

Immune-Related Proteins Induced in the Hemolymph After Aseptic and Septic Injury Differ in Honey Bee Worker Larvae and Adults

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We have employed the proteomic approach in combination with mass spectrometry to study the immune response of honey bee workers at different developmental stages. Analysis of the hemolymph proteins of noninfected, mock-infected and immune-challenged individuals by polyacrylamide gel electrophoresis showed differences in the protein profiles. We present evidence that in vitro reared honey bee larvae respond with a prominent humoral reaction to aseptic and septic injury as documented by the transient synthesis of the three antimicrobial peptides (AMPs) hymenoptaecin, defensin1, and abaecin. In contrast, young adult worker bees react with a broader spectrum of immune reactions that include the activation of prophenoloxidase and humoral immune responses. At least seven proteins appeared consistently in the hemolymph of immune-challenged bees, three of which are identical to the AMPs induced also in larvae. The other four, i.e., phenoloxidase (PO), peptidoglycan recognition protein-S2, carboxylesterase (CE), and an *Apis*-specific protein not assigned to any function (HP30), are induced specifically in adult bees and, with the exception of PO, are not expressed after aseptic injury. Structural features of CE and HP30, such as classical leucine zipper motifs, together with their strong simultaneous induction upon challenge with bacteria suggest an important role of the two novel bee-specific immune proteins in response to microbial infections. Arch. Insect Biochem. Physiol. 69:155–167, 2008. © 2008 Wiley-Liss, Inc.

KEYWORDS: *Apis mellifera*; antimicrobial peptides; hemolymph proteins; humoral immune response

INTRODUCTION

Because of their social lifestyle with a very high population density in their hives, honey bees are especially vulnerable to infection by pathogens. They spend most of their lives inside their nests in close contact, permanently feeding each other. Such extreme living conditions are believed to enforce the evolution of very effective strategies to combat pathogens and parasites. Like all insects, honey bees lack a classical adaptive immune system. Instead they have evolved several lines of defense mechanisms to cope with microbial

infections: (1) cooperative social behavior of individual group members to fight disease transmission within the colony (Cremer et al., 2007), (2) physical barriers such as the cuticle and epithelium of the gut, and (3) cellular and humoral immune responses constituted of the innate immune system.

The humoral immune reaction is mediated by four signaling pathways, i.e., the Toll, Imd, Janus kinase (JAK)/STAT, and JNK as best studied in the diptera *Drosophila melanogaster* (Lemaitre and Hoffmann, 2007). The pathways are usually activated by determinants that are conserved in the cell

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Abbreviations used: AMP, antimicrobial peptide; CE, carboxylesterase; PBS, phosphate-buffered saline; PO, phenoloxidase; PTU, N-phenylthiourea; RJ, royal jelly.

Contract grant sponsor: Deutsche Forschungsgemeinschaft, Sonderforschungsbereich; Contract grant number: 567.

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Received 15 April 2008; Accepted 22 July 2008

wall of microbes but absent in the host, such as lipopolysaccharides (LPS) present in Gram-negative bacteria, peptidoglycans (Gram-negative and Gram-positive bacteria), and β -1,3 glucan, a component of fungal cell walls. The complex signaling cascades regulate the transcription of target genes with conserved NF- κ B-like motifs that encode, for example, antimicrobial peptides (AMPs). With a few exceptions, AMPs are basic molecules of small size. According to their biochemical characteristics, they are classified in three main groups: (1) linear peptides without cysteine (e.g., cecropins); (2) linear peptides that are enriched in one amino acid, e.g., glycine or proline; and (3) peptides with an even number of cysteine residues resulting in several intramolecular disulfide bridges (Gillespie et al., 1997; Tenczek, 1998). Such immune peptides have been intensely studied in only a few insect species, including *Hyalophora cecropia*, *Bombyx mori*, *Drosophila melanogaster*, *Calliphora vicina*, *Anopheles gambiae*, and *Bombus pascuorum* (Rees et al., 1997; Chernysh et al., 2000; Tenczek, 1998; Vizioli et al., 2001; Hultmark, 2003; Wang et al., 2004).

