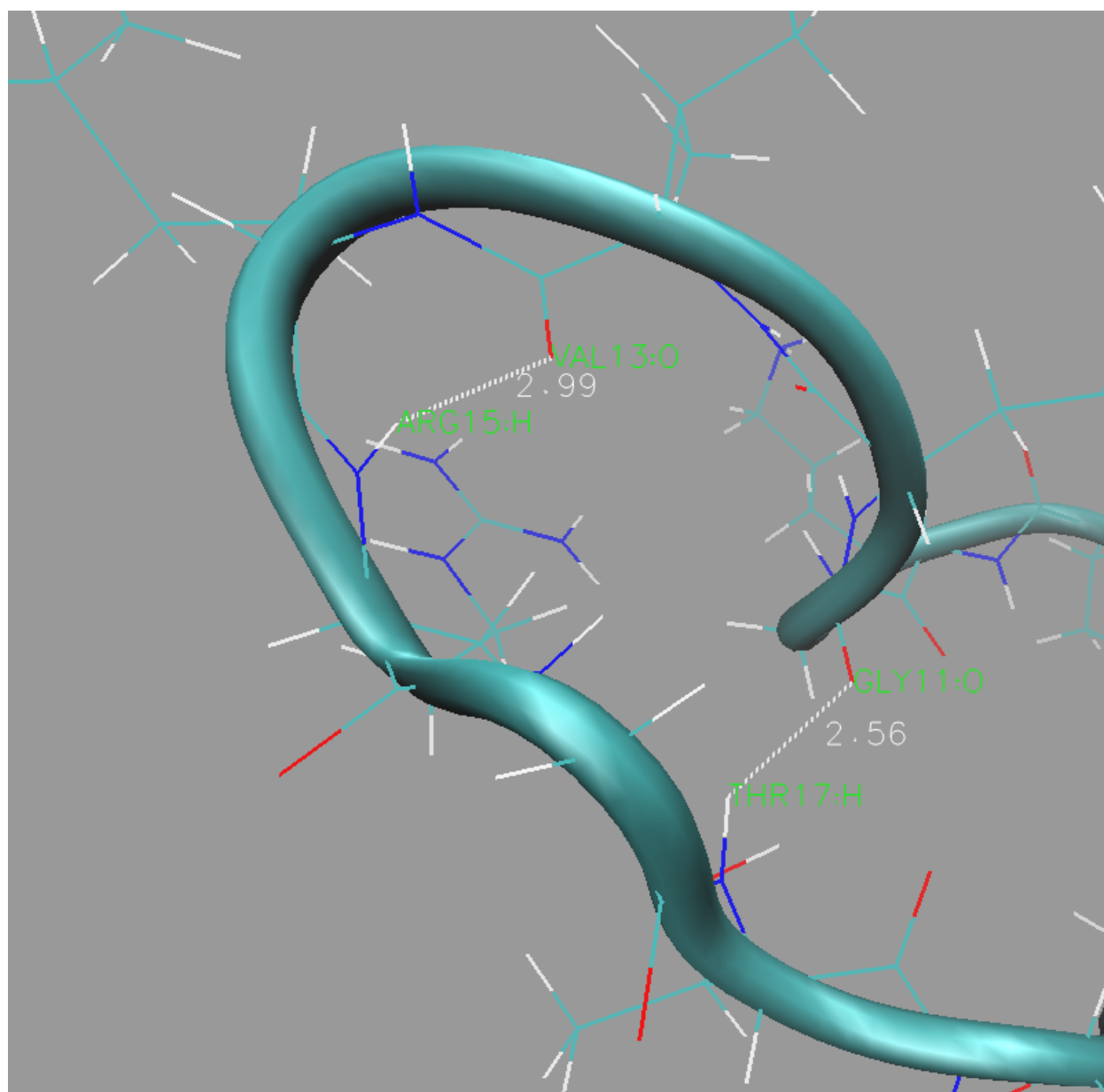


Figure 2.3.5. Pictorial representation of the hydrogen bonds present in SRP-3. Hydrogen bond constraints were added based on analysis of 2D ^1H - ^1H TOCSY temperature gradient NMR experiments. The lowest energy structure of SRP-3 is displayed with dotted lines indicating hydrogen bonds between Gly11 – Thr17, and Val13 – Arg15.

2.3.4. Secondary Structure Prediction and Structure Calculations

The proton chemical shifts are strongly dependent on the conformation and the state of the peptide's secondary structure (Wishart *et al.*, 1992). More specifically, the C α protons' chemical shift for all amino acids undergoes an upfield shift relative to the random coil value when arranged in a helical conformation, and a comparable downfield shift in a beta-strand conformation. To determine the preliminary secondary structure of SRP-3, the chemical shift index (CSI) values were calculated for each residue (Table 2) by taking the difference from the observed C α protons' chemical shift values with Wishart's "random coil" chemical shift values (Wishart *et al.*, 1994). The formula used is: $CSI = (\delta_{obs} - \delta_{ref})$, where δ_{obs} is the observed C α protons' chemical shift, and δ_{ref} is the Wishart C α protons' reference values. The CSI values are charted to observe any presence of secondary structural features (Figure 2.3.6). Consistent stretches of negative CSI values typically suggest the presence of helical conformations, whereas consistent positive stretches of CSI values are indicative of a beta-strand. From the charted CSI table for SRP-3, its conformation appears to be disordered throughout the length of the peptide as indicated by the inconsistent CSI values.

By measuring the intensity of NOE cross-peaks in the 2D ^1H - ^1H NOESY spectrum, distance constraints between specific protons were inferred and used to calculate the 3D structures of SRP-3. These distance constraints, along with other experimental data such as hydrogen bond constraints, were used as input into CYANA which then utilized computational algorithms to generate a set of structures that satisfy as many of the experimental restraints as possible. 200 such conformers were generated for SRP-3 and 20 of the lowest energy structures were analyzed for any restraint violations and validation against experimental data in each round of calculations. After the last round of structure calculations, the result displayed a 3D model of

SRP-3 which generated no violations greater than 0.3Å. The structural statistics for the 20 lowest energy structures of SRP-3 are summarized in Table 3. The superimposition of the 20 lowest energy structures as well as the lowest energy structure of SRP-3 are shown in Figure 2.3.7. These structures represent the most energetically favorable conformations of SRP-3. The NOE connectivity between specific protons of SRP-3, including both sequential and non-adjacent NOE connectivities, is summarized in Figure 2.3.8. Structural analyses conducted on SRP-1, and SRP-2 have unveiled shared structural patterns, notably beta-strands and beta-turns. Contrastingly, SRP-3 lack the propensities for both beta strands and beta-turn formation present in SRP-1 and -2. Although, the core region of SRP-3 is relatively stabilized by the disulfide linkage as well as hydrogen bonds.

Table 2. SRP-3 CSI (ppm) calculated values

Residue	HA (ppm)	Wishart Value (ppm)	CSI (ppm)
1PHE	4.397	4.66	-0.263
2LEU	4.075	4.17	-0.095
3ILE	3.849	3.95	-0.101
4LYS	3.886	4.36	-0.474
5SER	4.253	4.5	-0.247
6SER	4.43	4.5	-0.07
7GLY	3.881	3.97	-0.089
8CYS	4.632	4.65	-0.018
9PRO	4.407	4.44	-0.033
10LYS	4.232	4.36	-0.128
11GLY	4.099	3.97	0.129
12TYR	4.414	4.6	-0.186
13VAL	4.144	3.95	0.194
14LYS	4.241	4.36	-0.119
15ARG	4.236	4.38	-0.144
16GLY	3.821	3.97	-0.149
17THR	4.129	4.35	-0.221
18PHE	4.628	4.66	-0.032
19CYS	4.543	4.65	-0.107
20PHE	4.778	4.66	0.118
21PRO	4.427	4.44	-0.013
22ASP	4.396	4.76	-0.364
23GLU	4.174	4.29	-0.116
24ASP	4.066	4.76	-0.694
25TYR	4.445	4.6	-0.155
26ASP	4.395	4.76	-0.365
27TYR	4.256	4.6	-0.344

The CSI values were calculated from the difference between each residue's observed C α -H and its corresponding Wishart value (Wishart et al., 1992).

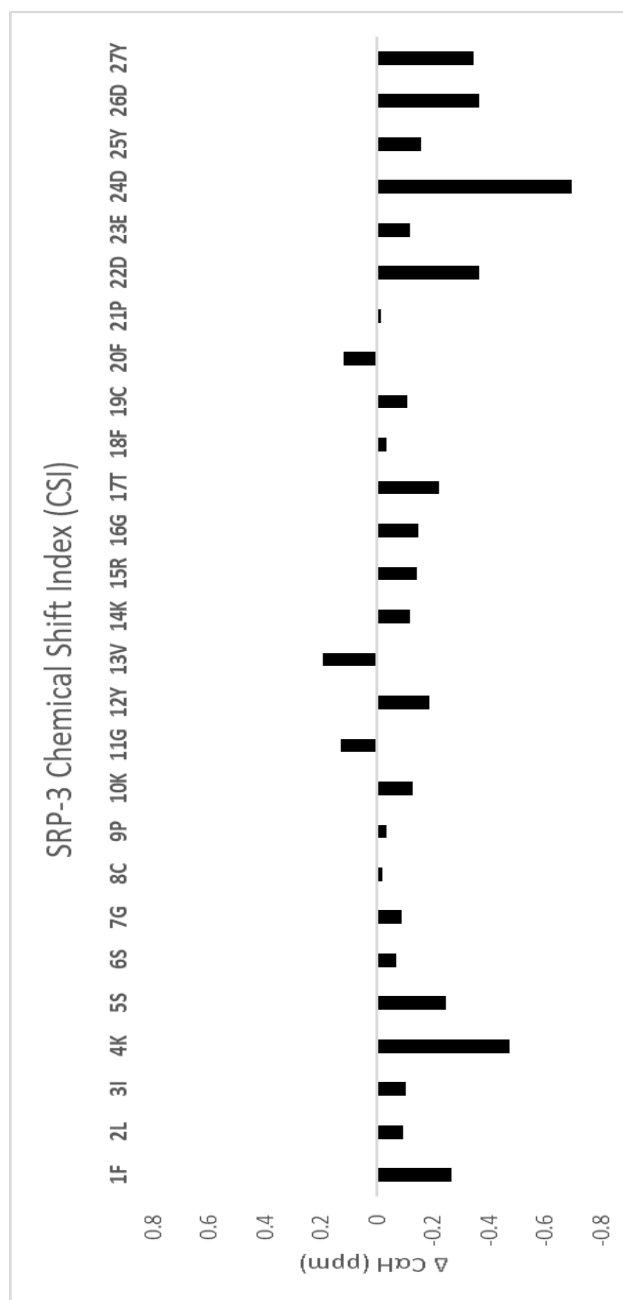


Figure 2.3.6. $C\alpha H$ chemical shift index of SRP-3. Consistent positive chemical shift index (CSI) values indicate the presence of beta-strands whereas consistent stretches of negative CSI values indicate the presence of alpha-helices or random coil conformations. Accordingly, SRP-3 shows no indication of beta-strands and alpha-helices.

Table 3. Structural statistics of the 20 lowest energy structures of SRP-3

NOE Constraints	Number
Total	186
Intra-residue	121
Sequential	37
Medium Range (<i+5)	17
Long Range ($\geq i+5$)	1
Disulfide Bonds	2
Hydrogen Bonds	4
Constraints per residue	6.89
Restraint Violations	Number
NOE > 0.3 Å	0
RMSD from the mean structure (residues 10-18)	Å
Backbone atoms	1.36 ± 0.34
Heavy atoms	3.01 ± 0.63
Ramachandran Analysis for the lowest energy structure	%
Residues in Allowed (>99.8%) Regions	
RMSD from Ideal Geometry	Å
Bond Angles	0.2
Bond Lengths	0.001
Number of close contacts (1.6 Å for H atoms, 2.2 Å for heavy atoms)	0

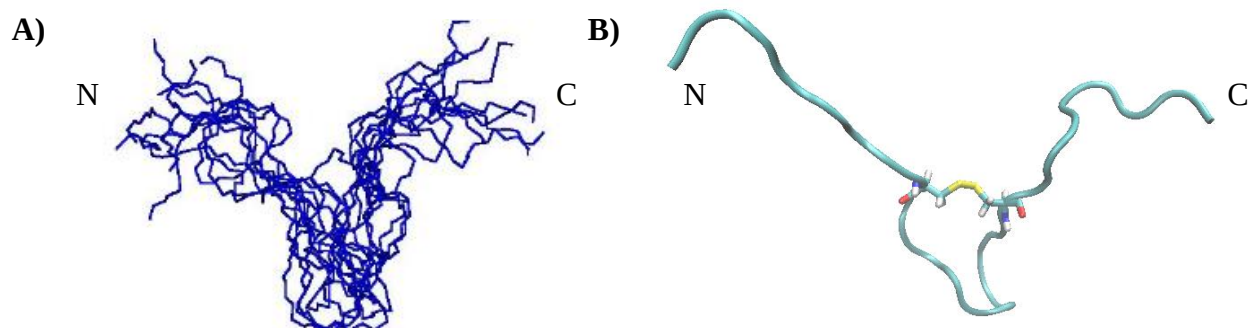


Figure 2.3.7. Structural representation of SRP-3. (A) Superimposition of the 20 lowest energy NMR structures of SRP-3. (B) Ribbon diagram for the lowest energy structure of SRP-3 with the disulfide bond depicted.

