CHARACTERIZATION, REGULATION AND BIOPHYSICAL STUDIES OF IMMUNE-RELATED PEPTIDES FROM *MANDUCA SEXTA*

by

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B.S., Al albayt University, 1999

M.S., Al albayt University, 2004

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Abstract

Insects secrete antimicrobial peptides as part of the innate immune response. Most antimicrobial peptides from insects have antibacterial but not antifungal activity. We have characterized an antifungal peptide, diapausin-1 from hemolymph of a lepidopteran insect, *Manduca sexta* (tobacco hornworm). Diapausin-1 was isolated by size exclusion chromatography from hemolymph plasma of larvae that were previously injected with a yeast, *Saccharomyces cerevisiae*. Fractions containing activity against *S. cerevisiae* were analyzed by SDS-PAGE and MALDI-TOF MS/MS and found to contain a 45-residue peptide that was encoded by sequences identified in *M. sexta* transcriptome and genome databases. A cDNA for diapausin-1 was cloned from cDNA prepared from fat body RNA. Diapausin-1 is a member of the diapausin family of peptides, which includes members known to have antifungal activity. The *M. sexta* genome contains 14 genes with high similarity to diapausin-1, each with 6 conserved Cys residues. Diapausin-1 was produced as a recombinant protein in *Escherichia coli*. Purified recombinant diapausin-1 was active against *S. cerevisiae*, with IC_{50} of 12 μM, but had no detectable activity against bacteria. Spores of some plant fungal pathogens treated with diapausin-1 had curled germination tubes or reduced and branched hyphal growth. Diapausin-1 mRNA level in fat body strongly increased after larvae were injected with yeast or with *Micrococcus luteus*. In addition, diapausin-1 mRNA levels increased in midgut and fat body at the wandering larval stage prior to pupation, suggesting developmental regulation of the gene. Our results indicate that synthesis of diapausin-1 is part of an antifungal innate immune response to infection in *M. sexta*. Biophysical analysis showed that diapausin-1 binds to the β-1,3 glucan component of the *S. cerevisiae* cell wall.

A second insect peptide investigated in this project was *M.sexta* stress-response peptide 1(SRP1), an immune-related peptide upregulated under different stress conditions including immune-challenge. Preliminary results for NMR structure determination are presented. Most of the amino acid residue spin systems were assigned, and we determined the connectivities of many amino residues as a first step to solve the NMR structure. The circular dichroism spectrum of SRP1 indicates that the peptide lacks alpha-helical structure and may contain beta strands and turns.

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Dedication

To my father, my mother, my wife Khadeeja, my children Lugein and Omar, my sisters and brothers and my two beloved uncles Abo Awwad and Abo Ashraf.

Chapter 1 - Literature review

Introduction

Living organisms need to evolve defensive mechanisms against invading organisms. Among these immune responses is the production of antimicrobial peptides (Aerts et al., 2008; Bahar and Ren, 2013). In general, antimicrobial peptides are small, cationic peptides, often produced from larger proteins after cleavage of a precursor protein, and may be constitutively produced or induced after infection (Van der Weerden et al., 2013; Yi et al., 2014). Plants and animals produce an arsenal of antimicrobial peptides and/proteins to encounter invaders (Selitrennikoff, 2001; Hegedüs and Marx, 2013). The functions of these antimicrobial agents are diverse, with activity against bacteria, some viruses, parasites and fungi. Antimicrobial peptides may have a wide spectrum of activity against many invading organisms, or their function can be restricted against one type of invader (Bulet et al., 2004).

Fungi live in all environments and in living organisms as well. They may cause severe harm to their host, and they may even lead to the host's death. Beside their role in fighting invaders, antifungal peptides have been exploited to synthesize antimicrobial drugs, or genes that encode these peptides have been introduced genetically into some important crops to produce plants that will more capably fight invading fungi (De Lucca and Walsh, 1999; Hegedüs and Marx, 2013; Nakatsuji and Gallo, 2012; Wu et al., 2013).

Plants, mammals, amphibians, fishes and insects produce many antifungal peptides. These peptides with non-enzymatic activity can be grouped based on structural homology and secondary structure into families (Bulet et al., 2004; De Lucca and Walsh, 1999; Van der Weerden et al., 2013; Wu et al., 2013).

Plant antifungal peptides

Plants produce a large number of antifungal peptides in many plant parts. The major plant antifungal peptides include: thaumatin-like proteins, plant defensins, Ib-AMPs, thionins, snakins, hevein-type peptides, knottin-type peptides, 2S albumin proteins, hairpinins, and lipid transfer peptides (Table 1.1).

Thaumatin-like proteins

These proteins are homologous to thaumatin, a sweet protein isolated from the African plant *Thaumatococuus daniellii* (Yun et al., 1997). Plant thaumatin-like proteins with antifungal activity include osmotin and zeamatin (Van der Weerden et al., 2013). Osmotin a 24 kDa protein isolated from *Nicotina tabacum*, has antifungal activity against several unrelated fungal genera such as *Aspergillus flavus*, *Aspergillus parasitica*, *Rhizoctonia solani*, *Macrophomina phaseolina*, and other species from *Bipolaris*, *Fusarium* and *Phytophthora* (Abad et al., 1996).

Zeamatin is a 22 kDa protein isolated from the seeds of *Zea mays* with antifungal activity against *C. albicans* (Roberts and Selitrennikoff, 1990). Proteins related to zeamatin have been isolated from seeds of other plants (Robert et al., 1990). The crystal structure of zeamatin showed that the protein has 13 β-strands, 11 of these strands form a β-sandwich that represents the core of the protein, and a short α-helix. This structure is maintained by 8 disulfide bonds (Batalia et al., 1996).

Plant defensins

Plant defensins are less than 50 amino acid residues long; their structure has a distinctive fold consisting of a triple-stranded anti-parallel β-sheet connected to an α-helix by three disulfide bridges, and the C- and N- terminals are connected by a disulfide bridge (Bruix et al., 1993; Van der Weerden and Anderson., 2013). Many plant defensins have antifungal activity, among of these, NaD1 from *Nicotina alata* (Lay et al., 2003), PhD1 and PhD2 from *Petunia hybrida* (Lay et al., 2003), Psd1 and Psd2 from *Pisum sativum* (Almeida et al., 2000), and Rs-AFPs 1-4 from *Raphanus sativus* (Terras et al., 1992). The antifungal activity of defensins has been exploited successfully to introduce defensin genes to some important plants such as rice, potato, tomato and other plants to resist plant pathogens (de Oliveira Carvalho and Gomes, 2009; Jha et al., 2009; Gao et al., 2000; Kaur et al., 2011). Furthermore, injection of Rs-AFP2 into mice increased their resistance to *Candida* infection (Tavares et al., 2008).

Ib-AMP1-4

Ib-AMP1-4 from *Impatiens balsamin* are considered the smallest antifungal peptides isolated from plants, only 20 amino acid residues long (Tailor et al., 1997). Ib-APMs are highly basic peptides, containing four cysteine residues forming two disulfide bridges to stabilize the peptide's structure (Patel et al., 1998). They have very potent antifungal activity against filamentous fungi and are less effective to yeast (De Lucca, 1998; Thevissen et al., 2005).

Thionins

Thionins were first isolated from wheat after an observation that wheat flour was toxic to bread yeast (Okada et al., 1970). Later, thionins were isolated from different plants (Rodriguez-Palenzuela et al., 1988; Vernon et al., 1985). Thionins are ~5 kDa cationic peptides rich in cysteines (Stec, 2006; Van der Weerden et al., 2013). Although sequence similarity maybe low, thionins share the same structural fold composed a two stranded β-sheet, two α-helices and a C-terminal coil. The structure is stabilized by three to four disulfide bridges (Stec, 2006). Many thionins have antifungal activity against plant pathogenic fungi (Asano et al., 2013; Bohlmann et al., 1988), however, the anti-yeast activity was reduced significantly when a conserved tyrosine residue was chemically modified (Keishiro et al., 1982).

Snakins

Snakins (SN) are small peptides of 63-66 amino acids, first isolated from potato (Segura et al., 1999; Berrocal-Lobo et al., 2002). Many snakin peptides have been isolated from a variety of plant species (Nahirnak et al., 2012). All snakins have 12 cysteine residues near the C-terminal, forming disulfides to stabilize the peptide (Nahirnak et al., 2012; Harris et al., 2014).

Hevein-type peptides

Hevein is a 30-43 amino acid chitin-binding domain first isolated from the rubber tree (Huang et al., 2004; Van Parijs et al., 1991). The structure consists of a triple stranded antiparallel

β-sheet and a helix on each side of the β-sheet (Van der Weeden et al., 2013; Huang et al., 2004).The antifungal activity of these peptides seems to be associated with the chitin binding activity (Kiba et al., 2003; Yokoyama et al., 2009). Some hevein-type peptides have potent activity against many fungi at concentration ranging from 1µg/ml to 100 µg/ml (Van den Bergh et al., 2002).

Knottin-type peptides

Knottins were isolated for the first time from the American pokeweed *Phytolacca americana*, named PAFPs, and found to have broad antifungal activity (Shao et al., 1999). The NMR structure of PAFPs has a three stranded antiparallel β-sheet, a flexible loop and four β-reverse turns; the knotted topology includes two parallel disulfide bridges threaded by a third one (Gao et al., 2001). Knottin-type antifungal peptides Mj-AMP1 and Mj-AMP2 isolated from *Mirabilis jalapa* have potent antifungal activity against tested plant pathogenic fungi, antibacterial activity against two tested Gram-positive bacteria, but were not active against tested Gram-negative bacteria (Cammue et al., 1992). Knottin-type peptides are characterized by a conserved sequence called a γ-core, β-hairpin motif, which is required for their biological activity (Sagaram et al., 2011; Yount et al., 2004).

2S albumin proteins

2S albumins are storage proteins in plant seeds, synthesized as 18-21 kDa precursors. This precursor is cleaved into small peptides of 3-4 kDa stabilized by disulfide bridges (Moreno and

Clemente, 2008; Puumalainen et al., 2006). Although these peptides draw attention as allergens, some of them have antifungal activity (Puumalainen et al., 2006; Pelegrini et al., 2006). Treatment of yeast cells with Ca-Alb isolated from *Caspiscum anuum* changed yeast cell morphology to an elongate shape and lead to formation of pseudohyphae in *Candida tropicalis*, *Candida albicans*, *Kluyveromyces marxiannus* and *Saccharomyces cerevisiae* (Ribeiro et al., 2012).

Hairpinins

Hairpinins are one of the newest plant antifungal peptides discovered. The first hairpinin was EcAMP1, which was isolated from kernels of barnyard grass *Echinochloa crus-galli* (Nolde et al., 2011). EcAMP1 is a 3 kDa peptide with a helix-loop- helix structure stabilized by two disulfide bridges with C1-C4 and C2-C3 connectivity (Nolde et al., 2011; Slavokhotova et al., 2014). EcAMP1 was active against tested fungi at concentrations ranging from 4~20 μM, and with a unique antifungal mode of action (described below) (Nolde et al., 2011). Recently, Sm-AMP-X isolated from the common chickweed, *Stellaria media*, revealed that both N- and C-terminals are necessary for the antifungal activity and the peptide integrity (Slavokhotova et al., 2014).

Lipid transfer peptides (LTP)

LTPs protein are mostly ~10kDa, with eight conserved cysteine residues, secreted or bound to plant cell walls (Sterk et al., 1991; Subirade et al., 1995). The primary function of LTP proteins is to transfer lipids between membranes (Arondel and Kader 1990, Kader 1996). LTP are classified into two families, LTP1 and LTP2, based on slight secondary structure differences (de

Oliveira Carvalho and Gomes, 2007). LTP1 family has four α-helices stabilized by four intrachain disulfide bridges, and a long random coil C-terminal containing a 3_{10} helix. On the other hand, LTP2 family has three α-helices, two single-turn helices and a carboxyl-terminal tail as in LTP1 (De Oliveira Carvalho and Gomes, 2007). Many LTP have antifungal activity. For example, the 9 kDa LTP protein isolated from mung bean (*Phaseolus mungo*) seeds has antifungal activity against *Fusarium solani*, *Fusarium oxysporum*, *Pythium aphanidermatum* and *Sclerotium rolfsii* (Wang et al., 2004). The 25 kDa LTP isolated from pearl millet seeds inhibited mycelium growth of both *Tricoderma viride* and *Rhizoctonia solant* (Velazhahan et al., 2001). Ha-AP10 LTP isolated from *Helianthus annuus L*. seeds caused 50% growth inhibition against *Fusarium solani* at 0.65 μM (Regente and De La Canal, 2000).

Mammalian antifungal peptides

Mammals synthesize many antimicrobial peptides in cells including neutrophils, keratinocytes, epithelial cells, mouth, tongue, and submucosal glands of the airways. Some of these antimicrobial peptides have antifungal activity against yeasts and filamentous fungi (Table 1.2).

Histatins

Histatins are small, cationic and histidine-rich peptides found in human saliva and some close primates. In humans, there are 12 histatins. Histatin 1 and 3 are produced from gene products with post-translational modifications, whereas the rest are cleavage products produced from large proteins (Van der Spek et al., 1989; Sabatini et al., 1993). Histatin 5 (24 amino acids) is the most potent antifungal histatin. It adopts random coil structure in aqueous solutions and α-helix in

hydrophobic environments (Raj et al., 1998). Histatin 5 has high activity against *C. albicans* (Tsai and Bobek, 1998). HIV patients have low concentration of histatin 5 and high fungal colonization, on the other hand, non HIV individuals have higher levels of histatin 5 and low fungal colonization (Torres et al., 2009). Furthermore, histatin 5 prevents *C. albicans* from adhering to epithelial cell surfaces, leading to low colonization on epithelial cell surfaces (Moffa et al., 2015).

Cathelicidins

Cathelicidins are cationic peptides with a conserved cathelin domain (~100 amino acids) in the N-terminal, but amino acid residues in the C-terminal are responsible for the antimicrobial activity of cathelicidins (Ramanathan et al., 2002; Zanetti et al., 1995). Although humans and a few mammals have a single cathelicidin, most other mammals have several cathelicidins with antifungal activity (Durr et al., 2006; Kosciuczuk et al., 2012). Cathelicin activity is stronger against yeast than for filamentous fungi (Benincasa et al., 2006). For example, sheep and rabbit cathelicidins SAMP29 and CAP18, respectively, showed anti-yeast activity against *Candida krusei*, *Candida tropicalis* and *Candida albicans* (Guthmiller et al., 2001). Human cathelicidin LL37 and its two truncations, LL13-37 and LL17-37, were able to inhibit the growth of *C. albicans* (Wong et al., 2011).

Defensins

Mammalian defensins have structural and functional similarities with defensins from plants, insects and fungi. Defensins are classified based on the structure and disulfide connectivity

into α-defensins, β-defensins and θ-defensins (Cederlund et al., 2011; Van der Weerden et al., 2013).

α-Defensins are composed of 29-35 amino acid residues forming a triple–stranded β-sheet structure stabilized by three disulfide bridges, and a cationic β-hairpin loop (De Smet and Contreras, 2005). Defensins isolated from rabbit granulocytes (NP1-5) were active against *C. albicans*. The anti-yeast activity was reduced in the presence of Ca^{+2} but not Mg^{+2} ions in the growth medium (Selsted et al., 1985). In humans, neutrophils express four α-defensins (HNP1-4), and only HNP3 was inactive against *C. albicans*. This difference in activity is due to the presence of acidic residues in the N-terminal region compared with cationic terminal in the other HNPs (Raj and Dentino, 2002; Wilde et al., 1989). Decreased expression of α-defensins in humans is associated with inflammatory bowel diseases (Kim, 2014).

β-Defensins contain 38-47 amino acid residues, with the same fold of α-defensins, but with different disulfide bridge pairing patterns (De Smet and Contreras, 2005). The first human β-defensin was isolated from plasma (Bensch et al., 1995). Several human β-defensins (HBD) with antifungal activity have been isolated from different tissues; HBD1-4 are expressed in epithelia and leukocytes, either constitutively or induced under immune challenge (Alekseeva et al., 2009; Selsted and Ouellette, 2005). HBD1-3 showed anti-yeast activity in concentrations ranging from 1.3 to 250 μg/ml against *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata* (Joly et al., 2004; Krishnakumari et al., 2009). A synthetic peptide consisting of the C-terminal 38 amino acids of bovine tracheal antimicrobial peptide (TAP) showed antifungal activity against *Aspergillus fumigatus* and *C. albicans* with minimum inhibition concentration of 400 μg/ml. This activity was increased with addition of amphotericin B in an additive effect (Lawyer et al., 1996).

θ-Defensins are cyclic octadecapeptides synthesized in the leukocytes of rhesus monkeys, the structure composed of two anti-parallel β-strands stabilized by three disulfide bridges (Tang et al.,1999). Rhesus theta defensin-1 (RTD-1) is synthesized from the C-terminal of two α-defensins in head to tail ligation (Tang et al., 1999). RTD-1has strong activity against *C. albicans* and *Cryptococcus neoformans*; two hours treatment with 2 to 4 μg/ml RTD-1 reduced the viability of tested organisms by 99% (Tang et al., 1999). RTDs1-6 isolated from neutrophils of monkeys were active against *C. albicans* at 1μM (Matejuk et al., 2010; Tongaonkar et al., 2011).

Lactoferricins

Lactoferrin, 80kDa, is an iron binding protein belonging to the transferrin family. Beside its role in transferring iron and fighting microorganisms by limiting iron levels essential for those microorganisms, lactoferrin acts as an antifungal protein (Farnaud and Evan, 2003). Human lactoferrin has iron-independent antifungal activity against oral isolates of *C. albicans* and *C. krusei* (Nikawa et al., 1993). Furthermore, many N-terminal derived peptides from lactoferrins, called lactoferricins, from humans and other mammals have antifungal activity (Gifford et al., 2005). For example, lactoferricin hLF representing amino acids 1-11 or 21-31 derived from N-terminal of human lactoferrin showed more antifungal activity against fluconazole resistant *C. albicans* than lactoferrin (Lupetti et al., 2000).

Amphibian antifungal peptides

The skin of many amphibian species, especially frogs and toads, are a rich source of antimicrobial peptides (Kumar et al., 2015). Amphibian antimicrobial peptides are named after the source of the peptides (Table 1.3), and can be classified based on structure into: α- helical structure devoid of disulfide bridges or peptides with a disulfide bridge in the C-terminal, forming a loop (Rinaldi, 2002). These peptides are mostly cationic, 8 to 50 residues, and with an amidated C-terminal (Conlon and Sonnevend, 2011; Rinaldi, 2002). Grahami 1 and grahami 2, are 21 amino acid residue peptides isolated from the spotted frog *Rana grahami*, with anti-yeast activity against *C. albicans* and minimum inhibitory concentration (MIC) of 7.5 μg/ml (Xu et al., 2006). Temporins, a family of peptides containing 10-14 amino acid residues isolated from the European red frog, *Rana temporaria*, were active against the chytrid fungus, *Batrachochytrium dendrobatidis*, which causes a decline in amphibians (Rollins-Smith, 2009; Rollins-Smith et al., 2003). The disulfide bridges in some amphibian peptides seem to be essential for the antifungal activity. For example, replacing the cysteine residues in Brevinin-1BYa with serine residues reduced dramatically the antifungal activity (Pal et al., 2006).

Insect antimicrobial peptides

Insects synthesize antimicrobial peptides (AMPs) to fight invading organisms. With a few exceptions, these AMPs are cationic with less than 50 residues (Jansen and Kogel, 2011). Most of these AMPs are synthesized as inactive precursors or pre-proteins (Bahar et al., 2005; Boman et al., 1989; Liu et al., 2000; Rayaprolu et al 2010; Veenstra, 2000). AMPs can be grouped, based on

sequence and structural homology, into three groups (Table 1.4): Cysteine – rich peptides, α-helical peptides, and peptides enriched with one or more specific amino acids (Bulet and Stocklin, 2005; Yi et al., 2014). Most of our knowledge about insect AMPs comes from the antibacterial peptides; little is known about insect antifungal proteins.

Cysteine-stabilized antifungal peptides

Cysteine-stabilized antifungal peptides are peptides of 4–6 kDa. Their secondary structure consists of an α–helix connected to a β-sheet (mixed α–helix/β-sheet), triple-stranded antiparallel β-sheets, or hairpin-like β-sheet structure (Bulet and Stocklin, 2005).

Insect antifungal peptides with an α–helix connected to a β-sheet include defensins and defensin- like peptides. Insect defensin tertiary structure is stabilized by three disulfide bridges with Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6 connectivity. Two of these bridges connect the α-helix to the β-sheet, forming a cysteine-stabilized alpha beta structure (CSαβ) (Ganz and Lehrer, 1995). Drosomycin, a defensin-like peptide, was the first insect antifungal peptide isolated from *Drosophila melanogaster* (Fehlbaum et al., 1994; Landon et al., 1997). Drosomycin has CSαβ structure stabilized by four disulfide bridges, with Cys1-Cys8, Cys2-Cys5 and Cys3-Cys6 and Cys4-Cys7 connectivity (Landon et al., 1997). Cysteine-stabilized antifungal peptides can be grouped into strict antifungal peptides and peptides with both antifungal and antibacterial activity. Strictly antifungal peptides include drosomycin (Fehlbaum et al., 1994; Landon et al., 1997), heliomycin from *Heliothis virescens* (Lamberty et al., 1999), gallerimycin from *Galleria mellonella* (Schuhmann et al., 2003), termicin from *Pseudacanthotermes spiniger* (Da Silva et al., 2003; Lamberty et al., 2001), galleria defensin from *G. mellonella* (Lee et al., 2004), and ARD1

from *Archeoprepona demophoon* (Landon et al., 2004). Peptides with both antifungal and antibacterial activity include *Bombus pascuorum* defensin (Rees et al., 1997), *Anopheles gambiae* defensin (Vizioli et al., 2001), coprison from *Copris tripartitus* (Lee et al., 2012), and Btdef from *Bemisia tabaci* (Wang et al., 2013).

In addition to antifungal peptides with CSαβ fold, there are some antifungal peptides with structure stabilized by disulfide bridges, but lacking the CSαβ fold, for example, diapause specific peptide (DSP) isolated from a leaf beetle, *Gastrophysa atrocyanea*. Compared to peptides with CSαβ fold, the DSP N-terminal has an extra helix, which is absent in antifungal peptides with CSαβ fold. This helix is connected to the second helix in the structure by a disulfide bridge, making the disulfide bridge connectivity different from those peptides with CSαβ fold. DSP has antifungal activity against *Trichophyton rubrum* but not against the entomopathogenic fungi *Beauveria bassiana* (Kouno et al., 2007; Tanaka et al., 2003).

Alo-3 and thanatin belong to triple–stranded antiparallel β-sheets and hairpin-like β-sheets peptide family, respectively. Alo-3 isolated from *Acrocinus longimanus* has antifungal activity against yeasts, and has six cysteine residues forming three disulfide bridges in the same connectivity as defensins (Barbault et al., 2003). Thanatin, composed from 21 amino acids with one disulfide bridge, isolated from *Podisus maculiventri*, has antifungal activity against yeasts and filamentous fungi (Fehlbaum et al., 1996).

α-helical peptides

α-Helical peptides include cecropins, moricins and some venom peptides (Andra et al., 2001). Cecropins are ~4 kDa peptides, which were the first AMPs isolated from insects (Hultmark et al., 1982). Cecropins from different insect orders have antifungal activity (Cavallarin et al., 1998; De Lucca et al., 1997, Ekengren and Hultmark, 1999; Vizioli et al., 2000). Papiliocin, stomoxyn and sarcotoxin pd are cecropins isolated from *Papilio xuthus*, *Stomoxys calcitrans* and *Paederus dermatitis*, respectively, with antifungal activity (Boulanger et al., 2002, Kim et al., 2010; Memarpoor- Yazdi et al., 2013). CecropinXJ isolated from *B. mori* has antifungal activity against many fungi, with MIC ranging from 0.78 μM against *Magnaporthe grisea* to 12.5 μM against *Rhizopus stolonifera* (Xia et al., 2013). Moricins, basic peptides found only in lepidopteran insects, have antifungal and antibacterial activity and are composed of a single α-helix (Brown et al., 2008; Dia et al., 2008; Hemmi et al., 2002). Three venom peptides, OdVP1-3, from *Orancistrocerus drewseni* have strong antifungal activity (Baek and lee, 2010).