One of the first antimicrobial peptides discovered in bees was royalisin found in royal jelly (Fujiwara et al., 1990) and also in the hemolymph of bacterially infected bees (Casteels-Josson et al., 1994). It was later termed defensin1 by Klaudiny et al. (2005), who identified the corresponding gene together with an additional defensin gene coding for defensin2. Casteels and colleagues analyzed hemolymph samples taken from noninfected and infected adults challenged with *Escherichia coli* suspensions. Factors present in immunized but not in control samples were further purified and subsequently characterized by amino acid sequence analysis. Four different types of antimicrobial peptides were identified: apidaecins (Casteels et al., 1989), abaecin (Casteels et al., 1990), hymenoptaecin (Casteels et al., 1993), and defensin1 (Casteels-Josson et al., 1994).

The recent sequencing of the honey bee genome (Honey Bee Genome Sequencing Consortium, 2006) stimulated a global analysis of each stage of immunity from recognition and signaling

pathways to effector molecules. Comparison with genomic data from other insects, i.e., *Drosophila melanogaster* and *Anopheles gambiae* revealed that honey bees possess homologues of peptidoglycan recognition proteins (PGRP-S and PGRP-L) and β -glucan recognition proteins (β GRP). Furthermore, homologues of members of each of the four signaling pathways implicated in humoral immunity response were identified (Evans et al., 2006). All the annotated bee AMPs were first characterized by protein sequencing as described above (Casteels-Josson et al., 1994) and, with the exception of defensin2 (Klaudiny et al., 2005), no further putative AMP was discovered by genomic search indicating the difficulty to detect novel AMPs by sequence similarity. This fact together with the understanding that novel classes of antimicrobial components might have escaped detection up to date, prompted us to initiate a proteomic analysis of immune peptides/proteins expressed in bees at different developmental stages after aseptic and septic injury. Here, we report that in adult bees, but not larvae, two novel proteins are induced in concert upon challenge with bacteria, one of which, i.e., a nonclassified protein with a molecular mass of about 30 kDa (HP30) appears to be honey bee-specific.

MATERIALS AND METHODS

Bacterial Strains and Media

The Gram-negative bacteria *Escherichia coli* (DSM 682) and *E. coli* B (DSM 613) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and the Gram-positive bacterium *Micrococcus flavus* was a gift from Dr. U. Rdest (Institute of Microbiology, Biocenter, Würzburg). The two *E. coli* strains were cultivated in self-made NB medium (5 g Nutrient broth, 5 g Bacto peptone, and 10 g NaCl per liter), whereas *M. flavus* was grown in LB medium (5 g Bacto yeast extract, 10 g Bacto tryptone, and 10 g NaCl per liter). All ingredients were purchased from Becton Dickinson (Heidelberg, Germany). The bacteria were grown to an absorbance of $A_{550} = 0.5$. After centrifuga-

tion, cells were washed two times, resuspended in phosphate-buffered saline (PBS) and diluted to the desired concentration with PBS.

Origin of Honey Bee Larvae and Adults

During the summer season, worker honey bee larvae and adult bees were obtained from three colonies with sister queens of *Apis mellifera carnica* maintained in the apiary of the BEEstation (University of Würzburg). To ensure a supply of larvae for the winter months (November to March), two colonies were placed in a flight room with the temperature set at 22°C under a diurnal illumination of 12 h light and 12 h dark and 58% relative humidity. Lighting was provided by fluorescent tubes.

In Vitro Rearing of Worker Bee Larvae

The basic diet was prepared according to Peng et al. (1992). Royal jelly (RJ) was purchased from Werner Seip (Butzbach, Germany). The RJ was lyophilized in a Freeze Dryer (CHRIST, Osterode, Germany) and kept as dried powder in a refrigerator.

