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Review

Proteomics and insect immunity

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Abstract

Insect innate immunity is both a model for vertebrate immunity as well as a key system that impacts medically important pathogens that are transmitted by insects. Recent developments in proteomics and protein identification techniques combined with the completion of genome sequences for *Anopheles gambiae* and *Drosophila melanogaster* provided the tools for examining insect immunity at a new level of molecular detail. Application of proteomics to insect immunity resulted in predictions of new roles in immunity for proteins already known in other contexts (e.g. ferritin, transferrin, Chi-lectins) and helped to target specific members of multi-gene families that respond to different pathogens (e.g. serine proteases, thioester proteins). In addition, proteomics studies verify that post-translational modifications play a key role in insect immunity since many of the identified proteins are modified in some way. These studies complement recent work on insect transcriptomes and provide new directions for further investigation of innate immunity.

Key words: phagocytosis; antimicrobial peptides; melanization; *Drosophila melanogaster*; *Anopheles gambiae*; 2D-PAGE; hemolymph

Introduction

Innate immunity refers to the first-line host defense against the early phases of microbial infection and is an evolutionarily ancient defense mechanism. Insects and vertebrates display considerable overlap in the intracellular signaling pathways that regulate innate immune responses (Salzet, 2001; Giot *et al*., 2003; Hultmark, 2003) and in some of the effector mechanisms used against microbes (e.g. phagocytosis, fluid lysozymes). Thus, discoveries made through research in the fruit fly, *Drosophila melanogaster* may be applicable to innate immunity in humans (Fallon *et al*., 2001).

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The study of innate immunity in insects has also garnered increasing attention because of the role of many insects in transmission of human disease agents. Understanding how the insect immune system interacts with pathogens may contribute to development of new strategies to block transmission of disease agents (Christophides, 2005). For example, cecropin is a protein originally identified for its antibacterial activity in lepidopteran insects but eventually shown to reduce malaria parasite development (Gwadz *et al*., 1989). As a result, transgenic mosquitoes were developed to overexpress cecropin in the midgut, resulting in significant decreases in the number of developing malaria parasites following infection (Kim *et al*., 2004).

The sequencing of the genomes coupled with EST projects for two dipteran species, *D. melanogaster* and the African malaria mosquito, *Anopheles gambiae,* provided new opportunities for studying immunity. New genes with candidate immune functions were quickly identified (Christophides *et al*., 2002) and microarrays were applied to survey transcriptome changes after bacterial, fungal or parasite infections (DeGregorio *et al*.,

2001; Irving *et al.,* 2001; Dimopoulos *et al*., 2002; Roxstrom-Lindquist *et al*., 2004). These studies have been fruitful in identifying a set of genes that can be tested for functional involvement in insect immune responses. However, mRNA-based approaches suffer some limitations. First, they can be misleading in estimating how much protein is present. Although changes in mRNA levels sometimes accurately serve as surrogates for changes in the respective protein levels, several studies have shown that this is not the case about 40-50 % of the time (Gygy *et al*., 1999; Ideker *et al*., 2001). A specific example of this type of problem can be seen in mosquito immunity where the antimicrobial peptide defensin displayed high levels of transcript that did not correlate with detectable peptide (Bartholomay *et al*., 2004). Second, mRNA analyses tell us nothing about whether the encoded proteins are active or not as the functions of proteins are often regulated by post-translational modification. For example, several proteolytic cascades are involved in insect responses to pathogens, with cleavage of a series of proteins necessary for activation of the end product. These functional modifications cannot be directly determined from DNA sequence information or mRNA levels. Third, mRNA cannot be used to profile changes in secreted proteins that occur in the hemolymph when the body cavity is invaded by pathogens or to identify components of extracellular reactions such as melanization or coagulation. Thus, a complementary approach for investigating immunity in greater detail is to focus on the proteins themselves. Proteomics is a tool for detecting changes in protein expression and modification in whole organisms and in specific cells, tissues, and fluids. This review will focus on the methods and applications of proteomics to insect immunity.

Methodologies

Definition of proteomics

The term “proteome" was coined in 1994 and defined as the entire protein complement expressed by a sample. Proteomics encompasses a broad set of disciplines aimed at understanding and monitoring proteins. This includes work correlating genetic sequence with three-dimensional protein structure and 3D structure with protein function, development of protein separation and protein profiling techniques, and investigation of protein-protein interactions.

Recent studies of insect immunity concentrate on “profiling/expression" and “functional" proteomics (Table 1). Profiling or expression proteomics focuses on the description of the whole proteome in a given tissue, body fluid, cell type, or organelle, and differential measurement of protein expression levels in samples collected under different conditions (Choudhary and Grant, 2004). Functional proteomics includes research approaches that directly analyze a subset of proteins, such as a family of sequence- or function-related proteins (Kocks *et al*., 2003), as well as those that characterize the protein’s biological functions, protein-protein or protein-DNA/RNA interactions, or post-translational modifications (Cai *et al*., 2004).

The tools of proteomics have been developing over the past three decades, but it was not until mass spectrometry began to be used for the identification of proteins in complex mixtures that the field really started to take off (Karas and Hillenkamp, 1988; Fenn *et al*., 1989). A summary of methodologies used for insect immunity studies will be presented next, with notes concerning limitations of the various procedures.

Protein separation

All proteomic technologies rely on the ability to separate a complex mixture so that individual proteins are more easily processed with other techniques. Two-dimensional gel electrophoresis (2-DE) is still the most widely used protein separation technology for insect immunity (O’Farrell 1975; Table 1; Fig. 1). In this approach, proteins are separated in the first dimension by isoelectric focusing using immobilized pH gradient strips. Then, these proteins are again separated, this time by molecular weight in standard polyacrylamide gel electrophoresis, resulting in a 2-dimensional display of proteins. The advantage of this method is that a large number (3,000 to 10,000) of proteins can be visually separated and those spots exhibiting changes between treatments can then be singled out for further exploration. Electrophoresis is followed by excision of specific spots, digestion, and protein identification (Fig. 1).