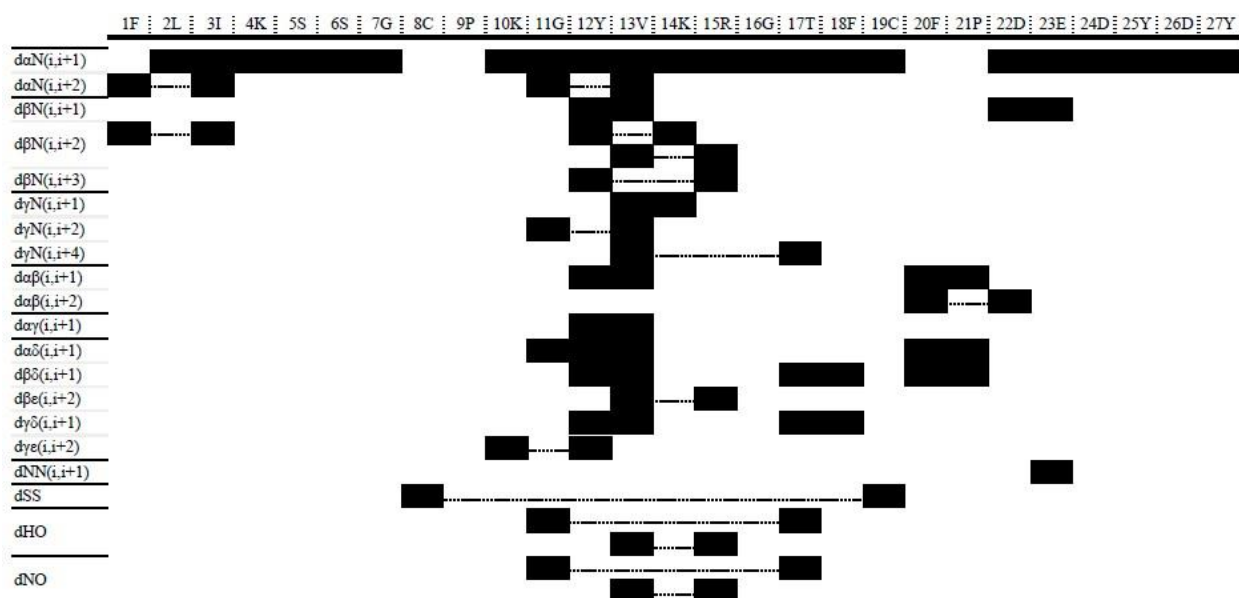


Figure 2.3.8. Summary of the NOE connectivities for SRP-3 in water. The bars indicate NOE connectivity between residues. The left column represents the distance between corresponding atoms. Denoted by dotted lines are non-sequential NOE connectivities.

2.4. Conclusions

Our results provide important insights as to the differences in receptor(s) preference that result in the nuanced variation of biological expression of AMP mRNAs observed in *M. sexta*. The NMR data suggests that SRP-3 displays a less propensity for beta-strands and beta-turn formation in contrast to SRP-1, and -2, explaining its diverse function than other SRPs. Although SRP-3 appears more disordered compared to those of SRP-1 and -2, the core region of SRP-3 remains relatively stable due to the presence of the disulfide bond and hydrogen bonds. The lack of beta-strands may have significant implications for its biological functions.

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Chapter 3 – NMR Structural Studies of Insect Cytokines: *Manduca sexta* Stress-Responsive Peptide-6

3.1. Introduction

In this chapter, the focus shifts to the investigation of *M. sexta* SRP-6 in the ongoing study of SRPs. SRP-6 is a 26-residue peptide (NMIVVPPNCPPGQQMGSDGVCRVVFN) stabilized by a disulfide bond between residues Cys9 and Cys21. *M. sexta* SRP-6 is a new type of cytokine, which inhibits the feeding and growth of larvae efficiently. After injection into the hemocoel, synthetic *M. sexta* SRP-6 efficiently suppresses the larval feeding behavior and affects growth (Jiang *et al.*, unpublished data). The bodyweight fold change by injection of SRP-6 is demonstrated in Figure 3.1.1. The growth inhibition by SRP-1 and SRP-2 contrastingly is not so significant. Both SRP-1 and SRP-2 exhibit well-defined structures consisting of two beta-strands and a beta-turn in their core region according to previous structural studies (Schrag *et al.*, 2017a; Schrag *et al.*, 2019). The structural analysis of SRP-4 also demonstrated two beta-strands and a beta hairpin (Miller *et al.*, unpublished data). Although SRP-3 follows a similar consensus sequence to the other SRPs, SRP-3 has been shown to have contrasting biological functions (i.e. antimicrobial peptide synthesis) from SRP-1 and -2 (Figure 2.1.3). The diverse roles played by the SRP family within the insect immune system make each of their structure-activity relationships compelling. Understanding the structural details of SRPs would provide valuable insights into the molecular mechanisms by which SRPs modulate the immune system.

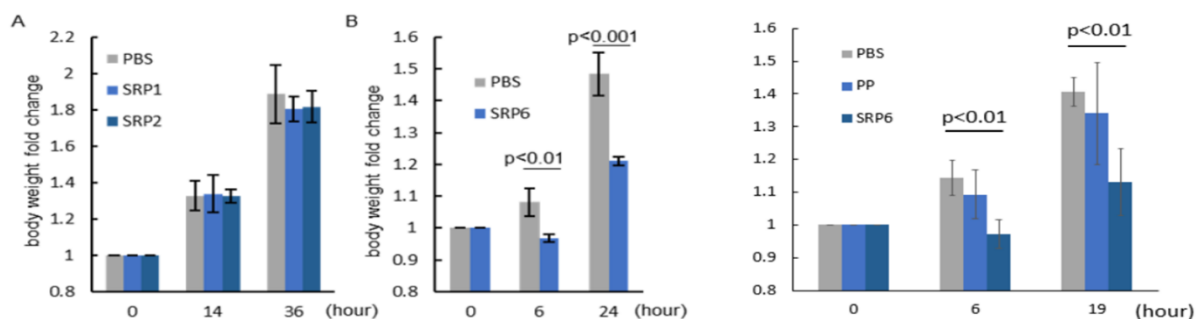


Figure 3.1.1. Bodyweight fold change over time by injection of SRP-1, -2, -6, and paralytic peptide (Jiang *et al.*, unpublished data). *M. sexta* larvae were injected with SRP-1, -2, -6, and paralytic peptide (PP) as well as phosphate-buffered saline (PBS) as the control. This unpublished data was provided by Dr. Haobo Jiang's lab at Oklahoma State University.

By using homo and hetero-nuclear 2D NMR spectroscopy, the solution structure of SRP-6 was elucidated. It is now understood that various SRPs exhibit diverse biological functions which may be dependent on the relative stability and secondary structural characteristics of the peptides' core region.

3.2. Materials and Methods for SRP-6's Structural Analysis

3.2.2. Peptide Synthesis

26-residue MsSRP-6 (NMIVVPPNCPGQQMGS DGVCRVVFN) was synthesized chemically using the standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and step-wise solid phase synthesis protocols. It was obtained from PepMic Ltd. in Jiangsu, China. The peptide underwent reverse-phase HPLC on a C18 column to achieve a purity level of above 97%. The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Applied Biosystems, Foster City, CA) provided additional confirmation of its mass and purity. Lyophilized SRP-6 was kept as a dry powder until NMR spectroscopy experimentation.

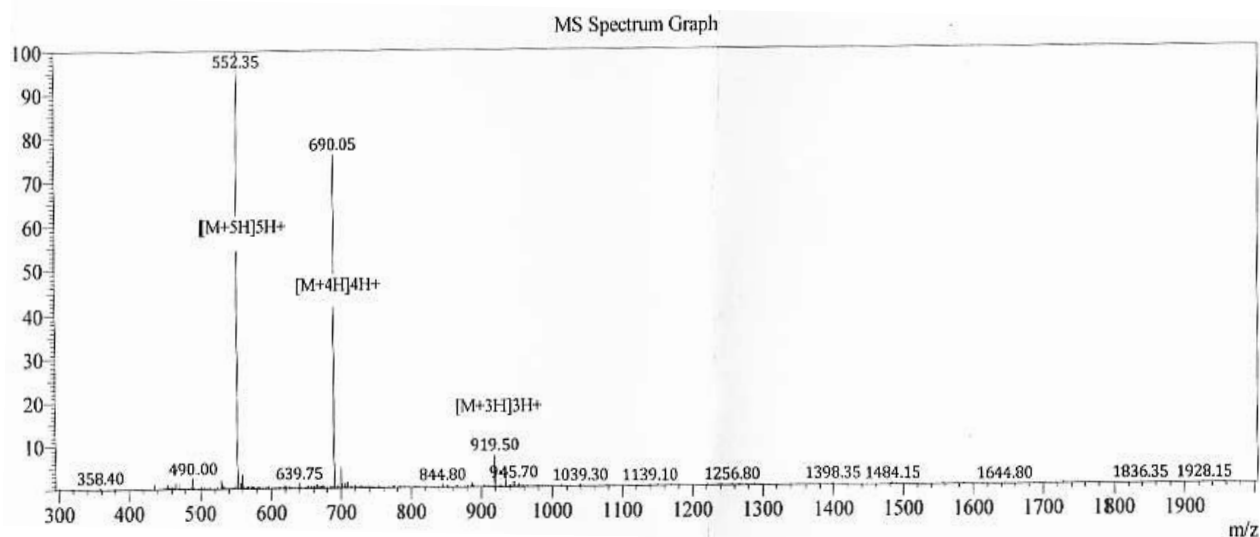


Figure 3.2.1. Mass spectrometry spectrum graph of SRP-6. The mass spectrometry analysis was performed on SRP-3 for its purity and molecular weight. SRP-6 shows a purity of >97% and the observed molecular weight of 2756.75 Da.

3.2.3. Circular Dichroism

Circular Dichroism (CD) analysis was performed with a Jasco-8155 spectropolarimeter (JASCO, Tokyo, Japan) using a 1mm path length cell over 190 to 250 nm range as previously described (Soulages *et al.*, 2001). The CD spectra data was acquired at 25°C every 1 nm, with a 2-second averaging time per point and a 1-nm band pass. SRP-6 samples were prepared in concentrations of 50 μ M in pure water with increasing volume/volume percentages (0, 10, 20, 30, 40, 50%) of 2,2,2-trifluoroethanol (TFE). The mean residue ellipticity was expressed as percent ellipticity.

3.2.4. Nuclear Magnetic Resonance Spectroscopy

SRP-6 was dissolved to a final concentration of 2.4 mM in 10% D₂O/ 90% H₂O and placed in a 5 mm Wilmad 535 NMR sample tube for NMR experimentation. All NMR experimental data were collected at Kansas State University's Biomolecular NMR Core Facility in the Department of Biochemistry and Molecular Biophysics. 5mm Pulse Field Gradient inverse

detection triple resonance HCN cryogenic probe was used for 2D ^1H - ^1H NMR experiments on a 14 Tesla Bruker AVANCE NEO (Bruker Biospin Corporation, Billerica, MA) NMR spectrometer operating at 600MHz for proton frequency. The same 2D NMR methodologies used to collect SRP-3 data, as described in chapter 2, were collected for SRP-6 as well, which includes 2D ^1H - ^1H COSY, 2D ^1H - ^1H TOCSY, and 2D ^1H - ^1H NOESY. These experiments were recorded at a temperature of 25°C with 512 increments of 2K data points. The 2D ^1H - ^1H NOESY experiments were performed with 100, 200, 300, and 400 ms mixing times, and 2D ^1H - ^1H TOCSY spectra were obtained by using MLEV-17 pulse sequence for isotropic mixing for 80 ms at a B1 field strength of 8 KHz. Additionally, temperature gradient experiments were performed through 2D ^1H - ^1H TOCSY data at 5, 10, 15, 20, 25, 30, and 35°C. Furthermore, 2D ^1H - ^{15}N HSQC and ^1H - ^{13}C HSQC NMR spectra were recorded as well. Water peak suppression was achieved by low-power irradiation of the water peak during relaxation delay. The external standard 2,2-dimethyl-2-silapentane-5-sulphonic acid (DSS) chemical shift (0.0ppm) at 25°C is used as a reference for the ^1H chemical shifts. Chemical changes for ^{15}N and ^{13}C were indirectly cited from DSS.

3.2.5. Chemical Shifts Assignments and Structure Calculations

CCPN 3.1 program (Skinner et al., 2016) and CARA 1.9 software (Keller, 2004) were used for peak picking and assigning proton chemical shifts. All data processing was done using Bruker TopSpin 4.1.4. The CYANA 2.1 program was used for structure calculations from the derived Nuclear Overhauser Effects (NOEs) constraints and chemical shift assignments (Güntert, 2004). VMD 1.9 program was used to visualize and analyze the calculated structures (Humphrey et al., 1996).