Young worker larvae were collected from a comb with a special grafting tool and transferred to a 24-well tissue culture plate (Greiner, Frickenhausen, Germany; No. 662160) that was kept on a warm pad (Thermolux, REPTILICA, Zirndorf, Germany) to prevent chilling of the larvae. An appropriate number of wells were filled with 300 µl of prewarmed diet. On the first day of culture, a maximum of 10 larvae were placed together in a single well. During the following 5 days, the number of larvae cultivated together was stepwise reduced according to their size. To ensure constant humidity, a piece of wet Whatman paper was placed between the top of the wells and the lid of the culture plate. The grafted larvae were maintained in an incubator at 35°C. Each day they were transferred to new tissue culture plates filled with fresh diet until the sixth instar day when the cultivation was normally terminated (Fig. 1A). The grafted larvae were examined under a stereo

microscope and the number of dead larvae was recorded. As determined previously, larval weight is a useful index of age (Thrasymonon and Benton, 1982) reflecting rather accurately the developmental stage. The mean weight of 10 (first instar), 5 (second instar), and 3 (third to sixth instar) larvae was determined on a microbalance.

Aseptic and Septic Wounding of Bee Larvae

Over a period of 2 years, five to six series of experiments were performed per season. For each series of experiments, a total of 30 larvae were divided into three groups: one group was kept as an untreated (noninfected) control, the second group was mock-infected with buffer and the third group was artificially infected with bacteria. Routinely, larvae were employed at the fourth instar stage with an average weight of 30 to 40 mg (Fig. 1). At this age, larvae are easy to handle and mortality after wounding is low. For injection, we used disposable calibrated (1–5 µl) glass capillaries (Servoprax, Hartenstein, Würzburg, Germany) with fine tips that were generated by a P-2000 laser-based micropipette puller (SUTTER Instrument, Novato, USA). Each larva was removed from the diet solution, gently rolled on paper tissue and then placed in a small dish under a stereo microscope. The larvae were injected dorsally with either 1 µl of phosphate-buffered saline (PBS) solution (0.15 M; pH 7.5) or with 1 µl of about 10³ viable *E. coli* 682 cells in PBS. Dose-dependent studies revealed an increased mortality rate at injections of more than 10³ cells (not shown). Immediately before injection, the capillary tip was dipped into a solution of 10 µg/ml N-phenylthiourea (PTU; P7629, Sigma Aldrich, Taufkirchen, Germany) to prevent prophenoloxidase activation at the site of wounding. The survival rate of aseptic and septic wounded larvae under these optimized conditions was 80–90%.

In Vitro Challenge of Adult Bees With Bacteria

Over a period of 2 years, six series of independent experiments were performed during

