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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 to 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, lebecins, 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.

Keywords

insect immunity; hemolymph protein; nanoLC-MS/MS; antimicrobial peptide; spectral counting; tobacco hornworm

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 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 μ l H₂O. As a control, larvae at the same stage were injected with 20 μ l 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 4000g 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 μ l 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 μ l 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 10,000g for 10 min at 4°C. The small peptide-enriched supernatants (150 μ l, control and induced, each with three biological replicates) were transferred to new tubes, dried in a SpeedVac, and redissolved at room temperature in 50 μ l 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 μ g 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 μ l 100 mM Tris-HCl (pH 8.3), and digested with 4 μ g/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).

2.2 LC-MS/MS analysis

The control and induced peptide samples were analyzed on an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a New Objective PV-550 nanoelectrospray ion source and an Eksigent NanoLC-2D chromatography system. Peptides were first collected by infusion using 1-micron infusion tips (New Objective) and further analyzed by trapping on a 2.5 cm ProteoPrepII pre-column (New Objective), followed by analytical separation on a 75 μ m ID fused silica column packed in house with 10 cm of Magic C18 AQ, terminated with an integral fused silica emitter pulled in house. Peptides were eluted using a linear 5–40% ACN gradient in 0.1% formic acid performed over 40 min at a flow rate of 300 nL/min. During each one-second full-range FT-MS scan (nominal resolution of 60,000 FWHM, 300 to 2000 *m/z*), six most intense ions were analyzed via MS/MS in the linear ion trap mode. MS/MS spectra were collected using a trigger threshold of 1,000 counts and monoisotopic precursor selection. Parental ions were rejected for MS/MS if their charge states were not assignable, if they were previously identified as contaminants on blank gradient runs, or if they had already been twice selected for MS/MS (data-dependent acquisition using a dynamic exclusion for 150% of the typical chromatographic peak width). Column performance was monitored using trypsin autolysis fragments and via blank injections between samples to assay for contamination.

2.3. Database construction

The polypeptide database (Msexta_040612, 1,306,670 entries) was established by *in silico* translation of DNA sequences in six open reading frames from the following sources: 1) *M. sexta* sequences in the NCBI RefSeq collection, 2) cDNA contigs from control and induced fat body and hemocytes (dataset 06CIFH09, Zhang et al., 2011), as well as those from midgut (Pauchet et al., 2010); 3) Cufflinks 1.0a, a genome-dependent assembly of *M. sexta* transcripts using RNA-Seq data (<http://agripestbase.org/manduca/>).

2.4. Polypeptide identification and validation

Centroided ion masses extracted by extract_msn.exe utility of Bioworks (v3.3.1) were used for database search with Mascot (v2.2.04, Matrix Science) and X! Tandem (v2007.01.01.1, <http://www.thegpm.org>). The following parameters were used for the search: no charge state deconvolution or deisotoping, trypsin digestion, maximum missed cleavage of 1, parental ion mass tolerance of 5.0 ppm, fragment ion mass tolerance of 0.60 Da, formylation or acetylation of polypeptide N-termini, Met oxidation, cyclization of glutamate to pyroglutamate, and iodoacetamide/acrylamide derivatization of Cys as variable modifications. Scaffold (v4.2.0, Proteome Software Inc.) was used to validate MS/MS-based peptide or protein identifications. Peptide identifications were accepted if they could be established at greater than 80% probability as specified by the Peptide Prophet algorithm (Keller et al. 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Polypeptides that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The reversed amino acid sequences of Msexta_040612 were used as a decoy polypeptide database to determine false discovery rate (FDR) as previously described (Elmore et al. 2012).

2.5. Sequence editing, statistical analysis, and ratio calculation

The accepted identifications were used to retrieve corresponding cDNAs to deduce their conceptual peptide/protein sequences that contained the identified peptides. In cases of partial open reading frames, identified polypeptides were manually extended by incorporating the genome and transcriptome information. The latter included Cufflinks 1.0b and *de novo* assemblies of the RNA-Seq data (Cao et al., unpublished data). For simplicity, molecular weights were calculated using entire polypeptide sequences deduced from the open reading frames. Signal peptide removal and precursor processing were not considered in the study. Polypeptides smaller or greater than 25 kDa are classified as peptides and proteins, respectively. To annotate the identities of novel polypeptide sequences, BLASTP search of the insect RefSeq collection at NCBI was performed under the default conditions using the improved polypeptide sequences as queries to identify their homologs in other species. To identify changes in peptide/protein expression, spectral counts for polypeptides in the CP or IP samples were normalized by multiplying each observed spectrum count value by the ratio of the average number of peptides identified in all the six samples divided by the total number of peptides identified in the sample the spectra were observed. For example, since the total numbers of peptides identified in CP1, CP2, CP3, IP1, IP2 and IP3