Peptides rich in one amino residue

Peptides rich in one amino residue include proline-rich peptides and glycine-rich peptides (Bulet and Stocklin, 2005). Proline-rich peptides with antifungal activity include lebocins and metchnikowin. Lebocins are synthesized as inactive precursor peptides and then cleaved by a furin-like enzyme at an RXXR consensus motif (Rao et al., 2012). Lebocins are upregulated in the fat body and the hemocytes in *M. sexta* larvae after immune challenge with *E. coli*, *Micrococcus luteus* and *S. cerevisiae*. The activity was near the N-terminus (Rao et al., 2012) not near the C-terminus as in *B. mori* (Chowdhury et al., 1995).

Metchnikowin from *D. melanogaster* has antifungal activity against the filamentous fungus *N. crassa* (Levashina et al., 1995). Transgenic barley with metchnikowin was resistant to plant pathogens (Rahnamaeian et al., 2009; Rahnamaeian and Vilcinskas, 2012). Glycine rich antifungal peptides include *M. sexta* gloverins (Xu et al., 2012), attacin B from *Hyphantria cunea* (Kwon et al., 2008) and a few other antifungal peptides, e.g Holotricin from *Holotrichia diomphalia* (Lee et al., 1995) and tenecin-3 from *Tenebrio molitor* (Kim et al., 2001). Although the first gloverins isolated from *H. gloveri* lack antifungal activity (Axen et al., 1997), *M. sexta* gloverin has antifungal activity against *S. cerevisiae* and *Cryptococcus neoformans*, and binds in vivo to the β-1,3 glucan laminarin (Xu et al., 2012). The only documented antifungal attacin is *Hyphantria cunea* attacin-B with antifungal activity against *C. albicans*. The expression of this attacin gene was upregulated in fat body, epidermis and hemocytes after injection with *E. coli*, *Citrobacter freundii*, or *C. albicans* (Kwon et al., 2008).

Insect antifungal proteins

Some insect proteins revealed antifungal activity, among these proteins are apolipophorin III and lysozyme. Apolipophorin III, ~18 kDa protein, is abundant in insect hemolymph plays a role in lipid transport in insects (Wiesner et al., 1997). Apolipophorin III isolated from *G. mellonella* has activity against *C. albicans* and the filamentous fungus *Fusarium oxysporium*. Furthermore, apolipophorin III caused change in cell morphology of *C. albicans* from the oval shape to filamentous, and caused low level of metabolic activity and increased vacuolization in *Fusarium oxysporium* (Zdybicka- Barabas et al., 2012).

Lysozyme is a cationic protein, 14 kDa, found in many insects (Yu et al., 2002). It has been reported that the antifungal activity of lysozyme is associated with its enzymatic activity by breaking the N-glycosidic bonds between the protein and polysaccharides or between β-1,4 linkages in fungal cell walls (Lee et al., 2010). Non-enzymatic activity has been reported for *G. mellonella* lysozyme against *C. albicans* by reducing metabolic activity and changing the topology of the cell surface (Sowa-Jasilek et al., 2014).

Manduca sexta antimicrobial peptides

Several antimicrobial peptides/proteins have been isolated from the tobacco hornworm, *M. sexta*, including: lysozyme (Mulnix et al., 1994), attacin (Kanost et al., 1990), cecropin (Dickinson et al., 1988), a lebocin-related precursor protein (Rayaprolu et al., 2010), moricin (Dai et al., 2008), gloverin (Zhu et al., 2003), and defensin (Genbank accession number: HQ400765). These are antibacterial peptides except gloverin and lebocin C, which both have antifungal and antibacterial activity. Gloverin has antifungal activity against both *S. cerevisiae* and *C. neoformans*. Gloverin mRNA was detected in epidermis, hemocytes, fat body, midgut and testis of *M. sexta* naïve larvae, and was upregulated significantly in fat body, hemocytes, and midgut after injection with *Staphylococcus aureus* or *E. coli* (Xu et al., 2012) Lebocin C has antifungal activity against *C. neoformans*. Lebocin C mRNA levels were upregulated in the fat body and hemocytes after injecting *E. coli*, *M. luteus* or *S. cerevisiae* (Rao et al., 2012).

Structure and components of fungal cell wall and plasma membrane

The fungal cell wall plays many roles in protecting fungi from the environment, adhesion, cell signaling, and invading other organisms. Fungal cell walls are composed of glucans, proteins, chitin, and other complex polymers (Selitrennikoff, 2001). Chitin represents less than 2% of the cell wall in *S. cerevisiae* and other fungi, found mainly in the bud scars (Brul et al., 1997). Glucans in fungal cell walls are a network of β-1,3-glucans with β-1,6-glucan branches, forming around 50% of cell wall components by weight (Van der Weerden et al., 2013). β-1,3- glucans link to N-acetylglucosamine (GlcNAc) via β-1,4 linkage, or to cell wall proteins called PIR (proteins with internal repeats). β-1,3-glucan binds to manoproteins on the outer layer of the cell wall, directly through an alkali-sensitive bond, whereas β-1,6-glucans bind to cell wall manoproteins via glycosylphosphatidylinositol (GPI) (Lesage and Bussey, 2006). Different forms of glycosylated proteins are present in fungal cell walls. For example, yeasts have manoproteins, whereas filamentous fungi have proteins bound to galactose, glucose or uronic acid (Schoffelmeer et al., 1999).

The plasma membrane plays a critical role in living organisms; it engulfs the cell components and has many important functions. In fungi, the plasma membrane lipid bilayer consists of glycerophospholipids, sphingolipids and sterols. The type of phospholipids in fungi are anionic phospholipids, particularly, phosphatidylserine and phosphatidylinositol (Smith and Lester, 1974; Thevissen et al., 2003). Sphingolipids have a ceramide backbone. Sphingolipids are classified based on type of head groups attached to ceramides as phosphosphingolipids or glycosphingolipids. The later class of sphingolipids can be neutral or acidic depending on the attached head groups. The most abundant neutral glycosphingolipids in most fungi are

glucosylceramide, however, both *S. cerevisiae* and *Schizosaccharomycyes pombe* lack this sphingolipid (Dickson and Lester, 1999). Acidic glycosphingolipids, in which ceramide is either attached to phosphate, sulphate or charged sugar, are found in many fungi (Dickson and Lester, 1999).

Phosphoglycosphingolipids are the most abundant acidic glycosphingolipids in fungi. *S. cerevisiae* possesses three types of phosphoglycosphingolipids, named inositol phosphorylceramides, mannosyl inositol phosphorylceramide and mannosyl diinositolphosphorylceramide, contributing about 20% of plasma membrane lipids (Patton and Lester, 1991). The difference in sphingolipid components between fungi and mammals have been used to design selective antifungal peptides that bind specifically to fungal sphingolipids (Thevissen et al., 2005). Sterols are found in all eukaryotic plasma membranes in different forms. In fungi, the sterol type is ergosterol. Ergosterol has been targeted by some antifungal drugs. For example, the antifungal Amphotercin B derived from the actinomycte *Streptomyces nodosus* has MIC of about 2 mg/L for many fungi (Ellis, 2002).

The mode of action of antifungal peptides

The mode of action of some antifungal peptides has been determined. Some of these peptides cause membrane permeablization. For example, drosomycin was the most studied insect antifungal peptide. It causes membrane permeabilization in tested fungi (Fehlbaum et al., 1994). In an *N. crassa* mutant in sphingolipids, the drosomycin activity was decreased, which may explain

that permeabilization depends on the interaction between drosomycin and sphingolipids in the plasma membrane (Gao and Zhu, 2008). Membrane permeabilization is also the mode of action of the plant antifungal protein zeamatins, leading to release of cytoplasmic material from *C. albicans* and *N. crassa* (Roberts and Selitrennikoff, 1990). Forming channels in lipid bilayer membranes is a characteristic of cecropin A and B (Efimova et al., 2014).

Binding specifically to plasma membrane components has been shown for some antifungal peptides. Heliomicin binds selectively to glucosylceramides isolated from *Pichia pastoris* but not to human glucosylceramides. Furthermore, *C. albicans* and *P. pastoris* mutants in glucosylceramides were unaffected (Thevissen et al., 2004). Plant defensins RsAFP2 and DmAMP1 isolated from radish and dahlia, respectively, bind to sphingolipids in the plasma membrane. When the sphingolipid mannose diinositolphosphoryl ceramide was disrupted from *S. cerevisiae*, the yeast was resistant to DmAMP1 (Thevissen et al., 2000). Similarly, when glucosylceramide was disrupted in *C. albicans* and *P. pastoris*, yeasts were resistant to RsAMP2 (Thevissen et al., 2004).

Some antifungal peptides target components of the cell wall. For example, *Pharbitis nil* antimicrobial peptide (Pn-AMP1), a plant defensin, binds to cell walls of *S. cerevisiae* and *C. albicans* and causes depolarization of the actin cytoskeleton, leading to changed yeast morphology. Yeast mutants in components of cell integrity pathway were more sensitive to Pn-AMP1, and yeasts with mutations in components of the α-1,6-mannosyltransferase complex, responsible for mannosylation of cell wall proteins, were resistant to Pn-AMP1(Koo et al.,2004). Binding to chitin is important to antifungal activity of some hevein–type peptides. For example, mutations in the chitin binding domain of Cy-AMP-1 from cycad decreased the antifungal activity compared with the native peptide (Yokoyama et al., 2009). On the other hand, hevein–type peptides EAFP1

and EAFP2 from *Eucommia ulmoides* showed antifungal activity against fungi with or without chitin in cell walls. This indicates that the chitin- binding activity is not important for the antifungal activity of those peptides (Huang et al., 2002).

The antifungal peptide EcAMP1 from kernels of barnyard grass E. crus-galli, has a unique way to enter Fusarium solani spores, by binding to cell wall components, and then internalizes to the cytoplasm without affecting the plasma membrane, leading to inhibited spore germination (Nolde et al., 2011). In a comparable unique entrance to fungal cells, human histatin 5 enters C. albicans by translocation, not endocytosis, causing loss of mitochondrial transmembrane potential, and killing the yeast (Helmerhorst et al., 1999; Jang et al., 2010).

The aim of this research

Because of lack of studies about antifungal peptides in *M. sexta*, and with only two peptides, gloverin and lebocin C, found to have antifungal activity (Rao et al., 2012; Xu et al., 2012), this study aimed to explore the presence of antifungal peptides/proteins in *M. sexta* hemolymph.. Finding new antifungal peptides/protein in *M. sexta* will help to understand its innate immune response to fungal infection. Disrupting this response could be used in future to control insects by making them more susceptible to fungal diseases and decrease their danger against crops. Since only gloverin has been shown to bind to β-1,3 glucan, another goal is to study the candidate target of the discovered antifungal peptide/protein.

Since natural resources have been mined, looking for new antimicrobial molecules because of the problem of multiresistant pathogens to available antimicrobial drugs, finding a new antifungal peptide/protein may be used in future to as an antifungal therapy or to identify new targets for future drug development for pathogenic fungi. Furthermore, insect antifungal peptides may be useful for generating transgenic plants resistant to some plant pathogenic fungi.

Tables

Table 1.1. A summary table for plant antifungal peptides/proteins and their activity against tested fungi.

Plant Antifungal peptides				
Family	Name	Source	Size (kDa)	Activity*
Thaumatin-like proteins	Osmotin	*Nicotina tabacum*	24	*Aspergillus flavus, Aspergillus parasitica, Rhizoctonia solani* , *Macrophomina phaseolina* and other species from *Bipolaris*, *Fusarium* and *Phytophthora*
	Zeamatin	*Zea mays*	22	*C. albicans*
	NaD1	*Nicotina alata*	~10	*F. oxysporum* and *Botrytis cinerea*
	PhD1	*Petunia hybrida*	~10	*F. oxysporum* and *B. cinerea*
	PhD2	*Petunia hybrida*	~10	*F. oxysporum* and *B. cinerea*
	Psd1 Psd2	*Pisum sativum*	~5	*A. niger, A.versicolor, F. oxysporum* , *F. moniliforme* and *Neurospora crassa*
	Rs-AFPs 1-4	*Raphanus sativus*	5	*Alternaria. Brassicola, B. cinerea* and *F. culmorum*

Plant defensins	NaD1	*Nicotina alata*	~10	*F. oxysporum* and *B. cinerea*
	PhD1	*Petunia hybrida*	~10	*F. oxysporum* and *B. cinerea*
	PhD2	*Petunia hybrida*	~10	*F. oxysporum* and *B. cinerea*
	Psd1 Psd2	*Pisum sativum*	~5	*A. niger , A. versicolor, F. oxysporum*, *F. moniliforme* and *N. crassa*
	Rs-AFPs 1-4	*Raphanus sativus*	5	*A. Brassicola, B. cinerea* and, *F. culmorum*
Impatiens balsamin antimicrobial peptides (Ib-AMP)	Ib-AMP1-4	*Impatiens balsamin*	~2	*A. flavus* and *C. albicans.*
Thionins	Thionins	Many Plants	~5	Many fungi were tested and *S. cerevisiae*.
Snakins	Snakin1 and 2	Potato ,Tomato , Arabidopsis and many other plants	~17	*B. cinerea* and many other fungal pathogens
Hevein-type peptides	WjAMP-1	*Wasabia japonica* *Hevea brasiliansis* and *Arabidopsis thaliana*	~ 21	*A. alternata, B. cinerea, F. solani,* and *Magnaporte grisea*
Knottin-type peptides	PAFPs	*Phytolacca americana*	4	*Alternaria panax*, *Fusarium* sp., and *Rhizoctonia solani*
	Mj-AMP1 Mj-AFP2	*Mirabilis jalapa*	4	Many fungi were tested

Hairpinins	*EcAMP1*	*Echinochloa crus-galli*	3	Many fungi were tested
	Sm-AMP-X	*Stellaria media*	~3	Many fungi were tested
Lipid transfer peptides (LTP)	LTP	*Phaseolus mungo*	9	*F. solani*, *F. oxysporum*, *Pythium aphanidermatum* and *Sclerotium rolfsii*
	LTP	pearl millet seeds	25	*Tricoderma viride* and *Rhizoctonia solant*
	Ha-AP10 LTP	*Helianthus annuus* L.	10	*F. solani*
Hairpinins	*EcAMP1*	*Echinochloa crus-galli*	3	Many filamentous fungi
Lipid transfer peptides (LTP)	LTP	*Phaseolus mungo*	9	*F. solani*, *F. oxysporum*, *Pythium aphanidermatum* and *S. rolfsii*
	LTP	pearl millet seeds	25	*Tricoderma viride* and *Rhizoctonia solant*
	Ha-AP10 LTP	*Helianthus annuus* L.	10	*F. solani*
Lipid transfer peptides (LTP)	LTP	*Phaseolus mungo*	9	*F. solani*, *F. oxysporum*, *Pythium aphanidermatum* and *S. rolfsii*
	LTP	pearl millet seeds	25	*T. viride* and *R. solant*
	Ha-AP10 LTP	*Helianthus annuus* L.	10	*F. solani*

* If many fungi were tested, a phrase of "many fungi were tested" is used in the activity. To have a list of the tested fungi, reader should refer to the reference in this chapter.

Table 1.2. A summary table for mammalian antifungal peptides/proteins and their activity against tested fungi.

Mammalian antifungal peptides					
Family	Name		Source	Size (kDa)	Activity
Histatins	Histatin 1 , Histatin 3 and Histatin 5		Human and some higher primates	1-4	*C. albicans , C. neoformans, A. fumigatus,* and *C. dubliniensis*
Cathelicidins	SAMP29		Sheep	3.2	*C. krusei*, *C. tropicalis* and *C. albicans*
	CAP18		Rabbit	18	*C. krusei*, *C. tropicalis* and *C. albicans*
	LL37 , LL13-37 and LL17-37		Human	2-4	*C. albicans*
Defensins	α-Defensins:	NP 1-5	Rabbit	~3.5	*C. albicans*
		HNP 1-3	Human	~3.5	*C. albicans*
		HBD1-3	*Human*	9	*C. tropicalis, C. parapsilosis, C. krusei,* and *C. glabrata*

	β-Defensins	Bovine tracheal antimicrobial peptide (TAP)	Bovine	~4	*A. fumigatus* and *C. albicans*
	θ-Defensins	Rhesus theta defensins (RTD)	Rhesus	2	*C. albicans* and *C. neoformans*
Lactoferricins	Human lactoferricin (hLF)		Human	~4	*C. albicans*

Table 1.3. A summary table for amphibian antifungal peptides/proteins and their activity against some tested fungi.

Amphibian antifungal peptides				
Family	Name	Source	Size (kDa)	Activity
Grahami	Grahami 1 and Grahami 2	*Rana grahami*	~1.9	*C. albicans*
Temporins	Temporin A	*Rana temporaria*	~1.5	*Batrachochytrium dendrobatidis*
Brevinin	Brevinin-1BYa	*Rana boylii*	~2.6	*C. albicans*, *C. tropicalis*, *C. krusei* and *C. parapsilosis*

Table 1.4. A summary table for insect antifungal peptides/proteins and their activity against some tested fungi.

Insect antifungal peptides				
Family	Name	Source	Size (kDa)	Activity*
Cysteine-stabilized antifungal peptides (with CSαβ fold)	Drosomycin	*Drosophila melanogaster*	~5	*A. brassicola, A. longipes, Ascochytu pisi, B. cinerea, F. culmorum, F. oxysporum , Nectria haematococca, N. crassa , Dichoderma hamatum* and *D. uiride*
	Heliomycin	*Heliothis virescens*	4.7	*A. fumigatus, F. culmorum, F. oxysporum, N. haematococca, N. crassa, T. viride C. albicans* and *C. neoformans*
	Gallerimycin	*Galleria mellonella*	8.4	*Metarhizium anisopliae*
	Termicin	*Pseudacanthotermes spiniger*	4.8	*F. culmorum, F. oxysporum, N. crassa, N. haematococca, T. viride C. albicans, C. neoformans, S. cerevisiae*
	Galleria defensin	*Galleria mellonella*	4.7	Fusarium oxysporum and Pyricularia grisea *T. viride, F. oxysporum , Pyricularia grisea , C. neoformans*

				C. albicans, and *Geotrichum candidum*
	ARD1	*Archeoprepona demophoon*	4.8	*A. fumigatus* and *C. albicans*
	Bombus pascuorum defensin	*Bombus pascuorum*	~5.2	*N. crassa*
	Anopheles gambiae defensin	*Anopheles gambiae*	~4	*N. crassa*
	Coprison	*Copris tripartitus*	~4.4	*A. flavus, A. fumigatus, A. parasiticus, C. albicans, C. parapsilosis, Malassezia furfur, Trichosporon beigelii* and *Trichophyton rubrum*
	Btdef	*Bemisia tabaci*	5	*C. albicans, Beauveria bassiana, M. anisopliae, B. cinerea* and *P. pastor*is
Diapause Family	Diapause Specific Peptide (DSP)	*Gastrophysa atrocyanea*	~5	*T. rubrum*
Cysteine-stabilized antifungal peptides(with	Alo-3	*Acrocinus longimanus*	4	*C. glabrata*
	Thanatin	*Podisus maculiventri*	~2.2	*N. crassa, B. cinerea, N. haematococca, T. viride, A. brassicola, F. culmorum. Ascochyta pisi, F. oxysporum, A. fumigatus*

					and *T. mentagrophytes*
α-Helical peptides	Cecropins	Cecropin	*Hyalophoru cecropia*	~4	*S. cerevisiae, Phytophthora infestans, F. oxysporum, F. moniliforme* *F. solani f. sp. Pisi, F. solani f. sp. Phaseoli,B. cinerea, M. grisea* *Trichoderma sp. Thielaviopsis basicola* and *Penicillium sp.*
		Papiliocin,	*Papilio xuthus*	4	*C. albicans*
		Stomoxyn	*Stomoxys calcitrans*		*F. culmorum, F. oxysporum, A. fumigatus, N. haematococca,* *N. crassa, T. mentagrophytes, T. viride, C. albicans, C. neoformans* and *C. glabrta*
		Sarcotoxin pd	*Paederus dermatitis*	3.6	*C. albicans, A. niger* and *A. fumigates*
		CecropinXJ	*Bombyx mori*		*A. alternate, P.digitatum, B. cinerea , R. stolonifer, P. italicum* and *M. grisea*
	Morcins	OdVP1-3	*Orancistrocerus drewseni*	1.5	*B. cinerea C. albicans*
Peptides rich in one amino residue	Lebocins		*M. sexta* *B. mori*	~4	*S. cerevisiae* and *C. neoformans*
	Metchnikowin		*D.melanogaster*	3	*N. crassa*
	Gloverin		*M. sexta*	14	*S. cerevisiae* and *C. neoformans*

	Attacin B	*Hyphantria cunea*	19.8	*C. albicans*
	Tenecin-3	*Tenebrio molitor*	~9	*C. albicans*
	Holotricin	*Holotrichia diomphalia*	~16.5	*C. albicans*

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Chapter 2 - Characterization and regulation of an antifungal peptide from hemolymph of an insect, *Manduca sexta*

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Introduction

Insects synthesize antimicrobial peptides as part of the immune response to infection by microorganisms (Casanova-Torres and Goodrich-Blair, 2013; Jiang et al., 2010; Hultmark et al., 1980; Memarpoor-Yazdi et al., 2013; Zhang et al., 2014). Some small α-helical peptides from insect hemolymph have antibacterial and antifungal activity. These include some cecropins (Boulanger et al., 2002; Cavallarin et al., 1998; De lucca et al., 1997; Ekengren and Hultmark, 1999; Kim et al., 2001; Vizioli et al., 2000; Xia et al., 2013) and moricins (Brown et al., 2008; Dai et al., 2008; Hemmi et al., 2002). Proline-rich peptides with antifungal activity include lebocins (Chowdhury et al., 1995; Rao et al., 2012) and metchnikowin (Levashina et al., 1995; Rahnamaeian et al., 2009; Rahnamaeian and Vilcinskas, 2012). Glycine-rich antifungal peptides include *Manduca sexta* gloverins (Xu et al., 2012), attacin B from *Hyphantria cunea* (Kwon et al., 2008), holotricin from *Holotrichia diomphalia* (Lee et al., 1995), and tenecin-3 from *Tenebrio molitor* (Kim *et al*., 1998).

Most insect antifungal peptides characterized so far are 4-6 kDa cysteine-stabilized molecules, with structures consisting of an α-helix connected to a β-sheet, triple-stranded antiparallel β-sheets, or two-strand hairpin-like β-sheet (Bulet and Stocklin, 2005). Insect defensins have in common an α-helical structure connected to a β-sheet, stabilized by three disulfide bridges, including two that connect the α-helix to the β-sheet, forming a cysteine-stabilized alpha beta structure (CSαβ) (Landon et al., 1997). Drosomycin, from *Drosophila melanogaster* contains four disulfide bridges and differs from the defensins in the pattern of disulfide connectivity (Landon et al., 1997). Drosomycin and some defensins are strictly antifungal (Da Silva et al., 2003; Fehlbaum et al., 1994; Lamberty et al., 1999; Lamberty et al., 2001; Landon et al., 2004; Landon et al., 1997; Lee et al., 1995), whereas some defensins have both antifungal and antibacterial activity (Rees et al., 1997; Vizioli et al., 2001; Hwang et al., 2009; Wang et al., 2009). An antifungal peptide named diapause specific peptide isolated from hemolymph of a leaf beetle, *Gastrophysa atrocyanea*, has three disulfide bridges, but its fold and disulfide connectivity differ from the defensins and drosomycin (Kouno et al., 2007; Tanaka et al., 2003). cDNA sequences similar in sequence to the beetle diapause specific peptide have been identified from some Lepidopteran species (Wan et al., 2012), and this group of peptides is known as the diapausin family (Tanaka et al., 2003; Tanaka and Suzuki, 2005; Kouno et al., 2007). Many antimicrobial peptides/proteins have been isolated from the tobacco hornworm, *Manduca sexta*, including: lysozyme (Mulnix and Dunn, 1994), attacin (Kanost et al., 1990), cecropin (Dickinson et al., 1988), a lebocin-related precursor protein (Rayaprolu et al., 2010), moricin (Dai et al., 2008), gloverin (Zhu et al., 2003), and defensin (Genbank accession number: HQ400765). These peptides are antibacterial peptides except gloverin and

lebocin C which both have antifungal and antibacterial activities (Xia et al., 2012; Rao et al., 2012).