3.3. Results and Discussion for SRP-6

3.3.2. CD Data and Preliminary Structural Analysis

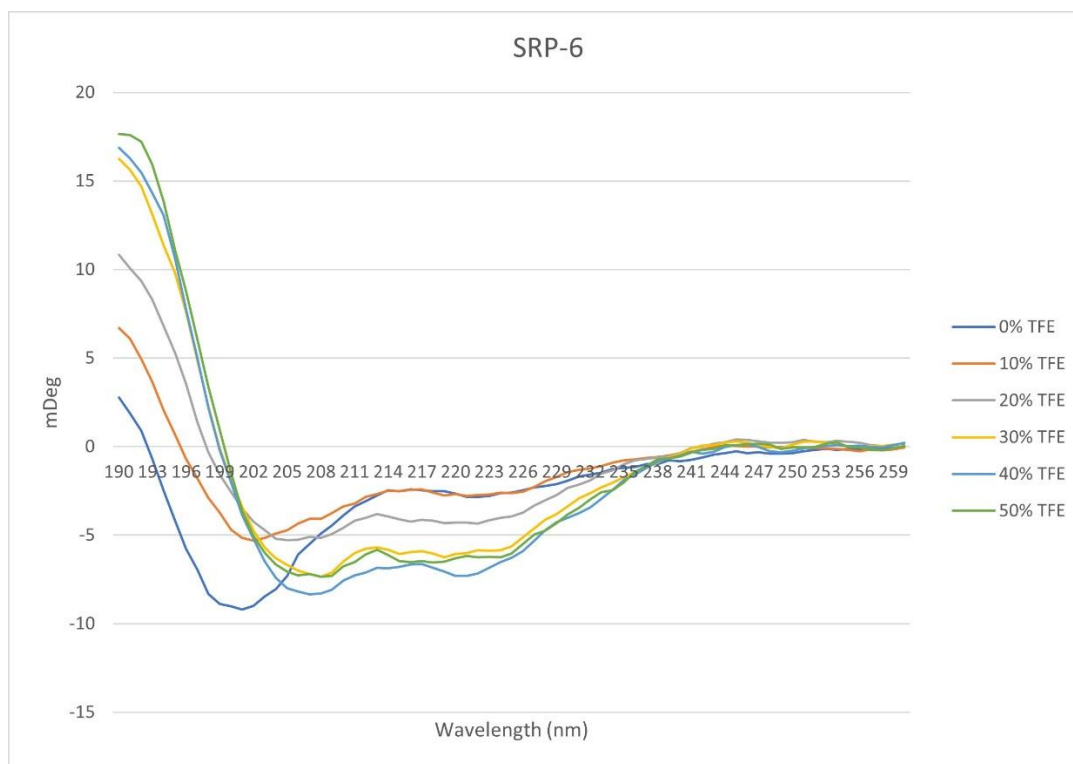


Figure 3.3.1. CD Spectra of SRP-6 in v/v percent TFE concentration. SRP-6 samples were prepared to a concentration of 50 μ M in pure water with increasing volume/volume percentages (0, 10, 20, 30, 40, 50%) of 2,2,2-trifluoroethanol (TFE). The mean residue ellipticity was expressed as percent ellipticity.

The CD spectrum indicated that SRP-6 was in random coil conformation in water. However, in the presence of TFE, the spectrum exhibited minima at 208 and 222 nm, particularly in the presence of 40% TFE in water, which may be indicative of α -helical conformation. Different solvent conditions are underway to gain further insights into the structure and dynamics of SRP-6.

3.3.3. NMR results and Chemical Shift Assignments

Proton chemical shifts were picked and assigned using the CCPN 3.1 program (Skinner et al., 2016) and the CARA 1.9 software (Keller, 2004). The fingerprint region of 2D ^1H - ^1H TOCSY was used to assign the amino acid resonances and associated proton chemical shifts as shown in Figure 3.3.2. A summary of proton chemical shift assignments is presented in Table 4.

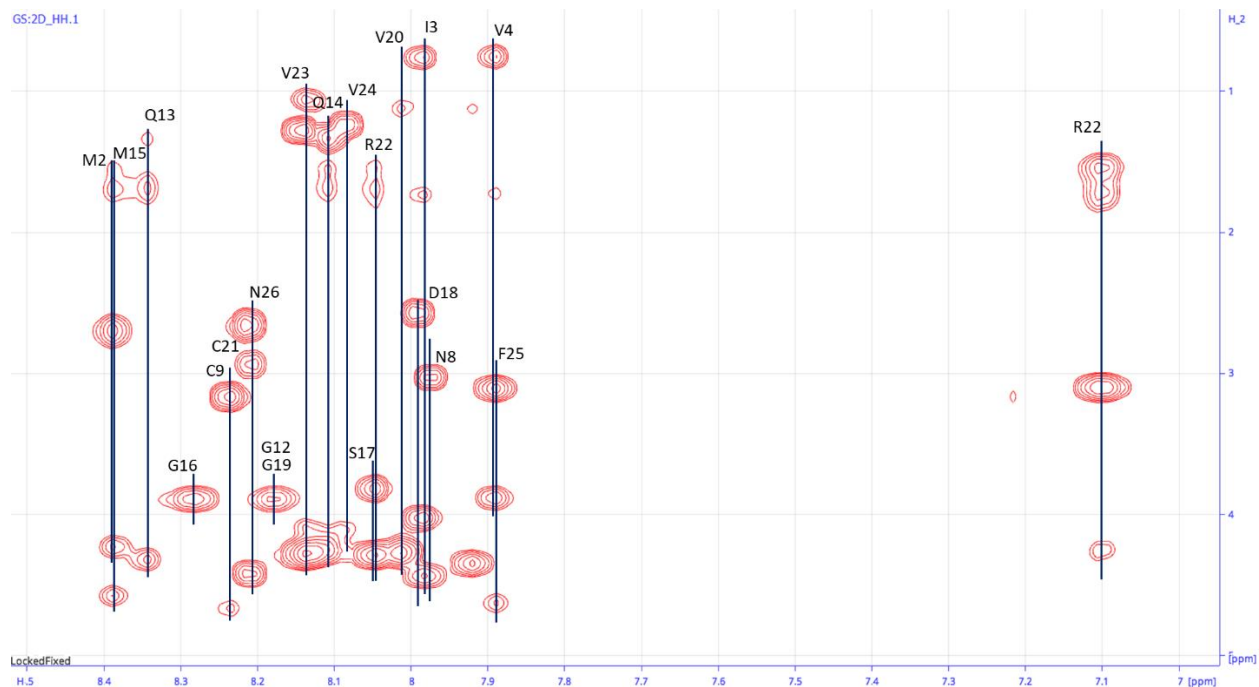


Figure 3.3.2. SRP-6 fingerprint region (NH-C α H/side chain protons) of 2D ^1H - ^1H TOCSY NMR spectrum. The vertical lines mark each individual amino acid spin systems of SRP-6.

Table 4. SRP-6 proton chemical shift (ppm) assignments

Residue	HN (ppm)	HA (ppm)	HB (ppm)	HD and others (ppm)
1ASN		4.427	2.949	
2MET	8.38	4.23	1.58	Hγ 2.65, 2.73
3ILE	7.98	4.37	1.73	Hδ 0.72 Hγ 1.31, 1.05, 0.76
4VAL	7.89	3.88	1.73	Hγ 0.76
5VAL	8.11	4.12	1.73	Hγ 1.34
6PRO		4.429	2.57	Hδ 2.89 Hγ 1.51
7PRO		4.273	2.57	Hδ 2.89 Hγ 1.51
8ASN	7.98	4.43	3.03	HNδ 7.05, 6.82
9CYS	8.23	4.66	3.16	
10PRO		4.3	1.673	Hδ 3.27 Hγ 1.52
11PRO		4.336	2.57	Hδ 3.26 Hγ 1.51
12GLY	8.186	3.89		
13GLN	8.34	4.32	1.34	Hγ 1.67, 1.75 Hϵ 8.14
14GLN	8.11	4.12	1.34	Hγ 1.67, 1.73 Hϵ 8.13
15MET	8.39	4.58	1.76, 1.67	Hγ 3.09 Hϵ 1.53
16GLY	8.28	3.89		
17SER	8.05	4.29	3.82	
18ASP	7.99	4.43	2.57	
19GLY	8.18	3.89		
20VAL	7.99	4.03	1.73	Hγ 0.76
21CYS	8.23	4.66	3.16	
22ARG	8.05	4.26	1.8	Hδ 3.1 Hγ 1.52 Hϵ 7.1
23VAL	8.14	4.17	1.73	Hγ 1.27
24VAL	8.09	4.08	1.73	Hγ 1.23
25PHE	7.89	4.63	3.1	Hδ 7.02 Hϵ 6.72
26ASN	8.21	4.42	2.94	

3.3.4. Secondary Structure Prediction and Structure Calculations

The conformation and secondary structure of peptides influence the proton chemical shifts (Wishart *et al.*, 1992). As such, the chemical shift index (CSI) values for SRP-6 were calculated by taking the difference between the observed C α proton chemical shifts for all amino acids within SRP-6 and the reference C α proton “random coil” chemical shift values (Wishart *et al.*, 1994). The formula used is: $CSI = (\delta_{obs} - \delta_{ref})$, where δ_{obs} is the observed C α protons’ chemical shift, and δ_{ref} is the Wishart C α protons’ reference values. As such, the CSI values are calculated for each residue of SRP-3 as shown in Table 5. The CSI values are then charted to observe any presence of secondary structural features as shown in Figure 3.3.3. The presence of consistent stretches of negative CSI values typically indicates the presence of helical conformations, while consistent positive stretches suggest the presence of beta-strand secondary structures. The charted CSI table for SRP-6 (Figure 3.3.3) indicates that the peptide’s conformation is disordered throughout its length, as evidenced by the inconsistent CSI values.

Using NOE-derived distance constraints obtained from the 2D ^1H - ^1H NOESY spectrum, the 3D solution structure of SRP-6 is calculated through the CYANA program which inputs these constraints along with hydrogen bond information to produce conformers that satisfy the given experimental restraints. A total of 200 conformers were generated and 20 of the lowest energy structures were used for analysis. The structural statistics for the 20 lowest energy structures of SRP-6 are summarized in Table 6 and the superimposition of the 20 lowest energy structures as well as the lowest energy structure of SRP-6 are shown in Figure 3.3.4. The NOE connectivity between specific protons of SRP-6, including both sequential and non-adjacent NOE connectivities, is summarized in Figure 3.3.5.

Table 5. SRP-6 CSI (ppm) calculated values

Residue	HA (ppm)	Wishart Value (ppm)	CSI (ppm)
1ASN	4.427	4.75	-0.323
2MET	4.23	4.52	-0.29
3ILE	4.37	3.95	0.42
4VAL	3.88	3.95	-0.07
5VAL	4.12	3.95	0.17
6PRO	4.429	4.44	-0.011
7PRO	4.273	4.44	-0.167
8ASN	4.43	4.75	-0.32
9CYS	4.66	4.65	0.01
10PRO	4.3	4.44	-0.14
11PRO	4.336	4.44	-0.104
12GLY	3.89	3.97	-0.08
13GLN	4.32	4.37	-0.05
14GLN	4.12	4.37	-0.25
15MET	4.58	4.52	0.06
16GLY	3.89	3.97	-0.08
17SER	4.29	4.5	-0.21
18ASP	4.43	4.76	-0.33
19GLY	3.89	3.97	-0.08
20VAL	4.03	3.95	0.08
21CYS	4.66	4.65	0.01
22ARG	4.26	4.38	-0.12
23VAL	4.17	3.95	0.22
24VAL	4.08	3.95	0.13
25PHE	4.63	4.66	-0.03
26ASN	4.42	4.75	-0.33

The CSI values were calculated from the difference between each residue's observed C α -H and its corresponding Wishart value (Wishart et al., 1992).

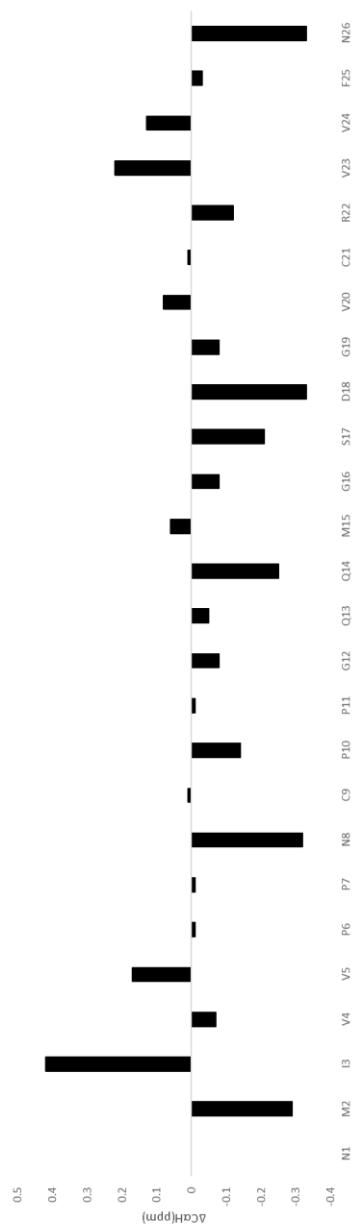


Figure 3.3.3. CaH chemical shift index of SRP-6. Consistent positive chemical shift index (CSI) values indicate the presence of beta-strands. Accordingly, SRP-6 shows no indication of beta-strands.