were 6,716, 6,779, 7,298, 7,723, 8,167 and 8,907, respectively (Table S1) and their average was 45,640/6, the observed spectral count (SC) of a polypeptide in one of the sample (say IP2) was normalized as $NSC_{IP2} = SC_{IP2} \times 45,640 / (6 \times 8,167)$. The normalized spectral counts were analyzed by Student's t-test to reveal statistically significant differences ($p < 0.05$) between the induced and control samples. Changes in polypeptide expression were estimated by dividing the average normalized spectrum count values from induced insects by the average normalized values observed in control insects: $(SC_{IP1}/7,723 + SC_{IP2}/8,167 + SC_{IP3}/8,907) / (SC_{CP1}/6,716 + SC_{CP2}/6,779 + SC_{CP3}/7,298)$. In calculating I/C ratios, when $SC_{CP1} = SC_{CP2} = SC_{CP3} = 0$, zero values in the denominator were all replaced with ones. Polypeptides were regarded as up-regulated if I/C > 1.67 , whereas polypeptides with I/C < 0.60 as down-regulated.

2.6. Correlation of peptide and mRNA levels as well as their changes after the immune challenge

Amino acid sequences of the identified polypeptide were used to search the database of CIFH09 contigs (http://darwin.biochem.okstate.edu/blast/blast_links.html) by TBLASTN. Raw numbers of CF, CH, IF, and IH reads assembled into the contig with the highest sequence identity to a query were normalized (Gunaratna and Jiang, 2013). To test if there is a direct correlation between mRNA and peptide *levels*, the normalized read numbers ($\log_2 \text{NRN}$) and corresponding normalized spectral counts ($\log_2 \text{NSC}$) were analyzed in the following pairs: CF-CP, CH-CP, IF-IP, and IH-IP. To test whether a correlation exists between mRNA and peptide *level changes*, the ratios of NRNs ($\log_2 \text{NRN}_{IF} / \text{NRN}_{CF}$ and $\log_2 \text{NRN}_{IH} / \text{NRN}_{CH}$) and the corresponding NSC ratios ($\log_2 \text{NSC}_{IP} / \text{NSC}_{CP}$) were examined in terms of F-P and H-P, respectively.

3. Results and Discussion

3.1. Peptidomics workflow, polypeptide identification, and semi-quantitative analysis

To identify peptides in *M. sexta* plasma, especially those involved in immune responses, we collected hemolymph from larvae injected with buffer or bacteria. After hemocyte removal, the plasma samples were treated with ACN to precipitate large proteins. The supernatants containing peptides and ACN-stable proteins were digested with trypsin to generate peptides to be analyzed by nanoLC-MS/MS. For each sample, we collected a total of 202,077 MS/MS spectra (33,397 in CH1, 32,083 in CH2, 32,129 in CH3, 33,781 in IF1, 34,940 in IF2, 35,747 in IF3). Of these, 45,590 or 22.5% matched spectra corresponding to 268 independent polypeptides (6,716 in CH1, 6,779 in CH2, 7,298 in CH3, 7,723 in IH1, 8,167 in IH2, and 8,907 in IF3) (Table S1). Here, we arbitrarily define peptides as polypeptides smaller than 25 kDa so that all attacins (21.7–24.4 kDa) are included in the analysis. At the stringency settings used, we identified few decoy sequences (peptide and protein FDRs: 0.0% and 0.4%), suggesting that each polypeptide was identified reliably. This success partly owes to the increase of available *M. sexta* cDNA sequences: 43 identified based on those deposited at GenBank over the years, 235 based on the fat body, hemocytes, and midgut transcriptome analyses (Zou et al., 2008; Pauchet et al., 2010; Zhang et al., 2011), and 118 based on genome-wide RNA-Seq data. Method and instrument improvements also contributed to the major increase in polypeptide identifications. The depth of penetration

into the plasma peptidome was also supported by the pre-fractionation employed: ACN precipitation substantially reduced abundant proteins (*e.g.* storage proteins, lipophorins) and removing these proteins enhanced the subsequent detection of peptides (Kay et al., 2008).

Spectral counting is widely adopted as a robust, label-free method for quantitative analysis of proteomes (Neilson et al., 2011). We used spectral counts (SCs), numbers of observed MS/MS spectra matching the theoretical spectra of peptides from polypeptides *in silico* digested with trypsin, to estimate their relative abundances. The SCs for individual polypeptides were normalized with the total and average counts of the corresponding sample (Section 2.5). The pairwise Pearson correlation coefficients demonstrated excellent data consistency within the control (C, 0.94–0.96) or induced (I, 0.94–0.96) group (Table 1). In comparison, the correlation coefficients between the C and I groups were only 0.55–0.65, indicating drastic differences in polypeptide levels.