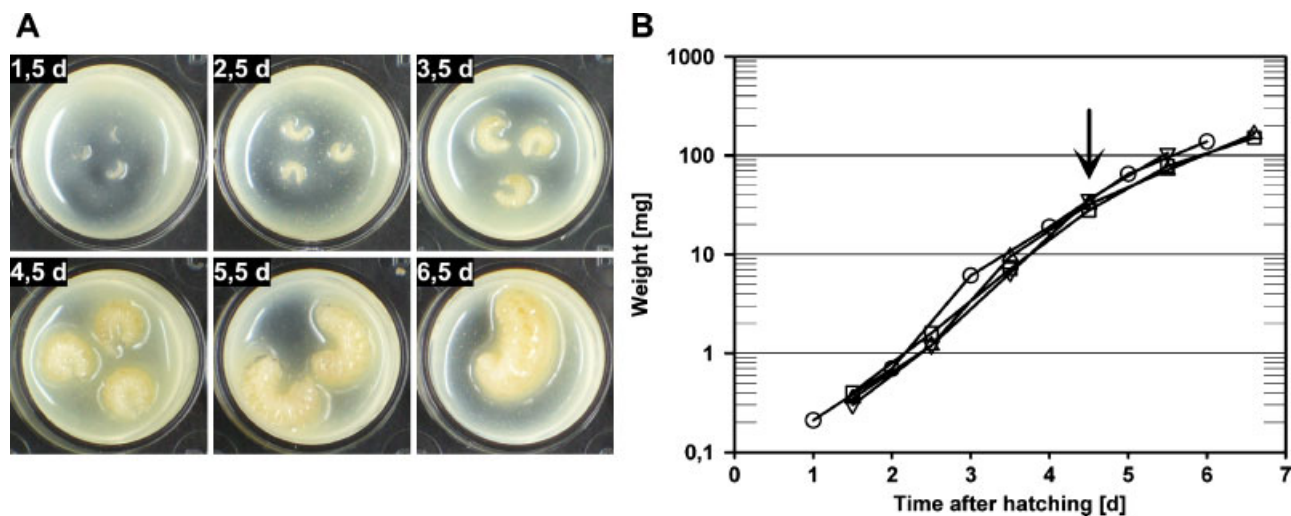


Fig. 1. In vitro cultivation of honey bee worker larvae. A: Development of in vitro reared larvae. First to second instar larvae were collected from brood combs and transferred into 24-well tissue culture plates supplemented with a mixed diet consisting of royal jelly, glucose, fructose, and yeast extract (Peng et al., 1992). Each day, surviving larvae were transferred to new culture plates. B: Growth rates of worker larvae until the prepupal stage. A subset of larvae from each experimental series was weighed each day, to ascertain their age. The four illustrated growth curves differ with respect to the origin of larvae: ○, □, larvae derived from colonies kept during the winter in a flight room; △, ▽, larvae derived from colonies kept during the summer at the apiary of the BEEstation (Würzburg). The arrow indicates the approximate time of artificial larval infection (see below).

two summer seasons. Freshly emerged bees were obtained from a caged comb placed in an incubator at 35°C about 20 days after the deposition of eggs by a queen confined on a comb without brood. For each series of experiments, a total number of 60 adult bees were divided into three groups, one of which was kept as an untreated control group. Shortly after collection, the remaining bees were chilled on ice in groups of 3 to 5 individuals. Subsequently, volumes of 1 µl buffer (PBS) or bacteria as indicated below were injected into the hemocoel with a fine-tipped glass capillary laterally between the second and third tergite. For septic wounding, viable *E. coli* 682 bacteria at a concentration of about 10^4 cells/µl were employed. The bees of each group of 20 individuals were kept in small metal boxes (5 × 7.5 × 10 cm) supplied with 45% (w/v) sucrose solution in an incubator at 35°C until hemolymph collection. The survival rate of aseptic and septic wounded adults 24 h post-injection was 90–100%.

Hemolymph Collection

At indicated times after septic and aseptic wounding, all challenged larvae or adult bees were bled by puncturing the abdomen with a fine-tipped calibrated glass capillary. The collected hemolymph (5–10 µl per adult bee and 20–30 µl per larvae, respectively) was transferred to reaction tubes containing 1 µl of a mixture of PTU (P-7629) and aprotinin (A-4529), a protease inhibitor (SIGMA ALDRICH, Taufkirchen, Germany), each at a concentration of 0.1 mg/ml to prevent melanization of the samples, which were kept at –20°C until further analyses.

Inhibition-Zone Assay

The inhibition-zone assay was employed to measure antimicrobial activity of all collected hemolymph samples from larvae or adult bees challenged with infectious agents. The test bacteria were either *E. coli* B (Gram-negative) or *M. flavus*

(Gram-positive). An aliquot (0.2 ml) of a fresh overnight culture was spread onto agar plates ($\text{Ø} = 9 \text{ cm}$) containing NB and LB medium, respectively. As soon as the bacterial layer had been adsorbed, 1.5 μl of undiluted hemolymph samples were applied as a droplet onto the plates with a pipette tip. After 24 h of incubation in an incubator at 37°C, the diameter of the clear zone of inhibition was measured and documented by photography.