Table 6. Structural statistics of the 20 lowest energy structures of SRP-6

NOE Constraints	Number
Total	198
Intra-residue	97
Sequential	58
Medium Range (<i+5)	32
Long Range ($\geq i+5$)	8
Disulfide Bonds	2
Hydrogen Bonds	2
Constraints per residue	7.62
Restraint Violations	Number
NOE > 0.3 Å	3
RMSD from the mean structure (residues 10-18)	Å
Backbone atoms	1.42 ± 0.39
Heavy atoms	2.36 ± 0.71
Ramachandran Analysis for the lowest energy structure	%
Residues in Allowed (>99.8%) Regions	
RMSD from Ideal Geometry	Å
Bond Angles	0.2
Bond Lengths	0.001
Number of close contacts (1.6 Å for H atoms, 2.2 Å for heavy atoms)	0

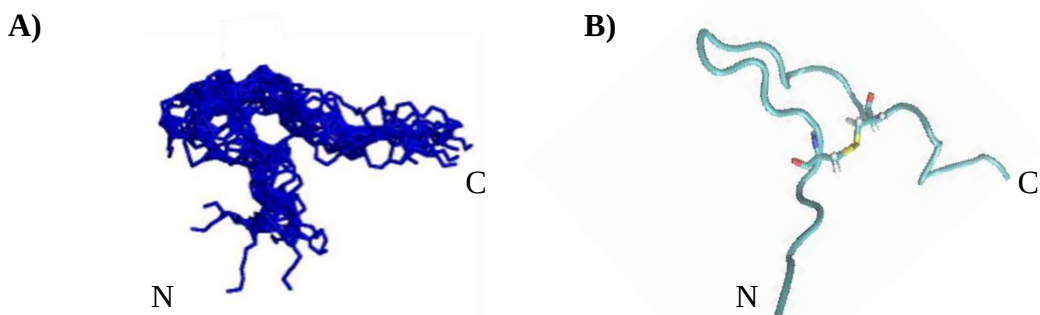


Figure 3.3.4. Structural representation of SRP-6. (A) Superimposition of the 20 lowest energy NMR structures of SRP-3. (B) Ribbon diagram for the lowest energy structure of SRP-6 with the disulfide bond depicted.



Figure 3.3.5. Summary of the NOE connectivities for SRP-6 in water. The bars indicate NOE connectivity between residues. The left column represents the distance between corresponding atoms. Denoted by dotted lines are non-sequential NOE connectivities.

3.4. Comparative Analysis of SRPs and its Homologs

The NMR structural data (CaH- CSI) of SRP-3 and SRP-6 clearly show that these stress-responsive peptides lack the propensities for both beta-strands and beta-turn formation necessary for the relative stability of the core region as observed in the case of SRP-1 and SRP-2 (Schrage *et al.*, 2017a; Schrage *et al.*, 2019). These structural differences of SRP-3 and SRP-6 from the other two peptides have significant implications for their biological function as shown (Figure 2.1.3) in the mRNA expression levels of AMPs by injection of SRP-1, -2, and -3 in *M. sexta* fat body.

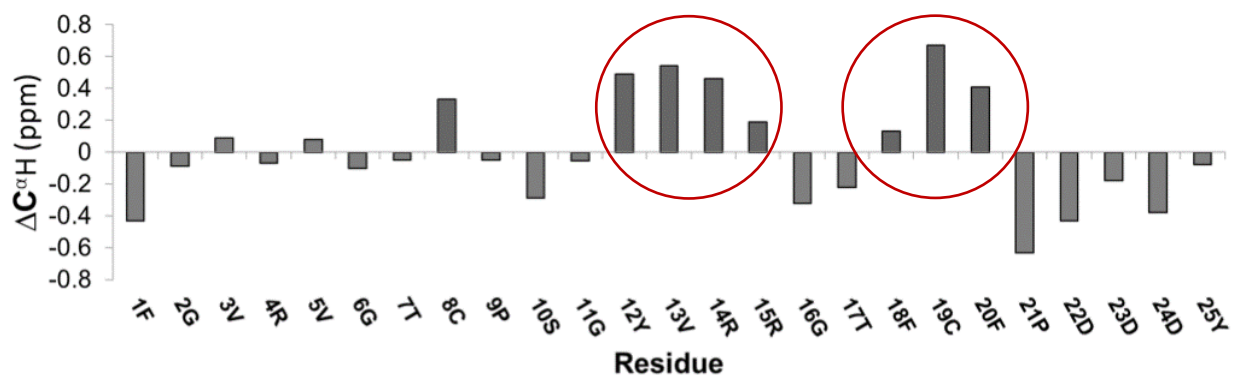


Figure 3.4.1a. CaH chemical shift index of SRP-1. (adapted from Schrage, *et al.*, 2019).

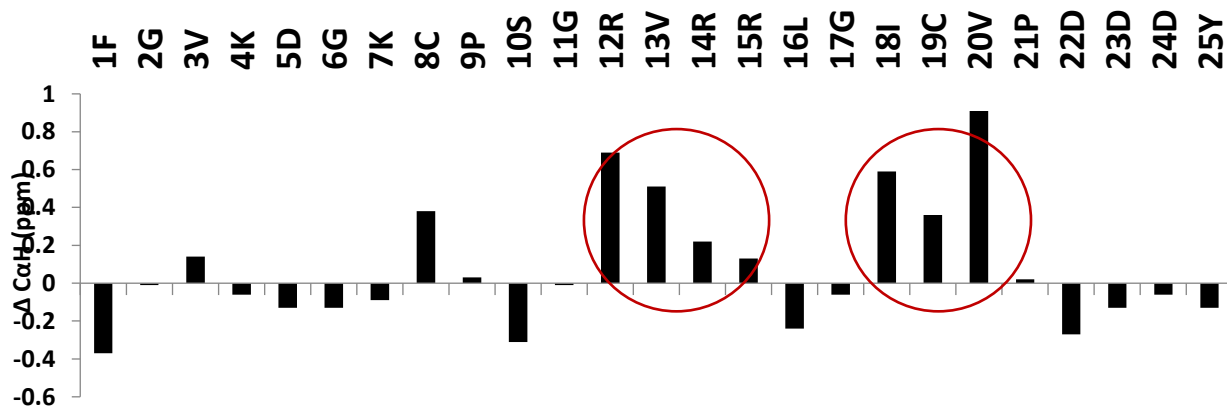


Figure 3.4.1b. CaH chemical shift index of SRP-2. (adapted from Schrage, *et al.*, 2017b).
Circled in red are the consistent positive CSI values that indicate the presence of beta strands.

The structural studies of *M. sexta* SRP-1 and -2 were conducted by former lab members (Schrage *et al.*, 2019; Schrage *et al.*, 2017a). The β -hairpin structure of the peptides involved in-depth analysis of 2D ^1H - ^1H NMR spectroscopy and NOE-derived distance constraints. SRP-1, a 25-residue peptide, exhibits β -strands encompassing residues Tyr12 – Arg15 and Phe18 – Phe20, coupled with a type-I β -turn from Arg15 – Phe18 (Schrage *et al.*, 2019). On the other hand, SRP-2, a 25-residue peptide, showcases β -strands from Arg12 – Arg15 and Ile18 – Val20, along with a type-I' β -turn from Arg15 – Ile18 (Schrage *et al.*, 2017a). Both peptides feature anti-parallel β -strands, each comprising four or three residues per strand, interconnected by a four-residue turn. Nevertheless, all SRPs' stability is maintained through a disulfide bond connecting the two cysteine residues present in all SRPs (Matsumoto *et al.*, 2012).

3.5. Conclusions

SRP-6 in water appears more disordered compared to those of SRP-1 and SRP-2. This may explain its functional difference from SRP-1 and SRP-2. We now know that the biological functions of different SRPs are diverse and that the relative stability and secondary structural features of the core region are likely necessary for their biological activity. The multiple functionalities of SRPs have been demonstrated (Schrage *et al.*, 2016; Shears and Hayakawa, 2019). Yet, the signaling function and regulation of SRPs in insects remains unclear. NMR experiments under different solvent conditions are underway to gain further insights into the structure and dynamics of SRP-6.

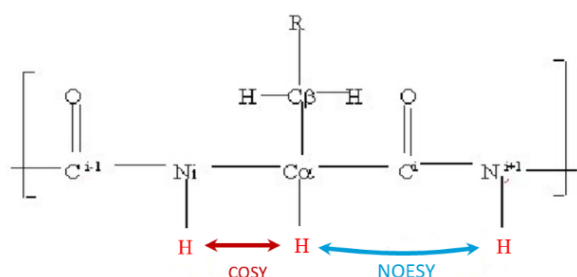
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Appendix A. The amino acid sequential connectivity assignments strategy and protocols

The amino acid sequential connectivity for the peptide's sequences was determined using NOEs cross peaks [$d_{\alpha N}(i, i+1)$] observed between the backbone amide proton of residue i and alpha protons of residue $(i+1)$. Further structural information were provided by assigning short, medium, and long-range proton-proton connectivities, including $d_{\alpha N}$, $d_{\beta N}$, and d_{NN} , in the 2D ^1H - ^1H NOESY spectra. The overlaying 2D ^1H - ^1H NOESY with 2D ^1H - ^1H COSY NMR spectra acquired under similar conditions allowed to confirm the amino acid sequential connectivities (Figure A.1). By starting with a known resonance, typically the amide proton of a specific amino



acid, sequential connectivity was walked through by identifying NOEs to adjacent residue's alpha proton as illustrated in Figures A.2 – A.6.

Figure A.1. Pictorial representation of how 2D ^1H - ^1H COSY and NOESY spectra are used to assign sequential residues. The red arrow indicates the through-bond $d_{\alpha N}$ connectivity that would be observed on the ^1H - ^1H COSY spectrum while the blue arrow represents the through-space [$d_{\alpha N}(i, i+1)$] connectivity that would be observed on the ^1H - ^1H NOESY spectrum.

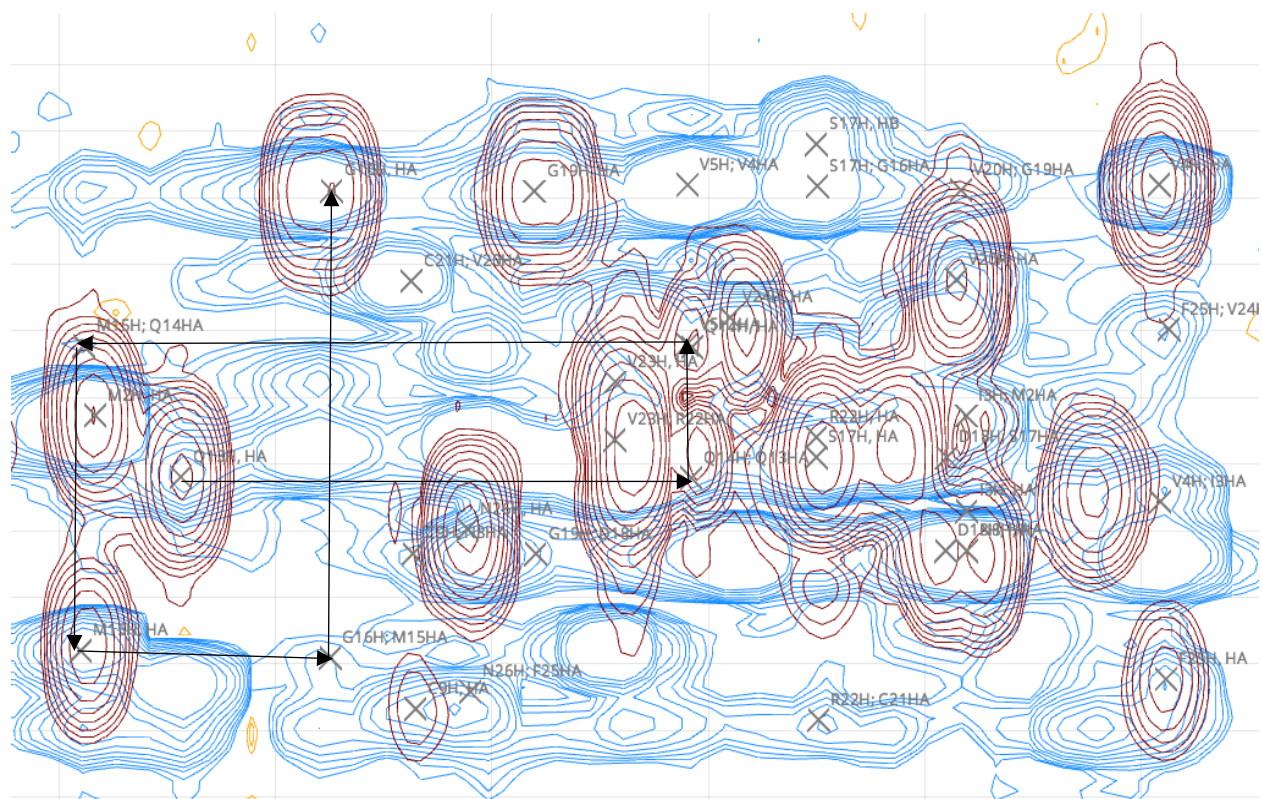
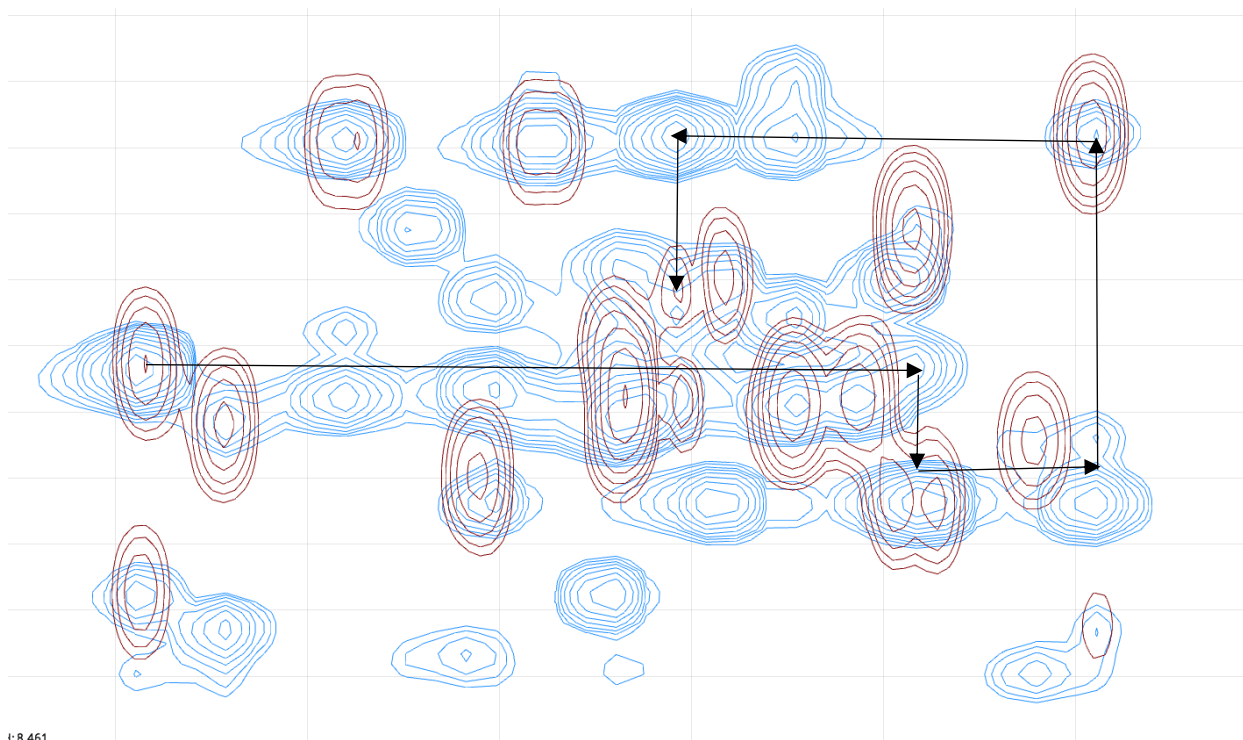