In this study we investigated antifungal activity in hemolymph of the caterpillar, *Manduca sexta*, which has been used as a model system for biochemical studies of insect immunity (Kanost et al., 2004; Kanost and Nardi, 2010; Ragan et al., 2009). We found that antifungal activity is induced in hemolymph after microbial exposure, and we isolated and characterized a 5 kDa antifungal peptide from the diapausin family. This peptide, *M. sexta* diapausin-1, affected the growth and morphology of yeast and fungal hyphae. Diapausin-1 mRNA level increased after injection of larvae with microorganisms and during normal development at the wandering larval stage prior to pupation.

Materials and methods

Insects

M. sexta larvae were fed with artificial diet and reared at 25 °C as described by Dunn and Drake (1983).

Sequence analysis

Sequence similarity searches were performed using Blast software (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Center for Biotechnology Information. Searches of the *Manduca sexta* genome sequence were carried out using a blast server

at the Manducabase web site (http://agripestbase.org/manduca/?q=blast). Multiple sequence alignment was done using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011). Signal peptides were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

Yeast injection and hemolymph collection

Saccharomyces cerevisiae strain SUB62 was grown in suspension in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C with shaking at 200 rpm to OD_{600} nm of ~ 0.6. Day two fifth instar *M. sexta* larvae were injected with 1.5×10^6 cells of *S. cerevisiae.* Uninjected larvae were used as control. After 24 h, hemolymph was collected from a cut proleg into a tube containing few crystals of diethyldithiocarbamate (DETC). The hemolymph was centrifuged at 7000×g for 25 min at 4 °C to remove hemocytes. The supernatant (plasma) was stored at -80 °C until use in protein preparations or assays.

Protein analysis

Protein concentration was measured by absorbance at 280 nm (calculated $\varepsilon = 7815\ M^{-1}\ cm^{-1}$). Hemolymph and recombinant protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS- PAGE), using 16% acrylamide gels and tricine buffer and stained with Coomassie brilliant blue R-250 (Invitrogen, USA). Recombinant proteins were analyzed by SDS-PAGE with NuPAGE 4-12% polyacrylamide Bis-Tris gels (Invitrogen, USA), and stained with Coomassie blue. Immunoblotting analysis was performed with mouse anti-His antibody as primary antibody (1: 2000 dilution) (Bio- Rad, USA), and goat-anti mouse conjugated to alkaline phosphatase (1: 3000 dilution) (Bio- Rad) as the secondary antibody.

Antimicrobial activity assays

Anti-yeast activity assay

Measurement of activity against yeast was performed as described by Fai and Grant (2009) with minor modifications. Briefly, *S. cerevisiae* grown in YPD medium at 30 °C to OD_{600} of about 0.6. The cultures were diluted with YPD to OD_{600} of 0.1. Samples (100 µL) of the diluted culture were added to wells of a 96-well plate. Samples of hemolymph or recombinant protein were added, and the final volume was brought to 200 µL with YPD medium. The plates were incubated 16 h at 30 °C with shaking at 200 rpm, and then OD_{600} was measured using a microplate reader (BioTek, USA).

Bacterial strains and antibacterial activity assays

Listeria fleischmannii (DSM24998), *Listeria grayi* (DSM 20601), *Listeria marthii* (DSM 23813), *Staphylococcus aureus* (DSM 2569), *Escherichia coli* (D31) and *Pseudomonas aeruginosa* (DSM50071) were used to assess the antibacterial properties of *M. sexta* recombinant diapausin-1 peptide. Growth inhibition assays were performed as described by Rahnamaeian et al (2015) and Tonk et al (2015) in diapausin-1 concentrations ranging from 0.03 µM to 250 µM using 384-well plates (Griener Bio One, Germany). Bacterial strains in mid-logarithmic phase were used for these assays. The initial OD_{600} nm of the bacterial culture was set to 0.01 for *Listeria* spp. and 0.001 for other strains. We used Brain Heart Infusion Broth (BHIB) medium for *Listeria* spp. and Tryptic Soy Broth (TSB) (Roth, Germany) for other strains (Tonk et al, 2014). The assays lasted 16 h and the OD_{600} was recorded every 20 min using an Eon™ Microplate Spectrophotometer (BioTek Instruments, USA). Each assay included an untreated control bacterial culture.

Antifungal activity assays

Fusarium culmorum and *Fusarium graminerum* were grown on Spezieller Nahrstoffarmer agar (SNA) medium for 7 days, and conidia were washed with 1 mL of sterile water. To assay antifungal activity ~50 conidia in 30 µL of sterile water were supplemented with 4 µM of diapausin-1, and the germination was examined after 24 h by phase contrast microscopy. To assess the effect of diapausin-1 against *Magnaporthe oryzae* spores, 1×10^5 spores were incubated with 15 µM of diapausin-1 for 4 h, then the spores were examined by the phase contrast microscopy. For *Beauveria bassiana*, 1×10^3 spores/well in a 96-well plate were incubated with 0, 10, 20 or 30 µM diapausin-1 in 200 µL B-medium (4% glucose, 2% yeast extract). The plates were incubated at 25 °C, 200 rpm for 24 h, and then OD_{600} was measured using the microplate reader and wells were examined using an inverted phase contrast microscope.

Size exclusion chromatography

A 20 mL sample of *M. sexta* plasma collected 24 h after injection of larvae with *S. cerevisiae* as described above was subjected to size exclusion chromatography on a column (120 × 2.5 cm) of Sephacryl S-200 (GE healthcare) and eluted with 20 mM Tris, 150 mM NaCl, pH 7.5 at 2 mL/min, with collection of 8 mL fractions. Protein content of the fractions was assessed by absorbance at 280 nm. Samples of each fraction (100 µL) were assayed for anti-yeast activity as described above.

Peptide mass fingerprinting

Size exclusion chromatography fractions 56-58, containing peptides with activity against yeast, were pooled and concentrated from 24 ml to 3.5 mL by vacuum centrifugation. Then, 10 µL

was added to 22 µL 5 mM dithiothreitol, 20 mM ammonium bicarbonate. The mixture was heated at 95 °C for 10 min, and then after reduced cysteine residues were alkylated by iodoacetate, 250 ng of proteomics-grade Trypsin Gold (Promega) was added, and incubated at 30 °C overnight. The digestion products were analyzed using a Bruker Daltonics Ultraflex III MALDI TOF/TOF Mass Spectrometer in MS mode at the core facility of the Biochemistry and Molecular Biophysics Department at Kansas State University (Dittmer et al., 2012). The peak lists were analyzed using Mascot software v 2.2.04 (Matrix Science Ltd.) and compared with protein sequences from the *M. sexta* official gene set 1.0 obtained from genome sequencing (http://agripestbase.org/manduca/), and with the NCBI nonredundant database (NCBInr 2008.10.24.08, restricted to Metazoa).

Preparation of recombinant diapausin-1

cDNA made to a sample of RNA isolated from fat body of *M. sexta* larvae collected 24 h after injection with 100 µg *M. luteus* was used as a template for PCR using forward (5′-GCC ATG GCTATCAACAACTG-3′) and reverse (5′-AAGCTTTTAACGTCTGTAACA-3′) primers containing NcoI and HindIII restriction sites, respectively, to amplify the sequence encoding the diapausin-1. The diapausin-1 cDNA and pET32a (Novagen, USA) were digested with NcoI and Hind III (New England Biolabs, USA). The digested fragments were separated by electrophoresis on a 1% agarose gel, purified using a QIAquick Gel Extraction Kit (Qaigen, USA), and then fragments were ligated using T4 DNA ligase (New England Biolabs, USA) and used to transform *E. coli* strain Rosetta gami (Novagen, USA). A single colony containing the expected sequence was grown in 1 L of LB medium, and recombinant protein expression was induced with 1mM isopropyl β-D-1-thiogalactopyranoside for 4 h. Cells were pelleted at 4000 × g for 20 min at 4 °C. The pellet was suspended in lysis buffer (0.05 M sodium phosphate buffer containing 10 mM imidazole and 300 mM NaCl, pH 8.0) and 50 µL inhibitor cocktail (P 8849, Sigma) per gram of

cell pellets, then cells were lysed by sonication and then centrifuged at 4000 × g for 20 min at 4 °C. The fusion protein consisting of diapausin-1 with an amino-terminal thioredoxin and 6-His tag was purified from the supernatant by nickel affinity chromatography. The supernatant was applied to Econo-column® (1.5 cm ID, 7 cm long) containing nickel beads (Bio-Rad, USA). The column was washed three times with 5 mL 20 mM imidazole, 300 mM NaCl in 0.05 M sodium phosphate buffer, pH 8.0. The fusion protein was eluted with 5 mL of 250 mM imidazole in 0.05 M sodium phosphate buffer, pH 8.0. Fractions were analyzed by SDS-PAGE with NuPAGE 4-12% polyacrylamide Bis-Tris gels (Invitrogen, USA). Fractions containing the fusion protein were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 7.4. The amino-terminal thioredoxin tag was removed by incubating 100 µg of fusion protein with one unit of recombinant enterokinase (Novagen, USA) at room temperature for 16 h according to the manufacturer's instructions. Then, nickel affinity chromatography was used to separate the His and thioredoxin tags sequence from the mature diapausin-1, which flowed through the column. Fractions containing diapausin-1 were concentrated, and buffer was exchanged to 10 mM sodium phosphate buffer pH 7.4, using Amicon centrifugal filters with 3000 Da molecular weight cutoff (Millipore, USA). The diapausin-1 was further purified by reverse phase chromatography on a C18 capillary column (75 µm inner diameter × 15 cm; PepMap, Dionex) using a linear acetonitrile gradient using buffer A (0.1% formic acid, 2% acetonitrile) and buffer B (0.1% formic acid, 80% acetonitrile) starting from 10% buffer B to 90% over 45 min at 2 mL/min.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR)

Day 2 fifth instar larvae were injected with 100 µg of dried *M. luteus* (Sigma, USA) suspended in PBS or with 1.5×10^6 cells of *S. cerevisiae* or left without injection as controls. After 24 h, the fat body was collected from each larva and washed three times with anti-coagulant saline (4 mM NaCl,40 mM KCl, 8 mM EDTA, 9.5 mM citric acid monohydrate, 27 mM sodium citrate, 5% sucrose, 0.1% polyvinylpyrollidone, 1.7 mM PIPES). Total RNA was isolated using TRI reagent (Sigma, USA). RNA (120 ng) was a template for reverse transcription using Oligo-dT primer and SuperScript™ II RT (Invitrogen, USA) following the manufacturer's instructions. Samples of cDNA (1 µL) were used in RT-PCR reactions for 30 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s followed by incubation at 72 °C for 4 min. The forward and reverse primers for diapausin were -:5′- GCCATGGCTATCAACAACTG -3′, and 5′- AAG CTT TTA ACG TCT GTA ACA -3′), respectively. Ribosomal protein small subunit 3 (rps3) was used as a control with forward and reverse primers 5′ - GCCGTTCTTGCCCGTTT -3′, and 5′- CGCGAGTTG ACTTCGGT- 3′) respectively. The PCR products were analyzed by electrophoresis on 1% agarose. For naive larvae in fifth instar feeding stage (day 2 and day 4) or wandering stage (days 0, 1, 2, 3) RNA was isolated from fat body and midgut. The cDNA, RT-PCR and analysis were performed as mentioned above. Quantitative RT-PCR (qPCR) was performed using IQ™ SYBER Green supermix (Bio-Rad, USA) in a CFX96 thermo cycler (Bio-Rad, USA). The cDNA template was diluted 25 fold (v/v) with nuclease-free water (Ambion, USA), then 25 µL reaction volume (12.5 µL 2 × SYBER Green master mix, 1 µL forward primer, 1 µL reverse primer and 10.5 µL cDNA template) was incubated at 95 °C for 3 min, followed be 40 amplification cycles with 3 steps: 95 °C 10 s, 52 °C for 30 s, 70 °C for 30 s. The primers for diapausin-1 were 5′-ACC ATG

GGC ATC AAC AAC TGG-3′ and 5′- CTCGAGACGTCGTAACAGTT-3′for both forward and reverse primers, respectively. The rps3 primers were the same as mentioned above. Relative expression of diapausin-1 mRNA levels were calculated using delta delta Ct (ΔΔCt) method (Livak and Schmittgen, 2001). Statistical analysis was carried out using GraphPad Prism software (GraphPad, USA).

Fluorescein isothiocyanate conjugated proteins

Diapausin-1 and bovine serum albumin (BSA) were conjugated with fluorescein isothiocyanate (FITC; Sigma, USA) as described in the manufacturer's instructions. The labeled proteins were isolated using PD 10 desalting column (GE healthcare, USA), and then the FITC-conjugated proteins were dialyzed against 10 mM sodium phosphate buffer, pH 7.4. *S. cerevisiae* cells were incubated with the FITC-labeled proteins each at 5 μM, for 8 h. After washing with 10 mM sodium phosphate buffer, pH 7.4, cells were examined by confocal laser microscopy.

Results

Purification of an antifungal peptide from *M. sexta* hemolymph

To investigate the presence of antifungal molecules in hemolymph of *M. sexta* larvae, we assayed activity against yeast in hemolymph from control larvae and from larvae 24 h after injection of *S. cerevisiae.* Plasma from larvae that had been injected with yeast had significant activity against growth of yeast in our bioassay (Fig. 1A), whereas plasma from control larvae did not. In SDS-PAGE analysis of these samples, plasma from larvae injected with yeast contained several protein bands at higher intensity than in controls, including a band at ~5 kDa (Fig. 1B).

To isolate and identify antifungal molecules, 20 mL of plasma from larvae collected 24 h after injection with yeast was separated by size exclusion column chromatography, and fractions were tested for activity against *S. cerevisiae* (Fig. 1C). Three fractions (56-58) had strong anti-yeast activity. In analysis of these fractions by SDS-PAGE, a single band was visible at ~ 5 kDa (Fig. 1D). These fractions were pooled, concentrated, and analyzed by mass spectrometry. The MALDI-TOF analysis of purified ~ 5 kDa peptide showed the sharp ms peak at (m/z) 5165.5, which matched with SDS-PAGE analysis (Appendix A). Proteomics analysis was performed to identify the 5 kDa peptide using MALDI-TOF MS/MS technology. Following MASCOT peptide search against NCBInr database, the spectra of tryptic fragments revealed significant matches with the N-terminal region including INNWVRVPPCDQVCSR from psychimicin (NCBI accession P83421), a peptide isolated from a lepidopteran insect, *Oiketicus kirbyi* (Figs. 2 and 3). Psychimicin is a member of the diapausin family of antimicrobial peptides (Pfam PF08036), named for its first representative, diapause-specific peptide from a beetle, *Gastrophysa atrocyanea*, which also has antifungal activity (Tanaka et al., 2003;Tanaka et al., 1998).

We used the psychimicin sequence for blast searching of official gene set 1.0 from the *M. sexta* genome sequencing project (http://agripestbase.org/manduca/), but no gene models had significant matches. However, when we searched using tblastn against the DNA sequences of the assembled *M. sexta* genomic scaffolds, we identified a region on scaffold 6084 with a strong match to the psychimicin sequence. MASCOT peptide analysis against scaffold 6084 showed that all of the predicted tryptic peptides were found from the tryptic mass spectra with high MS matching probability (Appendix A). This region contained a single exon encoding a predicted signal peptide of 23 residues, followed by a mature peptide of 45 residues, with mass of 5160.7 Da and theoretical pI of 8.5 (Fig. 3). This gene, named *M. sexta* diapausin-1 (synonym antifungal peptide-1)

(manually annotated by Haobo Jiang, Oklahoma State University) is designated as Msex2.15011 in official gene set 2.0 (http://agripestbase.org/manduca https://i5k.nal.usda.gov/Manduca_sexta).

Additional tblastn searching of the scaffold sequences revealed 13 additional single-exon genes in the diapausin family, which were not recognized as genes in official gene set 1 (Fig. 3). Genes 2-10 form a cluster on scaffold 1204 and have very similar sequences (He *et al*., 2015). Members of the diapausin family have six absolutely conserved Cys residues. An alignment of the *M. sexta* diapausin-1 amino acid sequence with the original diapause-specific peptide and some homologous lepidopteran sequences available in transcriptome databases is presented in Fig. 3. *M. sexta* diapausin sequence similarity ranges from 91% identity with psychimicin to 49% identity with *G. atrocyanea* diapause specific peptide. Calculated molecular weight of diapausin-1is 5169.8 which is slightly larger than the MALDI-TOF analysis of purified peptide (m/z^{+1} 5165.5). Since diapausin-1 is predicted to contain three disulfide bonds, the molecular mass difference could indicate the disulfide bonds in diapausin-1 structure.

cDNA cloning and antimicrobial activity of recombinant diapausin-1

Primers for PCR amplification of the region encoding the mature form of diapausin-1 (after removal of the secretion signal peptide) were designed based on the genomic sequence of gene Msex2.15011 and used to amplify the cDNA from RNA prepared from fifth instar larval fat body. The resulting cDNA (Genbank accession KT222965) matched exactly with the genomic sequence. It was cloned into expression vector pET32a, and a fusion protein of an amino-terminal 6-His tag followed by thioredoxin, fused with carboxyl-terminal diapausin-1 was expressed in *E. coli*. The 22.5 kDa fusion protein was purified by nickel-affinity chromatography and then cleaved with enterokinase to remove the fusion tag. Diapausin-1 was separated from the 6-His-thioredoxin by

passage through a nickel column, which retained the thioredoxin, and the 5 kDa diapausin-1 was collected in flow-through fractions (Fig. 4A). Diapausin-1 was further purified by reversed phase HPLC (Fig. 4B), and fractions were assayed for activity against *S. cerevisiae.* The major peak, representing ~50 % of the sample, was active against yeast. The sequence and total molecular weight of the expressed recombinant Diapausin-1 was confirmed by proteomics analysis. This recombinant diapausin-1 blocked yeast growth in a concentration dependent manner, with 50% inhibition at ~12 μM diapausin-1 (Fig. 5A). The activity of diapausin-1 was stable to heating at 90 °C for 10 min (Fig. 5B), perhaps due to structural stability provided by the three predicted disulfide bonds.

To examine binding of diapausin-1 to yeast cells, we incubated *S. cerevisiae* with fluorescently labeled (FITC) recombinant diapausin-1 or with FITC labeled BSA as a control. Cells were pelleted, washed with 10 mM phosphate buffer, pH7.4, and then visualized by confocal laser scanning microscopy. FITC-diapausin was detected at the surface of yeast cells, but not in their interior (Fig. 6). In contrast, FITC-BSA did not bind to the yeast cells at a detectable level. This result indicates that diapausin-1 may affect yeast by binding with a surface component, such as the cell wall or cell membrane. Recombinant diapausin-1 lacked detectable activity against tested bacterial species (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Listeria fleischmannii, Listeria grayi, Listeria marthii, Listeria innocua, Listeria welshimeri, Listeria seeligeri, Listeria rocourtiae, Micrococcus luteus*) (data not shown).

Effects of diapausin-1 on other fungi

We carried out assays to test the activity of diapausin-1 on growth and morphology in some species of ascomycete fungi. Diapausin-1 had no apparent activity against growth of the insect

fungal pathogen *Beauvaria bassiana* (data not shown). However, diapausin-1 did affect morphology and growth of some plant pathogenic fungi. When spores of *Magnaporthe oryzae* were treated with 15 μM diapausin-1and then allowed to germinate for 4 h, we observed that germination tubes were curled, with a corkscrew-like appearance, compared with the gently curved shape of the germ tubes from control spores not treated with diapausin-1 (Fig. 7). We examined the hyphal growth of two *Fusarium* species: *Fusarium culmorum* and *Fusarium graminearum* after treatment with diapausin-1. At 15 μM diapausin-1, no hyphal growth was apparent after 24 h (data not shown). Lower diapausin-1 concentration of 4 μM caused abnormal hyphal development after 24 h. In *F. culmorum*, germination was almost completely inhibited and few, very small germ tubes with constriction were observed. In *F. graminearum*, either strong constriction or destruction of septa was observed, which correlated with disintegration of conidia and loss of cell contents. In addition to ineffective germination, the hyphal length was significantly reduced compared with the controls, with frequent branching and changing in morphology (Fig. 8).

Change in diapausin-1 mRNA level after exposure to microorganisms and during development

Because we found that activity against yeast increased dramatically in hemolymph after injection of larvae with yeast, we carried out experiments to test whether this change is correlated with increased level of diapausin-1 mRNA. We analyzed RNA extracted from feeding stage fifth instar larval fat body 24 h after injection with *S. cerevisiae*, *M. luteus,* or uninjected control larvae. cDNA samples prepared from these RNA templates were analyzed by quantitative PCR (qPCR). Diapausin-1 mRNA levels increased about 18-fold compared with control after injection with M. *luteus* and increased more than 100-fold after injection of yeast (Fig. 9).

Because some hemolymph proteins involved in *M. sexta* immune responses are known to be expressed in the wandering larval stage just prior to pupation in the absence of an immune challenge (Russell and Dunn, 1996), we tested whether diapausin-1 may also be expressed with a similar developmental pattern. We analyzed RNA extracted from the fat body and the midgut of naïve larvae during the feeding stage of the fifth instar and on the first four days of the wandering larval stage. Diapausin-1 expression level was low in fat body and midgut of feeding stage larvae, but increased dramatically by day one of the wandering stage (Fig. 10), with about 100-fold increase in fat body and 18-fold increase in midgut. The diapausin-1 mRNA level increased further in midgut on wandering days 2 and 3, reaching ~50-fold greater than the level in fifth instar day 2.

Discussion

Insects can respond to infections by synthesizing antimicrobial peptides with activity against bacteria and/or fungi (Bulet et al., 2004; Casanova-Torres and Goodrich-Blair, 2013; Kanost et al., 2004; Yi et al., 2014; Rahnamaeian et al., 2015). Most known antimicrobial peptides from insect hemolymph are antibacterial, but some antifungal peptides have been discovered and characterized (Barbault et al., 2003; Fehlbaum et al., 1996; Kouno et al., 2007; Landon et al., 1997). *M. sexta* has been investigated extensively as a model organism for studies of innate immunity in insects (Kanost et al., 2004; Kanost and Nardi, 2010). Although many antibacterial peptides have been identified from *M. sexta* (Dai et al., 2008; Dickinson et al., 1988; Gorman et al., 2004; He et al., 2015; Zhu et al., 2003), gloverin and lebocin C are the only *M. sexta* plasma proteins so far shown experimentally to have antifungal activity (Rao et al., 2012; Xu et al., 2012).

In this study, we present the isolation, cDNA cloning, and characterization of an antifungal peptide, diapausin-1, isolated from *M. sexta* larval hemolymph after injection of the larvae with the yeast, *S. cerevisiae*. Diapausin-1, a slightly basic peptide of 45 amino acid residues, is a member of the diapausin family. The diapausin family includes antifungal peptides of 40-45 residues, with six conserved cysteines (Tanaka et al., 2003). The NMR structure of a beetle diapause-specific peptide, the first member of this family, contains a CSαβ - like motif stabilized by three disulfide bridges, which differ from the arrangement of disulfide bonds in insect defensins (Kouno et al., 2007). A search of the *M. sexta* genome revealed the presence of a family of genes that encode peptides similar to diapausin-1, all containing a predicted secretion signal peptide.