SDS Polyacrylamide Gel Electrophoresis

One-dimensional gel electrophoresis was carried out in vertical polyacrylamide gels (8.5 \times 13 \times 0.1 cm) containing 0.1% SDS with a 1.5-cm-long 5% stacking gel on top of the separating gel (Laemmli, 1970). Hemolymph samples were diluted with 2x concentrated sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 17% glycerol, and 0.8 M 2-mercaptoethanol), heated for 3 min at 95°C and subjected to electrophoresis at constant voltage (120 V). As a rule, two types of one-dimensional gels were run with the same hemolymph sample: 10% and 15% polyacrylamide/0.1% SDS gels for the separation of proteins in the range of 30–200 kDa and 3–30 kDa, respectively. For an even better resolution of small proteins, the SDS PAGE system according to Schagger and von Jagow (1987) was employed with some modifications. The composition of separating and stacking gel as well as sample buffer remained unaltered. However, the running buffer in the upper buffer tank was 0.1 M Tris, 0.1 M Tricine, pH 8.3, containing 0.1% SDS, whereas the buffer in the lower tank consisted of 0.2 M Tris-HCl, pH 8.9. Electrophoresis was carried out at constant current (25 mA) at room temperature.

For colloidal Coomassie staining, the gels were first fixed for 30 min in 0.85% o-phosphoric acid/20% methanol followed by staining overnight in a solution of Roti[®]-Blue (Roth, Karlsruhe, Germany) and 20% methanol according to the manufacturer's instructions. Gels were destained in 25% methanol.

Nano-HPLC-MS/MS Analysis

The excised gel slices were washed, dried and subjected to in-gel digestion with trypsin as recently described (Schönleben et al., 2007). The obtained peptide mixtures were eluted with 200 μl of 5% formic acid and pre-concentrated on a 100- μm I.D., 2 cm C18-column using 0.1% trifluoroacetic acid with a flow rate of 8 $\mu\text{l}/\text{min}$. The peptides were then separated on a 75- μm I.D., 15-cm C18-PepMap-column with a flow rate of 320 nl/min using an Ultimate 3000 nano-HPLC system (Dionex GmbH, Idstein, Germany). A binary gradient from 5% to 50% solvent B (solvent A: 0.1% formic acid; solvent B: 0.1% formic acid/84% acetonitrile) was applied for 1 h. The nano-RP-HPLC was directly coupled to an ion trap mass spectrometer (LCQDecaXP^{Plus}, ThermoElectron GmbH, Dreieich, Germany) acquiring repeatedly one full-MS and three tandem-MS spectra of the most intensive ions in the full-MS scan. The tandem-MS spectra were searched against the NCBI nr database using the Mascot Daemon and the Mascot algorithm (version 2.1; Matrixscience, London, UK) as reported in detail by Schönleben et al. (2007). Three to four independent MS/MS analyses were performed for the newly identified immune-related proteins presented in Table 1. For the remaining proteins, indicated in Figure 3, at least two MS/MS analyses were carried out. Sequence coverage by identified tryptic peptides was in the range of 20–45%.

RESULTS

In Vitro Reared Honey Bee Larvae Mimic Normal Development of Workers

A subset of larvae from each experimental series was weighed in order to ascertain their age. Until the fifth day after hatching, an exponential increase of the larval weight by almost a factor of 1,000 could be observed (Fig. 1B). The growth and survival rates were essentially the same for larvae derived from brood of "summer" or "winter" bees. The development of in vitro reared larvae until the prepupal stage appeared to be slightly delayed by

TABLE 1. Immune—Related Proteins Induced in Young Adult Bees After Bacterial Challenge

Protein	Accession (NCBI)	Calc masses (Da) ^a precursor/mature protein	pI ^a prec./mat. prot.	SP ^b	Pro ^c	Mowse score	Queries matched	Sequence coverage (%)
Phenoloxidase (PO)	gi 58585196	80.094/74.000	6.3/6.3	—	51	1063	35	32
Carboxylesterase (CE)	gi 66512983	65.249/63.327	6.1/6.0	18	—	1949	97	35
Hypothetical protein (HP30)	gi 66507096	30.421/28.700	6.4/6.1	17	—	2741	90	37
Peptidoglycan recognition protein (PGRP—S2)	gi 66522804	21.550/19.386	8.9/8.9	19	—	1857	59	45

^aMolecular weights and pI values were deduced from the ExPASy/ProtParam program.