Figure A.2. 2D ^1H - ^1H COSY (red) and NOESY (blue) overlaid (fingerprint region). This figure shows the NOE connectivity for Met2 – Ile3 – Val4 – Val5.



t: 8.461

Figure A.3. 2D ^1H - ^1H COSY (red) and NOESY (blue) overlaid (fingerprint region). This figure shows the NOE connectivity for Gln13 – Gln14 – Met15 – Gly16.

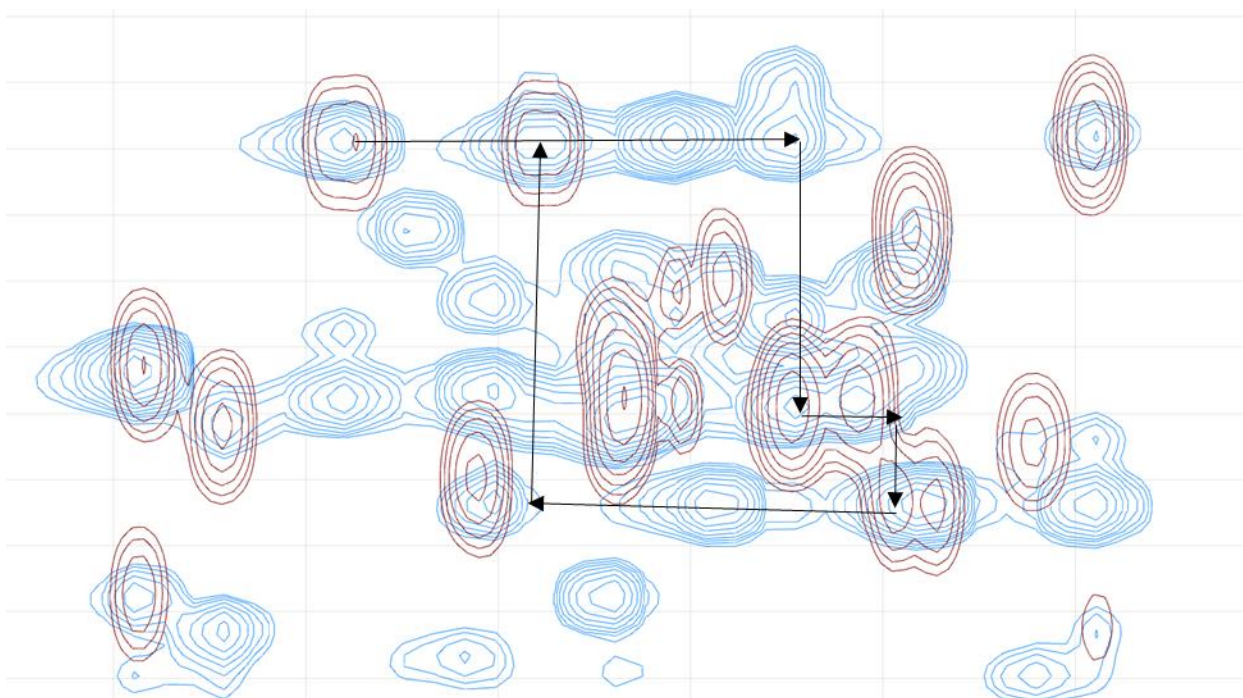


Figure A.4. 2D ^1H - ^1H COSY (red) and NOESY (blue) overlaid (fingerprint region). This figure shows the NOE connectivity for Gly16 – Ser17 – Asp18 – Gly19.

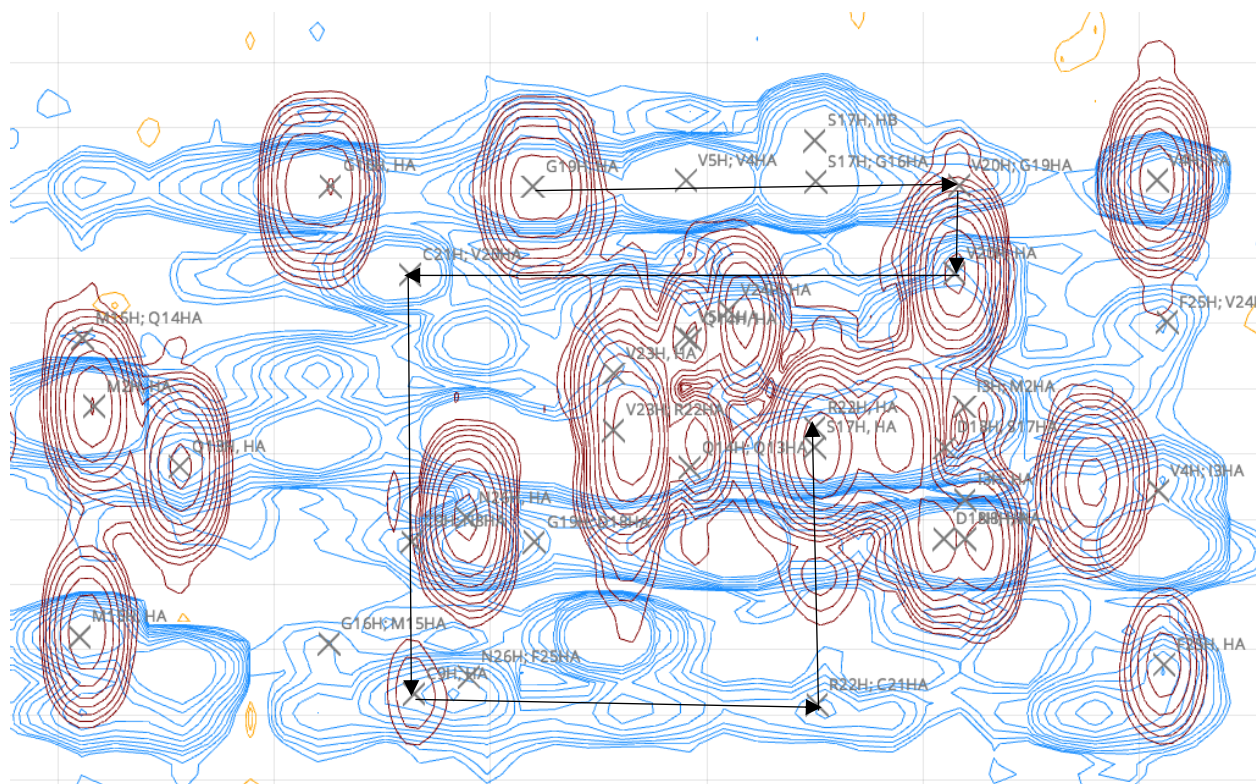


Figure A.5. 2D ^1H - ^1H COSY (red) and NOESY (blue) overlaid (fingerprint region). This figure shows the NOE connectivity for Gly19 – Val20 – Cys21 – Arg22.

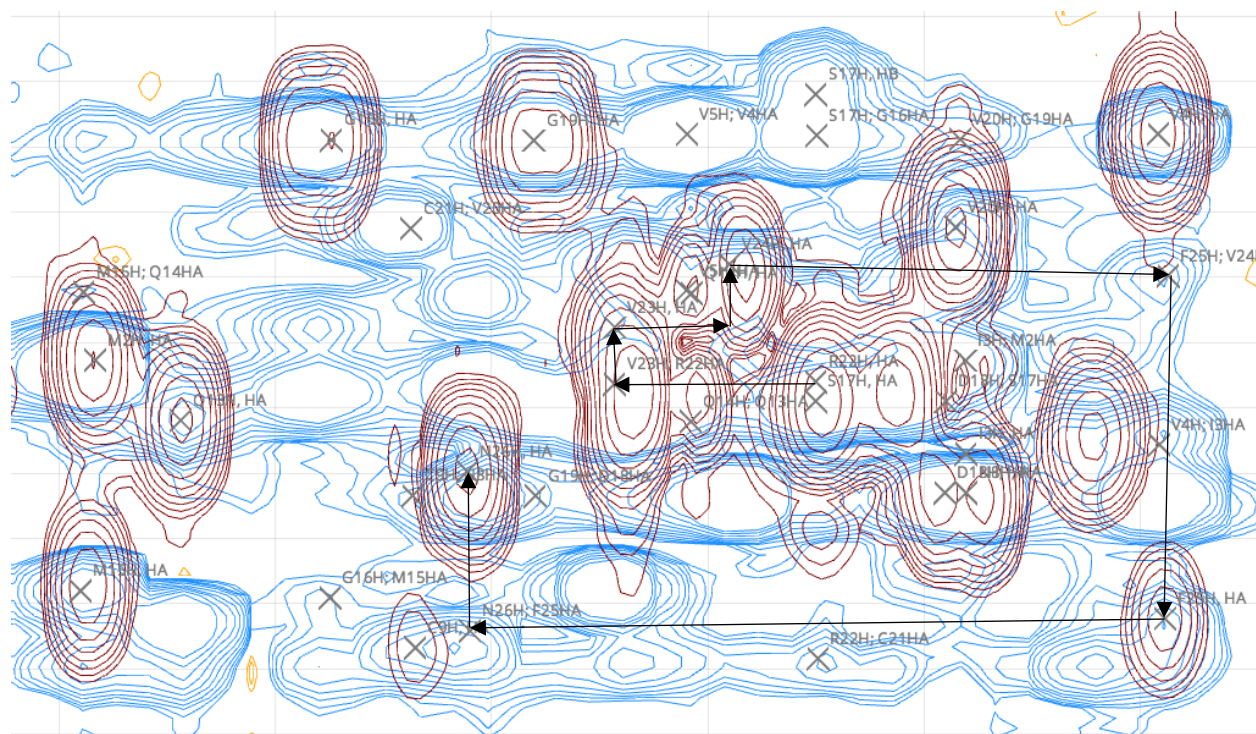


Figure A.6. 2D ^1H - ^1H COSY (red) and NOESY (blue) overlaid (fingerprint region). This figure shows the NOE connectivity for Arg22 – Val23 – Val24 – Phe25 – Asn26.

Appendix B. Preliminary NMR Structural Studies on *Manduca sexta* Pro-moricin-6 and Moricin-6

B.1. Introduction

Being devoid of an adaptive immune system, insects developed an efficient innate immune system that relies on various mechanisms including phagocytosis, activation of proteolytic cascades including melanization, as well as production of different antimicrobial peptides (AMPs) (Richman and Kafatos, 1996; Boman, 1998; Boulanger *et al.*, 2001). Antibacterial peptides are produced mainly from the fat body and hemocytes. Upon microbial infection, AMPs from the insect's humoral defense reactions are secreted into the hemolymph (Kanost *et al.*, 2004). Most AMPs are less than 10 kDa, show a great diversity of primary structures, and can be classified accordingly into four groups (Li *et al.*, 2012). Two of these groups, the cecropins and defensins, are well defined. Cecropins form two α -helices and are devoid of cysteines, whereas defensins contain an array of three or four stabilizing disulfide bridges and an α -helix linked to an antiparallel β -sheet. The other two families, proline-rich and glycine-rich peptides/polypeptides, are relatively heterogeneous (Bulet and Stocklin, 2005; Yi *et al.*, 2014).