3.2. Overview of the polypeptides identified in the ACN-treated plasma samples

As anticipated from the ACN pre-fractionation, 138 of the 268 polypeptides identified fall into the size range of peptides (Table 2). In contrast, 100 proteins are in the range of 25–65 kDa whereas 30 are 65–400 kDa. These polypeptides represent *ca.* 2/5 of IDs in the entire proteome of larval plasma (He et al., unpublished data). The SCs are low for some of the major proteins (*e.g.* apolipophorins I, II, and arylphorins), suggesting these large proteins are too abundant to be completely removed by ACN precipitation. On the other hand, the SCs for multicystatins (221 and 304 kDa), Leu-rich repeat protein (172 kDa), spondin (85 kDa), transferrin (75 kDa), and carboxylesterase CXE30 (75 kDa) were high (100–400/sample), indicating that these proteins were either fairly stable or cleaved to form smaller fragments soluble in 50% ACN – polypeptide processing was not considered in this study (Section 2.5). SDS polyacrylamide gel electrophoretic analysis of the 50% ACN supernatant (Fig. 1) showed no bands corresponding to proteins over 90 kDa after the extraction. A cutoff of 75 kDa was observed after human plasma had been treated with 60% ACN (Kay et al., 2008). Apparently, sizes, abundances and other features of polypeptides all affect their precipitation and subsequent MS detection. The detection of large proteins and drastic difference between 268 IDs (Table S1) and *ca.* 25 bands (Fig. 1, lane 3) emphasized the importance of our nanoLC-MS/MS analysis.

A majority (243) of the 268 observed polypeptides contain a signal peptide, consistent with their presence in plasma. However, twenty-five of them did not contain a leader peptide in their hypothetical sequences (Table S1). The lack of secretion peptide did not reflect incomplete open reading frames since all the transcripts and their genes had been extensively scrutinized. These “leaderless” polypeptides showed low spectral counts relative to the ones containing leaders (Table S1), suggesting that our hemolymph peptidome contained certain levels of polypeptides from cells via a non-classical secretion pathway or due to cell lysis. For instance, prophenoloxidase-1 is probably released from ruptured oenocytoids (Kanost and Gorman, 2008). The detected intracellular polypeptides include metabolic enzymes (*e.g.* triosephosphate isomerase, F16BP aldolase, enolase), redox proteins (*e.g.* thioredoxin, glutaredoxin, Cu-Zn superoxide dismutase), and others (*e.g.*

calmodulin, eIF-5A, transgelin, histone H1-like protein). The relative lack of contaminating cytoplasmic polypeptides validated the majority peptidome observed.

3.3. Up- and down-regulated polypeptides in response to the immune challenge

After the t-test and ratio calculation, we found 33 peptides and 17 proteins were significantly up-regulated ($>167\%$ of control, $p < 0.05$) (Table 3). As anticipated, 31 of these peptides are AMPs, proteinase inhibitors, or other defense proteins. In this paper, we only focus on the peptides (Table 3), since the same proteins (*e.g.* 46 kDa hemolin) were also found in a parallel proteomics study (He et al., unpublished data) and, without ACN precipitation, quantification of protein levels is likely more reliable in that analysis. We also identified 30 peptides and 41 proteins, which became less than 60% of the control ($p < 0.05$) after the immune challenge (Table 3). Among the down-regulated peptides are proteinase inhibitors, plasmatocyte spreading peptide precursor, antifungal peptide-5, Hdd1-like, superoxide dismutase, thioredoxin, lipid binding molecules (*e.g.* apolipoproteins III and D, chemosensory peptides, antennal binding protein-7, MD2-related lipid binding protein), and several conserved hypothetical peptides. In addition, we found a majority of the 268 polypeptides identified did not display significant changes in their abundances: 75 peptides and 72 proteins were detected at 60–167% of their control levels or beyond the cutoff values ($< 60\%$ or $>167\%$) but with no statistical significance ($p > 0.05$).