^bPredictions for signal peptides (SP) and cleavage sites were obtained with the TargetP program (Emanuelsson et al., 2000).

^cPutative length of pro-sequence.

12–24 h as compared to larval brood reared in the hive (Jay, 1963; Thrasyvoulou and Benton, 1982). However, we used the larval weight as indicator for the actual developmental stage in all further studies. The established in vitro cultivation of bee larvae has the advantage of a constant supply of individuals under sterile conditions independent of seasonal restrictions.

Wounding and Septic Injury of Bee Larvae With Gram-Negative Bacteria Results in the Synthesis of Antimicrobial Peptides

Hymenoptaecin is the major immune peptide whose synthesis is transiently induced for up to 24 h after aseptically wounding but continues to be expressed at increased rates for at least 48 h after challenge with viable *E. coli* cells (Fig. 2A). At this stage, larvae have reached the prepupal stage (Fig. 1). The induced synthesis of defensin1 follows a similar pattern as observed with hymenoptaecin, albeit at reduced synthesis rates. The quantitative determination of expressed abaecin is hampered by its weak staining. It could not be detected by Coomassie Brilliant Blue R250 staining and was only faintly visible by colloidal Coomassie staining.

The expression pattern of the three identified AMPs in the hemolymph of bee worker larvae after wounding and/or after challenge with *E. coli* (Fig. 2A) was observed consistently over a period of two years. In vitro reared larvae derived from brood of “summer” or “winter” bees showed similar growth and survival rates (Fig. 1B). Accordingly, we observed no difference in the response to aseptically and septic wounding with respect to the origin of larvae.

The presence of immune peptides in hemolymph samples confirmed by proteomic analyses was reflected by their antimicrobial activities in inhibition-zone assays. Employing *Micrococcus flavus* as a Gram-positive test bacterium revealed no activity in the hemolymph from noninfected larvae (Fig. 2B, samples 1 and 4) and weak or no activity in the hemolymph collected 24 h and 48 h p.i., respectively, from wounded larvae (Fig. 2B,