Diapausin-1 purified from hemolymph or produced as a recombinant protein had activity against *S. cerevisiae.* It was also active against some ascomycete filamentous fungi, although not the entomopathogen *B. bassiana,* which has perhaps adapted to lack susceptibility to insect antifungal defenses. Diapausin-1 lacked activity against all Gram-negative and Gram-positive bacteria that were tested *in vitro*. This specificity is similar to the well characterized insect antifungal peptide like drosomycin and few other insect antifungal peptides, which do not affect bacteria (Fehlbaum et al., 1994; Iijima et al., 1993). However, our data do not rule out the possibility that diapausin-1 contributes to antibacterial defenses by displaying combinatorial activities with other AMPs in *M. sexta*. Recent findings from bumble bees show at the mechanistic level that co-occurring AMPs can cooperate to fulfill immunity-related functions (Rahnamaeian et al, 2015).

Diapausin-1 appears to alter morphology when it restricts growth of fungal species. In germinating *M. oryzae*, the germination tube acquired a curled appearance not present in controls. With two *Fusarium* species, conidia germination was totally inhibited by 15 µM diapausin-1, and

after treatment with only 4 μM of peptide, hyphal growth was severely reduced, and the hyphae became much more branched and constricted with abnormal morphology. Abnormal hyphae and delayed growth were observed when low concentrations of drosomycin were tested against *Botrytis cinerea*, and extruded cytoplasm observed along the hyphae may indicate that drosomycin caused a partial lysis of the cells (Fehlbaum et al., 1994). In plants, antifungal defensins are grouped based on their effect on hyphal morphology into morphogenic or nonmorphogenic. Morphogenic peptides reduce hyphal growth and cause morphological change, whereas the nonmorphogenic peptides affect only hyphal growth (Ramamoorthy et al., 2007; Sagaram et al., 2011). Diapausin-1 bound to the surface of *S. cerevisiae* (Fig. 6). Perhaps interaction of diapausin-1 with some component of the fungal cell surface disrupts growth while causing distortion of cell shape.

Diapausin-1 mRNA levels increased dramatically in the fat body after larvae were injected with *M. luteus* or *S. cerevisiae*, with particularly strong induction of expression after exposure to the yeast (Fig. 9). The elevated expression of diapausin-1 is correlated with the appearance of anti-yeast activity in hemolymph after immune stimulation by injection of yeast (Fig. 1A). Because diapausin was first discovered in a leaf beetle as a hemolymph protein expressed during a quiescent stage, we tested whether *M. sexta* diapausin-1 might be expressed during development in the absence of microbial challenge, particularly at the time of pupation, when the insects might be vulnerable to infections. We found that diapausin-1 was strongly expressed in fat body and midgut during the wandering stage, just prior to pupation. This expression pattern is similar to other *M. sexta* immune proteins, which are upregulated in hemolymph and in midgut fluid, beginning at the wandering stage and persisting into the pupa. These include hemolin, lysozyme, and phenoloxidase, antimicrobial proteins and peptides (Russell and Dunn, 1996; Yu and Kanost,

1999) and a β- 1,3 - glucan recognition protein that can bind to fungal cell walls (Jiang et al., 2004 ; Takahashi et al., 2014). Expression of diapausin-1 in the midgut and fat body at pupation may be part of a developmentally upregulated immune protection, to help block infections during metamorphosis. Regarding structure and transient expression before pupation, diapausin-1 displays similarity with the antifungal peptide gallerimycin from the greater wax moth *Galleria mellonella* (Langen et al., 2006). Furthermore, drosomycin mRNA levels were up-regulated during developmental stages especially in the pre-pupa stages (Attrill et al., 2016). This model host has also been used to explore the induction of immune responses prior to pupation in Lepidoptera. The expression of a matrix metalloproteinases that mediates digestion of the extracellular matrix during metamorphosis has been found to produce fragments of collagen IV, which in turn function as danger signals that elicit immune responses (Altincicek and Vilcinskas, 2006).

We interpret the results of this study to conclude that diapausin-1 is an antifungal hemolymph peptide, which functions in the immune system of *M. sexta* larvae, likely providing protection against fungal infections. Diapausin-1 expression is strongly expressed after immune challenge and prior to metamorphosis. It is active against *S. cerevisiae* and some ascomycete fungi, disrupting growth and affecting fungal morphology. Further studies are needed to determine the molecular target of diapausin-1 and its mode of action.

Figures

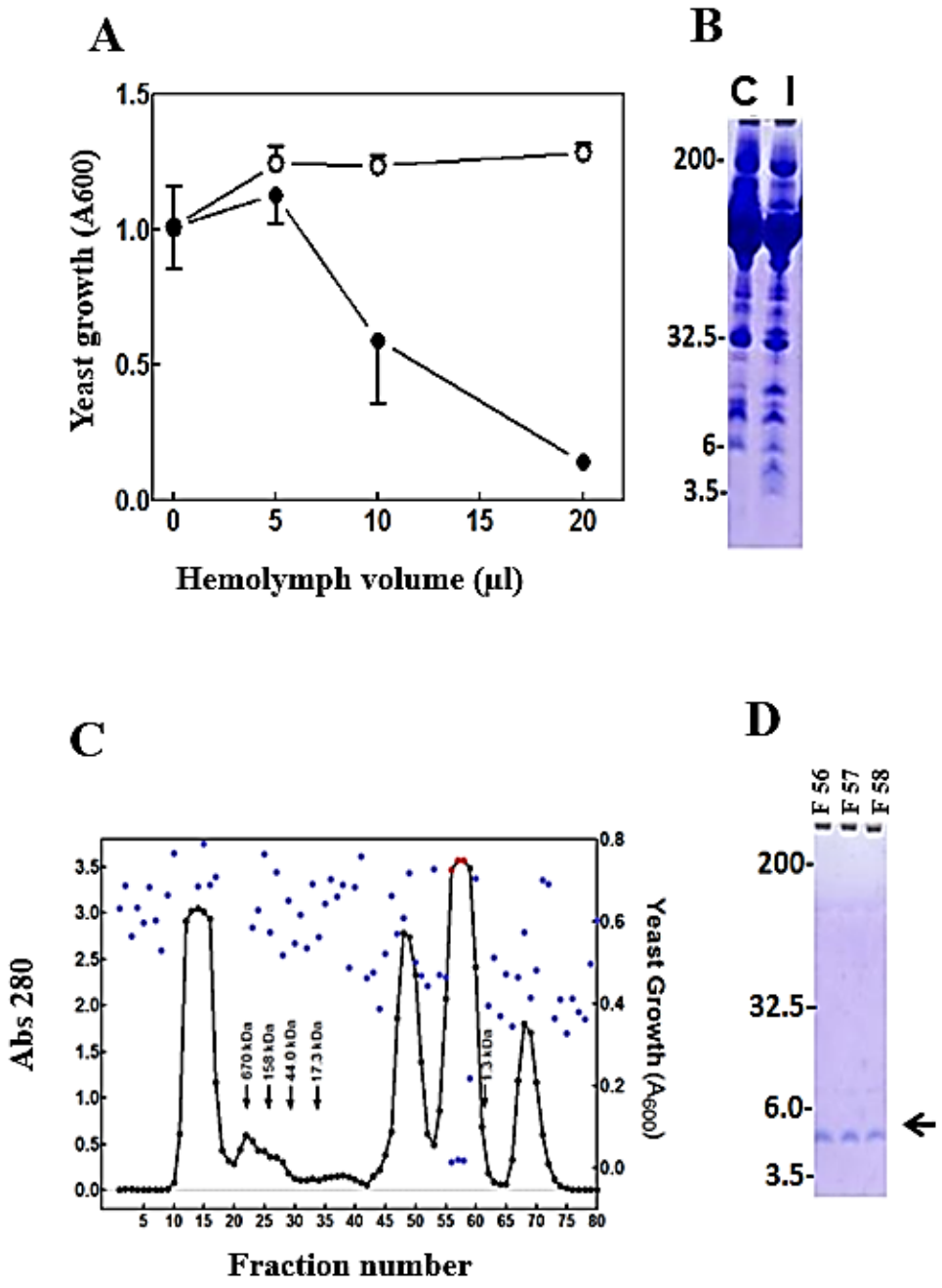

Figure 2.1 . *M. sexta* larval hemolymph contains inducible anti-yeast activity.

Day 2 fifth instar *M. sexta* larvae were injected with 1.5×10^6 cells of *S. cerevisiae* (induced); control larvae were uninjected. Hemolymph plasma was collected 24 h after injection. **(A)** Control (open circles) and induced (closed circles) plasma samples at different volumes were tested for anti-yeast activity as described in Materials and Methods. Error bars indicate standard deviation of technical replicates (n = 3). **(B)** Analysis of control (C) and induced (I) plasma samples (1.5 μL) by SDS-PAGE. **(C)** Separation of plasma by size exclusion chromatography. Hemolymph plasma (20 mL) from larvae collected 24 h after injection with *S. cerevisiae*was separated by size exclusion chromatography on a column of Sephacryl-S200, as described in Materials and Methods. Protein content of each fraction was monitored by measuring absorbance at 280 nm (black or red points). Activity of each fraction against yeast was assayed as described in Materials and Methods, with growth of yeast measured as turbidity, using absorbance at 600 nm (blue points). Arrows indicate the elution volumes of standard proteins. **(D)** Analysis of the fractions 56–58 with highest anti-yeast activity, by SDS-PAGE using a 16% acrylamide tricine gel. Size and positions of the molecular weight markers are indicated on the left. The arrow indicates the position of the band that was analyzed by mass spectrometry of tryptic fractions.

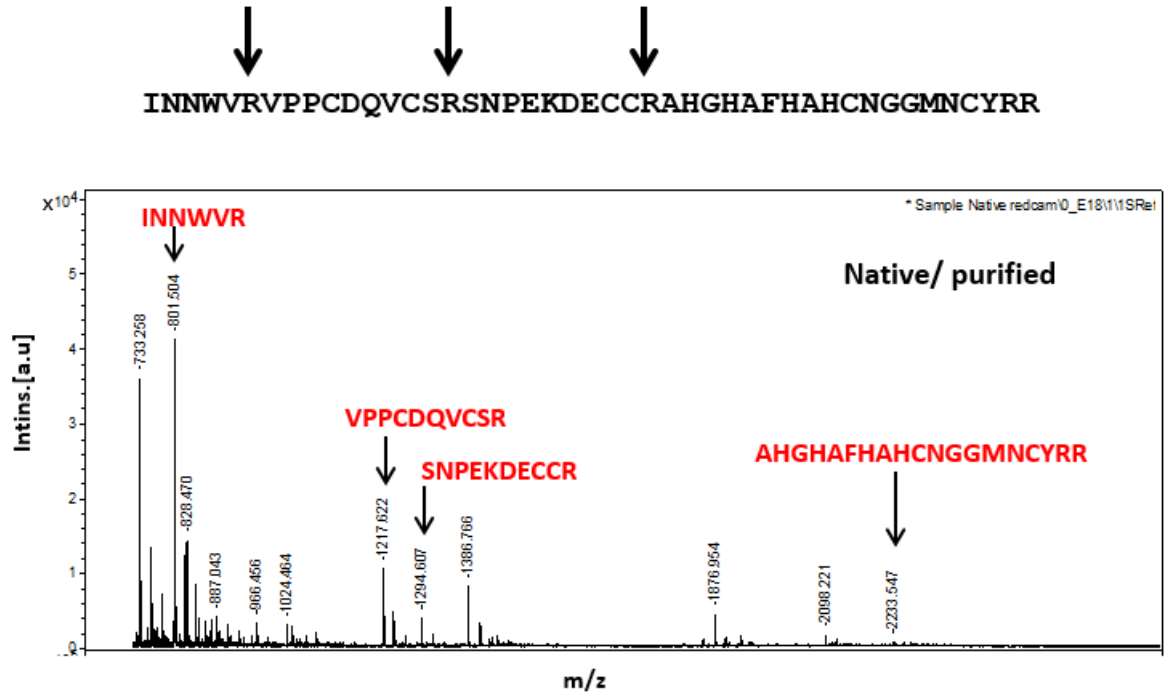

Figure 2.2. Identification of diapausin-1 by peptide mass fingerprinting.

The purified diapausin-1 peptide was digested with trypsin and then analyzed by MADLI-TOF/MS. The mass spectrum is labeled to show sequences of peptides represented by peaks with masses and fragmentation patterns that match predicted tryptic peptides from the mature diapausin-1 protein encoded in *M. sexta* gene Msex2.15011 found in the *M. sexta* genome sequence.

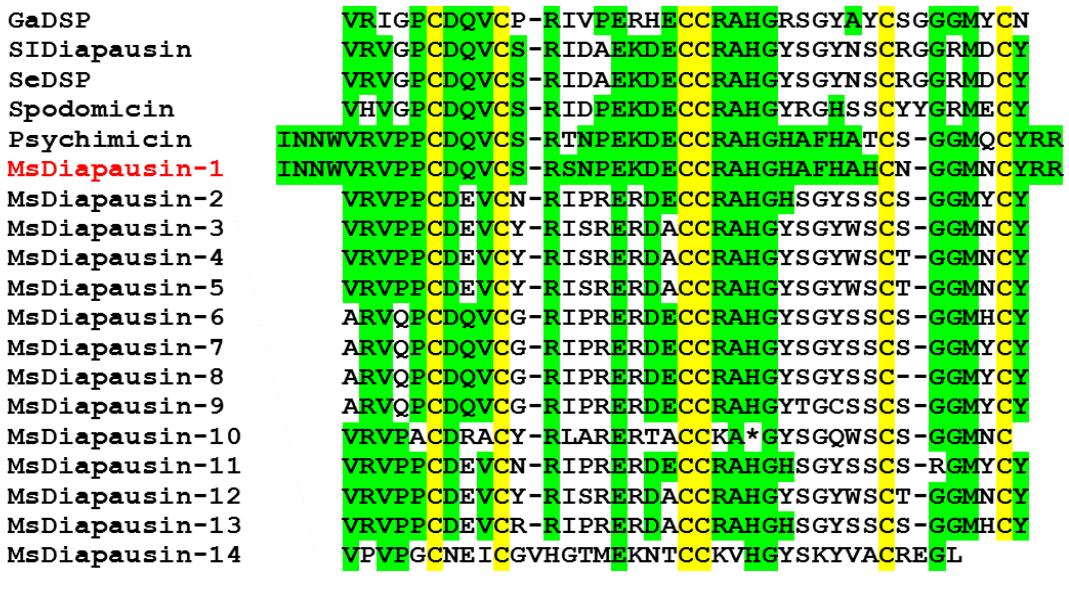

Figure 2.3. Alignment of the *M. sexta* diapausin-1 amino acid sequence with other members of the diapausin family from *M. sexta* and other insect species.

The sequence encoded by *M. sexta* gene Msex2.15011, diapausin-1 (NCBI accession number KT222965), after removing the secretion signal sequence, was aligned with mature peptide sequences of other members of the diapausin family present in the *M. sexta* genome (He et al., 2014) and several diapausin family members from lepidopteran species as well as the original member of this gene family, *G. atrocyanea* diapause specific peptide. Conserved Cys residues are highlighted in yellow. Other residues conserved with *M. sexta* diapausin-1 are highlighted in green. Accession numbers for the sequences retrieved from NCBI are: *Gastrophysa atrocyanea* diapause-specific peptide (GaDSP), Q8T0W8; *Spodoptera litura* diapausin (SlDiapausin), ABU96713; *Spodoptera exigua* diapause-specific peptide (SeDSP), ADM72854; *Spodoptera littoralis* spodomicin, P83411; *Oiketicus kirbyi* psychimicin, P83421.

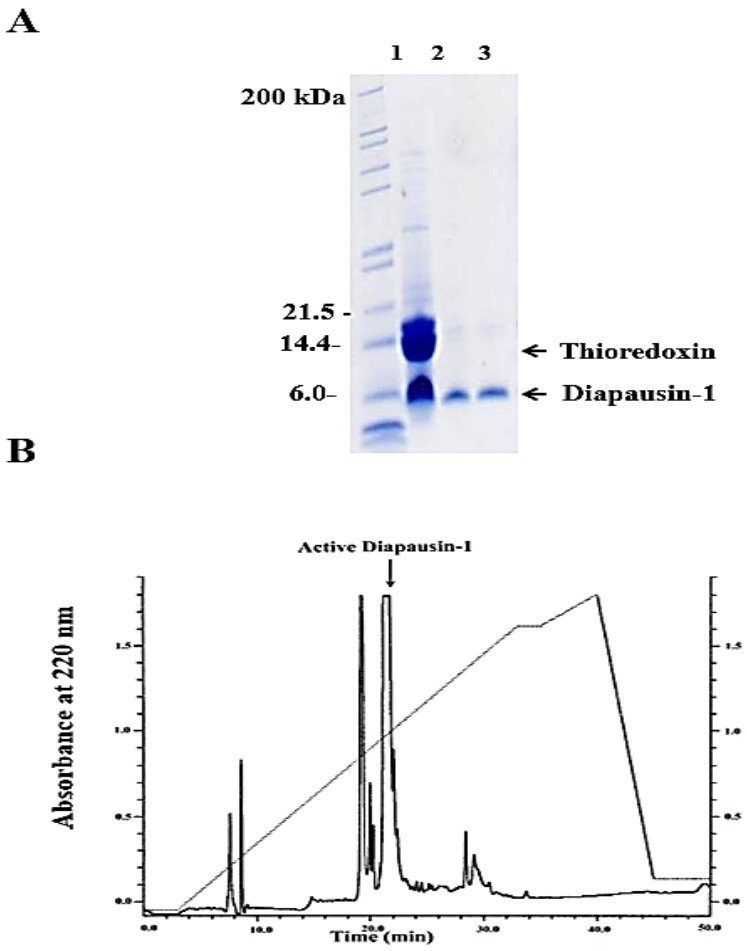

Figure 2.4. Isolation of recombinant diapausin-1.

(A) The 6-His-thioredoxin-diapausin fusion protein was cleaved with enterokinase, and 6-His-thioredoxin was removed by nickel-affinity chromatography. Lane 1: reaction mixture after enterokinase cleavage; Lanes 2 and 3: first two flow-through fractions containing diapausin-1. (B) Reverse HPLC separation of the Diapausin-1 after cleavage with enterokinase and purification with Ni-NTA chromatography. The arrow indicates the active diapausin-1 which confirmed with anti-yeast activity test (data not shown).

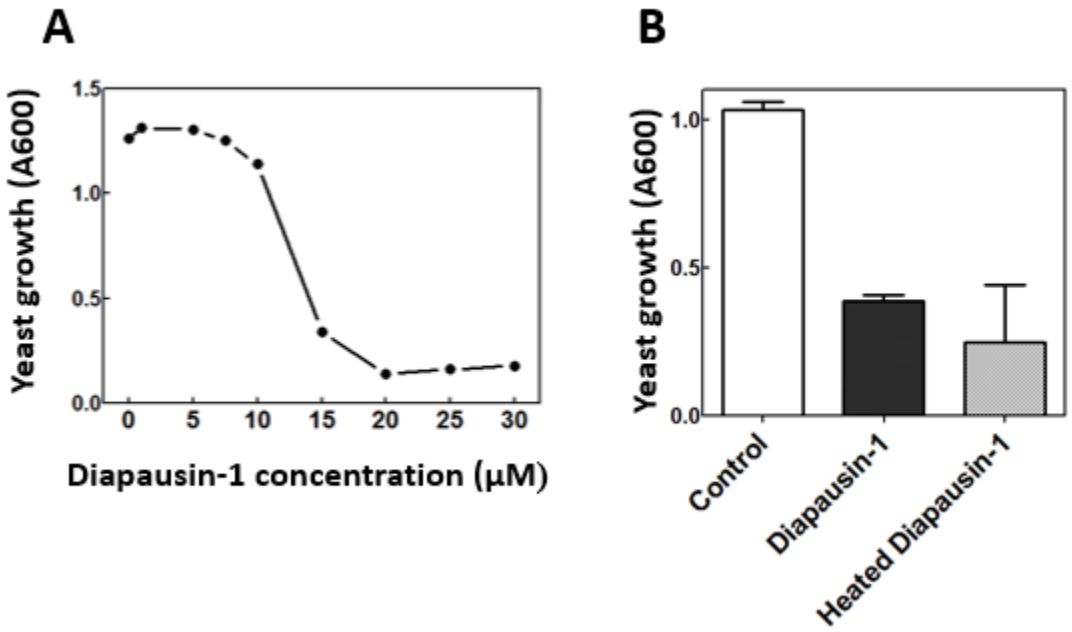

Figure 2.5. Activity of recombinant diapausin-1 against yeast.

(**A**) The concentration dependent activity of recombinant diapausin-1 in decreasing growth of *S. cerevisiae* was assayed by measuring turbidity at OD_{600} after a 12 h incubation of cultures in a 96 well plate, containing different concentrations of diapausin-1, as described in Materials and Methods. The Inhibitory concentration 50% (IC_{50}%) of recombinant diapausin-1 against *S. cerevisiae* was ~12 μM. (**B**) Heat stability of recombinant diapausin-1 was tested after treatment of the peptide at 90 °C or 25 °C for 10 min prior to assay against yeast at 12 μM diapausin-1. Bars represent mean ± standard deviation (n = 3). There was no significant difference in the activity of heated diapausin-1 compared with the unheated control (*t*-test, $t > 0.05$), indicating heat stability of this antifungal peptide.

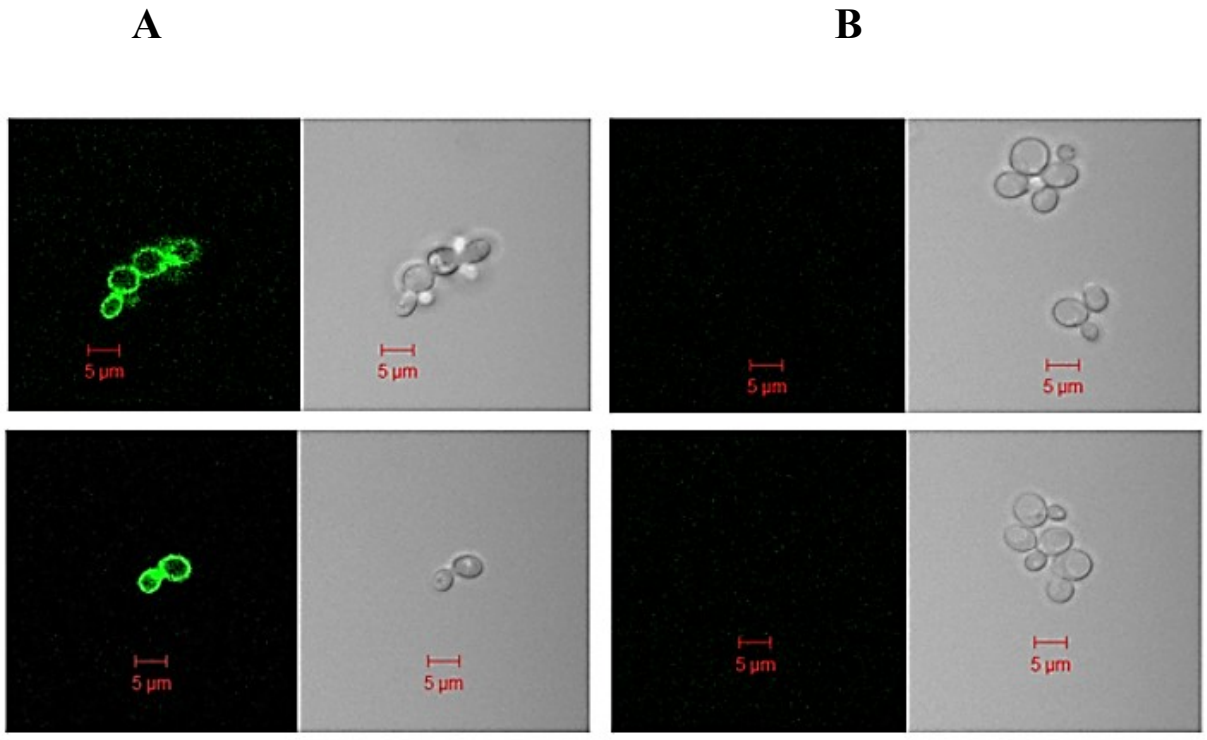

Figure 2.6. Binding of diapausin-1 to *S. cerevisiae*.