Insect AMPs are membrane-active and have an overall net positive charge with an α -helical conformation such as cecropin (Bulet and Stocklin, 2005). Moricin, initially isolated from *Bombyx mori* (Hemmi *et al.*, 2002), also consists of an α -helical conformation throughout its structure. Extensively identified in lepidopteran insects, moricin-like AMPs are active against both gram-positive and gram-negative bacteria (Hara and Yamakawa, 1995). *M. sexta* moricin-6 is a 38-amino-acid peptide isolated from the hemolymph of *M. sexta*. Along with moricin-6, pro-peptide pro-moricin-6 is a 42-amino-acid peptide also isolated from the hemolymph of the

tobacco hornworm. Both peptides are highly basic and their effects on bacterial membranes are predicted to be dependent on the N-terminal half of the peptide which is responsible for increased membrane permeability (Hara and Yamakawa, 1995). It is observed that after the liberation of the active peptide, moricin-6, by cleavage of the pro-region tetrapeptide, there was a substantial increase in bactericidal activity. In this chapter, we solve the tertiary structure of moricin-6 and pro-moricin-6 by NMR spectroscopy to investigate its biological functions and mode of action. A comparison of the two peptides provided insights into the structure-function relationships for AMPs with α -helical conformation.

B.1.2. Structure and Biological Function

As mentioned, moricin contains an α -helical conformation mostly throughout the entire peptide. They have a pre-pro-structure, that is, a signal peptide for secretion, a 2 or 4-residue pro-region, and a mature peptide (Figure B.1). The mature peptide, typically 35 – 39 amino acids in length, is predicted to form an amphipathic α -helix that disrupts bacterial cell membrane mainly after becoming functionally active by removal of the pro-region. *M. sexta* moricin-6 has a unique structural domain composed of a cluster of three basic amino acid residues in the hydrophilic C-terminus region while the N-terminus and core region of the peptide is relatively hydrophobic.

MsMor3	<u>MKLFSLFVVVF</u> AIMALMMGGTAA	KP	KGIGKAI	RGGKVI	KHGLGALGVIGTG	HEIYQ	EA	AKNR	G*
MsMor4	<u>MKLFSLFVVVF</u> AIMALMMGGTAA	NP	KGIGKAI	RGGKVI	KHGLGAIGVIGTG	HEIYQ	EA	AKNR	G*
MsMor5	<u>MKLFSLFV-VFAM</u> LALMMGGTSA	SP	KGIGKVI	RGGKVI	KHGLTAIGIAGTG	HEVYQ	EA	AKNR	G*
MsMor6	<u>MKLFGFLFVMLMA</u> VVALFVGTSQA	APEP	GRLSAI	KKGGKII	KKGLGVISAAGTA	HEVYSHV	KNRRN	*	
MsMor2	<u>MKFLNIFVIIMS</u> VFAMLSNISEA	AP	KGVGSA	LKTG		LRVIGAAGTA	H	DVYEHIK	HRNH G*
MsMor1	<u>MKLTSLFIFVIVA</u> LSLLFSSTDA	AP	GKIPVKA	IKQAG	KVIGKGLRA	INIAGTTHD	VVSFF	RP	KKKKH*

Figure B.1. Aligned sequences of *M. sexta* moricin-1, -2, -3, -4, -5, and -6. The signal peptides are underlined. Spaces separate the mature peptides, and the pro-region is segmented in the middle. Highlighted in color are charged amino acid residues.

M. sexta mature moricin-6, containing three arginines, six lysines, and one glutamic acid, is highly cationic with a calculated isoelectric point of 11.2. Moricin-6 is different in that it contains a 4-residue pro-region, whereas the other *M. sexta* moricins contain a 2-residue pro-

region. Nevertheless, most of the changes entail substitutions of residues with comparable chemical properties.

Previous NMR studies reveal that moricin contains an amphipathic N-terminal segment with one side of the helix carrying positively charged residues which may be responsible for interaction with the negatively charged bacterial membrane (Hemmi *et al.*, 2002; Oizumi *et al.*, 2005; Dai *et al.*, 2008). The solution structure of moricin also showed a curved helix throughout the whole length of the molecule containing eight turns.

The antibacterial activity of *M. sexta* pro-moricin-6 and moricin-6 was tested against *E. coli*. *M. sexta* moricin-6 showed the strongest antibacterial activity compared to pro-moricin-6 (Figure B.2). Mature moricin treatment significantly reduces colony formation. At 5 micrograms per ml, there is a 2.5-fold rise in specific activity, increasing to 31.2-fold at 10 micrograms per ml and 21.8-fold at higher concentrations. Consequently, a conservative estimate suggests a 15-fold enhancement in antimicrobial activity upon removal of the N-terminal tetrapeptide (APEP).

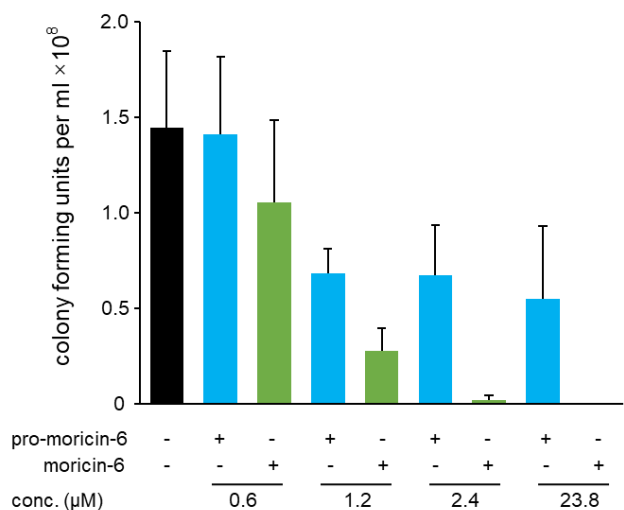


Figure B.2. Bactericidal activity of *M. sexta* pro-moricin-6 and moricin-6. Suspension of *E. coli* was plated on LB medium in the control (black) after incubation overnight at 37°C. The plates were then treated with pro-moricin-6 (blue) or moricin-6 (green) at varying concentrations (0.6, 1.2, 2.4, and 23.8 μM) for one hour at 37°C. The experiments were repeated thrice, and the statistical significance (*p*-value) is indicated above. For a *p*-value greater than 0.05, “n.s.” is indicated as not significant.

The biological data suggests that pro-moricin-6 is partially active compared to moricin-6. Therefore, we hypothesize that pro-moricin-6 becomes fully active after their N-terminal

tetrapeptide is removed. This leads us to investigate the structural differences between the pro-peptide and the mature peptide using 2D NMR spectroscopy.

Despite sequential differences between the different moricins, the structure remains conserved between the studied moricin homologs. NMR data suggests that *M. sexta* moricin-6's N-terminal region has more propensity for an amphipathic helical structure compared to pro-moricin-6. The absence of the N-terminal tetrapeptide pro-region may suggest a conformation of moricin-6 that allows for more equally large hydrophilic and hydrophobic faces in contrast to pro-moricin-6.

B.2. Materials and Methods

B.2.2. Peptide Synthesis

Both 42-residue MsPro-moricin-6 (sequence) and 38-residue MsMoricin-6 (sequence) were chemically synthesized (acquired from PepMic Ltd, Jiangsu, China) by automated protocols of step-wise solid phase synthesis using the standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The peptides were purified to >95% purity by reverse-phase HPLC on a C18 column. The mass and purity were further confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Applied Biosystems, Foster City, CA). The peptides were lyophilized and stored as a dry powder for NMR spectroscopy and circular dichroism experiments.

B.2.3. Circular Dichroism

CD experiments were performed on Jasco-8155 spectropolarimeter (JASCO, Tokyo, Japan) using a 1 mm path length cell over 190 to 250 nm range as described before (Soulages *et al.*, 2001). The spectra were acquired every 1 nm at 25°C, with a 2-second averaging time per point. Each spectrum was the average of five consecutive scans, followed by subtraction of the

CD signal of peptide-free solutions. Samples were prepared by dissolving the peptide to the concentration of 50 μ M in various solvents: water, 2,2,2-trifluoroethanol(TFE)/water solutions (from 10% to 40% (v/v) in TFE), and methanol.

B.2.4. Nuclear Magnetic Resonance Spectroscopy

The NMR samples were prepared by dissolving the peptides in 600 μ l of CD₃OH (Sigma-Aldrich, USA). The final peptide concentration for both *M. sexta* Pro-moricin-6 and Moricin-6 were ca. 1.2 mM and placed in a 3 mm Wilmad 335 NMR sample tube. All NMR spectra were obtained on a 14 Tesla Bruker AVANCE NEO (Bruker Biospin Corporation, Billerica, MA) NMR detection triple resonance HCN cryogenic probe in the phase-sensitive mode by TPPI and States-TPPI. The following spectra were recorded with 16 ppm spectral widths in *t*₁ and *t*₂ dimensions at 25°C: 2D ¹H-¹H Total Correlation Spectroscopy (TOCSY) with MLEV mixing sequence, recorded with mixing time of 80 ms; 2D Nuclear Overhauser Effect Spectroscopy (NOESY), recorded with mixing times of 100, 200, 300, and 400 ms; and 2D ¹H-¹H Correlation Spectroscopy (COSY). These experiments were recorded with 512 and 2048 data points in *t*₁ and *t*₂ dimensions. 2D ¹H-¹H Double-Quantum-Filtered Correlated Spectroscopy (DQF-COSY) was recorded using 800 and 4096 complex points in *t*₁ and *t*₂ dimensions. Water suppression was performed using WATERGATE pulse sequence. The data were processed using Topspin 4.1.4 (Bruker). Exchanging amide protons were determined by collecting temperature gradient 2D ¹H-¹H TOCSY experiments at 12.5, 15, 20, 25, 30, and 35°C, recorded with the same parameters as 2D ¹H-¹H TOCSY at 25°C. For all 2D homonuclear NMR spectra, 32 scans were used. The ¹H dimensions were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 25°C.

2D heteronuclear NMR experiments were also recorded to obtain additional information on amide groups and proton-bearing carbons. The following spectra were recorded using echo/anti-echo gradient selection at 25°C: 2D ^1H - ^{15}N Heteronuclear Single Quantum Coherence (HSQC), recorded with time domains of 2048 and 256 complex points for ^1H (F2) and ^{15}N (F1), respectively; and 2D ^1H - ^{13}C HSQC and 2D ^1H - ^{13}C HSQC-TOCSY, both recorded using 1024 and 256 complex points for ^1H (F2) and ^{13}C (F1), respectively. ^{15}N and ^{13}C chemical shifts were referenced indirectly from DSS. For peak sensitivity improvement on HSQC NMR spectra, the number of scans were increased to 64, 128, and 256 for 2D ^1H - ^{13}C HSQC, 2D ^1H - ^{13}C HSQC-TOCSY, 2D ^1H - ^{15}N HSQC, respectively. Water peak suppression was obtained by low-power irradiation of the water peak during relaxation delay. The Computer-Aided Resonance Assignment (CARA) software (<https://cara.nmr.ch/>) (Keller, 2004), as well as the Collaborative Computational Project for NMR (CCPN) software (Skinner et al., 2016), were used for peak-picking and assignment.

B.2.5. Chemical Shift Assignments

2D ^1H - ^1H TOCSY was first analyzed for intraresidue resonance assignments. 2D ^1H - ^1H COSY was used for identifying C α H chemical shift assignment. 2D ^1H - ^{15}N HSQC was used in the identification of the amide proton chemical shifts. Overlapping peaks were further clarified by 2D ^1H - ^{13}C HSQC analysis due to the broad range of the carbon chemical shift. Each amino acid has a characteristic proton chemical shift pattern. Therefore, the identification of each amino acid side chain spin system was observed in the fingerprint region of 2D ^1H - ^1H TOCSY (Figures B.3 and B.4). The identification of neighboring residues in the amino acid sequence was determined using the sequential assignment procedure (Wüthrich, 1986), which involves overlaying cross-peaks of 2D ^1H - ^1H COSY and NOESY spectra acquired under similar

conditions. Sequential residues were identified by following the *i* residue's NH/H α COSY peak to the *i*+1 residue's H α /NH NOESY peak. 2D ^1H - ^1H NOESY acquired with 400 ms mixing time was used for the assignment of NOE cross-peaks. The volume of the cross-peaks determines the distances between pairs of proton spins. Thus, the greater the peak volume, the closer the protons are through space. By calculating the volume of NOE cross-peaks, we can derive proton-proton distance constraints classified into three ranges, strong (1.8-2.7 Å), medium (1.8-3.4 Å), and weak (1.8-5.0 Å).