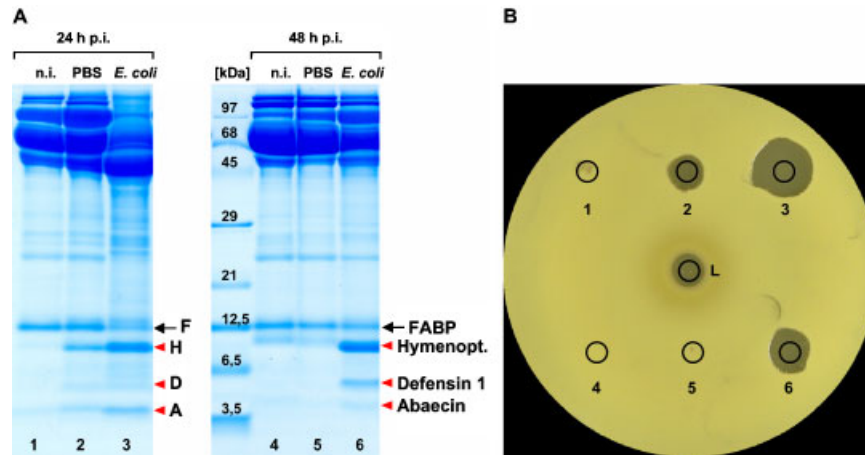


Fig. 2. Characterization of immune peptides transiently expressed in bee larvae. A: Gel electrophoretic analysis of hemolymph proteins. Fourth instar larvae were mock-infected with buffer or challenged with *E. coli*. Hemolymph samples were collected from a total of 8 individual larvae per group 24 and 48 h post-injection (p.i.) followed by separate gel analysis for each replicate. An aliquot of these samples (i.e., 1.5 μ l) was mixed with dissociation buffer and applied onto a 15% polyacrylamide/0.1% SDS gel. A representative hemolymph sample of each group is shown. The running buffer contained Tricine-HCl buffer according to Schägger and von Jagow (1987). Gels were stained with Coomassie Brilliant Blue G250. The induced antimicrobial peptides are indicated by arrowheads. The arrow identifies fatty acid binding protein (FABP) that is constitutively synthesized in worker larvae. For comparison, the protein pattern of untreated larvae (n.i.) is shown. B: Zone-inhibition assay for the detection of antimicrobial activities in the hemolymph of infected larvae. An aliquot of fresh overnight cultures of the Gram-positive bacterium *Micrococcus flavus* was spread on an agar plate. Hemolymph aliquots (1.5 μ l) derived from the same samples as analysed by gel electrophoresis in A: were directly applied onto the agar plate with pipette tips and the plate was subsequently incubated overnight at 37°C. As a positive control, lysozyme (L) at a concentration of 5 μ g/ μ l was placed in the center of the agar plate.

samples 2 and 5). Strong inhibitory activity was detected in the hemolymph of larvae challenged with *E. coli* cells (Fig. 2B, samples 3 and 6) as deduced from the size of the inhibition zones. A similar pattern was observed with the Gram-negative *E. coli* B bacterium (not shown).

Differential Induction of Immune-Related Proteins in the Hemolymph of Bee Larvae and Adults

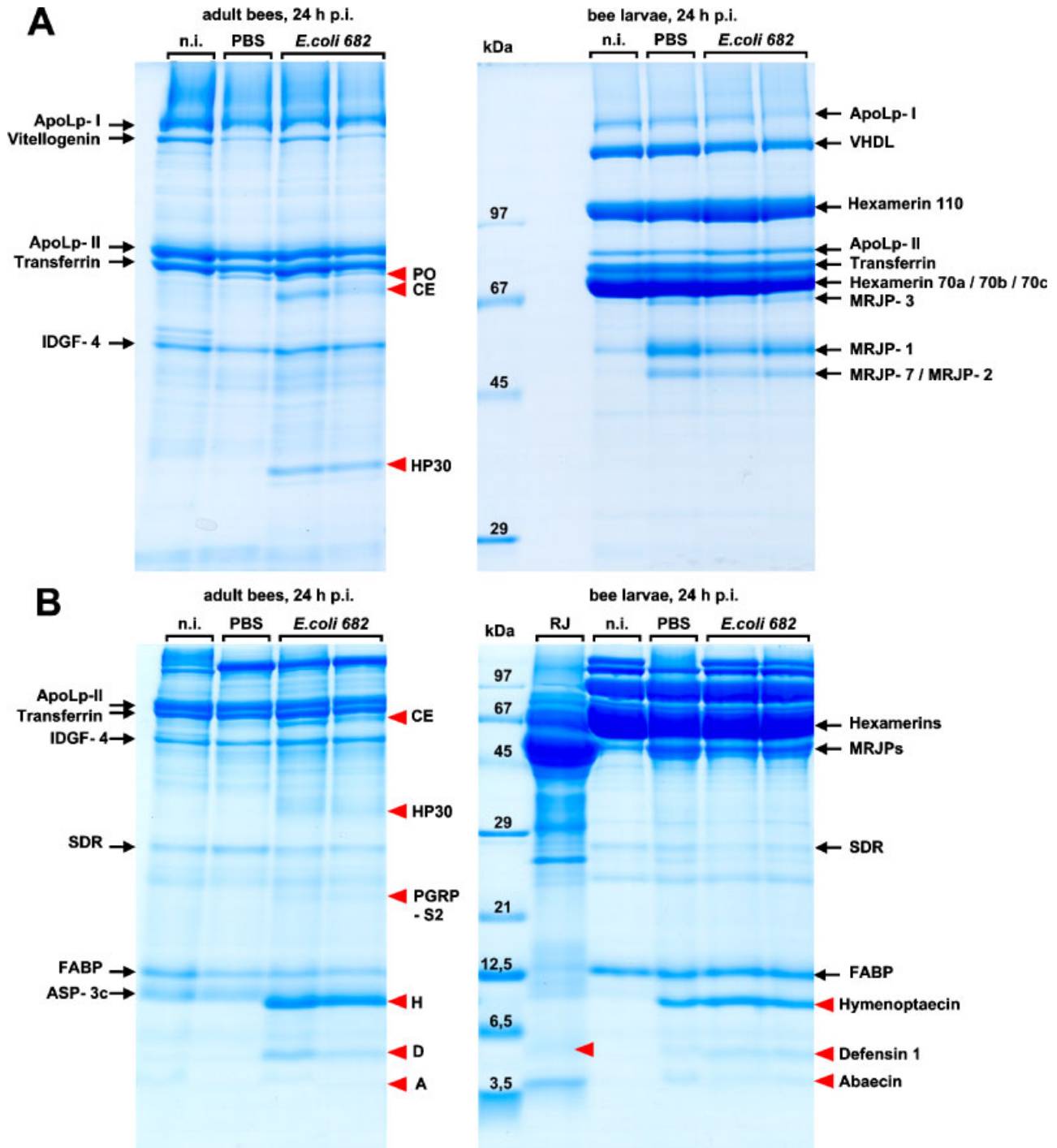
Fourth instar bee larvae induced the humoral defense peptides hymenoptaecin, defensin 1 and abaecin in response to bacterial challenge (Fig. 2). The same three antimicrobial peptides were synthesized in the hemolymph of adult bees infected with viable bacteria (Fig. 3B). None of these immune peptides was detected in the hemolymph of aseptically wounded adults. In con-