A number of problems have to be confronted when using 2-DE as a protein separation and expression profiling technique. For example, proteins present at low concentrations, those of very high or very low molecular weight, or membrane proteins are generally not detected on the gels. Detection of some of these proteins can be improved by pre-separation fractionation and processing of tissue and cell extracts or by altering the conditions of the 2-DE (Fig. 1B; Chevallet *et al*., 1998), but pre-separation requires additional manipulation to make quantification possible. Insect tissues such as the fat body that have high lipid contents may also require pre-separation fractionation (e.g. Stadler and Hales, 2002). Perhaps most frustrating, the reproducibility of 2-DE experiments can be poor, requiring many replicates to ensure confidence in the results. However, several tools have reduced some of the variability associated with 2-DE. Immobilized pH gradients are commercially available and replace the unstandardized tube gels used in older protocols. 2D-DIGE (see below) provides internal standards and reduces the non-biological variability associated with standard 2-DE. Companies have introduced software that greatly facilitates 2-D gel image analysis. Such programs generally automate the alignment of spots on one gel with corresponding spots on another, facilitating analysis even when gel distortions occur. An alternative to 2-DE that is likely to become more widely used in the future is MudPIT (Multidimensional Protein Identification Technology), which couples two-dimensional chromatography of peptides in mass

Table 1. Summary of proteomics studies in insect immunity

	Insect	Samples	Treatment(s)	Methods	Ref
Profiling proteomics	*Drosophila melanogaster*	3th instar larval hemolymph	LPS, *M.luteus*, *S. cerevisiae*	2D-DIGE	Vierstraete *et al*., 2005
		3th instar larval hemolymph	No	2DE, MALDI-TOF	Vierstraete *et al*., 2003
		3th instar larval hemolymph	*M. luteus*, *S. cerevisiae*, LPS.	2D-DIGE, MS	Vierstraete *et al*., 2004a, b
		3th instar larval hemolymph	*M. luteus*, *E. coli*, *B. bassiana*	2DE, MALDI-TOF	Levy *et al*., 2004a, b
		3th instar larval hemolymph	*D. pneumoniae*, *N. catarrhalis*, *S. aureus*, *K. pneumoniae*, *H. influenza*, *S. pyogenes*	2DE, MALDI-TOF	Guedes *et al*., 2005
		3th instar larval hemolymph	Hemolymph clot	2DE, MALDI-TOF	Karlsson *et al*., 2004
		mbn-2 cell line	LPS	2DE, MALDI-TOF	Loseva and Engstrom, 2004
	Anopheles gambiae	Adult 4a rr and L3-5 strains, hemolymph	Bacteria (*M. luteus* and *E. coli*) Sephadex beads	2DE, MS	Paskewitz and Shi, 2005 Chun *et al*., 2000
		Adult G3 strain female salivary gland	Blood meal	SDS-PAGE, LC-MS/MS	Kalume *et al*., 2005
		G3 strain male and female midgut	Sugar-fed or blood meal	2DE	Prevot *et al*., 2003
	Anopheles stephensi	Female midgut from mosquitoes of different susceptibility to *P. falciparum*	Sugar-fed or blood meal	2DE	Prevot *et al*., 1998
	Aedes aegypti	Larval tissues	*V. culicis*	2DE, MALDI-TOF	Biron *et al*, 2005
		Fat body	Eclosion or blood meal	2DE	Shih and Fallon, 2001
		RKF, LVP, rLVP strain female thoracic tissue	Sucrose meal, blood meal or *B. malayi*	SDS-PAGE, 2DE	Wattam and Christensen, 1992
	Bombyx mori	5th instar larvae hemolymph, midgut and fatbody	LPS	2DE, MALDI-TOF	Wang *et al*., 2004
Functional proteomics	*Drosophila melanogaster*	*Drosophila* S2 cell line	DCG-o4	SDS-PAGE MS	Kocks *et al*., 2003
	Anopheles gambiae	Adult female midgut, 4a-3A cell line	Purified *P. berghei* ookinetes bind annexins	MALDI-TOF, 2DE	Kotsyfakis *et al*., 2005

spectrometry-compatible solutions directly to tandem mass spectrometry (2D-LC-MS/MS), allowing for the identification of proteins from highly complex mixtures (Fig. 1C). A useful aspect of this technique is the ability to analyze proteins when the amount of starting material is too small for 2-DE. This technique has been used by Levy *et al*. (2004b) to investigate small peptide (1-11 kDa) profiles in hemolymph from immune challenged and naïve *Drosophila* adults and by Florens *et al.* (2002) to investigate mosquito-stage proteins of the malaria parasite, *Plasmodium falciparum*.

Protein modification

Almost all proteins are modified following translation. Specialized methods have been developed to study phosporylation (phosphoproteomics; Salih, 2005) and glycosylation (glycoproteomics; Hirabayashi *et al*., 2002) but these have not yet been applied to studies of insect immunity. However, changes due to glycosylation and protein spot was quantified by its staining intensity. In this approach, protein mixtures are often prepared from two different samples and resolved by separate 2D gels for subsequent comparison of protein expression or changes in protein modification. Gels can be compared by eye but are now usually digitized and analyzed using imaging software. For reliable densitometric analysis, image acquisition needs to be done by calibrated gel scanners with a wide dynamic range. Several software packages that can analyze this input exist and have greatly facilitated spot quantification and comparison.