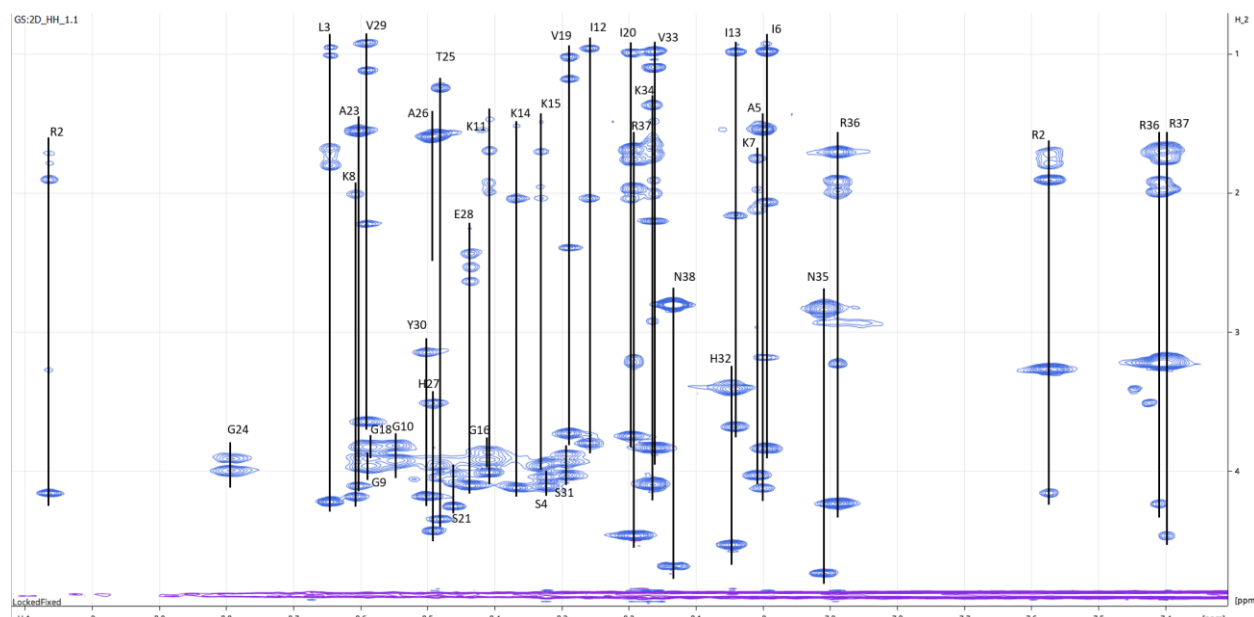


Figure B.3. 2D ^1H - ^1H TOCSY fingerprint region of moricin-6 collected in CD_3OH at 25°C . The fingerprint region represents NH-C α H, NH-C β H, NH-C γ H, NH-C δ H and NH-C ϵ H crosspeaks. The vertical lines indicate the spin system of the corresponding residue.

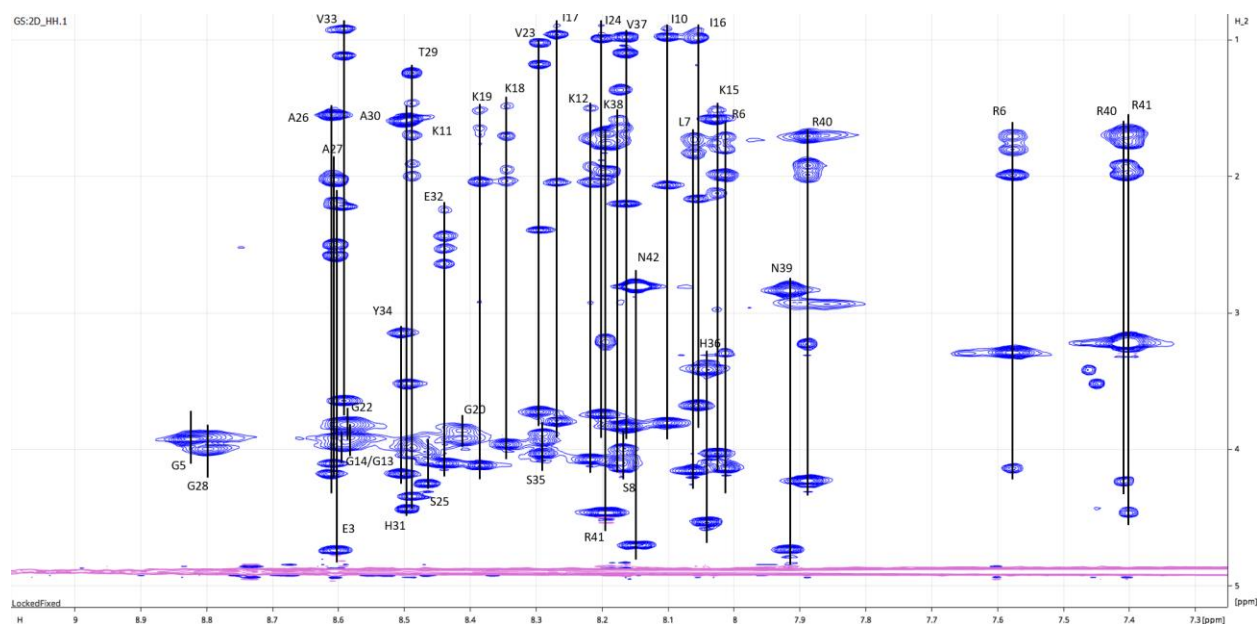


Figure B.4. 2D ^1H - ^1H TOCSY fingerprint region of pro-moricin-6 collected in CD_3OH at 25°C .

B.3. Results and Discussion for Pro-moricin-6 and Moricin-6

B.3.2. CD Data and Preliminary Structural Analysis

The CD spectra were acquired for both moricin-6 and pro-moricin-6 in aqueous solution, in TFE/water (10% to 50% (v/v) in TFE), and methanol at 25°C. Both peptides demonstrate a largely random coil conformation in water (Figures B.5 and B.6). However, in the presence of TFE, CD spectra showed two minima at 208 and 222 nm for both peptides, typical of an α -helical conformation. The helical content increased with increasing concentration of TFE up to 50% (v/v). The CD spectrum in methanol showed a spectrum similar to that in TFE, indicating that moricin-6 and pro-moricin-6 adopted an α -helical conformation in methanol. Notably, the CD spectra data indicates that moricin-6 has a higher propensity for α -helical characteristics than pro-moricin-6. This conformational difference may be attributed to the cleavage of the tetrapeptide of pro-moricin-6. Furthermore, the conformational transition from a random coil in water to an α -helix in hydrophobic environments such as methanol and TFE is common to cecropin-like AMPs, and solution structures of moricin homologs have been determined in hydrophobic media by NMR (Dai *et al.*, 2008). Therefore, NMR data were collected in CD₃OH for both peptides to determine the solution conformation and their structural difference.

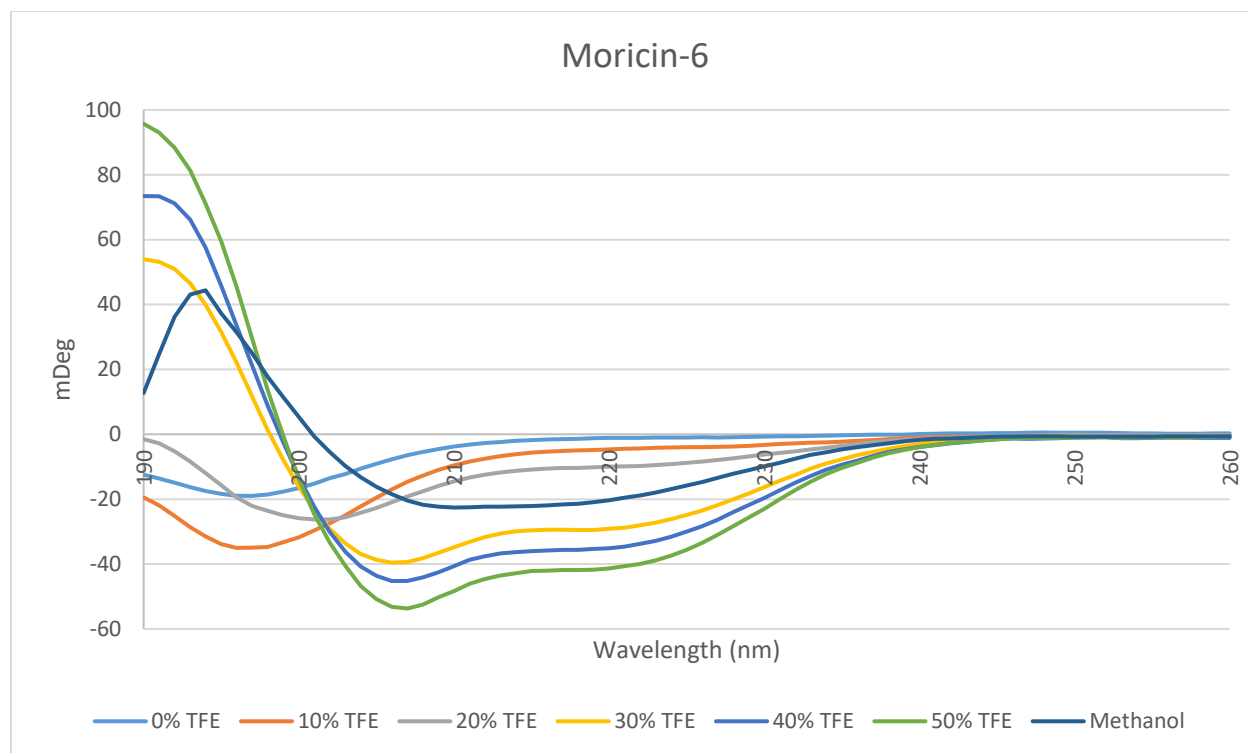


Figure B.5. CD spectra of moricin-6 in water, TFE/water (v/v) or methanol solution.

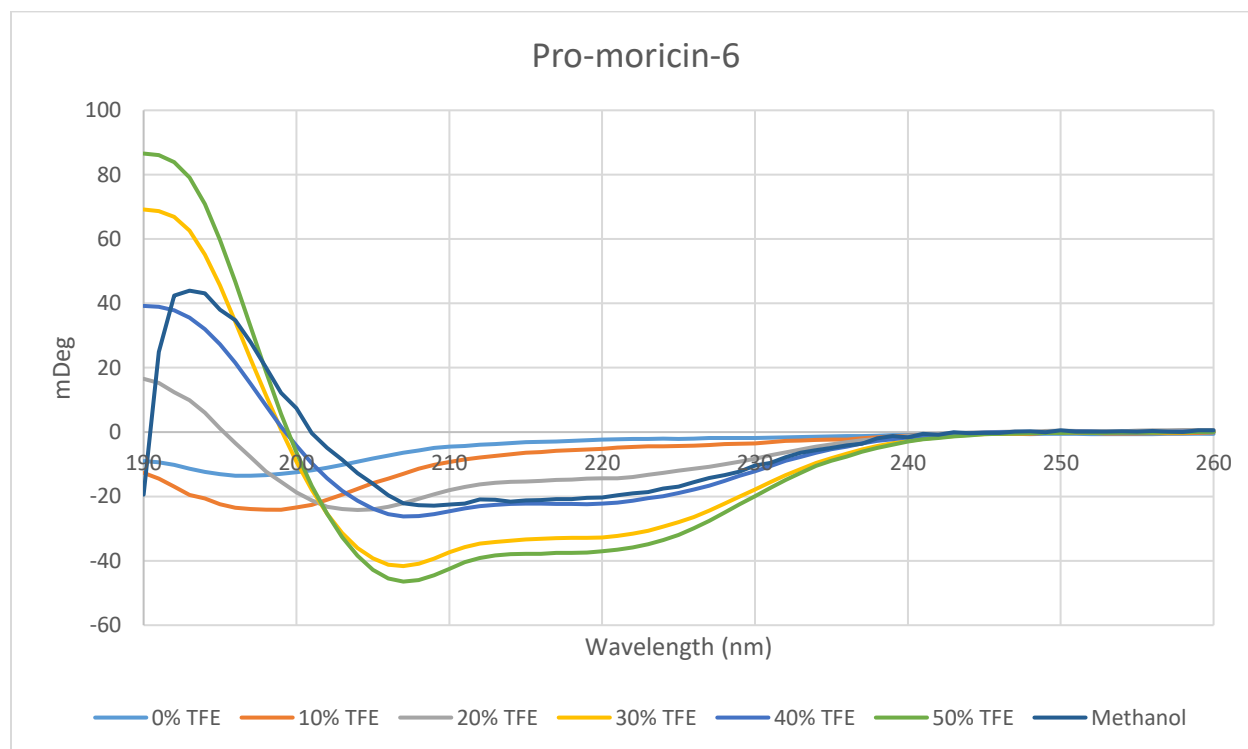


Figure B.6. CD spectra of pro-moricin-6 in water, TFE/water (v/v) or methanol solution.

B.3.3. NMR Results and Chemical Shift Assignments

Proton resonance assignments were obtained for both moricin-6 and pro-moricin-6 using spin system identification and sequential assignment (Figures B.3 and B.4) from 2D NMR spectra recorded at 25°C. The resonance assignments for the backbone and side-chain protons were completed except for the amide proton of the first N-terminal residue of moricin-6 (Gly1) and pro-moricin-6 (Ala1) (Figure B.3 and B.4). Residues Gly22 to Asn42 of pro-moricin-6, and Gly18 to Asn38 of moricin-6 display near-identical proton chemical shift values while Ala5 to Leu21 of pro-moricin-6 contrasts heavily with the proton chemical shift values of moricin-6 N-terminal region. With the C-terminus residues of moricin-6 and pro-moricin-6 displaying similar chemical shift values, it suggests that the C-terminal region of both peptides is structurally alike, while the differences between the two peptides lie in the N-terminal region as shown in Table 7. This suggestion is also supported by qualitative analysis of the 2D NMR spectra overlays (Figure B.7). Overlaying the 2D ^1H - ^1H TOCSY fingerprint region of moricin-6 and pro-moricin-6, there are noticeable differences in the chemical shifts between residues of the N-terminus regions between both peptides. A similar observation was made when overlaying the 2D ^1H - ^1H NOESY NH-NH region of the two peptides (Figure B.8). NH-NH NOEs are characteristics of α -helical secondary structures. Cleavage of the N-terminal tetrapeptide of pro-moricin-6 caused a significant downfield shift of Arg2 of moricin-6. Moricin-6 also displayed extra NOEs for N-terminal residues when comparing the 2D ^1H - ^1H NOESY NH-NH regions of the two peptides.