Studies of the fat body, hemocyte, and midgut transcriptomes uncovered ten attacin contigs (Gunaratna and Jiang, 2013; Pauchet et al., 2010). We now know some of the contigs are hybrids of eleven highly similar genes in the genome (He et al., unpublished data). Based on their coding sequences, we identified ten attacins in the plasma peptidome, whose levels increased 66 to 163 fold after the immune challenge (Table 3). There is a family of 14 genes in the genome (He et al., unpublished data), which encode polypeptides homologous to a beetle antifungal peptide (AFP) named diapausin (Kouno et al., 2007). Our peptidomic analysis identified eight potential AFPs: 1, 4, 10 through 13 increased 1.8 to 12.9 fold after inoculation; 2 did not change; 5 decreased significantly. Similarly, cecropin-1, 5, 6 and 7 increased 2.3 to 27.1 fold whereas cecropin-8 did not change much. Identification of these closely related peptides demonstrated the capabilities of nanoLC-MS/MS on the Orbitrap mass spectrometer. Other up-regulated AMPs include lebecins B, C, D (9.7–37.2 fold), gallerimycin-1 (21.8 fold), gloverin (3.7 fold), and lysozyme-like protein-2 (1.9 fold). Like cecropin-8, AFP2 and AFP5, some AMPs did not significantly increase ($p > 0.05$) at 24 h after the challenge, such as defensin-like peptides, whey acidic protein homologs (WAP-1, 2 and 9), and lysozyme. We have examined other immune peptides (Table 3). Peptidoglycan recognition protein-1, 2, 5 (3.8 fold) and Reeler-1 (21.4 fold) may recognize pathogens; plasmatocyte spreading peptide and uENF2 may act as cytokines to induce cellular responses; proteinase inhibitor (PI-like, 30.6 fold), CP8, cysteine proteinase inhibitor, and trypsin inhibitor A may inhibit microbial or host proteinases. Additionally, we found some up-regulated peptides that are not known to be immunity-related. They are hypothetical peptides 06199 (17.0 fold), 55808 (8.6 fold), KGM20797 (1.7 fold), and ID-155 (13.8 fold), and a homolog of heat shock protein 25.4 (11.9 fold).

Of all the defense molecules identified in this study, nineteen AMPs were significantly induced (I/C: 1.8 to 163.4; average ratio: 61.3) after the immune challenge. Their high induction allowed us to detect half of the 77 AMPs that have been identified based on genomic data (He et al., unpublished results). On the other hand, attacins-6, cecropin-2, 3, 4, 9 through 15, and others may be present at concentrations below the detection limit. In the case of moricins, their Lys-richness might cause them to become short trypsinolytic peptides not suitable for LC-MS/MS detection.

3.4. Correlations of peptide and mRNA levels and level changes

In this study, we used the published fat body and hemocyte transcriptome data to construct the polypeptide database for mass data analyses and were thus able to compare transcript levels to peptide levels in induced and control samples (Table 3). We were able to match mass spectra of the peptide samples to 138 peptide sequences deduced from their cDNAs. Using these and corresponding spectra counts, we attempted to first correlate the mRNA and peptide levels after the immune challenge. As shown in Fig. 2, there was a positive correlation between control fat body and control plasma (CF-CP: 0.62) and between IF and IP (0.39). However, when we plotted the IH and CH read numbers with the peptide data, the coefficients (IH-IP: -0.13; CH-CP: 0.11) did not indicate either positive or negative correlation. Perhaps, due to its sheer volume and major contribution to plasma factors, the low to moderate positive correlation exists between fat body mRNA and polypeptide levels. In contrast, hemocytes do play crucial roles in cellular immune responses but, due to lower cell number, their contribution to the plasma peptide pool seems limited, as suggested by the near zero values.

We then tested if there is a stronger correlation between the mRNA and peptide *level changes* after the immune challenge. Of the 138 peptides, 107 and their respective contigs were identified for retrieving the read numbers from CF and IF. Similarly, 70 peptides had reads in CH and IH. The logarithm scatter plots of IH/CH-IP/CP and IF/CF-IP/CP (Fig. 3) demonstrated reasonable correlations (0.67 and 0.76), indicating that most peptides showed the same tendency of change with their mRNAs. Only a small number of peptides showed mRNA level increases but peptide level decreases or vice versa, and the deviations from origin (I/C = 0) were mostly small in these cases.

3.5. Concluding remarks

By taking the gel-free approach, we performed a mass spectrometric analysis of the polypeptides in ACN-treated plasma samples from *M. sexta* larvae injected with buffer and bacteria. The state-of-art of Orbitrap mass spectrometer and rich sequence information from this model species allowed the identification of 268 hemolymph polypeptides, over half of which fell into the realm of peptides ($M_r < 25$ kDa). The label-free, spectral counting method revealed 33 peptides with 1.67 to 200 fold abundance increases after the immune challenge. These included attacins, cecropins, defensins, diapausins, gallerimycin, gloverin, lebecins, and lysozymes. Some of the antimicrobial peptides (*e.g.* attacins) had highly similar amino acid sequences. The correlations between transcript and hemolymph peptide *levels* were better for fat body (0.62 and 0.39) than they were for hemocytes (0.11 and -0.13). Stronger correlations (0.76 and 0.67) were found for the *degree of changes* between

induced and control samples of the two tissues and plasma. Future functional analyses of plasma proteins after the immune challenge is necessary to provide a better overview of the proteomic changes and their correlations with the mRNA level variation after the immune challenge.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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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Highlights

1. Identification of 138 peptides ($M_r < 25$ kDa) and 130 proteins ($M_r > 25$ kDa) in hemolymph of *Manduca sexta* larvae;
2. Detection of 33 and 30 peptides up- and down-regulated to $>5/3$ and $<3/5$ of the control levels after immune challenge;
3. Discovery of antimicrobial peptides, proteinase inhibitors, and other defense molecules at the protein level;
4. Good correlation between plasma peptide and transcript *levels* in fat body; stronger correlation between their *level changes*.