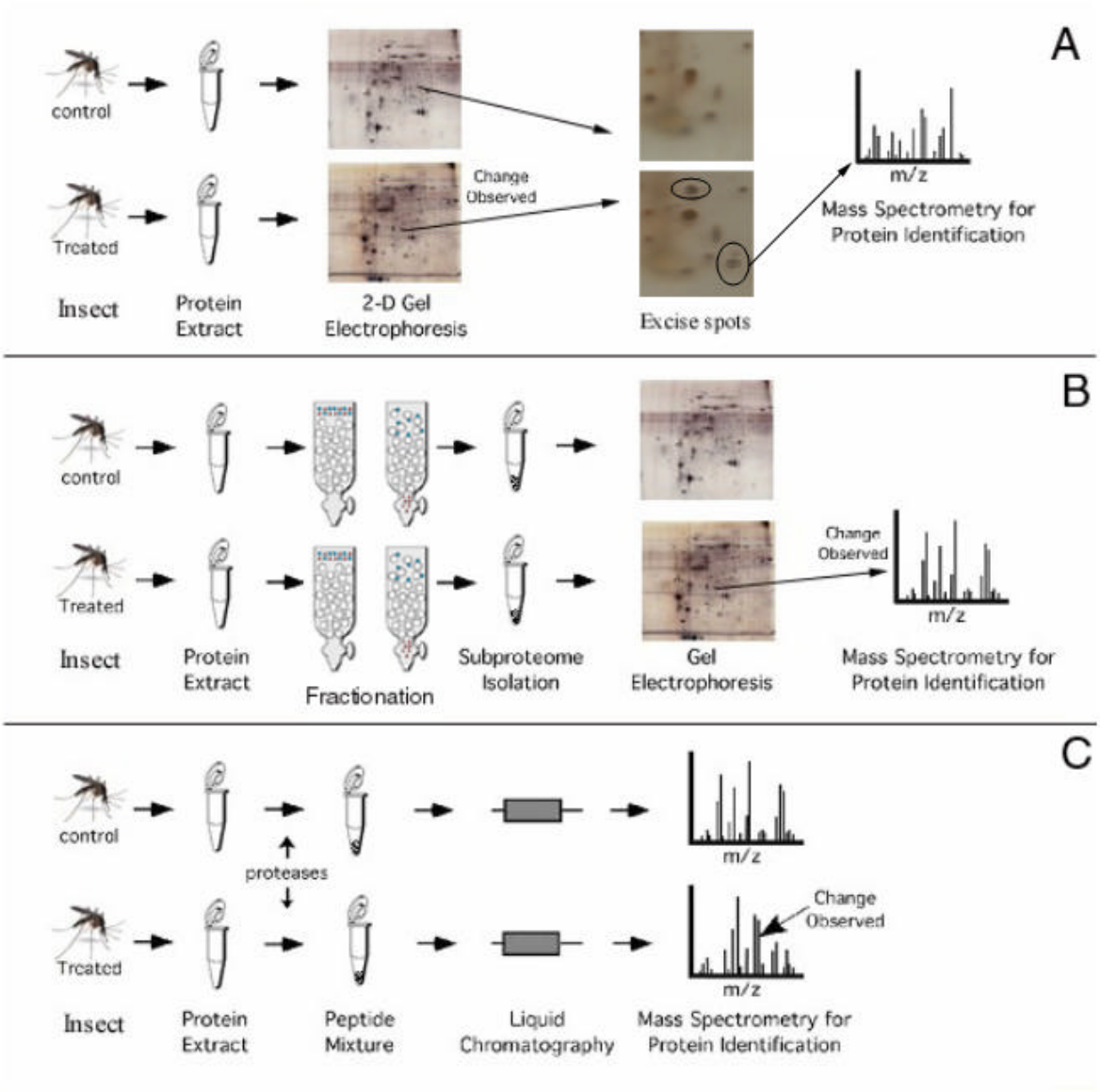

Fig. 1 Strategies for proteome analysis. (A) Analysis of whole proteomes by two-dimensional gel electrophoresis (2D-PAGE). In this approach, a protein extract is prepared from two different samples and the proteins are resolved by 2D gel electrophoresis. Proteins that differ by some variable then are selected for identification by mass spectrometry (MS). Although this method allows for the selection of relevant proteins, few low-copy proteins can be identified. (B) Analysis of whole proteomes by MS. In this approach, all cellular proteins are converted to peptides. The peptides are resolved by liquid chromatography and analyzed by MS. This method allows for the identification of low-abundance proteins but, since there is no selection for relevant proteins, all proteins must be analyzed by MS. (C) Analysis of sub-proteomes. In this approach, a protein extract is separated into individual sub-proteomes by fractionation or specialized affinity chromatography and proteins are resolved by 1D or 2D-PAGE. This allows for the enrichment of low-copy proteins and their selection for further analysis (Graves and Haystead, 2003).

Protein quantification

Traditionally, visualization of spots in a 2D gel was by Coomassie or silver staining.

Proteomics analysis utilizing 2-DE protein separation is frequently criticized as being low-throughput, in part due to the time-consuming process of image analysis that is necessary to determine differential protein expression. This process can be laborious due to gel-to-gel variations that confound the analysis process. Through the use of fluorescent dyes to label protein samples prior to 2-DE, the DIGE (difference gel electrophoresis) technique allows multiple samples to be co-separated and visualized on one 2D gel (Tonge *et al*., 2001; Fig. 2). The protein extracts, for example one control and one treated, are labeled with different fluorescent dyes (e.g. Cy2, Cy3), then combined and separated by 2-DE. In this example, two images of the gel would be captured – using the Cy2 and Cy3 excitation wavelengths. The images are then merged, and differences between them can be determined using image analysis software. The method minimizes the gel-to-gel variation implicit in 2-DE but cannot eliminate variation due to differences in the extraction or labeling steps. The dyes are reported to produce a linear response to protein concentration over five orders of magnitude, have enhanced sensitivity in comparison with other commonly used protein stains, and are compatible with MS analysis. The 2D-DIGE method was used successfully by Veirstraete and colleagues (2004a,b; 2005) to profile changes in the larval hemolymph of *D. melanogaster* following immune challenge.

Protein identification

Protein identification is now an essential part of almost every proteomics experiment. Some of the studies of insect immunity incorporated N-terminal sequencing or sequencing of proteolytic fragments from spots by Edman degradation to generate sequences for database searches (Chun *et al*., 2000). Currently, however, the most commonly used identification approach is MS. MS can rapidly, and with high sensitivity, determine masses and structures of peptides. Software is available to use the two different types of data generated by mass spectrometers to search sequence databases. Protein identification using peptide mass fingerprinting is an effective technique when studying organisms with completed genomes. Using the programs Mascot (www.matrixsciences.com), ProFound (http://prowl. rockefeller.edu/) and PeptIdent (www.expasy.org/tools/ peptident.html), one can analyze the peptide mass profiles produced by MS. FindMod (www.expasy.org/ tools/findmod/) is used to find modifications for analysis of unmatched peptide masses.

A second method for protein identification is based on the use of sequence data created by tandem mass spectrometers. This information can be used to search databases of translated protein sequences as well as nucleotide databases such as expressed sequence tag (EST) sequences. The ability to search nucleotide databases is an advantage when analyzing data obtained from organisms whose genomes are not yet completed, but for which a large amount of expressed gene sequence is available.

Data analysis

To make the most of the wealth of proteomics data being produced around the world, it is important to establish standards for storing and reporting proteomic data enable comparisons across platforms and research groups. The Universal Protein Resource, or UniProt, (http://www.pir.uniprot.org/) was recently established by NIH as a centralized database of protein information such as function, classification and cross-reference. UniProt combines the resources from the major annotated protein databases SwissProt and TrEMBL from the European bioinformatics Institute (EBI) and the Swiss Institite for Bioinformatics (SIB) as well as the Protein Sequence Database (PSD) from the Protein Information Resource (PIR) (http://pir.georgetown.edu/) (Apweiler *et al*., 2004).

For *Drosophila melanogaster* or *Anopheles gambiae*, gene identity and predicted protein function are found at the FlyBase Report (http://flybase.bio.indiana.edu/) or AnoBase (www.anobase.org/AnoBase/Genes/Ano-Xcel) (Loseva and Engstrom, 2004; Ribeiro *et al*., 2004).

Application of proteomics to the study of insect immunity

Mechanisms of insect immunity

In this section, we will briefly introduce proteins that have known or likely functions in insect immunity to place them in context when discussing the results of proteomics studies. Insect innate immunity is based on the recognition of microbial molecules, such as LPS, peptidoglycans, or β-1,3-glucans, by specific receptors with the subsequent activation of immune effector responses. Non-microbial surfaces (e.g. Sephadex beads) also elicit responses. Proteins that are considered to have a recognition or opsonizing function include GNBP, TEP and PGRP. Recognition leads to activation of cellular and/or humoral effector mechanisms. These include phagocytosis by hemocytes, encapsulation or nodulation of pathogens by hemocytes, activation of proteolytic cascades leading to localized melanization and hemolymph clotting, and synthesis of a battery of AMPs. The latter process can occur in most insect tissues including the fat body, hemocytes, respiratory system, cuticular and midgut epithelia, Malpighian tubules, and male and female genital tracts (Tzou *et al.,* 2000).

