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Semi-quantitative analysis of changes in the plasma peptidome of *Manduca sexta* larvae and their correlation with the transcriptome variations upon immune challenge

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ABSTRACT

The tobacco hornworm, *Manduca sexta*, has been used as a biochemical model for studying insect physiological processes. While the transcriptomes of its fat body, hemocytes, midgut, and antennae have been examined in several studies, limited information is available for proteins in tissues, cells, or body fluids of this insect. In keeping pace with the *M. sexta* genome project, we launched a pilot study to identify differences in the peptidome of cell-free hemolymph samples from larvae injected with buffer or a mixture of bacteria. At 24 h after injection, plasma was collected and treated with 50% acetonitrile to precipitate large proteins. The supernatants, containing peptides (<25 kDa) and other stable proteins (>25 kDa), were digested with trypsin and analyzed by nano-liquid chromatography and nano-electrospray tandem mass spectrometry (nanoLC-MS/MS) on an LTQ Orbitrap XL mass spectrometer. Known *M. sexta* cDNA sequences and gene transcripts from the draft genome were translated *in silico* to generate a database of polypeptides (*i.e.* peptides and proteins) in this species. By searching the database, we identified 268 hemolymph polypeptides, 50 of which showed 1.67–200 fold abundance increases after the immune challenge, as judged by significant changes in normalized spectral counts between the control and induced plasma. These included a total of 33 antimicrobial peptides (attacins, cecropins, defensins, diapausins, gallerimycin, gloverin, lebocins, lysozymes), pattern recognition receptors, and proteinase inhibitors. Although there was no strong parallel (correlation coefficients: −0.13, 0.11, 0.39 and 0.62) between plasma peptide levels and their transcript levels in control or induced hemocytes or fat body, we observed the mRNA *level changes* in hemocytes and fat body concurred with their peptide *level changes* with correlation coefficients of 0.67 and 0.76, respectively. These data suggest that fat body contributed a significant portion of the plasma polypeptides involved in various aspects of innate immunity after the bacterial injection.

1. Introduction

Innate immunity is the first-line defense of multicellular organisms against microbial infection. Like other invertebrates, insects rely entirely on this response system since they lack adaptive immunity found in vertebrates (Gillespie et al., 1997; Lemaitre and Hoffmann, 2007). Insect innate immune responses are triggered by wounding or microbes recognized by proteins that bind to the aberrant tissues or pathogen surface moieties. These interactions either induce cellular responses (*e.g.* phagocytosis, nodulation, and encapsulation) (Strand, 2008) or activate an extracellular serine proteinase system that generates phenoloxidase, spätzle, and other signaling molecules (Jiang et al., 2010; Park et al., 2010). Phenoloxidase is key to melanization, a pathogen-killing mechanism often associated with encapsulation (Kanost and Gorman, 2008; Zhao et al., 2011). Spätzle, a Toll ligand, binds to the receptor on immune cells to up-regulate gene transcription through an intracellular signal transduction pathway (Valanne et al., 2011). The induced polypeptides (*i.e.* proteins and peptides) either replenish

Abbreviations: CP, control plasma from larvae injected with buffer; IP, induced plasma from larvae injected with bacteria; NSC, normalized peptide MS/MS spectral count; CF, IF, CH and IH, control (C) and induced (I) fat body (F) and hemocyte (H) mRNAs; NRN, normalized cDNA read number; FDR, false discovery rate; RA, relative abundances; ARN, adjusted read numbers; ACN, acetonitrile; AMP, antimicrobial peptide; HP, hypothetical polypeptide; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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those constitutively present in plasma prior to infection or represent new defense molecules which control the proliferation of pathogens that survived in the initial battle with the host immune system. Among these induced polypeptides are antimicrobial peptides that destroy the pathogens (Bulet et al., 2004; Reddy et al., 2004). Genetic and biochemical studies have revealed a framework of the insect immune system and some molecular mechanisms underlying specific responses (Lemaitre and Hoffmann, 2007; Ragan et al., 2009). Genome and cDNA sequences are utilized to examine this system in different insects from the perspectives of gene evolution (comparative immunogenomics), transcriptional regulation (microarray or RNA-Seq analysis), and proteomic changes. Due to limited sequence information and technical hindrance, high-quality proteomic investigations have been performed in only a few insects such as *Drosophila melanogaster* (Veraksa, 2010). In *Manduca sexta*, 58 non-redundant polypeptides have been identified in larval plasma using one- and two-dimensional gel electrophoresis (Furusawa et al., 2008).