Table 7. Pro-moricin-6 and moricin-6 proton chemical shift (ppm) assignments comparison

Pro-moricin-6												Moricin-6												
Residue #	Residue	NH	H α	H β	H β 2	H γ	H γ 2	H δ	H δ 2	He		Residue #	Residue	NH	H α	H α 2	H β	H β 2	H γ		H γ 2	H δ	H δ 2	He
1	ALA		4.267	1.522								2	PRO											
3	GLU	8.601	4.73	2.198		2.022	2.117	3.64	3.729			4	PRO											
5	GLY	8.821	3.917					3.828				5	GLY			3.919	3.834							
6	ARG	8.007	4.13	1.984		1.703	1.795	3.291				6	ARG	9.061	4.149		1.903			1.784	1.7	3.262		7.569
7	LEU	8.058	4.15	1.829		1.719						7	LEU	8.641	4.208		1.798			1.678		1.011	0.943	
8	SER	8.164	4.13	3.982								8	SER	8.32	4.102		4.036							
9	ALA	8.416	3.919	1.525								9	ALA	7.997	4.114		1.539							
10	ILE	8.028	4.089									10	ILE	7.991	3.835		2.064		1.331/1.239	0.976	0.916			
11	LYS	8.486	3.982	1.905	1.993	1.236	1.463	1.697		2.916		11	LYS	8.007	4.023		2.115			1.743		1.968		2.964
12	LYS	8.214	4.067	1.917		1.495		1.716		2.925		12	LYS	8.604	4.169		2.004							
13	GLY	8.595	3.956									13	GLY	8.582	3.957	3.903								
14	GLY	8.581	3.904									14	GLY	8.545	3.917	3.81								
15	LYS	8.341	3.957	1.951	2.027	1.484		1.705		2.936		15	LYS	8.407	4		1.986	1.919		1.463		1.687	2.92	
16	ILE	8.268	3.784	2.037		1.236		0.952				16	ILE	8.254	3.794		2.034			1.171	0.956	0.902		
17	ILE	8.054	3.677	2.156		1.178		0.974				17	ILE	8.036	3.678		2.157			1.175	0.98	0.928		
18	LYS	8.022	4.023	2.115		1.515	1.573	1.744		2.97		18	LYS	8.366	4.117		2.038			1.508		1.642		2.905
19	LYS	8.381	4.114	2.037		1.514	1.64	1.687	1.76	2.916		19	LYS	8.326	3.957		2.031	1.949		1.486		1.695		2.927
20	GLY	8.415	3.85									20	GLY	8.407	3.91	3.85								
21	LEU	8.097	3.795	2.065		0.971		1.203				21	LEU	8.416			2.065			0.971		1.203		
22	GLY	8.583	3.812									22	GLY	8.583	3.823									
23	VAL	8.294	3.716	2.384		1.009	1.167					23	VAL	8.286	3.717		2.382			1.172	1.015			
24	ILE	8.197	3.735	2.031		1.238		0.986				24	ILE	8.194	3.741		2.034			1.23	0.98	0.89		
25	SER	8.461	4.246	4.009								25	SER	8.458	4.25		4.076	4.013						
26	ALA	8.608	4.171	1.541								26	ALA	8.601	4.103		1.548							
27	ALA	8.606	4.097	2.02/1.541								27	ALA	8.601	4.103		1.548							
28	GLY	8.794	3.983									28	GLY	8.788	3.988	3.904								
29	THR	8.487	4.034	4.339		1.58						29	THR	8.477	3.956		4.34			1.235				
30	ALA	8.491	3.951	1.58								30	ALA	8.488	4.42		1.582							
31	HIS	8.492	4.43	3.507								31	HIS	8.488	4.42		3.502							
32	GLU	8.434	4.097	2.233	2.429	2.53	2.637					32	GLU	8.434	4.101		2.424	2.249		2.624	2.529			
33	VAL	8.588	3.636	2.217		0.908	1.104					33	VAL	8.588	3.637		2.22			1.115	0.918			
34	TYR	8.501	4.169	3.142								34	TYR	8.498	4.172		3.141							
35	SER	8.287	4.023	3.872	3.929							35	SER	8.288	4.022		3.929	3.876						
36	HIS	8.037	4.529	3.409								36	HIS	8.042	4.526		3.393							
37	VAL	8.161	3.823	2.189		0.969	1.087					37	VAL	8.161	3.824		2.196			1.09	0.979			
38	LYS	8.168	4.089	1.64	1.73	1.364				2.909		38	LYS	8.161	4.09		1.995	1.905		1.477	1.355	1.715	1.637	2.917
39	ASN	7.914	4.729	2.827								39	ASN	7.908	4.732		2.839							
40	ARG	7.888	4.224	1.917	1.984	1.703		3.224		7.407		40	ARG	7.886	4.221		1.979	1.917		1.693		3.225		7.407
41	ARG	8.192	4.457	1.961		1.677	1.756	3.213		7.398		41	ARG	8.189	4.459		1.971			1.755	1.686	3.209		7.394
42	ASN	8.148	4.691	2.796								42	ASN	8.135	4.679		2.793							

The regions where pro-moricin-6 and moricin-6 display near-identical chemical shifts values are highlighted in blue.

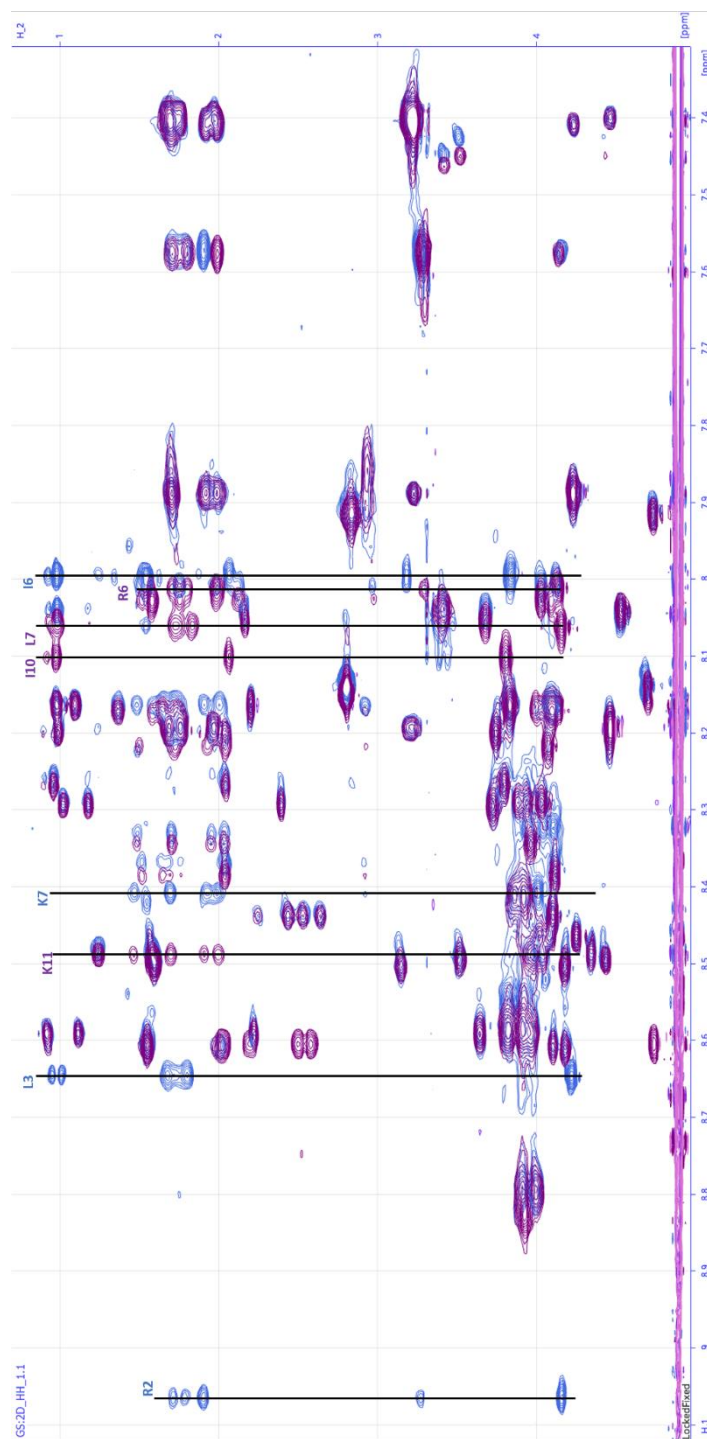


Figure B.7. 2D ^1H - ^1H TOCSY (fingerprint region) overlay of moricin-6 (blue) and pro-moricin-6 (purple) in CD_3OH at 25°C . The vertical lines indicate spin systems that have significant variations in chemical shifts between the two peptides. Of those indicated: Arg2, Leu3, Ile6 and Lys7 of moricin-6; and Arg5, Leu7, Ile10, and Lys11 of pro-moricin-6.

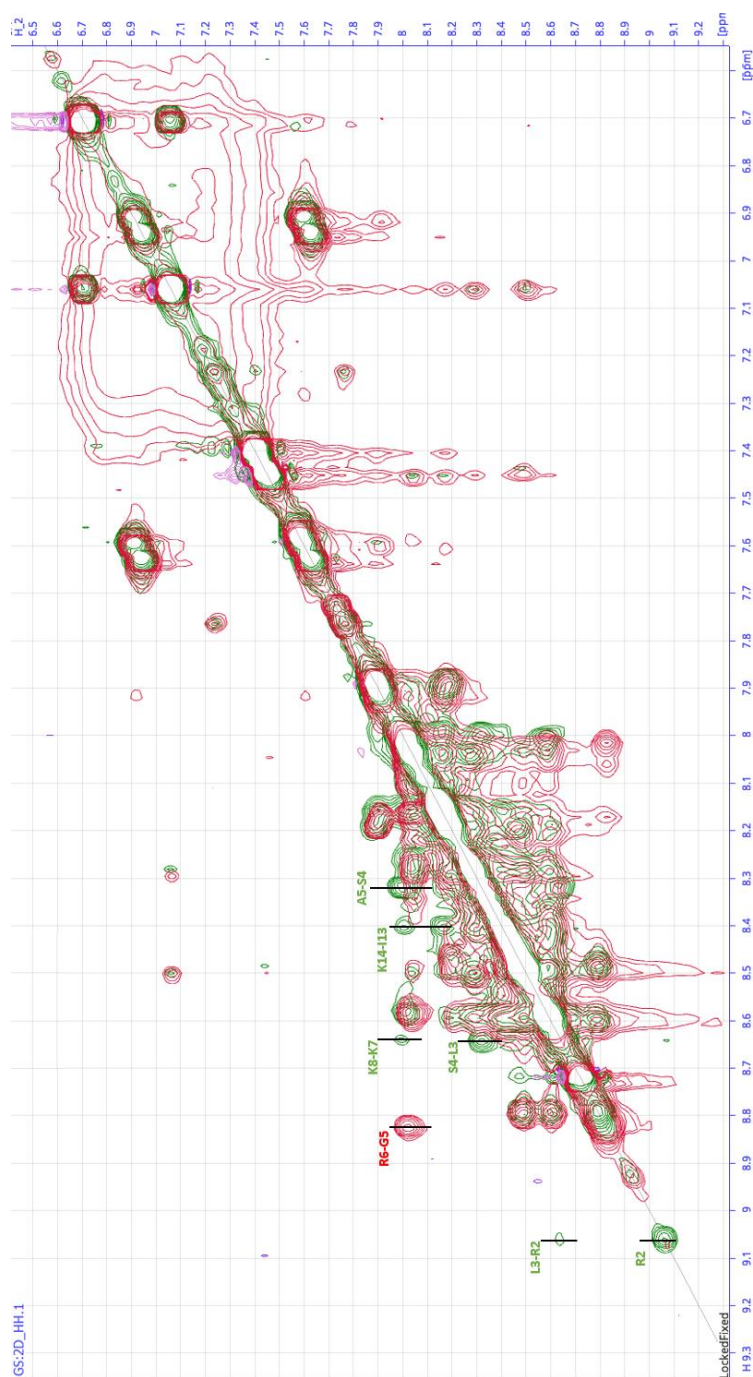


Figure B.8. 2D ^1H - ^1H NOESY (NH-NH region) overlay of moricin-6 (green) and pro-moricin-6 (red) in CD_3OH at 25°C . The vertical lines indicate NOE crosspeaks that are isolated in the 2D NMR spectra overlay. Prominent NOEs are observed for the N-terminal residues of moricin-6 (Arg2, Leu3, Ser4, Ala5, Lys7, Lys8, Ile13 and Lys14).

B.4. Conclusions

The removal of the N-terminal tetrapeptide (APEP) from pro-moricin-6 leads to a substantial 15-fold increase in specific activity, underscoring the critical role of this cleavage event in enhancing antimicrobial potency. Notably, the ^1H chemical shift values in the C-terminus portion of both moricin-6 and pro-moricin-6 exhibit near-identical patterns, indicating structural similarity in this region. Conversely, significant differences in the ^1H chemical shift values are observed in the N-terminus region of both peptides, implying distinct structural characteristics in this segment. The overlay of the ^1H - ^1H 2D NOESY NH-NH region reveals an abundance of NH-NH NOEs for moricin-6, particularly in the N-terminus residues, suggesting increased structural interactions and stability. Furthermore, analysis of NOE connectivity data demonstrates continuous strong NH-NH NOEs, indicative of alpha-helical secondary structure, more prominently in the backbone of moricin-6 compared to pro-moricin-6. This preliminary NMR data collectively suggests that moricin-6 exhibits a higher propensity for alpha-helical characteristics in its overall structure, reinforcing its role as an antimicrobial peptide with enhanced biological activity.

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