The activation of AMP synthesis is the best described of these effector processes. In *Drosophila*, there are several groups of AMPs that act mainly against either Gram positive bacteria, Gram negative bacteria or fungi. Two distinct signaling pathways, Toll and imd (immune deficiency), control their expression. The Toll pathway is activated by an extracellular proteolytic cascade, where serine proteases are important. Serine proteases can be regulated by inhibitors, including serpins and Kunitz types.

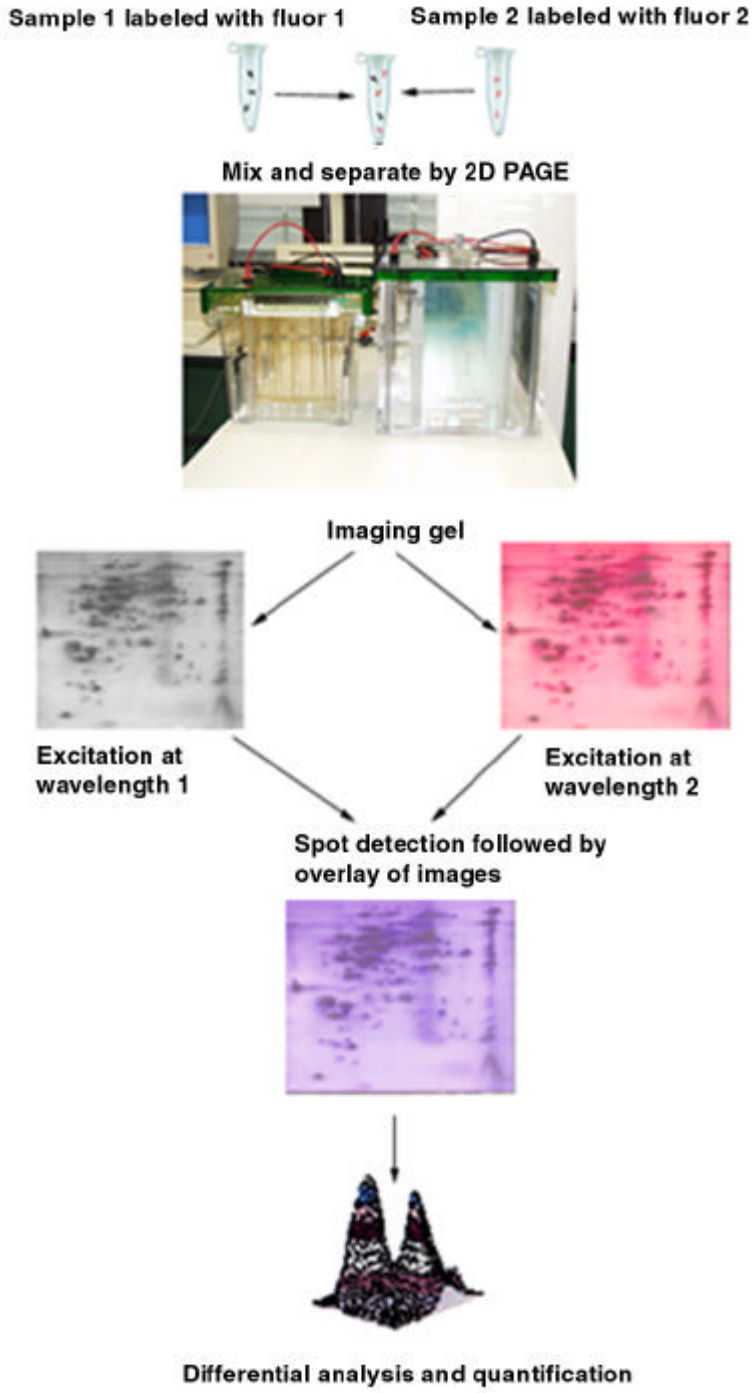

Fig. 2 Schematic representation of the 2D-DIGE (differential in gel electrophoresis) method. Two samples for comparison are individually labeled with distinct fluorochromes. The two samples are mixed and separated on the same 2D gel to reduced gel-to-gel variation. The resulting gel is imaged twice using the two different wavelengths for the two fluors. Image analysis software is used to detect spots in each image, overlay gels, and quantify differences.

2002) and, with a few exceptions, specific targets have not been identified for them. Some members of a subgroup called the clip-domain serine proteases, as well as their inhibitors, are important in activating the Toll pathway and in localized melanization responses. Mosquitoes are known to melanize malaria parasites, nematode worms, yeast, microsporidial spores, bacteria and Sephadex beads. The melanin pathway depends on the activity of phenoloxidase, which exists in a zymogen form prior to cleavage by an activating serine protease. Phenoloxidases and serine proteases are also involved in hemolymph clotting (Karlsson *et al*., 2004). Interestingly, a serpin mutant suggests that Toll activation is linked to the melanization pathway in *Drosophila* (Green *et al*., 2000; Ligoxygakis *et al*., 2002).

Expression profiling proteomics

Protein expression profiling of insect immune responses has been initiated for several insect species and tissues (Table 1) and many differentially expressed proteins have been identified by MS. Most of these studies have been carried out in *D. melanogaster* (Uttenweiler-Joseph *et al*. 1998; Guedes *et al.,* 2003; Sabatier *et al*. 2003; Vierstraete *et al*. 2003, 2004a,b, 2005; Engstrom *et al*., 2004; Levy *et al.,* 2004a,b; Guedes *et al*., 2005). Other taxa where immunity has been investigated by proteomic methods include the mosquitoes, *A. gambiae* (Chun *et al*. 2000; Paskewitz and Shi 2005) and *Aedes aegypti* (Wattam and Christensen, 1992; Biron *et al*., 2005), the silkworm, *Bombyx mori* (Wang *et al*., 2004), and the locust, *Odaleus australis* (Stadler and Hales 2002). Overall, we are lacking in genomic and proteomic information for hemimetabolous insects as well as broad coverage of the holometabolous orders.

There are significant and extensive methodological differences between the above mentioned proteomics studies, including types of challenge agents (single species or mixes of living bacteria, LPS, bacterial lysates, yeast, filamentous fungi, microsporidia, picorna-like virus, Sephadex beads) as well as the method of introduction of the agent (feeding, external exposure, injection), the length of incubation time after exposure, the tissue examined (hemolymph, hemocyte-like cells in culture, fat body, midgut, thorax, whole insect larvae), the method of tissue collection, and the stage of the insect (larvae vs adult). Standardizing these aspects would provide better ability to compare innate immunity across taxonomic groups. Nevertheless, taken together these studies identify some patterns in proteins that are affected by immune challenges and provide a framework for future investigations.