FITC-labeled diapausin-1 (A) or FITC-labeled bovine serum albumin (B), each at 5 µM incubated with *S. cerevisiae* for 8 h and then examined by confocal laser scanning microscopy (left panels) or phase contrast microscopy (right panels) to detect fluorescent protein associated with the yeast.

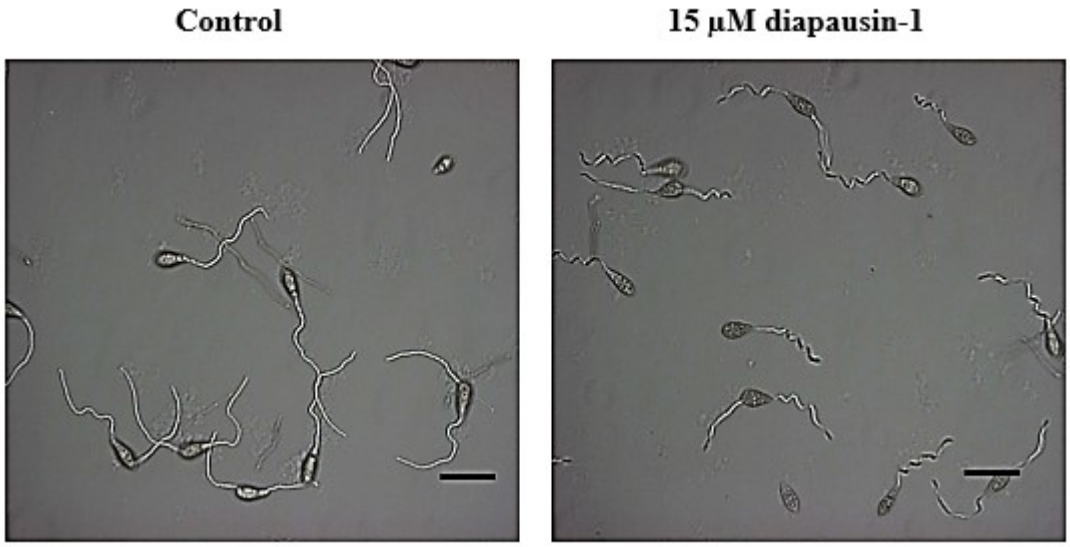

Figure 2.7. Diapausin-1 alters morphology of *M. oryzae* germination tubes.

M. oryzae spores were incubated with 15 µM diapausin-1 for 4 h, and then examined by phase contrast microscopy. Scale bars indicate 50 µm.

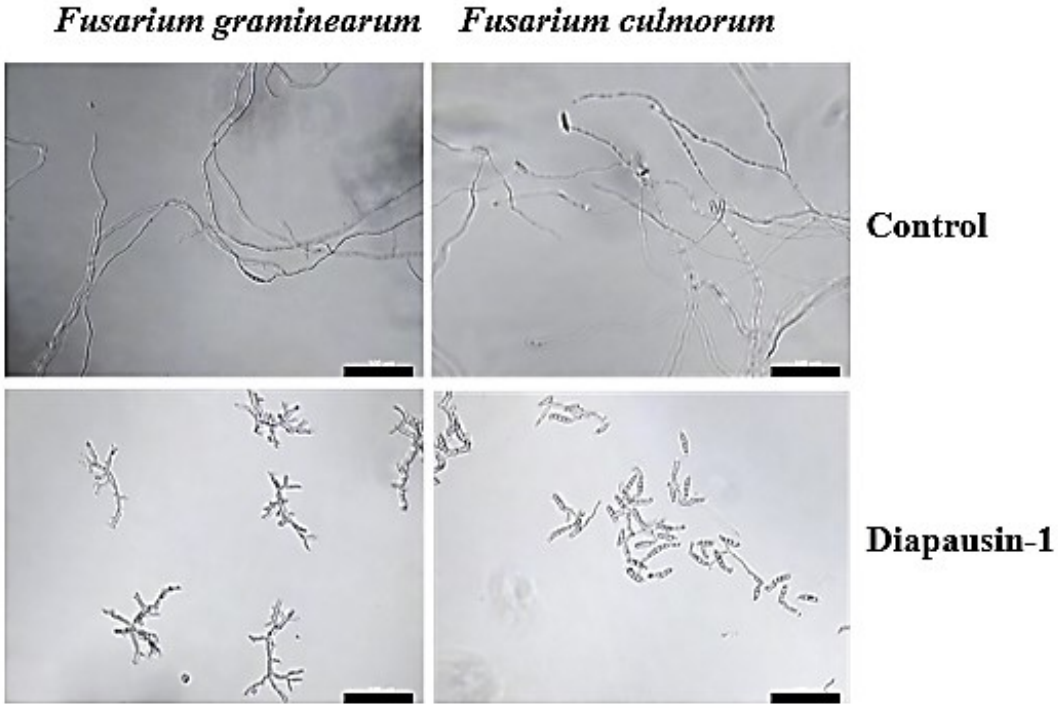

Figure 2.8. Diapausin-1 alters Fusarium hyphal morphology.

Spores of *F. graminearum* or *F. culmorum* were incubated in medium with 4 μM diapausin-1 for 48 h or in medium alone as a control. Resulting hyphae were visualized by phase contrast microscopy. Scale bars indicate 100 μm.

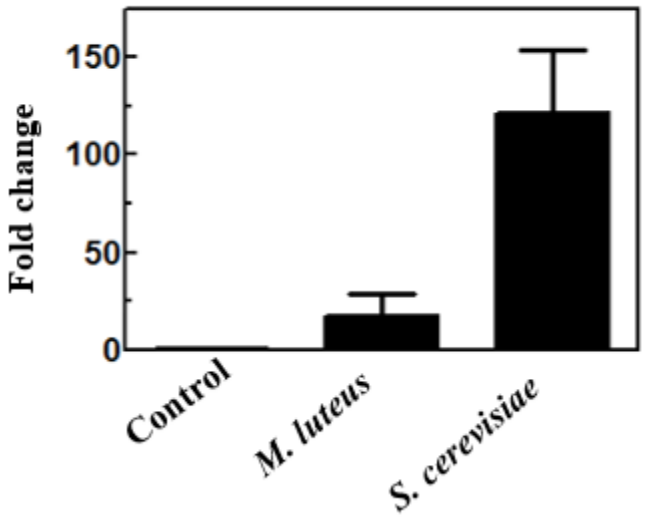

Figure 2.9. Diapausin-1 levels in the fat body of *M. sexta* after injection with *Micrococcus luteus* or *S. cerevisiae*.

Total RNA isolated from the fat body of three individual larvae for each treatment was used to synthesize cDNA, which was analyzed by qPCR, relative to the level of mRNA for ribosomal protein subunit 3 (RPS3). The bars represent the mean ± standard error of the mean, n = 3.

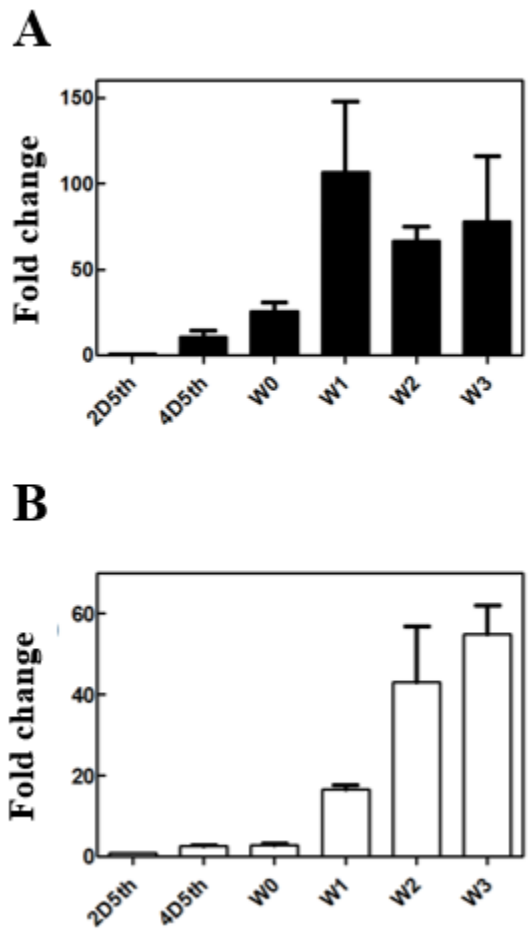

Figure 2.10. Diapausin-1 mRNA levels in fat body and midgut of naïve larvae increase at the wandering stage before pupation.

Total RNA isolated from the fat body (A) or midgut (B) of three individual larvae at each time point was used to synthesize cDNA, which was analyzed by qPCR to determine level of diapausin-1mRNA relative to ribosomal protein S3 mRNA. The relative expression level on fifth instar day 2 was set as 1.0.2D5th = day 2 of fifth instar; 4D5th = day 4 of fifth instar, W0 to W3 indicates wandering larval stage days 0–3. The bars represent the mean ± standard error of the mean for three individual larvae.

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Chapter 3 - Diapausin-1 interaction with the cell wall of *Saccharomyces cerevisiae*

Introduction

Insects produce antimicrobial peptides (AMP) as part of their innate immune response (Hoffman, 1995; Yi et al., 2014). Although this response in insects is not specialized compared with adaptive immunity in higher organisms, it represents a fast and efficient response to eliminate or stop invading pathogens (Hultmark, 1993; Hoffman et al., 1996; Tsakas and Marmaras, 2010; Vilcinskas, 2013).

AMPs are low molecular weight, often cationic and diverse in structure (Bulet et al., 1999; Zasloff, 2002; Chowdhury et al., 1995). They can have a wide spectrum of activity against bacteria, or they may be specialized against only one group of bacteria (Otvos, 2002). Some AMP have both antibacterial and antifungal activities, whereas others are strictly antifungal (Bulet and Stocklin, 2005; Fehlbaum et al., 1996; Fehlbaum et al., 1994; Lemaitre et al., 1997). Antifungal peptides and other antimicrobial peptides are produced in insects in the fat body, hemocytes, and the midgut (An et al., 2010; Kanost et al., 2004; Russell and Dunn, 1996; Xu et al., 2012). The first step to produce an antifungal peptide is recognition of invading fungi by receptor molecule in the hemolymph A family of receptors that bind to fungi is known as β-1,3-glucan recognition proteins (βGRPs), which bind to β-1,3-glucan on the invading fungal cell walls (El Chamy et al., 2008; Takahashi et al., 2014; Royet et al., 2005). Those receptors have an amino-terminal β-1,3-glucan binding domain and a carboxyl-terminal glucanase-like domain. The binding between β-1,3-glucan and its binding domain initiates a serine protease cascade leading to expression of antifungal and/or antibacterial peptides via the Toll pathway or can stimulate activation of the

immunodeficiency signaling pathway (IMD) (Hultmark, 2003; Bernnan and Anderson, 2004; Tanji et al., 2007).

A peptide from the leaf beetle, *G. atrocyanea* is expressed at diapause initiation and has antifungal activity against *Trichophyton rubrum* but not against the entomopathogenic fungus *Beauveria bassiana.* This peptide was named diapause-specific peptide (DSP) (Tanaka et al., 1998; Tanaka et al., 2003). Homologous peptides have been isolated from a few other insects (Wan et al., 2012), and peptides in this family are called diapausins.

Previously, we isolated a 45-residue antifungal peptide from *M. sexta* hemolymph, *Manduca sexta* diapausin-1 homologous to other members of the diapausin family (Al Souhail et al, 2016). *M. sexta* diapausin-1 at low micromolar concentrations has activity against *S. cerevisiae* and some filamentous fungi and is expressed in response to infection and under developmental regulation at the prepupal stage. Diapausin-1 binds to the surface of yeast cells. In this chapter, we will aim to test a hypothesis that diapausin-1 functions through binding to β-1,3-glucan in fungal cell walls.

Materials and Methods

M. sexta diaupsin-1 expression, purification, and antifungal activity

Diapausin-1 was expressed in *E. coli* and purified as described in chapter 2. The anti-yeast activity assay was performed using the same method described in chapter 2.

Pull-down assay

A pull-down assay was performed to study interaction of diapausin-1 with insoluble polysaccharides: curdlan from *Alcaligenes faecalis* (Sigma), zymosan from *S. cerevisiae* (Sigma), peptidoglycan *M. luteus* (Wako chemicals), and starch from wheat (Department of grain science, Kansas State University). Purified recombinant diapausin-1 (50 μg) was incubated with 0.5 mg of polysaccharide in 100 μL of 10 mM phosphate buffer, pH 7.4, for 10 min at room temperature. The mixture was centrifuged at 10,000 × g for 5 min, and supernatant was collected as the unbound fraction, and saved for subsequent analysis. The pellet was washed twice with 500 μL of the same buffer and centrifuged at 10,000 × g for 5 min. Each time, the supernatant (wash 1 and wash 2) was collected and saved for further analysis. The bound diapausin-1 was eluted from insoluble carbohydrates by adding 100 μL of 2× SDS/PAGE sample buffer containing β-mercaptoethanol and heating for 10 min at 95 °C. Equal volumes all samples (unbound fraction and the two wash fractions) were added to the same volume of sample buffer for SDS-PAGE analysis. SDS-PAGE was performed using pre-made 4-12% Bis-tris (Novagen), followed by InstantBlue (Expedeon) staining.

Surface plasmon resonance analysis

A surface plasmon resonance (SPR) experiment to examine binding of diapausin-1 to β-1,3-glucan was conducted using a Biacore 3000 (GE Healthcare) instrument. All analyses were carried out at 25 °C and a flow rate of 20 μL/min. Diapausin-1 (100 μg/mL in 10 mM phosphate, pH 7.4) was immobilized on a CMD 500 M sensor chip (Xantec bioanalysis, Germany) using standard amine coupling chemistry. A total 42.5 RU of diapausin-1 was immobilized on the chip surface. A reference surface created by activation followed by immediate quenching with 1.0 M

ethanolamine (pH 8.5) was used in the experiment as a blank. A two-fold dilution series (10 μM−10 nM) of the soluble β-1,3-glucan laminarin (5.5 kDa) from *Laminaria digitate* (Sigma) was injected over the immobilized diapausin-1 for 2 min, followed by 5 min of dissociation phase in a running buffer (20 mM Hepes, pH 7.4, 50 mM NaCl and 0.005% Tween-20). Regeneration was achieved by two 30-s pulses of 7.5 mM glycine (pH 2.2), 1.25 M NaCl. Kinetic analysis of the diapausin-1 and laminarin binding was performed by fitting the data to a 1:1 Langmuir model of binding and fitting R_{max} locally. BIAevaluation software 4.1.1 (GE Healthcare) was used to process all SPR data and conduct kinetic analysis. Steady-state curve fitting was performed by using GraphPad Prism 5.0 software.

Results

Our previous results indicated that *M. sexta* diapausin-1 is an antifungal peptide, lacking detectable antibacterial activity, and that FITC-conjugated diapausin-1 binds to the surface of *S. cerevisiae* (Al Souhail, 2016). Experiments were performed to test the hypothesis that diapausin-1 binds to β-1,3-glucan in fungal cell walls. In a pull-down assay, diapausin-1 was incubated with zymosan, a preparation of *S. cerevisiae* cell walls components. The pull-down assay result showed that the ~6 kDa diapuasin-1 band was present in the bound fraction (Fig. 3.1, lanes 1-4), and very little peptide was detected in the unbound or wash fractions. On the other hand, after diapausin-1 was incubated with peptidoglycan, diapausin-1 was present mainly with the unbound fraction, and a small amount in the first wash fraction, but not in the bound fraction (Fig 3.1, lanes 6-9). Because peptidoglycan contains cross-linking pentapeptides, it stains with Coomassie, and was visible as a smear of heterogeneous size on SDS-PAGE analysis (Fig. 3.1, lane 9). The results of

this experiment are consistent with an interpretation that diapausin-1 binds to yeast cell walls but not to bacterial peptidoglycan.

A major component of *S. cerevisiae* cell walls is β-1,3-glucan (Levin, 2005; Orlean, 2012). To test whether diapausin-1 binds to β-1,3-glucan, another pull-down assay was performed using an insoluble β-1, 3-glucan, curdlan. Starch, an insoluble α-1,4-glucan, was used as a negative control. When diapausin-1 was incubated with curdlan, it was eluted entirely in the bound fraction (Fig. 3.2, lane 4) and was not detected in the unbound or wash fractions (Fig. 3.2, lanes 1-3). Diapausin-1 did not bind detectably to starch (Fig.3.2, lanes 6-9), indicating that diapausin does not bind non-specifically to polysaccharides. This result indicates that diapausin-1 binds preferentially to β-1,3-glucan.

To further evaluate the binding between diapausin-1 and β-1,3-glucan, surface plasmon resonance experiment was performed. Diapausin-1 was immobilized on the surface of a chip using amino coupling chemistry, and then laminarin at concentrations ranging from 10 μM to 10 nM was injected for 2 min, followed by a 5 min dissociation phase. (Fig 3. 3). Fitting the data to a 1:1 Langmuir model of binding yielded a dissociation constant of ~100 nM laminarin. This result indicates a significant binding interaction between diapausin-1 and laminarin, a β -1,3-glucan, and is consistent with the nearly complete binding of diapausin-1 to zymosan and curdlan in the pull-down assays.

Discussion

Insects can produce an arsenal of antimicrobial peptides secreted into hemolymph to protect themselves from invading microorganisms. These peptides share many characteristics, including low molecular weight, most of them are cationic, and last for several hours to days, with a wide-spectrum of activities (Bult and Stocklin, 2005; Hoffmann, 1999; Li et al., 2012; Reddy et al., 2004). Most known insect antimicrobial peptides are active against bacteria, but the number of antifungal peptides discovered is increasing rapidly (Wang et al., 2016).

M. sexta diapausin-1 is a 45 amino acid residues peptide expressed in the fat body after immune challenge and in the fat body and midgut of naïve larvae at the prepupal stage just before metamorphosis (Al Souhail, 2016). Diapausin-1 has antifungal activity against yeast and some tested ascomycete filamentous fungi. FITC-conjugated diapausin-1 was detected at the surface of *S. cerevisiae* cells. To investigate the binding target(s) of this peptide, a pull-down assays were performed, which demonstrated that diapausin-1 binds to zymosan, an isolated cell wall preparation from the yeast *S. cerevisiae* .Diapausin-1 binding to zymosan is consistent with its interaction with a component of the yeast cell wall, which may be a clue toward its mechanism of action in blocking fungal growth and survival. Diapausin-1 did not bind to bacterial cell wall peptidoglycan, consistent with its lack of activity against bacteria (Al Souhail et al., 2016). Yeast cell walls have many components, including β-1,3-glucan , mannans, and chitin, but the major structural polysaccharide is β-1,3-glucan (Lesage and Bussey, 2006; Orlean, 2012). To investigate if β-1, 3-glucan is a candidate binding partner to diapausin-1, I performed a pull-down assay with insoluble β-1,3-glucan, curdlan, or with insoluble α-1, 4-glucan, starch as a negative control. The result from this assay showed that diapausin-1 bound to β-1,3-glucan but not to α-1,4-glucan,

showing a preferential binding to β-1,3-glucan. Glucans comprise 30-60% of the dry weight of the yeast cell wall and includes some β-1,6 cross-links. (Orlean, 2012). Insects sense invading fungi via the interaction between β-1,3-glucan on the fungal cell wall and pathogen recognition receptors called β-1,3-glucan recognition proteins (βGRPs) (Kanost et al., 2004; Ochiai and Ashida, 2000). This binding between the pattern-recognition protein and β-1,3-glucan initiates a serine protease cascade that triggers prophenoloxidase activation and melanization of pathogens as well as activation of transcription factors to stimulate expression of antimicrobial peptides via the toll pathway (Buchon et al., 2009; Lemitre and Hoffmann, 2007).

The mode of action of some insect antifungal peptides has been reported. The first isolated insect antifungal peptide was drosomycin from *Drosophila melanogaster* (Fehlbaum et al., 1994). This 44-residue peptide is expressed in the fat body after immune challenge via the Toll pathway (Zhang and Zhu, 2009). Several genes encoding peptides homologous to drosomycin have been identified on the 3rd chromosome of *D. melanogaster*, and five of these peptides have antifungal activity, although drosomycin was the most effective of these peptides against all tested fungi (Yang et al., 2006). Several mutations introduced in drosomycin displayed significantly decreased antifungal potency against tested fungi. Furthermore, mutations in aspartate 1, arginine 20 and lysine 38 appeared to most strongly decrease drosomycin antifungal activity, indicating that electrostatic binding of drosomycin to a target molecule may be important in its mode of action (Zhang and Zhu, 2010). Those mutations may explain why drosomycin has selectivity for cell membrane components such as sphingolipids (Gao and Zhu, 2008). Membrane sphingolipids appear to be the target of some antifungal peptides from plants (Ferket et al., 2003). Poacic acid, a fluorescent plant-derived antifungal agent, has anti-yeast activity against S. *cerevisiae* with IC_{50} of 324 μM. Poacic acid localizes to the cell wall by binding to β-1,3-glucan and causes cell lysis

(Piotrowski et al., 2015). This hypothesis was confirmed by several methods, such as the binding of the this agent to the entire cell surface and not to the budding region which is rich in chitin, the lack of ^{14}C-glucose incorporation in the yeast cell wall after treatment with poacic acid, and finally staining protocols to exclude binding to mannoprotein in the cell wall (Piotrowski et al., 2015). Furthermore, the mode of action was not by targeting the glucan synthase enzyme, which can be targeted by echinocandins that bind to the catalytic subunit, FKSp (Balashov et al., 2006; Johnson and Edlind, 2012). In addition to affecting *S. cerevisiae*, poacic acid binding to β-1,3-glucan caused fungal growth inhibition for *Sclerotinia sclerotiorum* and *Alternaria solani* as well as the oomycete *Phytophthora sojae* (Piotrowski et al., 2015).

Based on our current and previous results I hypothesize that the binding of Diapausin-1 to β-1,3-glucan may be the direct cause which affects the growth *S. cerevisiae* and filamentous fungi. This hypothesis needs to be investigated further using additional methods.

Figures

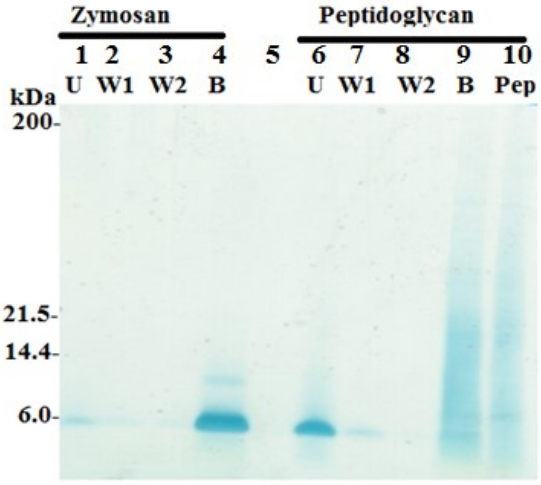


Figure 3.1. Pull-down assay to investigate binding of diapausin-1 to zymosan or bacterial peptidoglycan.