Fig. 1. SDS polyacrylamide gel electrophoretic analysis of the control plasma (CP) samples separated on 10% (left panel) and 15% (right panel) gels

Total proteins (lane 1), 50% ACN precipitate (lane 2) and supernatant (lane 3), all equivalent to 1.5 μ l of plasma, were loaded along with the M_r markers (lane M), whose sizes are indicated in kDa on the left.

Fig. 2. Comparison of the mRNA and peptide abundances

Left panels: normalized read numbers (NRNs) of specific transcripts in the control (CF, *upper*) and induced (IF, *lower*) fat body (●) and their corresponding normalized spectral counts (NSCs) in control (CP, *upper*) and induced (IP, *lower*) plasma are plotted in log₂ scale, representing the relative mRNA and peptide levels respectively. *Right panels* are the same as *left panels*, except that the NRNs of mRNA in the control (CH, *upper*) and induced (IH, *lower*) hemocytes (◆) are shown. The dataset include all identified peptides with corresponding reads in the CF, IF, CH, or IH libraries (Zhang et al., 2011). Transcripts whose NRNs are 0 are adjusted to 1, so that log₂NRN is equal to 0. The correlation coefficients for all the comparisons are indicated in the panels.

Fig. 3. Comparison of plasma peptide and mRNA level changes in fat body (left panel) or hemocytes (right panel)

The peptide and mRNA level changes are represented by ratios of normalized spectral counts (NSC_{IP}/NSC_{CP}) and normalized read numbers (NRN_{IF}/NRN_{CF}) of fat body (●) or hemocyte (◆) transcripts in $\log_2$ scale, respectively. The dataset include all identified peptides with corresponding reads in the CF, IF, CH, or IH libraries (Zhang et al., 2011). Transcripts whose NRNs are 0 are adjusted to 1 so that meaningful ratios can be shown.

Table 1

Pearson pairwise correlation among biological replicates

	CP1	CP2	CP3	IP1	IP2	IP3
CP1	1.00	0.95	0.94	0.60	0.55	0.55
CP2		1.00	0.96	0.64	0.58	0.59
CP3			1.00	0.65	0.58	0.59
IP1				1.00	0.94	0.96
IP2					1.00	0.94
IP3						1.00

Table 2

Numbers of proteins/peptides identified in different size ranges

<i>M_r</i> (kDa)	5–10	10–15	15–20	20–25	25–35	35–45	45–55	55–65	65–100	100–400	Σ
peptide IDs	29	43	29	37			24	9	16	14	138
protein IDs					24	43					130

Table 3

Identification and semi-quantification of 138 peptides from *M. sexta* plasma

ID ^a	Name	CP1, CP2, CP3 ^b	IP1, IP2, IP3 ^b	p-value ^c	Protein (I/C) ^d	mRNA (IF/CF) ^e	mRNA (IH/CH) ^e
Immunity-related peptides: effectors and others							
168	attacin1	0, 0, 0	138, 0, 164	0.119	91.6	83.6	16.6
80	attacin2	0, 0, 0	116, 121, 117	0.000	99.1	43.3	223
73	attacin3	0, 0, 0	94, 103, 118	0.000	87.7	52.0	20.2
166	attacin4	0, 0, 0	179, 201, 205	0.000	163	78.1	8.70
208	attacin5	0, 0, 0	87, 121, 159	0.002	101	329	nd
164	attacin7	2, 3, 2	146, 201, 211	0.000	66.3	108	41.6
167	attacin8	0, 0, 0	158, 190, 193	0.000	151	108	41.6
291	attacin9	0, 0, 0	89, 130, 177	0.003	109	103	nd
253	attacin10	0, 0, 0	137, 165, 177	0.000	133	83.6	16.6
208a	attacin11	0, 0, 0	88, 126, 168	0.002	105	220	nd
121	AFP1	0, 0, 0	11, 23, 12	0.018	12.9	1.53	3.56
275	AFP2	7, 4, 9	7, 8, 9	0.960	1.01		
276	AFP4	9, 2, 8	11, 17, 17	0.074	1.98	12.5	3.60
207	AFP5	4, 4, 6	0, 0, 0	0.001	0.00		
277	AFP10	0, 0, 0	13, 15, 17	0.000	12.5		
278	AFP11	0, 0, 0	8, 12, 12	0.001	8.89	4.50	nd
279	AFP12	0, 0, 0	8, 18, 16	0.008	11.6	4.50	nd
117	AFP13	10, 8, 7	20, 17, 17	0.018	1.81	7.65	3.56
107	cecropin1	5, 4, 5	10, 13, 15	0.002	2.26	24.5	34.4
100	cecropin5	0, 0, 4	43, 42, 37	0.000	27.1	78.0	nd
115	cecropin6	2, 0, 0	12, 16, 18	0.001	18.6	17.3	11.9
269	cecropin7	0, 0, 0	6, 4, 9	0.007	5.25	18.0	nd
270	cecropin8	0, 0, 0	2, 0, 0	0.374	0.60	6.00	nd
286	defensin1	2, 0, 2	0, 1, 2	0.552	0.61	1.00	2.40
124	defensin-like	0, 0, 0	11, 4, 0	0.201	4.42		
91	gallerimycin1	0, 1, 1	17, 17, 17	0.000	21.8	127	8.31