Hemolymph profiles

Hemolymph is a critical immune fluid in insects. It contains hemocytes and is a transport tissue for effector molecules like the fat body-produced AMPs. Most proteomic data are from this fluid. Several studies provide reference maps of identified hemolymph proteins that are not immune responsive but may be useful for other studies (Guedes *et al.,* 2003; Vierstraete *et al*., 2003; Karlsson *et al*., 2004; Paskewitz and Shi, 2005). The hemolymph reference maps contain constitutively expressed proteins from several groups: storage and transport proteins, metabolic proteins, cytoskeletal proteins, defense/immune proteins, antioxidant and stress proteins, and novel proteins. There are relatively few proteins in common between some of the *Drosophila* studies, reflecting the methodological issues described above.

Many of the identified proteins in the reference maps of *Drosophila* hemolymph are intracellular metabolic proteins while more than half of those identified in *A. gambiae* are related to immunity and appear to be secreted proteins. Cellular proteins occur in these samples because hemocytes and other contaminants are generally not removed (but see Karlsson *et al*., 2004 for serum versus plasma maps). In *A. gambiae*, fat body can be a major hemolymph contaminant, so care must be taken in attributing changes in cellular proteins to the hemocytes. In addition, some of the hemocyte types are known to be quite labile (e.g. crystal cells in *Drosophila*, oenocytoids in *Anopheles*) meaning that they break down and quickly release their contents when their environment changes. We attributed finding phenoloxidases in hemolymph and plasma to this feature, since oenocytoids produce POs and they do not contain signal peptides (Paskewitz and Shi 2005). Other cellular proteins that are abundant in labile hemocytes might also exhibit this pattern. Cellular protein profiles may also be affected by changes in hemocyte behavior after infection. Microbial challenge can result in mobilization of sessile hemocytes, so the relative cellular content of immune-challenged versus unchallenged hemolymph may not be equal.

Some considerations as to controls

Controls for immune-induced hemolymph samples must be carefully chosen since the manner of introduction of the immune challenge is often traumatic. Pricking an insect with a needle dipped in a pellet of bacteria is the most common challenge, although some studies use feeding or external exposure to simulate more natural conditions. We found that several of the proteins we had labeled as “wound-induced” proteins in *A. gambiae* were probably a specific result of damage to the thoracic musculature during aseptic wounding, rather than a generalized response to damaging the cuticle that would include hemolymph clotting and wound healing. Comparison with mosquitoes wounded in the abdomen did not produce the same group of proteins and the thoracic wound samples contained some proteins of obvious muscle origin (Paskewitz and Shi, 2005). Damage to tissue in vertebrates can also lead to the release of cellular proteins into circulation (Alleyne *et al.,* 2001; Renz *et al*., 2001)

If defined numbers of microbes or aliquots of surface molecules are to be injected, it is critical to use a sterile solution of the suspension buffer for the controls. Vierstraete *et al*. (2004a) report that they injected LPS in a solution that contained 1 % ethanol but did not use ethanol in the controls. This may explain the fact that the strongest induction they observed was for alcohol dehydrogenase in the LPS-injected samples.

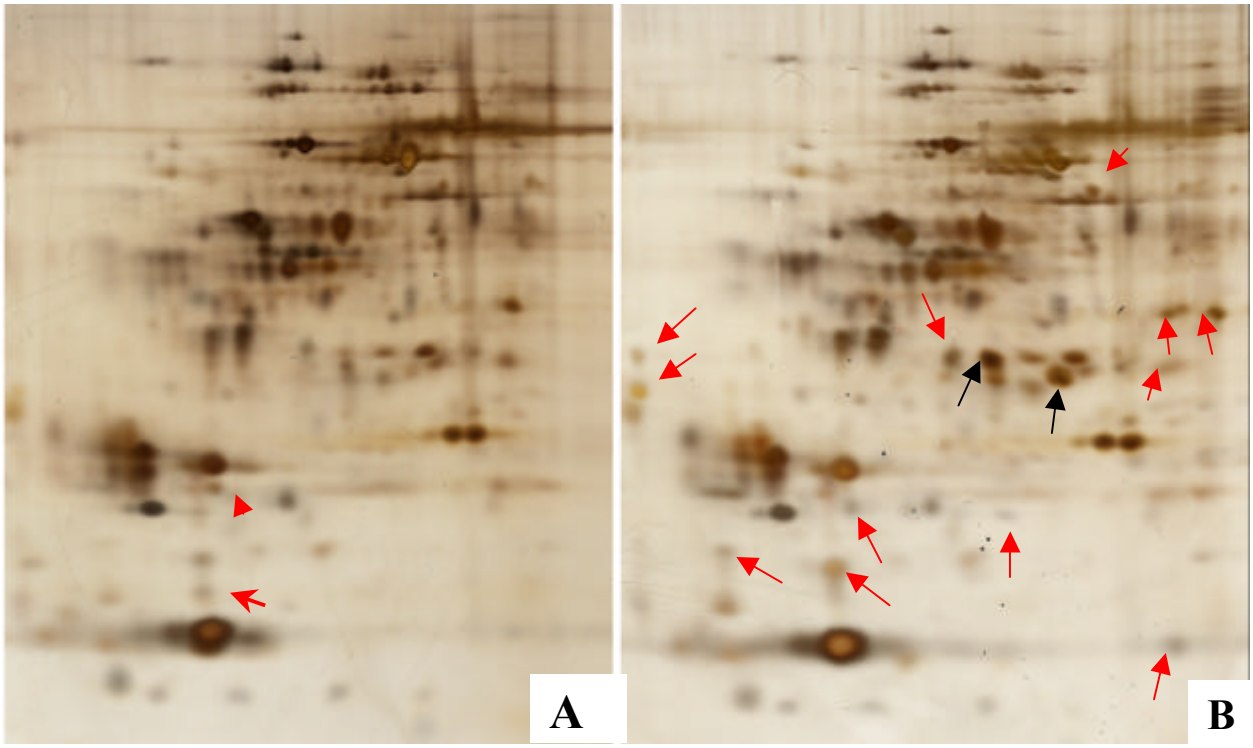

Fig. 3 Two dimensional gel profiles of *Anopheles gambiae* hemolymph incubated *in vitro* in the absence (A) or presence (B) of heat-killed bacteria (*M. luteus and E. coli*). Black arrows indicate the chi-lectins BR1 and BR2 in the bacterially-induced sample. Red arrows indicate some of the other proteins altered after exposure to bacteria in this sample.

Guedes *et al*. (2005) tried to overcome the problems of injection by feeding bacterial lysates to larvae. They combined lysates from six different bacterial species and incorporated them into the feeding medium. This procedure might result in relatively low activation of the immune system, since only the digestive and exoskeletal systems would be directly exposed to bacterial products and bacteria are a normal part of the larval feeding environment. The controls for this experiment were not exposed to a change in their normal feeding medium. It is possible that a change in food quality could induce stress or cause metabolic shifts in an insect as it does in vertebrate animals. Indeed, most of the induced proteins identified were metabolic or stress-related. AMP production could be a useful marker for verifying immune induction under these conditions.