A proteome is an entire set of polypeptides comprising the structural, metabolic, and regulatory machinery of a cell, a tissue, or an organism. Since there is no strong correlation between mRNA and protein levels (Chen et al., 2002; Tian et al., 2004) and because proteins play direct roles in biochemical processes, substantial efforts have been made to identify proteins in specific tissues or cells, measure their levels at various life stages, and explore protein interactions associated with certain physiological states (Rotilio et al., 2012; Gingras et al., 2005). Mass spectrometry (MS)-based proteomic research using a shotgun approach has dominated the field of proteomics for identifying polypeptides and measuring their levels in complex mixtures (Yates et al., 2009). This approach typically comprises sample pre-fractionation, trypsin digestion, peptide separation by nanoscale liquid chromatography (nanoLC), electrospray ionization of resolved peptides, and MS/MS analysis of peptides and their fragments (Feng et al., 2008). Accurate determination of a peptide mass in the 1st MS and its fragment masses in the 2nd MS, combined with searching of a species-specific database of trypsinolytic peptides deduced from cDNA coding regions, allows identification of the parental protein. For measuring and comparing levels of proteins identified in different samples, several methods (*e.g.* AQUA, ICAT, iTRAQ, ion intensity, spectral counting) have been developed (Becker and Bern, 2011; Zhu et al., 2010), one of which uses the total number of fragmentation spectra that map to peptides of a specific protein for quantitative analysis (Lundgren et al., 2010). The rationale behind this technique is quite simple: the more abundant proteins are, the higher frequencies their fragmentation spectra will be. As the method relies on the number of mapped MS/MS spectra, its optimization favors total protein identification (Bantscheff et al., 2007). Consequently, this simple method has been used in a wide range of differential proteomic studies. Due to its low accuracy for rare proteins (Old et al., 2005), however, spectral counting is only considered as a semi-quantitative method.

As a part of the proteome, the peptidome represents the whole set of peptides (*i.e.* low M_r polypeptides) in a cell or tissue. Many peptides are too small for two-dimension electrophoresis, too large for *de novo* sequencing, and often masked by abundant proteins (Baggerman et al., 2004). Heating, acid, or organic solvent treatment (Ziganshin et al., 2011; Merrell et al., 2004) can greatly reduce large proteins while retaining heat-, acid-, and solvent-stable bioactive peptides (*e.g.* neuroregulators, hormones, antibiotics) for peptidomic analysis. As hallmarks of insect immunity, antimicrobial peptides (AMPs) have been extensively characterized by biochemical methods (Bulet et al., 2004) and peptidomic techniques (Brown et al., 2009). We have been studying the innate immune system of a biochemical model insect *M. sexta* and found AMP genes were highly expressed in fat body and hemocytes in response to bacteria injected into the larvae (Zhu et al., 2003; Zou et al., 2008; Zhang et al., 2011; Gunaratna and Jiang, 2013). To identify these molecules in plasma samples and quantify differences in their levels between buffer- and bacteria-injected larvae, we adopted the shotgun approach to explore the *M. sexta* peptidome. To ensure all attacins are covered in this study, we define peptides and proteins as polypeptides smaller and larger than 25 kDa, respectively. Since quantitative transcriptomic data of fat body and hemocytes from naïve and induced larvae were available (Zhang et al., 2011; Gunaratna and Jiang, 2013), we examined possible correlations between transcript levels in these tissues versus peptide abundances in plasma. We also analyzed whether there was a positive correlation between *changes* in mRNA levels in fat body or hemocytes from larvae injected with buffer or bacteria and peptide level *differences* in control and induced larval plasma. In addition, we discovered differentially expressed polypeptides previously not known to be involved in immune responses.

2. Materials and methods

2.1. Insect rearing, pathogen injection, and plasma sample preparation

M. sexta eggs, kindly provided by Dr. Michael Kanost's group at Kansas State University, were highly similar to the ones used for genome and transcriptome sequencing (personal communication). Hatched larvae were reared on an artificial diet as described by Dunn and Drake (1983). Each day 1, 5th instar larva was injected with a mixture of *Escherichia coli* (1.3×10^7 cells), *Micrococcus luteus* (13 mg), and curdlan (13 mg, insoluble β-1,3-glucan from *Alcaligenes faecalis*) in 20 μl H_2O. As a control, larvae at the same stage were injected with 20 μl of sterile phosphate buffered saline. At 24 h after injection, prolegs of the insects were cut and hemolymph was collected into clean tubes containing a few crystals of phenylthiourea and 1 mM *p*-aminobenzamidine to block melanization and proteolysis. After centrifugation at 4000*g* for 5 min at 4 °C to precipitate hemocytes, the supernatants were transferred to clean tubes, and equal volume of the plasma samples from three induced insects were pooled as "IP1", standing for induced plasma-1. In the same way, a combined plasma sample "CP1" (for control plasma-1) was prepared using three control larvae injected with the buffer. The two mixtures were aliquoted (100 μl per tube) and stored at −80 °C. This experiment was repeated twice on different days to obtain "CP2", "CP3", "IP2", and "IP3". The control and induced plasma samples (100 μl each) were thawed on ice and mixed with equal amount of chilled 100% acetonitrile (ACN) by vortexing. After incubation on ice for 2 h, the suspensions were centrifuged at 10,000*g* for 10 min at 4 °C. The small peptide-enriched supernatants (150 μl, control and induced, each with three biological replicates) were transferred to new tubes, dried in a SpeedVac, and redissolved at room temperature in 50 μl buffer (8 M urea, 100 mM Tris-HCl, pH 8.5). Polypeptide concentrations were determined using the bicinchoninic acid assay (Bio-Rad) with bovine serum albumin as the standard. Samples (*ca.* 30 μg each) were reduced with 5 mM Tris(2-carboxyethyl)phosphine at room temperature for 20 min and then alkylated with 10 mM iodoacetamide for 15 min in the dark at room temperature. Subsequently, the samples were diluted with 150 μl 100 mM Tris-HCl (pH 8.3), and digested with 4 μg/ml sequencing grade modified porcine trypsin (Promega) overnight at 37 °C. After digestion, samples were acidified to 1% formic acid (MS grade) and desalted using OMIX C18 affinity media as recommended by the manufacturer (Agilent).