ID ^a	Name	CPI, CP2, CP3 ^b	IPI, IP2, IP3 ^b	p-value ^c	Protein (I/C) ^d	mRNA (IF/CF) ^e	mRNA (IH/CH) ^e
230	gloverin	23, 14, 24	90, 84, 97	0.000	3.74	143	97.4
211	lebocin B	6, 0, 10	64, 55, 61	0.001	9.66	57.6	1.19
213	lebocin C	0, 1, 2	37, 41, 38	0.000	33.4	142	7.12
212	lebocin D	0, 0, 0	44, 43, 46	0.000	37.2	452	0.90
47	WAP1	10, 10, 6	12, 8, 8	0.715	0.91	20.4	7.63
116	WAP2	0, 0, 0	0, 6, 8	0.120	3.77	3.57	nd
113	WAP9	6, 7, 2	7, 6, 6	0.883	1.05	0.68	nd
11	lysozyme	9, 17, 11	4, 9, 15	0.240	0.62	1.51	1.24
134	lysozyme-like2	58, 56, 55	114, 128, 138	0.000	1.88	3.50	58.80
76	PI-like	0, 0, 0	32, 37, 41	0.000	30.6	70.9	27.3
110	CP8	46, 51, 44	44, 46, 41	0.056	0.78	0.80	nd
64	Cys PI	33, 20, 23	15, 14, 18	0.048	0.52	2.04	1.89
120	Trypsin inhibitor A	37, 35, 38	19, 29, 25	0.002	0.56	2.55	nd
247	PGRP1	60, 67, 55	71, 65, 74	0.720	0.97	5.00	26.1
272	PGRP2	0, 0, 0	0, 0, 1	0.374	0.26	151	4.70
182	PGRP5	4, 5, 5	18, 22, 24	0.000	3.82	4.50	10.1
79	Reeler1	0, 0, 0	16, 23, 39	0.010	21.4	97.9	3.02
205	PSP precursor	33, 36, 34	24, 26, 23	0.002	0.60		
282	uENF2	1, 2, 4	0, 0, 3	0.249	0.34	2.60	nd
267	HP55808	0, 0, 0	5, 15, 11	0.023	8.58	13.0	7.80
84	HP06199	0, 0, 0	27, 17, 16	0.009	17.0	40.3	nd
63	HSP25.4	0, 0, 0	2, 19, 23	0.079	11.9	56.1	nd
88	HSP25.4	0, 0, 0	2, 0, 5	0.167	1.89	13.8	nd
Peptides not known to associate with immunity: mostly insignificant up or down regulation							
155	no hit	2, 0, 0	7, 17, 10	0.027	13.8	16.5	nd
87	HP KGM16590 REPAT32	9, 10, 11	27, 27, 16	0.058	1.99	11.7	0.53
75	HP KGM20797	0, 0, 0	2, 3, 1	0.029	1.70	6.40	5.34
185	HP	0, 0, 0	0, 11, 0	0.374	3.11	7.65	nd
114	PI1	16, 23, 28	25, 33, 33	0.403	1.14	5.78	1.19
222	silk PI	9, 14, 10	19, 13, 8	0.915	1.04		