Peptidomic studies of hemolymph

Most proteomic studies are not designed to capture low molecular weight proteins. Unfortunately, many of the AMPs fall into this category. Uttenweiler-Joseph *et al*. (1998) and Levy *et al*. (2004a) report results of using HPLC and MALDI-TOF for analysis of bacterially-induced peptides (1-15 kDa) in adult *Drosophila* hemolymph. Of the 28 induced peptides that were characterized, ten were AMPs exhibiting post-translational modifications and one was a Kunitz type serine protease inhibitor; many of the others are hypothesized to have a chemokine function (Levy *et al.,* 2004a). Additional Kunitz type inhibitors were described in the reference maps of larval hemolymph (Vierstraete *et al*., 2003). The targets of these inhibitors are not known. Similar methodology has been applied to peptides induced in the hemolymph of *A. aegypti* after bacterial challenge (Lowenberger, 2001). Several AMPs (defensins and cecropin) were identified as well as novel peptides. The identification of a large number of peptides with unknown functions in *Drosophila* and *Aedes* indicates that much remains to be done to fully characterize even this narrow aspect of immunity.

Proteomic studies of hemolymph

Approximately 130 larger proteins that are described as immune-induced have been documented in larval or adult hemolymph from *D. melanogaster* by 2-DE and protein identification methods (Levy *et al*. 2004a, b; Vierstraete *et al*. 2004a,b; Guedes *et al*. 2005). In general, the studies identify the following groups of proteins as regulated by the immune treatments: i)

immune-responsive proteins that have functional roles that are somewhat or well defined; ii) immune-responsive proteins that have hypothesized functions that can be tested; iii) cellular proteins that are involved in stress responses; iv) proteins that appear following injury; v) metabolic proteins; and vi) proteins that are not similar to any other proteins in databases and as yet have no hypothesized functions. Catalogs of these proteins can be found in the studies listed above and not all of these groups will be discussed in detail in this review. Here we will first consider some of the patterns suggested by these studies. An example of a 2D-PAGE separation of hemolymph proteins is provided in Fig. 3.

In studies of adult hemolymph following bacterial challenge, a similar number of proteins were affected in *Drosophila* and *Anopheles*. For example, 50 of 350 (14 %) silver-stained spots were up- or down-regulated in *Drosophila* (Levy *et al*., 2004a, b) while 14 of 280 (5 %) silver-stained spots were upregulated in *A. gambiae* (Paskewitz and Shi, 2005). By comparison, a much larger number of spots were said to be specifically regulated in adult fruit flies by 72 h after fungal exposure (Levy *et al*., 2004a, b). Comparison of a subset of 42 proteins regulated following fungal exposure showed that twelve of the proteins were also affected by bacterial infection. Three of the twelve were upregulated following both types of challenge (α-amylase distal; HSP20-like chaperone; Fructose 1,6, bisphosphate aldolase) while nine were regulated oppositely (ProPO-AE CG16705; DNase II CG7780; Aldehyde dehydrogenase; Glyceraldehyde 3 phsophate dehydrogenase; enolase; cathepsin L; transferrin; ferritin; Obp99c). In larval *Drosophila* hemolymph, proteins that were immune-regulated were compared at 25 min after injection of *Micrococcus luteus* or *Saccharomyces cerevisiae*. Thirteen were upregulated with either challenge agent. Three additional proteins were regulated only following *Saccharomyces* and two were regulated only following *Micrococcus* inoculation. Some proteins were present as multiple spots, and these were often differentially regulated between challenges. For example, several different ferritin heavy chain spots were identified, each upregulated after a different type of challenge agent (Vierstraete *et al*., 2004b).

In *Drosophila* larval hemolymph, 131 of approximately 289 (45 %) silver-stained spots were altered following exposure to a diet that included bacterial lysates (Guedes *et al.,* 2005). The majority of the 71 proteins that were identified were cellular proteins involved in metabolism and stress responses.

Since only 20 % of the immune-regulated proteins in *A. gambiae* and 14 % of those in *Drosophila* adult hemolymph were identified, we don't yet have a complete picture of the changes that are occurring at the protein level at any time after infection of adults. However, one study did identify 94 % of the proteins found to be upregulated in larval fruit fly hemolymph by 25 min after immune challenge (Vierstraete *et al*., 2004b). This study provides a good baseline for examining the overall profile of the types of immune proteins that are quickly secreted or processed following infection, and that would not be detectable by transcript analysis.

TEPs are complement-like proteins characterized by a conserved thioester motif that enables covalent binding to target surfaces. These proteins appear to be fundamental to a number of immune processes in insects. Reverse genetics clearly demonstrate that *A. gambiae* TEP1 regulates phagocytosis of bacteria and killing of malaria parasites in this mosquito (Levashina *et al*., 2001; Blandin *et al*., 2004). Among the proteins that increase in *Drosophila* hemolymph upon infection are other members of the family, including TEP2 following *M. luteus* or LPS injection and TEP4 after *Beauvaria bassiana* exposure (Levy *et al*., 2004b; Vierstraete *et al*., 2004a,b). TEP2 was especially sensitive to *M. luteus* injection; this was the strongest upregulation seen in this study of larval proteins. The rapid appearance of TEP2 (within 25 min) means that other recognition processes occur to trigger its release or cleavage in the hemolymph. All of the TEP proteins identified in the proteomics studies were smaller than predicted based on gene sequences and probably represent cleaved forms.

The only other recognition/opsonizing protein identified by proteomics is a protein related to gram negative binding proteins. This was the most strongly upregulated protein seen in samples taken following fungal infection of *Drosophila* adults (GNBP3; Levy *et al*., 2004b).

Serine proteases and serine protease inhibitors play important roles in modulating and amplifying the Toll signaling and melanization activation pathways. Four different clip-domain serine proteases were identified following fungal/yeast infection in larval or adult *Drosophila*. One, CG5390, was found within 25 min of challenge with *Saccharomyces cerevisiae* but not after *M. luteus* or LPS inoculation (Vierstraete *et al*. 2004b). Three others (CG1102, CG16705 and CG9372) were induced 72 h after *B. bassiana* exposure (Levy *et al*., 2004a, b). Two clip-domain serine proteases were identified in the *A. gambiae* proteomic studies but neither was reliably immune-induced (Chun *et al*., 2000; Paskewitz and Shi, 2005). Finally, three other serine proteases (not clip domain SPs) were upregulated in hemolymph when high doses of bacterial lysates were fed to *Drosophila* larvae (Guedes *et al*., 2005). The significance of this observation is not clear but some serine proteases have direct antibacterial activity (Tsuji *et al*., 1998).