Lanes 1,2,3,4 represent dipausin-1 binding to zymosan, a preparation of *S. cerevisiae* cell walls. The pull-down assay shows that diapausin-1 was eluted in the bound fraction (B), and was nearly absent in the unbound fraction (UB) and the two wash fractions (W1 and W2). When diapausin-1 was incubated with peptidoglycan, most of the peptide, detected as a band at ~6 kDa, was eluted in the unbound fraction, whereas the other fractions (W1, W2 and B) bind very little diapausin-1. Peptidoglycan alone (lane 10) was used as a control to compare its staining with the peptidoglycan plus diapausin-1 mixture (lane 9).

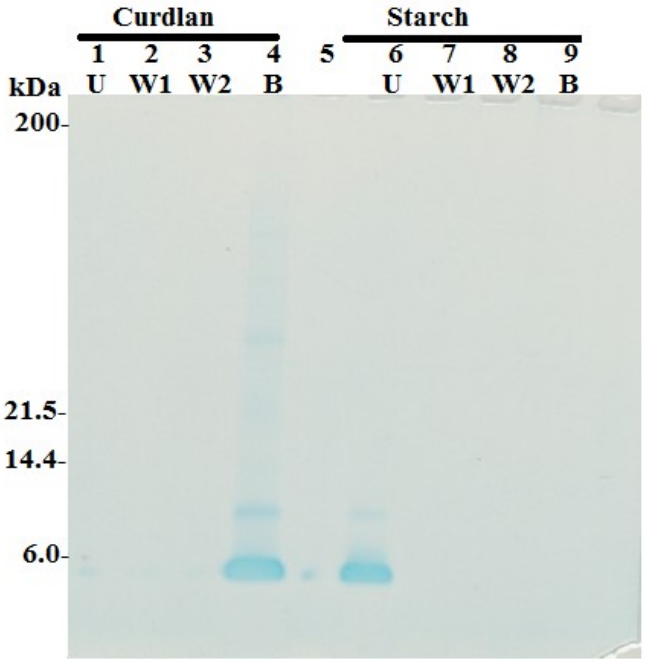
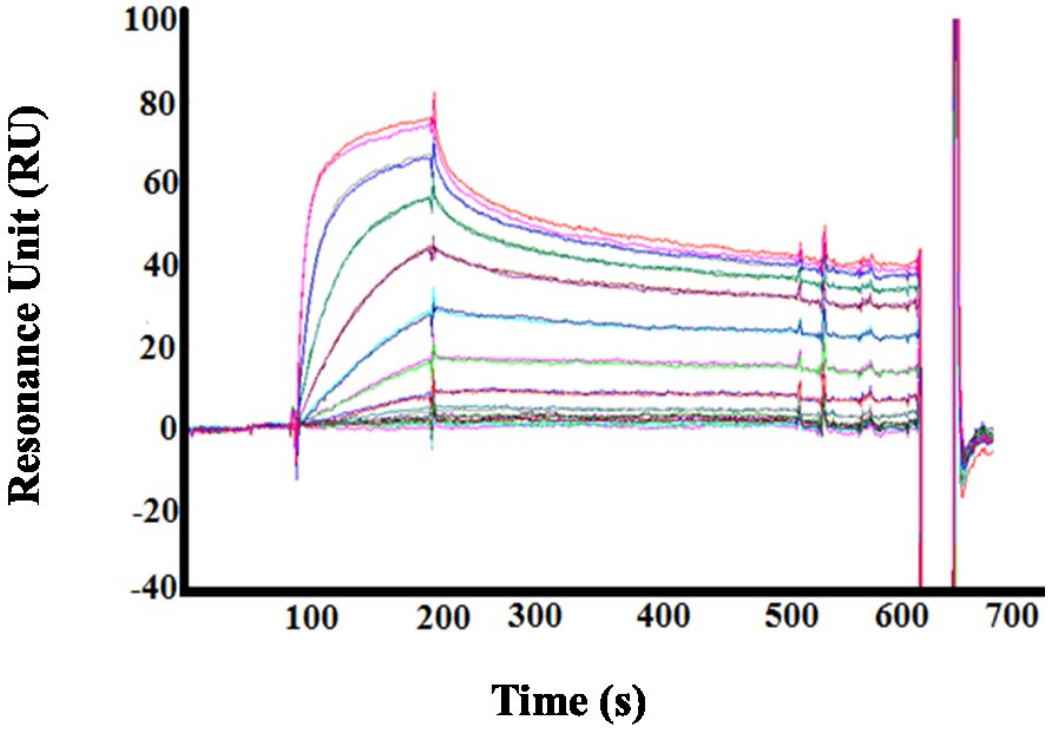


Figure 3.2. Pull-down assay to investigate binding of diapausin-1 to curdlan or starch.

Curdlan was incubated with diapausin-1 peptide (lanes 1-4), and then unbound, wash, and bound fractions were separated as described in Materials and Methods. Diapausin-1 eluted with the bound fraction (B, lane 4), and was not detectable in the unbound fraction (U, lane 1) or wash fractions (W and W2, lanes 2 and 3). When starch was incubated with diapausin-1 (lanes 6-9), the peptide was detected only in the unbound fraction (lane 6).

A


Resonance Unit (RU)
100
80
60
40
20
0
-20
-40
100
200
300
400
500
600
700
Time (s)

B

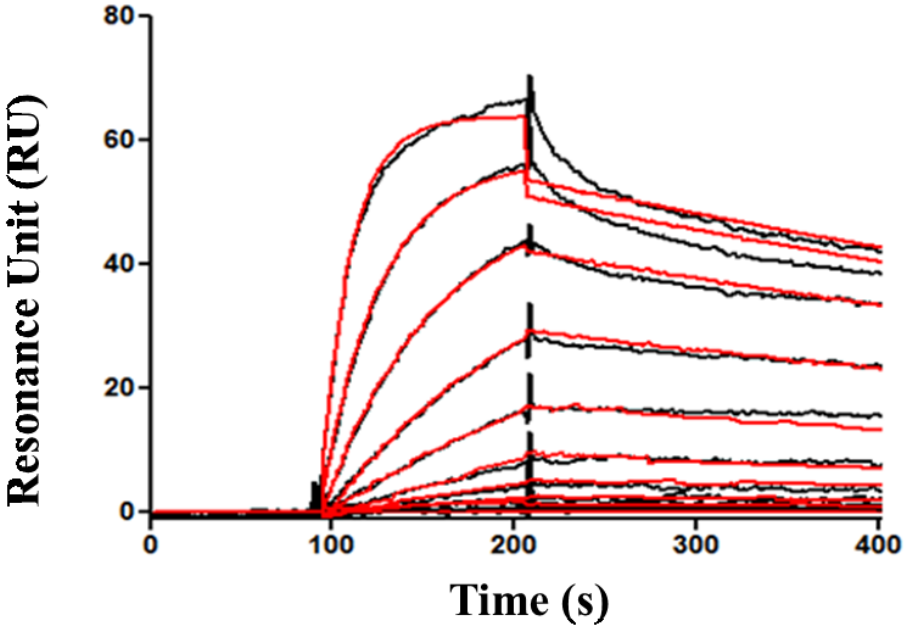

Resonance Unit (RU)
80
60
40
20
0
0
100
200
300
400
Time (s)

Figure 3.3. SPR analysis of diapausin-1 binding to soluble β-1,3-glucan (laminarin).

(A) Diapausin-1 was immobilized on the surface of the chip, then a series of two-fold serial dilutions of laminarin from 10 μM to 10 nM was injected for 2 min followed by 5 min of dissociation phase in a running buffer of 20 mM Hepes, pH 7.4, with 150 mM NaCl, 0.005% Tween 20, and 5 mM $CaCl_2$. (B) Using the same data, the black line represents the resonance units at each concentration. Curve fitting for kinetic analysis (red traces) yielded a binding constant of ~100 nM.

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Chapter 4 - Preliminary results for structure determination of *M. sexta* stress-responsive peptide 1 (SRP1) using two-dimensional NMR spectroscopy

Introduction

Although little is known about insect cytokines, the number of discovered cytokines and cytokine-like factors is increasing (Matsumoto et al., 2012). Among those molecules are: Spaetzle, unpaired-3, eda-like cell death trigger (eiger), sex peptides, plasmatocytes spreading peptide (PSP) and stress-responsive peptides (SRP) (Agaisse et al., 2003; Strand and Clark, 1999; Mabery and Schneider., 2010; Nakatogawa et al., 2009; Peng et al., 2005; Weber et al., 2003; Yamagushi et al., 2012). A family of cytokines discovered first in lepidopteran insects was the ENF peptides, which are ~25 residue peptides that all shared the consensus Glu (E)-Asn (N)-Phe (F) at the N-terminus. (Strand et al., 2000; Tufail and Takida, 2012). ENF peptides have diverse biological activities including paralytic activity, hemocyte-spreading, growth blocking and mitogenic activities (Aizawa et al., 2002; Kamimura et al., 2001). The first isolated ENF peptide was the growth-blocking peptide (GBP) (Hayakawa, 1990).

Insect cytokines can regulate hemocyte-mediated immune responses, including formation of a hemocyte capsule or nodule to trap invading pathogens. To accomplish this task, hemocytes must change from non-adhesive to adhesive, to bind the invading microorganisms (Yamagushi et al., 2012; Russo et al., 1996). Recognition of wounding or infection triggers the release of ~25 amino residue peptides such as PSP, GBP and paralytic peptides (PPs) by proteolysis of larger,

inactive precursors that pre-exist in the hemolymph. PPs have been isolated from the hemolymph of lepidopterans including *M. sexta*, *Spodoptera exigua* and *Heliothis virescens*. PP peptides have multiple functions including rapid paralysis, blockage of growth and development, or stimulation of plasmatocyte spreading and aggregation (Ishii et al., 2015; Nakahara et al., 2003; Skinner et al., 1991; Wang et al., 1999). *M. sexta* has two PPs, PP1 and PP2. When PP1 was injected in *M. sexta* larvae, it reduced bleeding from wounds and stimulated the aggregation and the spreading of the plasmatocytes (Wang et al., 1999). Active PP2, 23 amino residues, was pro-duced from a 107 amino acid precursor protein. Its mRNA was detected in the fat body and was not increased after bacterial injection (Wang et al., 1999). When bacteria or small parasites invade insects, hemocytes aggregate around the invaders, then PSP attracts more plasmatocytes to surround the invading pathogen, and form many layers of cells to form a nodule around the invading microorganisms, which then become melanized (Clark et al., 1997; Clark et al., 1998; Clark et al., 2001; Wang et al., 1999). Finally, the nodule is attached to insect body wall or other organs (Gonzalez-Santoyo and Cordoba-Aquilar., 2012; Horohov and Dunn., 1983; Lavine and Strand, 2002).

SRP peptides similarly cause the hemocytes to become adhesive. These peptides result from cleavage of a larger protein precursor to release the carboxyl-terminal active peptide consisting about 25 amino residues. The first SRP cDNA was identified in the fat body of *Hyphantria cunea* after immune challenge with bacteria, using polymerase chain reaction-based differential display. Since this peptide had no homology for known peptides at that time, it was named Hdd23 (Shin et al., 1998). Later, a homologous cDNA was isolated from *Spodoptera litura* parasitized by *Microplitis manila*. The mRNA encodes a pre-pro-peptide consisting of 119 amino acid residues, including 25 C-terminal amino acid residues that constitute the functional cytokine-like peptide. Since this gene and other homologous genes are expressed under different stress

conditions such as parasitization, wounding, ligation, and temperature change, these cytokine-like peptides were classified as stress-responsive peptides (Qiao et al., 2014; Yamaguchi et al., 2012).

Recently, expression profiles of *Helicoverpa armigera* revealed SRP in midgut and hemocytes, but not in the fat body or epidermis. Furthermore, SRP mRNA levels in hemocytes were upregulated after immunization with *E. coli*. When the SRP gene expression was decreased by double–stranded RNA (dsRNA), phenoloxidase at the transcriptional level was decreased as well. Furthermore, when larvae were injected with dsRNA followed by bacterial injection, both phenoloxidase activity and nodule formation decreased significantly compared to uninjected larvae. These studies indicated that SRP may be involved in insect innate immunity in regulating phenoloxidase activity and nodule formation (Qiao et al., 2014).

M. sexta transcriptome analysis showed the upregulation of a Hdd23 mRNA, the old name for SRP, in the fat body and hemocytes in larvae that had been injected with bacteria, but not in control larvae (Gunaratna and Jiang, 2013). The *M. sexta* genome has several SRP genes, but there are no published studies about their biological functions or their structures. Given the possible role of SRP in insect innate immunity and the lack of published SRP structures, it will important to solve the structure of at least one of the *M. sexta* SRPs to better understand the structure-function relationship and to compare their structure with known structures of cytokine peptides like PP, PSP and GBP.

Materials and methods

Sequence analysis

M. sexta paralytic peptide 1(Ms-PP1), plasmatocytes spreading Peptide-1 (Ms-PSP1) and growth blocking peptide (Ms-GBP) sequences were obtained from Yu et al., (1999). The full length SRP protein sequence from *Helicoverpa armigera* (Ha-SRP), *Spodoptera litura* SRP (Sl-SRP), Hy-phantria cunea (Hc-SRP) and *Bombx mori* (Bm-SRP) were obtained using Blast software (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Center for Biotechnology Infor-mation using *M. sexta* SRP1(Ms-SRP1) as a query. Searches of the *Manduca sexta* genome for *M. sexta* SRP2 sequence was carried out using a blast server at the Manducabase web site (http://agripestbase.org/manduca/?q=blast) using *M. sexta* SRP1(Ms-SRP1) as a query. Multiple sequence alignment was done using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011). Signal peptides were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

M. sexta SRP1 peptide preparation:

The 25- residue *M. sexta* SRP1 (FGVRVGTCPSGYVRRGTFCFPDDDY) was obtained from Professor Haobo Jiang in the Department of Entomology and Plant Pathology at Oklahoma State University, Stillwater, OK. The peptide was synthesized by solid-phase Fmoc chemistry (Chinapeptide. Ltd, China). The linear peptide was cyclized by formation of a disulfide bond, then purified by reverse-phase (C-18) high-performance liquid chromatography and characterized by

MALDI-TOF mass spectrometry. The peptide was dissolved in 90 % deionized and triple distilled water (DI-H_2 O) and 10 % D_2 O to a final concentration of 2 mM for NMR data collection.

NMR Spectroscopy

The 1D and 2D ^{1}H- ^{1}H NMR experiments were performed with the 11.75 Tesla Varian 500 MHz VNMRS system (Varian Inc. now Agilent Inc., Palo Alto, CA), operating 499.84 MHz for ^{1}H frequency. The NMR data were acquired at the Biomolecular NMR facility of the Biochemistry and Biophysics Department at Kansas State University, using a 5 mm cryogenic triple resonance inverse detection pulse field gradient probe at 25 °C. The NMR samples were prepared by dissolving SRP1 in triple distilled water containing 10% D_2O. 2D ^{1}H-^{1}H Total Correlation Spectroscopy (TOCSY) and Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were performed in phase-sensitive (States-TPPI) mode using 2000t_2 and 256t_1 data points with spectral width of 12 ppm in each dimension, and 16 transient per increment. Spin-lock time of 100 ms at B1 field strength of 7 KHz was used for 2D ^{1}H- ^{1}H TOCSY experiments using MLEV- 17 pulse sequence (Bax and Davis, 1985), and mixing times of 100, 300 and 500 milliseconds (ms) were used for 2D ^{1}H- ^{1}H NOESY experiments. Linear prediction and zero filling to 4K data points in t2 dimension were used during NMR data processing. The suppression of the solvent peak (HOD) was achieved using the WATERGATE pulse scheme during acquisition (Piotto et al., 1992) and the residual water peak (4.72 ppm) was used as reference for chemical shift as-signments. NMR Data processing was done using VnmrJ2.3a (Varian Inc. now Agilent Inc., Palo Alto, CA) and analyzed using Sparky- NMR Assignment and Integration software (Goddard and Kneller, 2004).

For distance constraint determination, NOE cross peaks were classified as strong, medium, weak or very weak based on the observed number of contour lines.

Structure calculations

2D ^{1}H- ^{1}H NOESY experiments were performed with 100, 300 and 500 ms mixing time. Initially NOESY spectrum acquired at 500 ms mixing time was used to get dis-tance restraints for the preliminary structure calculations. The NMR spectral data were analyzed with Sparky software (Gooddard and Kneller, 2004). The obtained distances were then used to calculate peptide structures using the Crystallography and NMR System (CNS) software version 1.1 (Brunger et al., 1998). NOE cross peaks were converted into inter-proton distance upper bounds using the following classifications: strong (2.7 Å), medium (3.5 Å), weak (4.0 Å), and very weak (5.0 Å). Upper distance limits for NOEs involving methyl protons and nonstereo-specifically assigned methylene protons, were corrected appropriately for center averaging by adding 1 Å to the maximum distance constraint (Wüthrich et al., 1983).

Circular Dichroism (CD) analysis

To get preliminary information about secondary structure in SRP1. The peptide was dissolved in 300 µl triple-distilled and deionized water (DI-H_2O) at 100 µM for a CD experiment. CD measure-ments were performed using a Jasco J-815 spectropolarimeter (Jasco Inc., Tokyo, Japan) using a cylindrical quartz cuvette with 0.1 mm path length (Fisher Scientific, Waltham, MA). Spectra were recorded from 260 to 190 nm wavelength range. The spectra were acquired

using 1.0 nm, 0.2 nm step resolution, 50 nm/min scan speed, and 2 second response time for three scans. CD scans of DI-H2O were used as a baseline, and were subtracted from the average of SRP1 scans

Homology model

A homology model of SRP1 was generated using I-TASSER software (Roy et al., 2010), using the *M. sexta* PP1 NMR structure (PDB: 1HRL) as a template. The resulting structure was viewed using pymol software (www. pymol.org).

Results

Sequence alignment analysis

In order to examine conservation of sequences between different full length SRP pro-teins from different insect species, we aligned *M. sexta* SRP1with SRPs from several lep-idopteran species. All of the proteins have a sequence consistent with pre-pro-proteins of ~118 amino residues, with a predicted signal secretion peptide (Fig. 4 .1). There are nine absolutely conserved amino acid residues, including an arginine residue at position 94, which is the predict-ed serine protease cleavage site to generate the active C-terminal peptides (Yamagushi et al., 2012). Furthermore, there are two conserved cysteine residues in the C-terminal peptide. The conserved glycine residue occurs two positions before the first cysteine residue, and this cysteine is followed by a conserved proline.

SRP proteins are near the same size of ENF family of cytokines, which result from proteolytic cleavage in the C-terminus of the protein, yielding an active peptide of ~ 23-25 amino residue and contain two conserved cysteine residues with spacing similar to the SRP peptides. Because of the aforementioned similarities, we carried out an alignment between the mature SRPs and members of the *M. sexta* ENF peptide family (PP1, PSP1 and GBP), to evaluate whether SRPs may be homologous with ENF family peptides. The alignment showed that both mature SRPs and mature ENF peptides share the two cysteine residues in different species. These residues have been found to form a disulfide bridge in ENF peptides, PP1, PSP and GBP (Aizawa et al., 1999; Volkman et al., 1999; Yu et al., 1999). The alignment also showed that the glycine two amino residues before the first cysteine is conserved in all mature peptides as well. In addition, there are six amino acid positions conserved in six or more of mature peptides (Fig.4. 2).

Structure calculations and the homology model

A synthetic SRP1 peptide was used for structural studies. A 2D ^{1}H- ^{1}H TOCSY spectrum was used to identify the spin systems of the amino acids in the SRP1. We identified amino acid residue spin systems using the fingerprint regions of TOCSY spectra containing NH-C^{α} and side chain protons region (Fig. 4.3) except spin systems for the first phenylalanine (1F), because the amino group proton was exchanged with D_2O, and spin systems for prolines 9 and 21(9P and 21P). These three amino acids were identified in the C^{α} proton region (2.5-5 ppm) of the TOCSY spectrum (Fig. 4. 4). Most other spin systems in SRP1 were distinct and were easy to assign. However, NMR signals of some spin systems overlapped. For example, 18F and 20F, and 12Y

and 25Y spin systems were aligned on top of each other. Also, we observed that NMR signals for Cα-protons of 2G and 6G resonated very close to each other (Fig. 4.3).

We used C^{α}-proton chemical shift values obtained from the spin system assignments to calculate the chemical shift index (CSI) (Table 4. 1). CSI was calculated by subtracting the corrected reference values of Cα-proton chemical shift values from the experimental ones. We plotted the CSI values versus residue number to get information about the secondary structures for SRP1. In general, if there is a negative CSI value for four or more amino acids in sequence, then it is predicted to have either a helical conformation or a turn. If the three or more amino acids in a row showed a positive CSI value, then this sequence may form a β-strand (Wishart et al., 1992). We observed a regions of SRP1 with negative CSI values. This region contains amino acid residues 11G -16G indicating that this region may represents either a helix or a turn. On the other hand, a stretch of amino residues 17T-21P showed positive CSI values, indicating the presence of β-strand, whereas both N-terminal and C-terminal amino residues were found to be unstructured (Fig. 4. 5).

Then we overlaid 2D ^{1}H- ^{1}H TOCSY and NOESY spectra of SRP1 acquired under similar experimental condition to determine the sequential assignment for the amino acid residues (Fig. 4. 6 and Fig. 4. 7). The NOE cross-peaks were divided into intraresidual, sequential (i+1), medium range (≤ 5 amino acids apart), and long range (≥ 6 amino acid apart). Using this overlay, we were able to determine amino acid residue sequential connectivity (Fig. 4. 6 and Fig. 4. 7). Furthermore, we were able to characterize the overlapping cross peaks found in the TOCSY spectrum by overlaying NOESY and TOCSY spectra. A few amino acid residue connectivities were difficult to determine because of the overlapping of some NOEs peaks and the poor phasing for others (Fig. 4. 6). In this case, we used our best judgment to resolve those issues.

In order to get more information about the secondary structure of SRP1, we performed a CD spectrum analysis. The CD analysis showed a minimum around 208 nm with small shoulder (Fig. 4. 8). This spectrum differs from typical minima in folded or random coil proteins. In a folded protein, α-helix structures should have two distinct minima at 222 nm and 208 nm, whereas β-sheet should show a single minimum around 218 nm. On the other hand, random coil structures have minima between 190 nm and 200 nm (Greenfield, 2006).

We performed a first round of structure calculations using CNS software to yield 10 initial structures. Structures obtained from these calculations were aligned using Pymol software. Only three structures showed alignment. However, this alignment did not indicate a well-defined secondary structure, and all the three structures were aligned in a region which has a turn structure (data not shown). These structures had many NOE distance violations. Another round of 20 structure calculations also showed many NOE distance violations (data not shown), suggesting a need for rechecking of proton chemical shift and NOE cross peaks assignments for SRP1.

To have an insight of a potential structure of SRP1, we made a homology model using I-TASSER using the structure of *M. sexta* PP as a template. The model includes a main secondary structure motif in SRP1 of two antiparallel strands.The first strand is composed of amino acid residues 13V-15R, and the second strand is composed of amino acid residues 18F-20F (Fig.4.9). The peptide fold is consistent with existence of a disulfide bridge between the cysteine residue in the unstructured region near the N-terminus of SRP1 and the cysteine residue in the second beta strand near the C-terminus. This hypothetical fold of SRP1 is similar to the fold of PP, which includes a short antiparallel two-strand β-sheet connected to an unstructured N-terminal region by a disulfide bridge, and with N-terminal and the C-terminal unstructured segments.

Discussion

Known insect cytokines are small peptides produced by proteolytic cleavage of a larger protein precursor, and play an important role in regulating insect innate immunity (Ishii et al., 2015; Wang et al., 1999; Weber et al., 2003). A group of peptides called SRPs was discovered recently, with biological activity in innate immunity and larval development, and expressed under different stress conditions (Yamaguchi et al., 2012). These peptides share common features with ENF peptide family including: i) the production from pre-pro-peptides~119 residues. ii) The active peptide located at the C-terminus and resulting from proteolytic cleavage after a conserved arginine residue. iii) The presence of two conserved cysteine residues located in a motif which can be represented as C-x (2)-G-x (4, 6) G-x (1, 2) C-[KR] (Matsumoto et al., 2012; Qiao et al., 2014; Yamaguchi et al., 2012).