ID ^a	Name	CPI, CP2, CP3 ^b	IPI, IP2, IP3 ^b	p-value ^c	Protein (I/C) ^d	mRNA (IF/CF) ^e	mRNA (IH/CH) ^e
106	PI (TIB)-like	24, 31, 25	23, 24, 24	0.056	0.74	1.24	1.19
108	PI (TIB)	35, 18, 20	17, 20, 25	0.287	0.70	12.8	nd
74	Kazal PI	56, 53, 49	39, 46, 44	0.010	0.68	0.55	1.96
2	repat32	35, 20, 25	24, 23, 28	0.294	0.78	2.17	1.55
176	REPAT31	38, 32, 35	25, 30, 30	0.008	0.68	4.00	nd
66	Spz1-like1	11, 17, 15	6, 16, 12	0.171	0.66	1.53	2.18
154	dorsal-like	13, 16, 18	32, 45, 0	0.594	1.43	27.4	2.40
34	FK506 BP	0, 2, 0	1, 9, 2	0.299	4.93	1.22	1.35
82	not hit	0, 0, 2	0, 4, 5	0.262	3.84	4.42	nd
161	HP KGM07285	3, 9, 6	8, 9, 9	0.513	1.21		
72	HP KGM09811	13, 9, 10	11, 9, 16	0.730	0.93	3.06	1.58
184	HP	42, 41, 44	39, 43, 46	0.001	0.84	1.70	nd
261	HP KGM06199	22, 13, 15	18, 13, 17	0.382	0.80		
140	HP KGM21589	13, 13, 22	15, 14, 16	0.270	0.79	1.00	3.60
78	HP KGM06199	45, 27, 28	19, 36, 28	0.241	0.69	1.94	nd
112	no hit	0, 0, 0	0, 7, 3	0.186	2.75	5.10	nd
90	transgelin/calponin	2, 1, 1	0, 7, 2	0.559	1.86	0.55	1.38
158	Lopap biliverdin-BP	9, 5, 5	15, 6, 5	0.759	1.17	0.80	nd
60	Pro isomerase B	20, 42, 30	40, 34, 52	0.583	1.14	1.14	1.09
52	unknown pr.	10, 14, 13	14, 15, 16	0.836	1.02	20.1	0.00
95	cuticle pr. CPR3	7, 8, 9	12, 8, 7	0.858	0.96	1.15	nd
122	no hit	7, 2, 5	1, 12, 3	0.954	0.96	0.40	1.50
126	cold-related pr.	13, 14, 12	15, 14, 15	0.532	0.95		
216	no hit	6, 0, 7	7, 0, 7	0.905	0.91		
97	profilin	5, 3, 8	0, 13, 3	0.831	0.84	1.19	2.71
187	P-ethanolamine BP	4, 10, 11	11, 7, 5	0.565	0.79	0.50	nd
98	thioredoxin peroxidase	8, 15, 18	12, 18, 8	0.497	0.79	0.58	1.30
54	cyclophilin Pro isomerase	13, 15, 12	12, 14, 11	0.118	0.78	0.94	1.01
70	destabilase	24, 25, 21	16, 25, 24	0.115	0.77	2.41	1.19
85	NDPK, abnormal WD-like	27, 21, 30	23, 24, 21	0.061	0.74	0.76	1.36
192	diapause bioclock pr.	7, 5, 8	6, 4, 7	0.137	0.71	33.0	nd

ID ^a	Name	CPI, CP2, CP3 ^b	IPI, IP2, IP3 ^b	p-value ^c	Protein (I/C) ^d	mRNA (IF/CF) ^e	mRNA (IH/CH) ^e
198	no hit	30, 41, 42	31, 28, 33	0.029	0.69		
169	MD2-related lipid-BP	5, 8, 7	6, 3, 9	0.343	0.75	0.10	nd
175	MGD2b	7, 5, 8	2, 11, 2	0.417	0.64		
228	antennal BP7	14, 24, 19	20, 16, 23	0.484	0.87		
209	antennal BP6	27, 15, 27	17, 26, 28	0.507	0.86		
132	OBP/ABP1	50, 52, 43	43, 50, 46	0.091	0.80		
144	OdorantBP	74, 72, 71	44, 65, 63	0.010	0.66	0.29	nd
86	OBP	17, 44, 21	22, 19, 14	0.263	0.57	1.98	nd
254	chemosensory pr.	27, 41, 44	31, 34, 32	0.106	0.73		
149	chemosensory pr.4	6, 9, 7	6, 5, 7	0.084	0.68		
255	chemosensory pr.	76, 66, 64	52, 51, 56	0.010	0.65	0.60	nd
59	bombyrin, apolipD	87, 79, 73	61, 61, 72	0.013	0.68	0.88	nd
265	apolipoproteinD-like I	96, 86, 89	57, 61, 81	0.004	0.61	0.60	nd
44	lysosomal thioreductase	32, 28, 25	21, 17, 27	0.035	0.64	2.82	0.00
81	retinoic acid BP	3, 13, 19	6, 15, 3	0.435	0.59	0.58	1.81
233	insectanin B	198, 166, 176	160, 168, 155	0.034	0.75	1.45	0.00
256	insectanin A	115, 121, 138	96, 100, 87	0.003	0.64	1.40	0.00
159	insectanin	0, 6, 0	1, 0, 0	0.446	0.15	9.00	nd
10	eIF-5A	1, 4, 7	0, 0, 0	0.073	0.00	0.71	0.92
29	calmodulin	0, 10, 4	0, 0, 0	0.192	0.00	1.02	1.28
83	glutaredoxin	6, 0, 2	0, 0, 1	0.258	0.10	0.51	0.70
77	Spz1-like2	8, 6, 6	0, 9, 0	0.194	0.38	4.08	3.56
215	carboxypeptidase inhibitor	28, 31, 30	21, 25, 27	0.002	0.69	0.30	0.60
125	MBF2/REPAT31	11, 12, 11	12, 10, 10	0.088	0.79		
173	HP KGM07587	1, 0, 2	0, 0, 0	0.150	0.00		
96	HP KGM07842 CXE19	2, 7, 6	4, 1, 5	0.289	0.56	1.49	1.19