Inhibitors of serine proteases called serpins are also important in immunomodulation. Two serpins (SRPN2 and SRPN15) were identified in *A. gambiae*, both as constitutively expressed proteins (Paskewitz and Shi, 2005). Two *Drosophila* serpins (CG1857, CG6687) were significantly upregulated in adult flies on fungal but not bacterial infection. One of them (Nec, CG1857) had previously been shown to regulate the induction of the Toll pathway through inhibition of the activation of Spaetzle, the Toll ligand (Levy *et al*., 2004a, b). A serpin was also found in hemolymph of injected silkworm larvae at 24 h post inoculation (Wang *et al*., 2004). Serpins were not found in the studies of the fruitfly larval hemolymph taken at 25 min after challenge (Vierstraete

et al., 2004a,b).

In addition to recognition factors and immuno-modulators, some of the known effector proteins have also been identified. As noted above, many of the AMPs are small molecules and need to be examined by special methods. These studies have shown that post-translational modifications are frequent and biochemical analysis indicates that the modifications affect the antimicrobial activity of the peptides. Phenoloxidases are another effector category. In *A. gambiae* there are nine PO genes, while *Drosophila* has only three. The contribution of the individual PO gene products to immunity has not yet been examined. Proteomics pinpointed the PO6 protein as strongly upregulated at one timepoint (24 h) following bacterial injection in *A. gambiae* (Paskewitz and Shi, 2005). One of the *Drosophila* POs was down-regulated upon feeding of bacterial lysates. Both of these observations could be reconciled by considering processing of PPO. That is, the down-regulated spot might represent PPO and the upregulated spot might be activated PO6. PO2 was identified as a constitutive protein in *A. gambiae* hemolymph that was not altered on bacterial injection.

The proteomics studies pointed to post-translational modifications of proteins involved in iron metabolism as a potentially fruitful area for investigation. Paskewitz and Shi (2005) found decreases in both ferritin subunits at 6 and 24 h after a bacterial challenge. Levy *et al.* (2004b) reported that ferritin was down-regulated in fungally challenged adults but unchanged after bacterial infection, although a more complex pattern is indicated in Levy *et al.* (2004a). Vierstraete *et al.* (2004b) found a large increase in the amount of a spot corresponding to the ferritin heavy chain homologue in larval hemolymph 25 minutes after *M. luteus* challenge. The spot is described as “shifted” indicating that post-translational modifications occurred. Additional information on multiple forms of the light and heavy chains of ferritin can be found in Levy *et al*. (2004a) and Vierstraete *et al*. (2004b). The biology of ferritin in relation to immunity is not at all clear but it might serve to sequester iron from invading microorganisms (Yoshiga *et al*., 1997).

Tsf is an iron transport protein that also occurs in the hemolymph. The Tsf protein was found to be upregulated upon treatment of mosquito cells with bacteria (Yoshiga *et al*., 1997) and during encapsulation of filarial worms in *A. aegypti* (Beerntsen *et al*., 1994). Proteomics studies also identified differences in Tsf during fungal infections (Levy *et al*., 2004a). In addition to an increase in Tsf production, fungal infection induced proteolytic cleavage of Tsf. In vertebrates Tsf fragments have been linked to immunity, acting directly as antimicrobial peptides or as inducers of nitric oxide production by macrophages. A role in sequestering iron is also possible (Yoshiga *et al*., 1997).

Another group of proteins ripe for investigation belong to a group called Chi-lectins. We identified two of these proteins (BR1 and BR2) by proteomic analysis of *A. gambiae* as they were very strongly induced by bacterial infection (Shi and Paskewitz, 2004). The two new spots represented C-terminal peptides and we found that both proteins are converted to smaller forms in hemolymph *in vivo* or *in vitro* on exposure to bacteria. We could identify this processing as early as 5 min after incubation of hemolymph with bacteria *in vitro*. A related protein, *Drosophila* DS47 behaves similarly (Shi and Paskewitz, 2004). BR2 and DS47 also can be processed on exposure to peptidoglycan alone but not LPS (Shi and Paskewitz, 2004). Other members of this group are called imaginal disc growth factor proteins (IDGFs) in *Drosophila*, because of a role in stimulating proliferation of an imaginal disc cell line in conjunction with insulin (Kawamura *et al*., 1999). Vierstraete and colleagues (2004b) reported that two spots identified as chain A of IDGF2 were significantly regulated by 25 min after either yeast or bacterial challenge, while DS47 was down-regulated after bacteria only. Levy *et al.* (2004a) found that spots corresponding to IDGF2 and IDGF3 were down-regulated after fungal or bacterial infections, respectively. Again, differences in direction of regulation likely reflect processing, with down-regulated spots corresponding to the full-length proteins and upregulated spots representing the processed forms. This group of proteins is particularly interesting because vertebrates have members of the family that are also immune responsive but not yet well-understood (Houston *et al*., 2003). Some act as growth factors while others promote migration of immune cells (Owhashi *et al.,* 2000; Chang *et al*., 2001; Hung *et al*., 2002; Recklies *et al*., 2002). A related molecule in *Manduca sexta*, HAIP, inhibits aggregation of hemocytes *in vitro* (Kanost *et al*., 1994).

Finally, the proteomics studies have identified several types of proteins that belong to categories not previously known to have immune functions. For example, an odorant binding protein (OBP99c) was upregulated after fungal infection of *Drosophila* adults (Levy *et al*., 2004a). Odorant binding proteins (OBP) bind small hydrophobic molecules and function in chemoreception. OBPs are found in the antennae but some members of the group appear in the hemolymph (Paskewitz and Shi, 2005). Two pherokines, proteins related to the OBP family, were also induced by viral or bacterial infections (Sabatier *et al.,* 2003). Another group of proteins that was found in to be immune-induced in both larval and adult *Drosophila* contains members with a phosphatidylethanolamine binding domain. The PEB family is widespread but little is known about the function of this group. One possibility is in coordinating regulation of the various signaling pathways but see Levy *et al*. (2004a) for other possible functions.

Hemocytes (blood cells)

Hemocytes play important roles in immunity in all insects but there is a great deal of variation in cell types and number between taxa. In general, an insect will have several different types of hemocytes, each of which has a specialized function. Some hemocytes are capable of phagocytosis of foreign targets, including latex beads, bacteria, and malaria sporozoites. Larger targets can elicit encapsulation responses, where layers of hemocytes adhere to the target surface and enclose it.

This process resembles some types of granuloma formation in vertebrates. Hemocytes also produce some of the effector molecules for humoral immunity, including components of the PO cascade and some of the AMPs.