The *M. sexta* genome has several genes encoding SRP proteins, and none of them has been investigated yet. To gain insight into the function of stress response peptides, we initiated structural studies on SRP1 peptide, using solution NMR spectroscopy. In this study we were able to identify the spin systems for many amino acids unambiguously in TOCSY spectrum. On the other hand, a few amino acids have the same spin system, for which it was difficult to distinguish these amino acids from each other. When we overlaid TOCSY and NOESY spectra, we were also able to identify the connectivities between many amino acids, however, again we faced another problem in which some NOE peaks were broad and close to each other, and we could not confirm the connectivity of some amino acid residues. All these problems resulted in many structure violations when we performed structure calculations. This kind of problems may be due to transit problems

in the NMR spectroscope, or the peptide synthesis has something wrong. For future study on *M. sexta* SRP1, we need a new synthesized peptide sample and/or using more powerful NMR instrument.

Although the data presented in this chapter did not result in a well-defined structure, the analysis gave a clue that the structure of *M. sexta* SRP1 may have a conformation similar to, PP1, GBP and PSP1 peptides, based on the presence of conserved residues in the peptide core. Furthermore, the CD spectrum analysis also indicated that this peptide is lacking helical structure and may have a kind of β-sheet and a turn in its core structure. Furthermore, a homology model for SRP1 showed an antiparallel β-sheet consisting of two antiparallel strands, the first composed of amino residues 13V -15R and the second composed of 18F- 20F, with a potential disulfide bridge connecting the first cysteine residue in the unstructured region near the N-terminus with the second cysteine in the strand near the C-terminus (Fig. 4.9). Those observations may indicate that SRP1 is homologous to ENF peptides and may have a fold similar to those observed for *M. sexta* PP1, GBP from *Pseudaletia separate* and PSP1 from *Pseudoplusia includes*, that have been solved by NMR. The GBP solution structure is composed of a short anti-parallel β-sheet containing amino acid residues 11-13 and 19-21, and a type II β-turn, whereas PSP1 has a β-hairpin and a type II β-turn in the same region as GBP (Aizawa et al., 1999; Volkman et al., 1999). The solution structure for *M. sexta* PP1 has a short β-sheet with two strands composed of four amino acid residues each and a type I β-turn in the same core region as in both GBP and PSP1 (Yu et al., 1999). The conserved disulfide bond has an important role in the activity of those peptides. When the cysteine residues were replaced with alanine in PSP from *P. includes*, the plasmatocyte spreading activity was eliminated (Clark et al., 2001a). Furthermore, replacing the conserved 13R reduced the activity dramatically (Clark et al., 2001b). Interestingly, those mutated residues were conserved in

most of the SRP peptides, PSP, PP and GBP. Furthermore, those peptides have charged residues in the C-terminus (7-23 amino residues) and hydrophobic residues in the unstructured N-terminus, indicating that charged residues may be involved in binding to receptors especially 13R (Clark et al., 2001b). In GBP, the replacement of 3F with alanine or tyrosine reduced the biological activity severely.

All those structures share the same fold of the C-terminal domain of mammalian epidermal growth factor (EGF) (Aizawa et al., 1999; Miura et al., 2002; Yu et al., 1999; Volkman et al., 1999). Furthermore, all those three peptides including SRP peptides in *M. sexta* and other insects SRP share the same conserved motif C-x (2)-G-x (4, 6) G-x (1, 2) C-[KR] (Matsumoto et al., 2012). This indicates that similar core peptides could be responsible for structure similarities.

Collectively, studying the structure-function relationship is needed for SRP peptides to investigate their biological activities. In this regard, our preliminary data are a start toward future high-resolution NMR studies on SRP1 peptide.

Figures

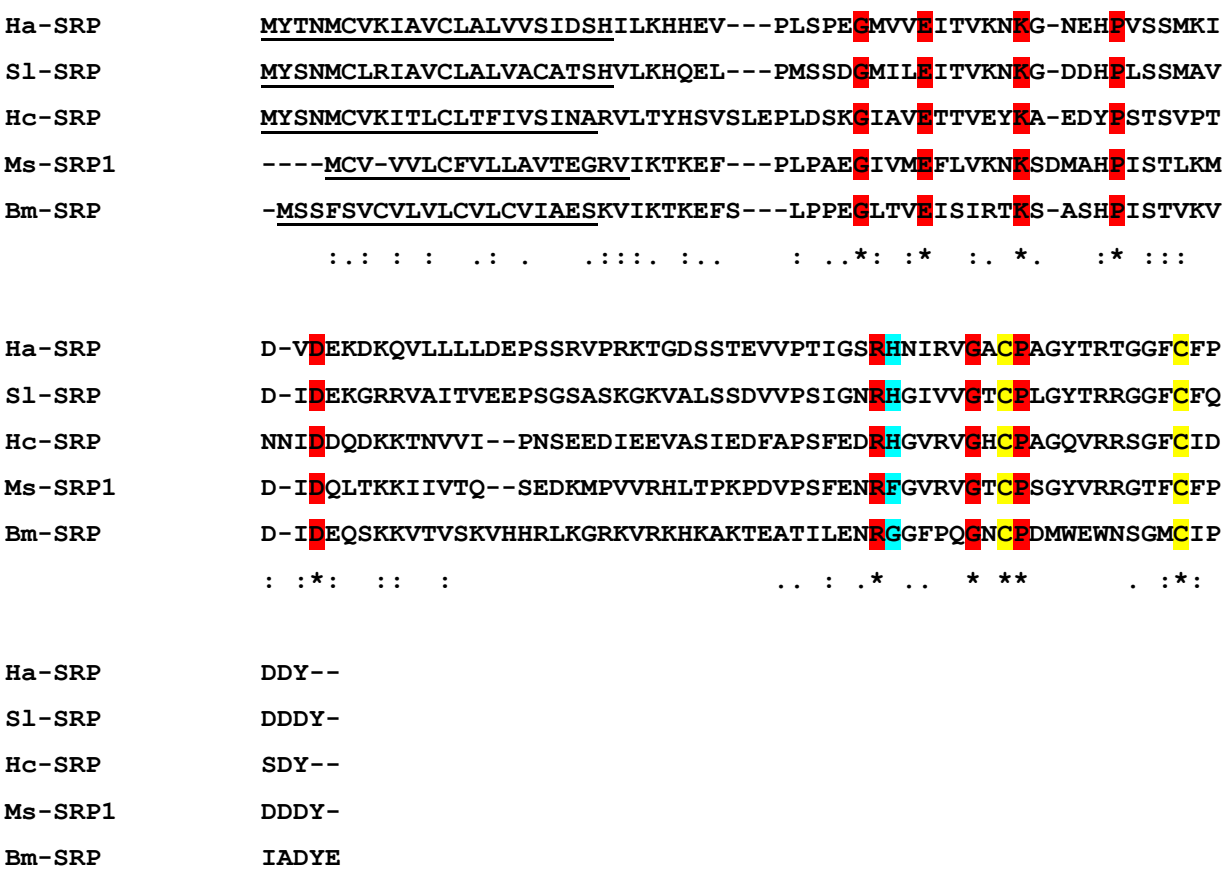

Figure 4.1. Alignment of the amino acid sequences of SRP pro-proteins from different insects.

Full length SRP proteins from *H. armigera* (Ha-SRP), S. *litura* SRP (Sl-SRP), *H. cunea* (Hc-SRP), *B. mori* (Bm-SRP) and *M. sexta* SRP1(Ms-SRP1) were aligned to identify conserved amino acids. The predicted signal secretion peptide was underlined, conserved amino residues other than cysteines are shaded in red, whereas conserved cysteine residues are shaded in yellow. Positions marked with: indicate conservative substitutions in all of the sequences. The predicted N-terminus of the active peptides is shaded in blue.

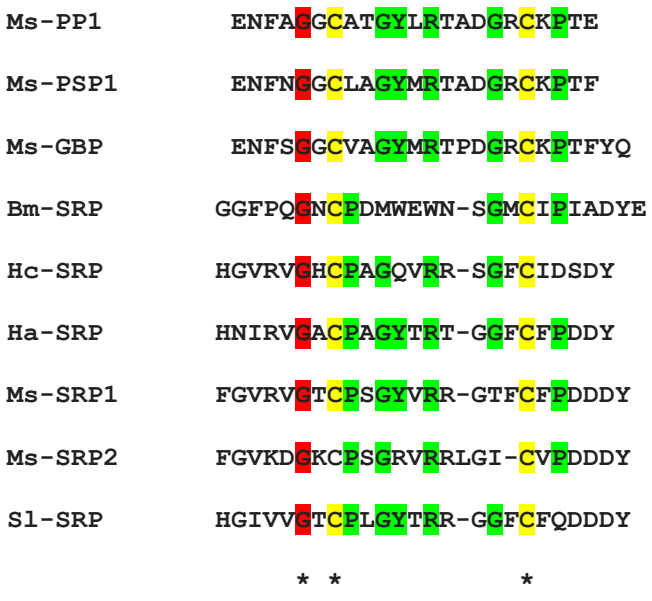

Figure 4.2. Alignment of mature SRPs from several lepidopteran insects and ENF peptides from *M. sexta*.

Absolutely conserved glycine residues are highlighted in red, whereas cysteines are highlighted in yellow. Amino residues conserved in six or more mature peptides are highlighted in green.

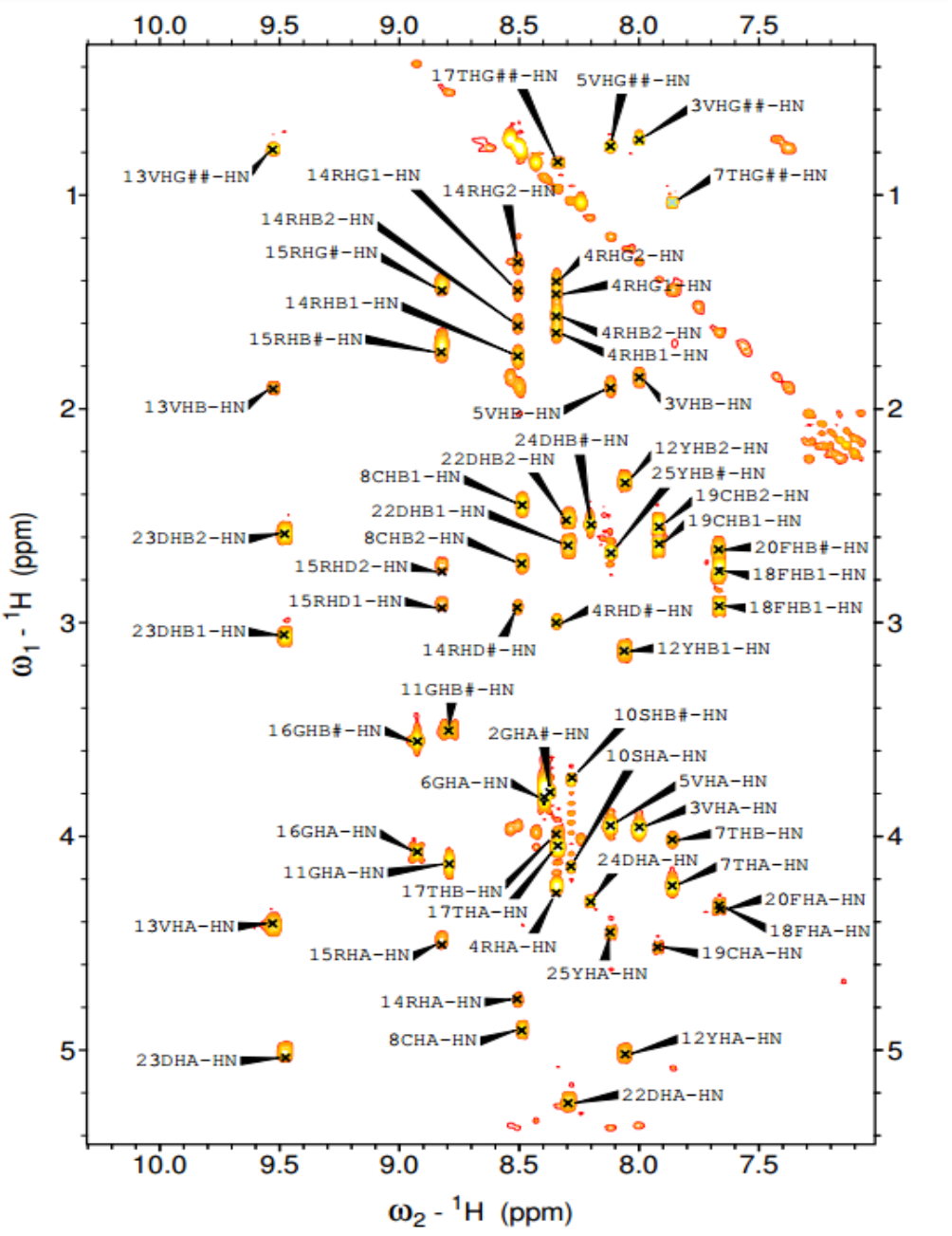

Figure 4.3. 2D ^{1}H-^{1}H TOSCY spectral fingerprint region showing NH-C$^{\alpha}$/side chain protons of amino acids for *M. sexta* SRP1.

Assignments were labeled using X-PLOR nomenclature (HA= H^{α}, HN= H^{N}, HB= H^{β}, HG= H^{γ}, HD= H^{δ}) in all spectra.

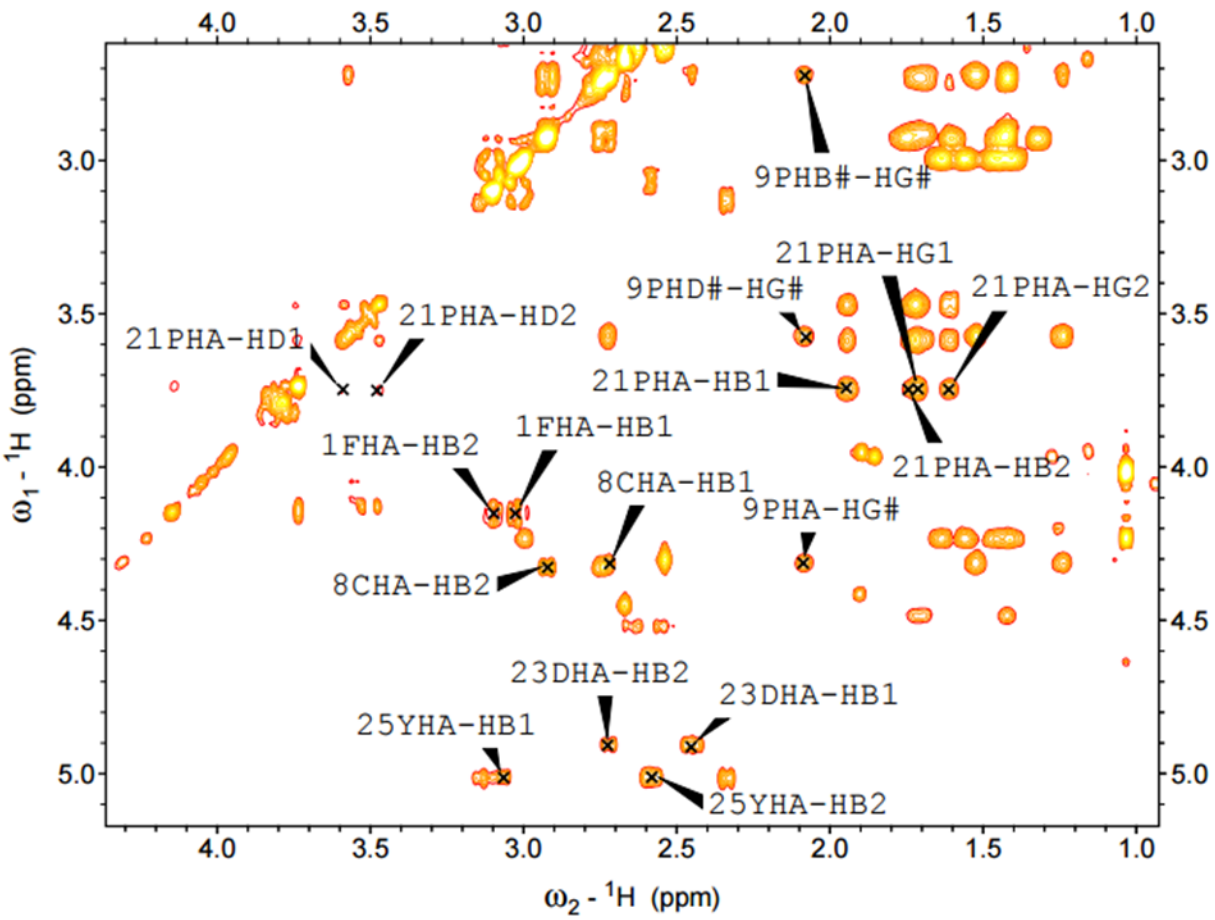

Figure 4.4. 2D ^{1}H-^{1}H TOSCY fingerprint region containing C$^{\alpha}$ and side chain proton resonances to identify 1F, 9P, 21P and other amino residues for *M. sexta* SRP1

Table 4.1. Preliminary proton chemical shifts (ppm) assignments for the amino acids residues of *M. sexta* SRP1.

Residue	NH	αH	βH	Others
1F	-	4.14	3.01, 3.10	-
2G	8.37	3.78	-	-
3V	7.99	3.96	1.85	0.73
4R	8.34	4.23	2.99	1.43, 1.56, 1.63
5V	8.11	3.95	1.89	0.77
6G	8.39	3.77	-	-
7T	7.86	4.23	4.01	1.03
8C	8.48	4.9	2.44, 2.71	-
9P	-	4.31	2.08, 2.72	3.75, 1.24, 1.52
10S	8.28	4.14	3.73	-
11G	8.79	4.12	3.49	-
12Y	8.06	5.01	2.32, 3.13	-
13V	9.52	4.41	1.9	0.78
14R	8.5	4.75	2.92	1.31, 1.44, 1.60,
15R	8.82	4.48	2.73, 2.91	1.42,1.69 , 6.8
16G	8.92	4.07	3.55	-
17T	8.34	4.04	3.98	0.84
18F	7.66	4.32	2.66, 2.74	-
19C	7.91	4.51	2.55, 2.62	-
20F	7.66	4.32	2.75, 2.91	-
21P	-	3.74	3.46, 3.58	1.6, 1.72, 1.94
22D	8.29	5.23	2.52, 2.63	-
23D	9.47	5	2.58, 3.06	-
24D	8.2	4.3	2.53	-
25Y	8.08	4.45	2.67	6.64, 6.91

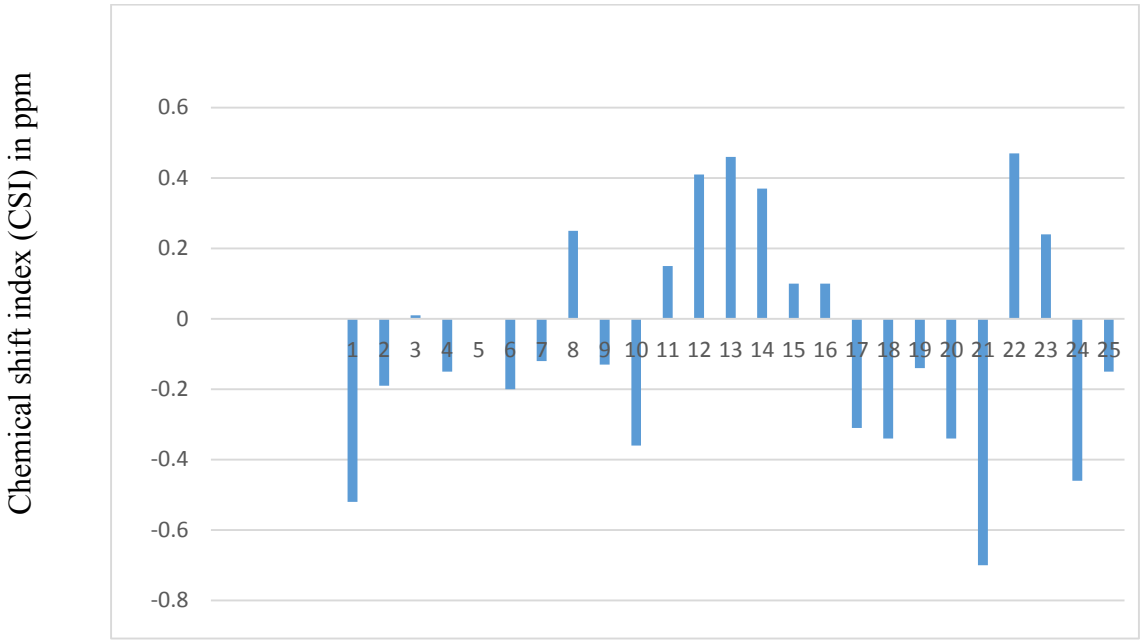

Figure 4.5. Chemical shift index (CSI) in ppm for C^{α} protons of *M. sexta* SRP1.

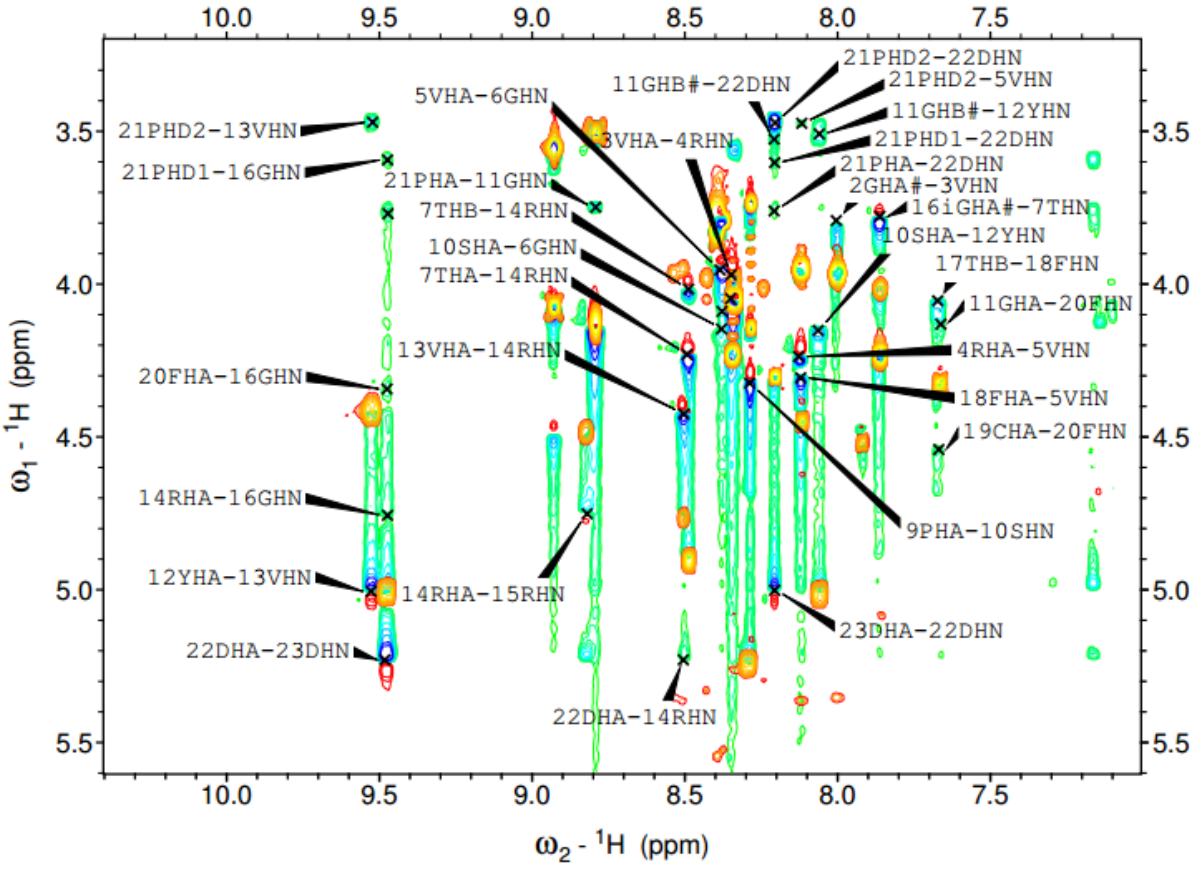

Figure 4.6. 2D ^{1}H-^{1}H NOESY (blue and cyan) TOSCY (red) spectral fingerprint regions (NH-C$^{\alpha}$ H) overlaid for sequential assignments.