Down regulated peptides not known to associate with immunity

180	HP KGM17056	54, 55, 55	33, 44, 41	0.001	0.60	0.70	nd
109	REPAT32-like	61, 56, 54	47, 38, 37	0.012	0.60		
99	Kunitz PI	23, 27, 20	19, 15, 16	0.032	0.60	0.75	1.19

ID ^a	Name	CPI, CP2, CP3 ^b	IPI, IP2, IP3 ^b	p-value ^c	Protein (I/C) ^d	mRNA (IF/CF) ^e	mRNA (IH/CH) ^e
12	secreted pr.	28, 22, 24	21, 17, 14	0.032	0.60	0.74	1.13
92	MD2-related lipid-BP	55, 59, 58	29, 46, 40	0.003	0.56	1.76	0.79
179	apolipo III	242, 195, 221	141, 128, 164	0.004	0.55	0.60	0.30
65	Ecdys.-reg 16 k pr.	21, 16, 15	12, 12, 10	0.030	0.55	2.17	0.59
190	no hit	9, 9, 12	5, 9, 5	0.028	0.54	1.30	1.30
89	r-glutamyl cyclotransferase-like	36, 32, 28	20, 13, 28	0.026	0.53	1.12	nd
42	tenacin-like KGM17335	23, 23, 21	7, 15, 21	0.029	0.53	0.84	nd
62	HP KGM08446	14, 11, 12	10, 6, 7	0.022	0.53	1.45	0.81
105	HP KGM06638	115, 124, 152	90, 77, 71	0.004	0.52	0.66	0.00
150	chemosensory pr.1	51, 59, 67	42, 29, 37	0.005	0.52		
71	apolipoD-like1	32, 33, 35	17, 15, 28	0.003	0.50	0.71	nd
163	HP secreted	75, 55, 57	34, 30, 37	0.011	0.45	0.60	0.60
224	PI4	28, 24, 24	5, 19, 17	0.023	0.44		
102	Hdd1-like	19, 18, 25	4, 19, 8	0.045	0.42	nd	0.84
238	acyl-CoA:diazepam BP	13, 16, 14	6, 5, 7	0.001	0.35	0.80	1.60
217	no hit	15, 8, 13	3, 1, 7	0.023	0.25		
227	antennal BP7	4, 5, 6	1, 0, 2	0.003	0.16		
69	Cu-Zn SOD	4, 8, 12	4, 0, 0	0.050	0.15	0.82	1.37
103	thioredoxin	5, 5, 9	0, 3, 0	0.019	0.14	0.94	1.26
151b	chemosensory pr.3	6, 7, 11	0, 3, 0	0.010	0.11		
151a	chemosensory pr.2	6, 7, 11	0, 3, 0	0.010	0.11		
118	HP KGM01763	4, 8, 6	0, 0, 2	0.013	0.09	2.10	1.50
94	larval cuticle pr. 16/17	12, 13, 12	0, 0, 2	0.000	0.04	0.08	nd

^a Identification number of peptides ($M_r < 25$ kDa), with the intracellular ones shaded light cyan;

^b Raw counts of matching MS/MS spectra from the triplicate CP and IP samples.

^c Results of the Student's t-test conducted using normalized spectral counts of peptides from the induced and control samples.

^d I/C values calculated using average normalized spectral counts of the induced and control samples according to $(SC_{IP1}/7723 + SC_{IP2}/8167 + SC_{IP3}/8907)/(SC_{CP1}/6716 + SC_{CP2}/6779 + SC_{CP3}/7298)$. If $CP1 = CP2 = CP3 = 0$, the three raw counts are all adjusted to 1 to avoid getting a ratio of infinity. When sum of the raw counts for one peptide in either sample is zero, the I/C value is shaded green. For peptides with $p < 0.05$, I/C values greater than 1.67 or less than 0.60 are shown in red font and bold blue font, respectively.

^e Relative abundances (RAs) and adjusted read numbers (ARNs, shaded green) of mRNAs in fat body (IF/CF) and hemocytes (IH/CH) (Zhang et al., 2011). RA >5 or ARN >10: red font; RA <0.2 or ARN <0.1: bold blue font. nd: undetected. Detailed information of these peptides can be found in Table S1.