Cellular responses were examined in many of the hemolymph studies, since neither hemocytes nor other cellular contaminants were separated from the plasma component. Cellular responses were also examined by using a *Drosophila* cell line called mbn-2 for analysis of cytoplasmic and nuclear proteins that changed following exposure to LPS (Loseva and Engstrom, 2004). The mbn-2 line is hemocyte-like and retains its ability to phagocytose bacteria and the signaling systems necessary to activate the genes coding for AMPs. In this proteomic profiling study, 24 intracellular proteins were identified as regulated (up or down) or modified in response to immune challenge. At 30 min following LPS administration, proteins that regulate post-translational modifications and traffic over the nuclear membrane were up-regulated including lamin Dm (*Lam*), nuclear porin p62 (*Nup62*), calmodulin (*Cam*), and the receptor of activated protein kinase C1 (Rack1). After 6 h of LPS treatment, proteins that are directly involved in effecting an immune response proteins were differentially regulated. These included actin-binding/cytoskeletal remodeling proteins and lysosomal proteases (Cathepsin L, D, K), (Loseva and Engstrom, 2004). Examination of immune-challenged hemolymph resulted in the identification of a few of the same proteins as well as others likely to be involved in the same processes (Vierstraete ***et al***., 2004a,b; Guedes ***et al***., 2005).

Changes in cytoskeletal proteins and their regulators are likely markers of activation of cells in preparation for phagocytosis. Lysosomal cathepsins are also related to the phagocytic function since they localize to the phagolysosome in mammals and in *Drosophila*. Cathepsin D (CG1548) was found in seven isoforms and LPS treatment of cells resulted in the disappearance of four of these (Loseva and Engstrom, 2004) while LPS treatment of larvae resulted in upregulation of a Cathepsin D spot (Vierstraete ***et al.,*** 2004b). Cathepsins were further characterized in *Drosophila* S2 cells using a functional proteomics technique whereby these enzymes are labeled covalently in an activity dependent manner. Chemical tagging allowed the investigators to follow increases in cathepsin activity within the phagolysosome after phagocytosis of latex beads (Kocks ***et al***., 2003).

In addition to the direct immune responses described above, proteomic studies reveal that infection causes shifts in homeostasis that alter proteins involved in metabolic and redox processes. Oxidative stress results when the production of reactive oxygen species exceeds the ability of the animal to remove them. Both transcript and proteome analyses indicate that oxidative stress occurs in insects during infection. Members of the peroxiredoxin family (CG12405 and CG1633) were regulated in mbn-2 cells (Loseva and Engstrom, 2004) and in *Drosophila* hemolymph following bacterial and fungal challenge (Vierstraete ***et al***., 2004b) or after feeding on bacterial lysates (Guedes ***et al***. 2005). Glutathione S-transferase was also upregulated in all cases of infection and may have a protective role against oxidative stress (Vierstraete ***et al.,*** 2004b; Guedes ***et al***., 2005). Two forms of GST were also identified as upregulated in *A. gambiae* following wounding (Paskewitz and Shi, 2005) and GST activity increased in hemolymph sampled following immune challenge (data not shown).

A large number of cellular proteins involved in carbohydrate and amino acid metabolism were also described as immune-responsive in hemolymph and cell line studies. Possible involvement of these proteins in responses to oxidative damage or in shifts in energy/biosynthetic pathways is discussed in the relevant articles (Levy ***et al***., 2004a; Vierstraete ***et al***., 2004b; Guedes ***et al***., 2005).

Other tissues

Because of their important role as developmental sites for vector-borne disease agents, mosquito midguts, salivary glands, and thoracic tissues are also potential targets for studying immune responses to parasites.

Work is in progress on identification of midgut proteins that are altered on malaria parasite infection. Other proteomic studies of the mosquito midgut have been undertaken on strains of *Anopheles stephensi* that exhibited different susceptibility to the human parasite, *Plasmodium falciparum* (Prevot ***et al***., 1998). Whether susceptibility is governed by immune factors is not known but 29 differences in spot patterns were identified following blood feeding in the susceptible line. Additional work was done to describe spots that differed between male and female mosquitoes and following blood feeding (Prevot ***et al.,*** 2003).

Nematode worms such as *Brugia malayi* occupy a different developmental location in mosquitoes, traveling from the midgut to the thoracic musculature. Wattam and Christensen (1992) compared different strains of *A. aegypti* and reported that thoracic muscle of refractory strains (rLVP and RKF) produced seven polypeptides in response to a blood meal, whereas a susceptible strain (LVP) did not exhibit this pattern. Subsequent work identified mosquito transferrin as a protein altered in this tissue upon worm infection (Yoshiga ***et al***., 1997).

The proteome of *A. aegypti* larvae was examined following infection by a microsporidian parasite, *Vavraia culicis* (Biron ***et al.,*** 2005). Samples contained the head, thorax and part of the abdomen of larvae that were sampled at either 5 or 15 days following infection. Fifteen proteins that were upregulated following infection were identified by peptide mass fingerprinting. Among others, these included heat shock protein co-chaperone Cdc37, proteins involved in protein biosynthesis, an OBP, a nitrophorin, and a GST. Additional proteins that were suppressed included proteins involved in ornithine metabolism, nitric-oxide synthase, GSTs, and an OBP.

Future perspectives

As an important complement to genomics, proteo-

mics allows for the examination of the entire complement of proteins in an organism, tissue, or cell-type. Current proteomics technologies not only identify protein expression, but also post-translation modifications and interactions of immune response proteins in insect. Successful protein profiling with identifications has only recently been applied to insect immunity and only the two dipterans with completed genomes have been used as models. Clearly, many milestones have yet to be reached. In the future, increased use of LC-MS/MS will increase the sensitivity, resolution, dynamic range and throughput of proteomics. Future studies will take advantage of the availability of new genomes and better identification technologies so that we will be able to compare proteomic results across taxonomic groups and life stages, leading to advances in our understanding of the evolutionary history of innate immunity. Within model organisms, reverse genetics will allow us to inactivate key immune regulators through RNA interference and to examine the effects on proteome profiles. Identification of post-translational modifications suggests experiments designed to identify activators or changes in efficacy of the modified molecules. Protein-protein interactions and subcellular locations will provide more precise information about the functions of the unknown proteins that are induced by infection. In short, we predict satisfying application of the methods of proteomics to questions of insect immunity on all fronts within the coming decades.

Abbreviations

2-DE: 2 dimensional electrophoresis; 2D-DIGE 2: dimensional differential in gel electrophoresis; AMP: antimicrobial peptide; BR1: bacterially responsive protein 1; BR2: bacterially responsive protein 2; EST: expressed sequence tags; GNBP: gram negative binding protein; GST: glutathione S transferase; HPLC: high performance liquid chromatography; HSP20: heat shock protein 20; IDGF: imaginal disc growth factor; Imd: immune deficiency protein; LPS: lipopolysaccharide; MS: mass spectrometry; MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight; Nec: necrotic; OBP: odorant binding protein; PEB: phosphatidylethanolamine binding protein; PGRP: peptidoglycan recognition protein; PPO: prophenoloxidase; PO: phenoloxidase; SRPN: serpin; TEP: Thioester protein; Tsf: transferring.

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