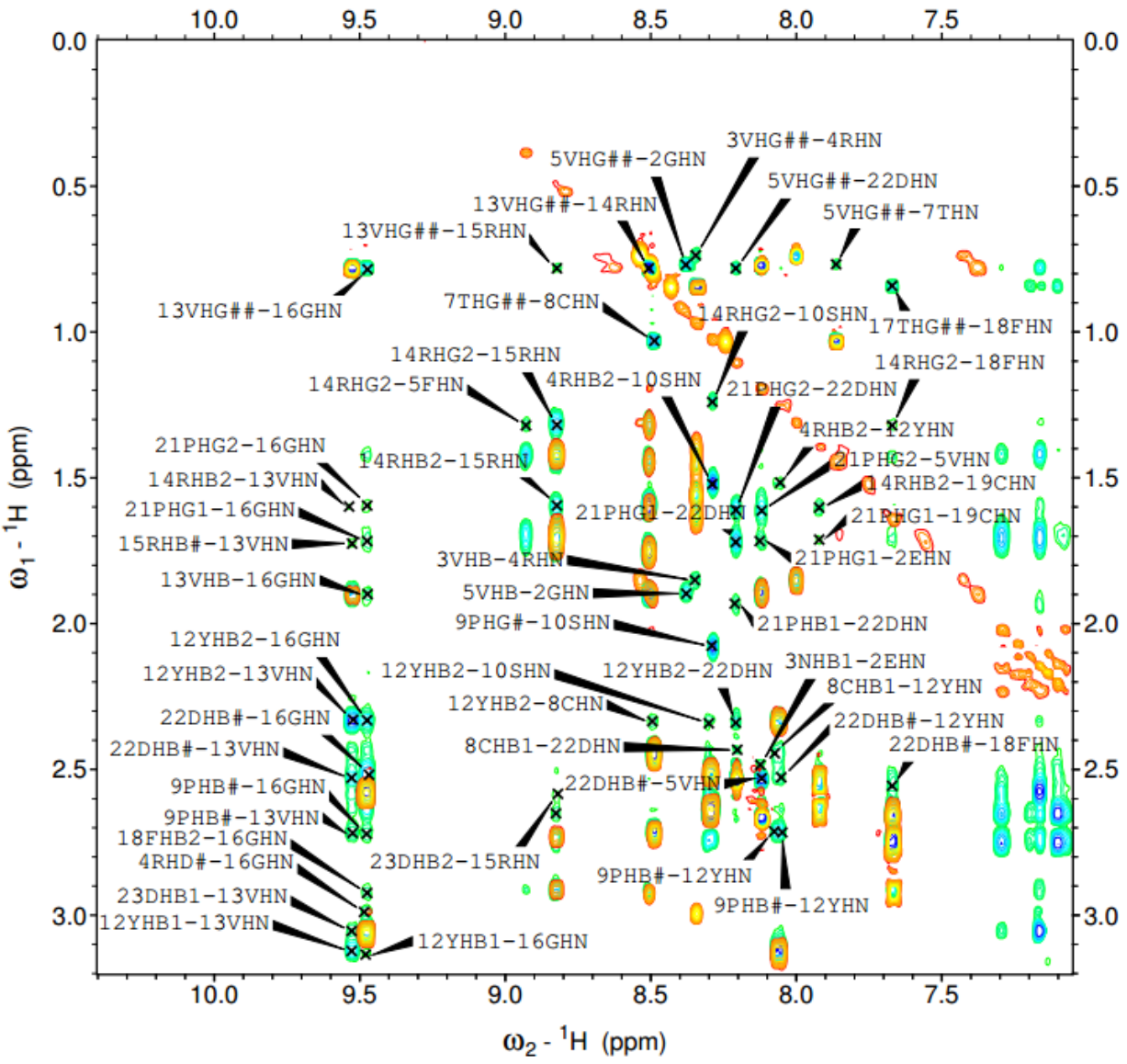

Figure 4.7. 2D ^{1}H-^{1}H NOESY (blue and cyan), and TOSCY (red) spectral finger- print regions (NH-side chain protons) overlaid for sequential assignments.

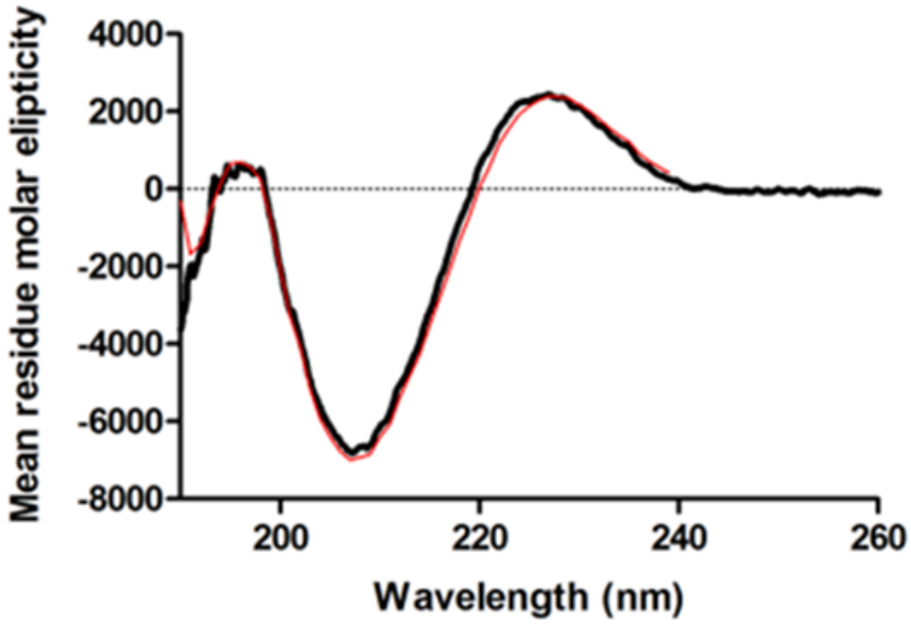

Figure 4.8. Circular dichroism (CD) spectrum for *M. sexta* SRP1

The line represents the actual mean residue molar elipticity of three scans after subtracting the solvent baseline. The black curve is the real data, wherease the red curve represents the smoothed data.

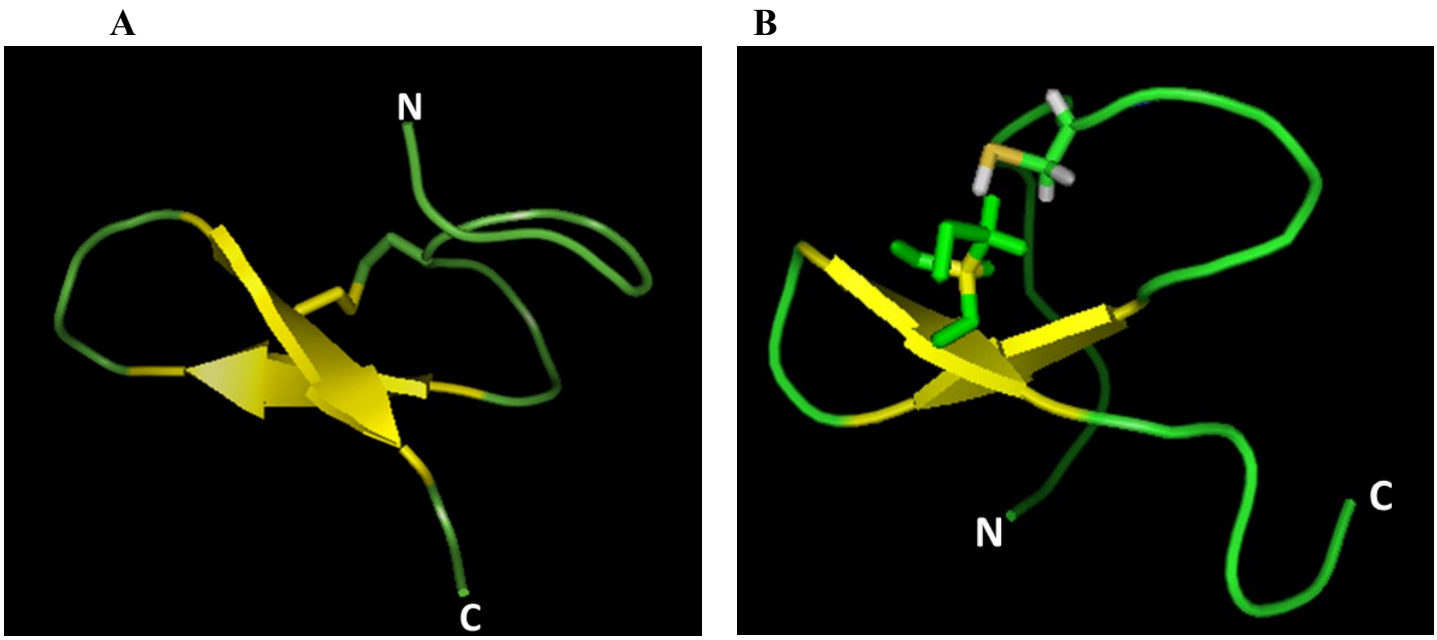

Figure 4.9. Comparison between *M. sexta* paralytic peptide NMR structure and a homology model of *M. sexta* SRP1.

(A) *M. sexta* paralytic peptide NMR structure (PDB: 1HRL**),** which is composed of a short anti-parallel β-sheet at Tyr 11- Thr14 and Arg18- Pro21 shown in yellow. The disulfide bridge in PP is represented in a stick model. (B) *M. sexta* SRP1 homology model generated using I-TASSER using *M. sexta* PP as a template. The two predicted strands in the homology model (13V-15R, and 18F-20F) are shown in yellow, wherease side chains of the two cysteine residues in the homology model are shown in a stick model.

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Appendix A -

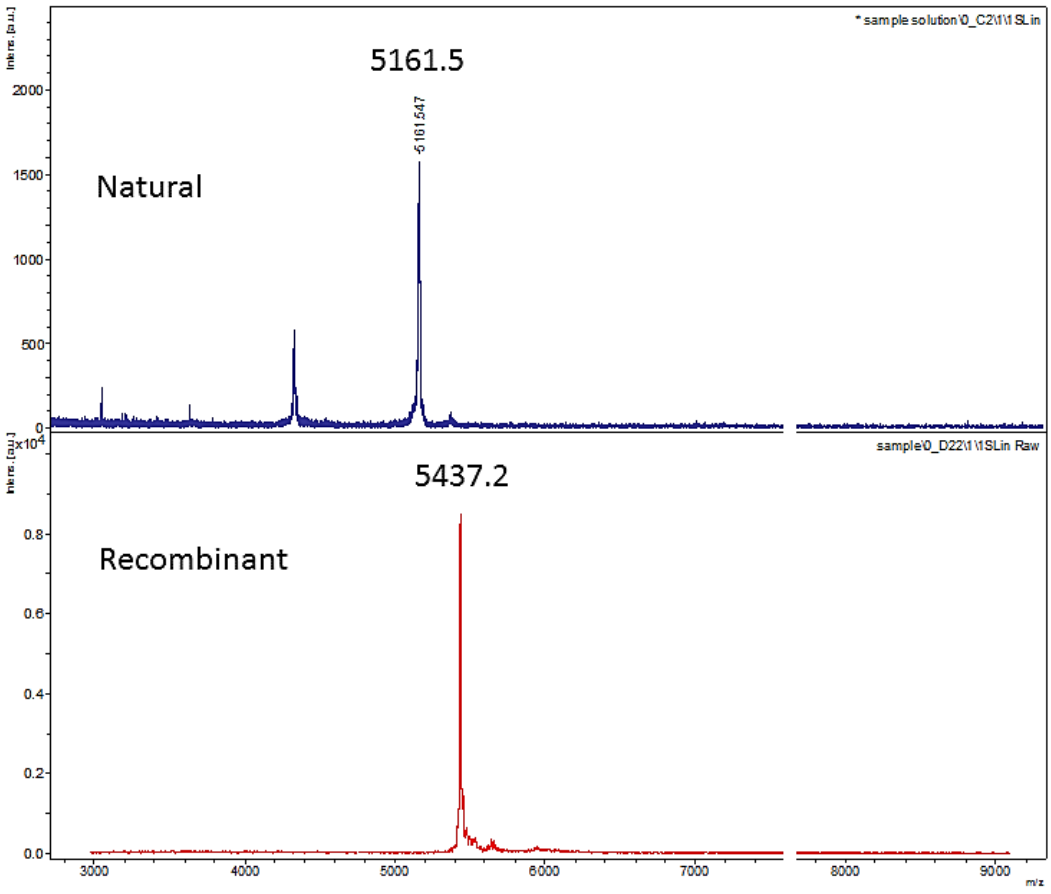


Figure A.1 . MALDI-TOF mass spectrometry analysis of diapausin-1 purified from hemolymph (natural) and from *E. coli* (recombinant).

Intact molecular mass of purified diapausin-1 peptide (top panel), and recombinant peptide expressed in *E. coli* (bottom panel).

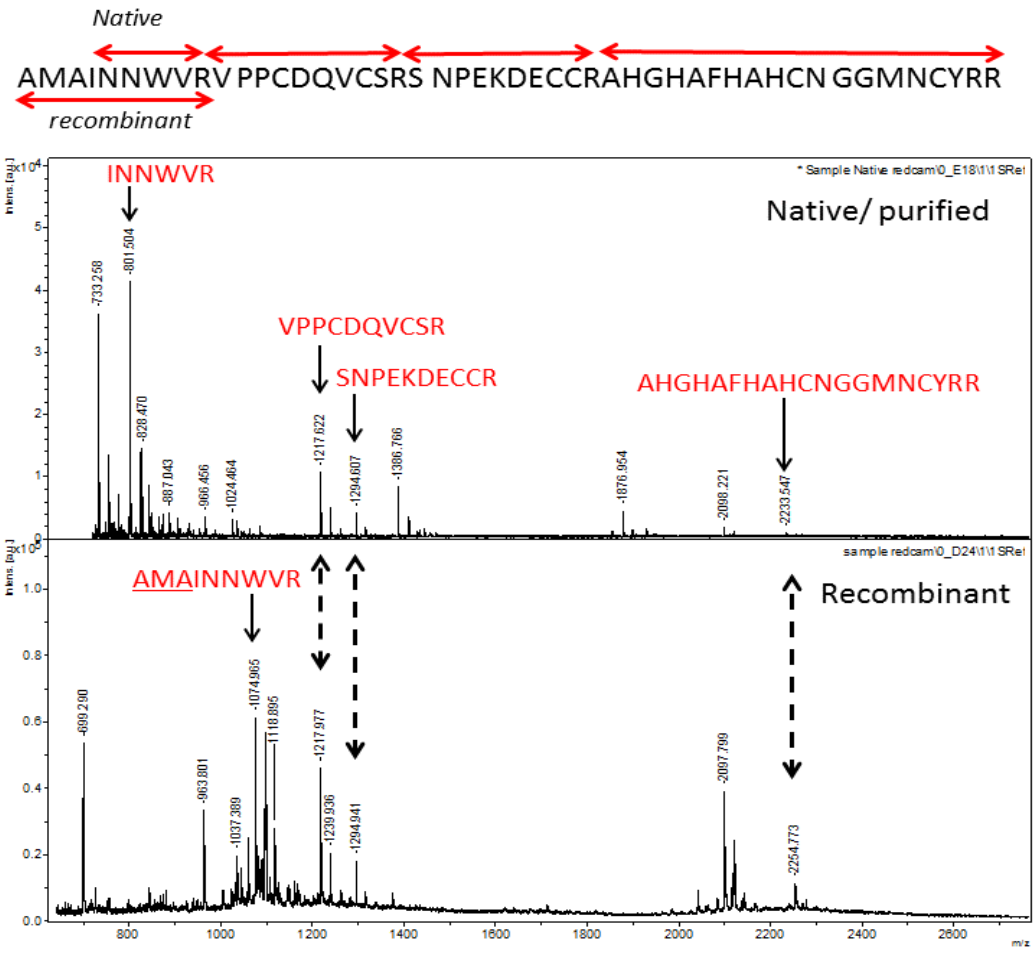
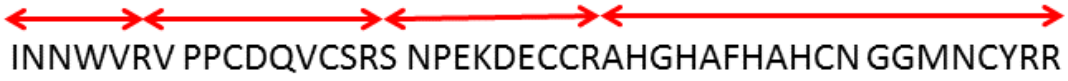


Figure A.2.Validation of (natural) native and recombinant diapausin-1 peptides isolated from *M. sexta* using MALDI-TOF/ MS.

The peptide sequence for both native and recombinant peptides is shown in top. The arrows indicate to trypsin cleavage sites. Both of peptides were digested with trypsin and analyzed by MADLI-MS/MS, as described in the Materials and Methods. Both arrows point toward the size of the tryptic fragments of the peptide in both native and recombinant peptides. The sequence of the fragment showed in red in upper panel in native and recombinant peptides, respectively.

A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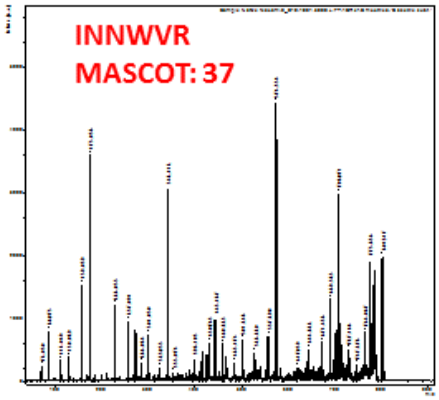
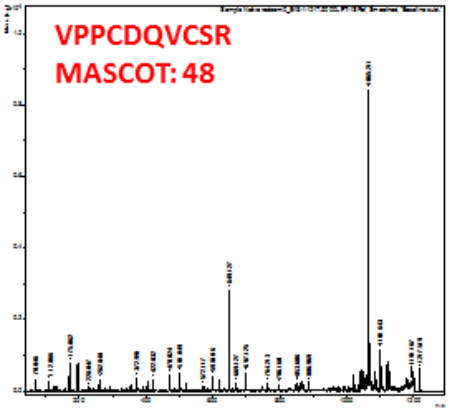

INNWVR
MASCOT: 37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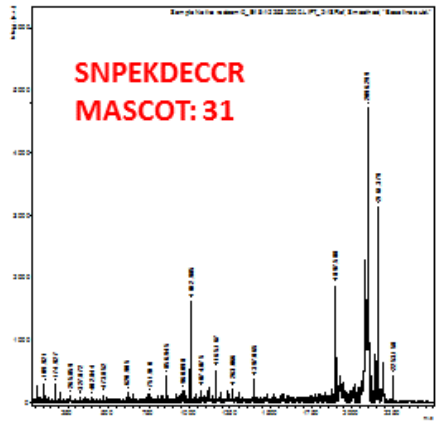
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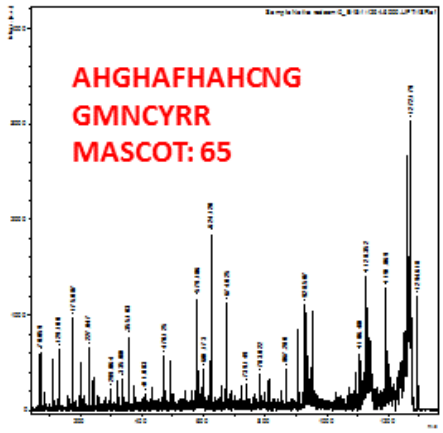
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B

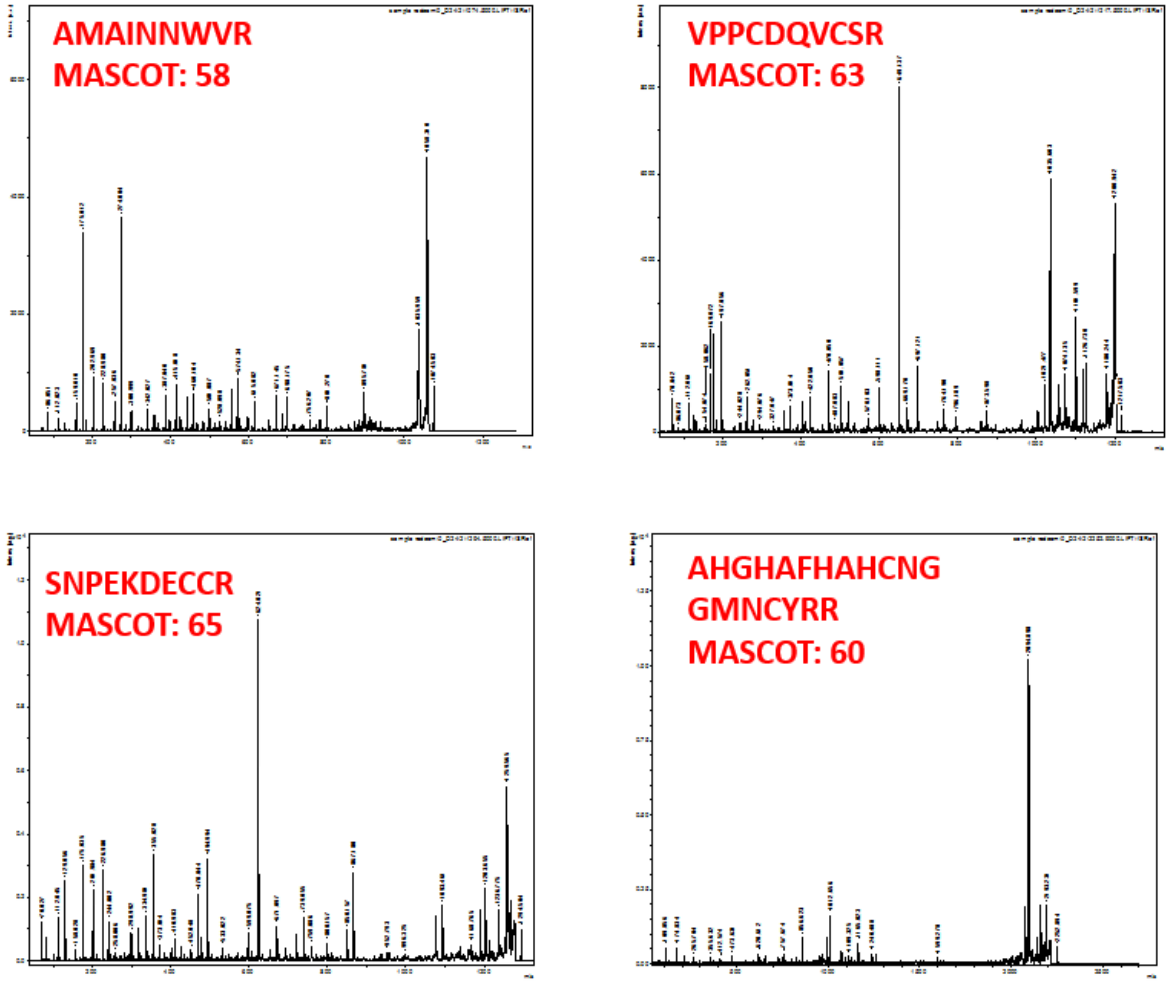

Figure A.3. Validation of isolated and recombinant diapausin-1 by MALDI-TOF- MS/MS.

Purified (A) and recombinant (B) peptides were digested with trypsin, and the resulted fragments and analyzed using MALDI-TOF- MS/MS. MASCOT was used to analyze the tryptic fragments. Validated fragments are highlighted in red in each panel.