trast, bee larvae responded to aseptic injury with a strong transient synthesis of hymenoptaecin (Figs. 2A and 3B).

At the beginning of our studies, we focused our attention on the identification of small peptides in the range of 2–15 kDa, but later shifted our interest to larger proteins. The observed protein patterns revealed (1) proteins that were differentially expressed in larvae and adult bees and (2) novel immune-related proteins induced only in adults (Fig. 3A). In freshly emerged worker bees (1–2 days old), apolipoprotein (ApoLp) I and II (the latter lacking the N-terminal amino acids 1 to 747), vitellogenin, transferrin, and imaginal disc growth factor 4 (IDGF-4) are prominent hemolymph proteins, whereas in fifth instar worker larvae four classes of hexamerins (i.e., hexamerin 110, hexamerin 70a, hexamerin 70b,

and hexamerin 70c) that serve as source of amino acids for tissue reconstruction during pupal development (Burmester and Scheller, 1999) and a very high-density lipoprotein (VHDL) of ~175 kDa (Shipman et al., 1987) constitute the

bulk of hemolymph proteins. Unexpectedly, members of the major royal jelly proteins (MRJPs), components of the royal jelly (Drapeau et al., 2006) were detected in the hemolymph of larvae. Contamination by the larval diet



can be excluded since larvae were thoroughly washed in PBS buffer and subsequently wiped with soft paper towels before collecting hemolymph samples.

In the hemolymph of young adults challenged with viable *E. coli* bacteria, three proteins of about 22, 35, and 65 kDa were induced whose synthesis was not observed after wounding. Additionally, phenoloxidase with an approximate mass of 75 kDa was detected in adult bees after aseptic and septic wounding. None of these proteins was found in infected bee larvae (Fig. 3). We identified the 22-kDa protein as peptidoglycan recognition protein-S2 (PGRP-S2), the 35-kDa protein as a hypothetical protein (HP30) with a calculated mass of about 30 kDa and the 65-kDa protein as carboxylesterase (Table 1).

DISCUSSION

Honey Bee Larvae Respond With a Humoral Immune Reaction to Bacterial Challenge

So far, little information is available about the immune response of bee larvae. Evans (2004) studied the defense reaction of bee larvae orally infected with spores of the bee pathogen *Paenibacillus larvae*. Individual first instar larvae exposed to a high concentration of spores in the

diet showed a significant increase of abaecin transcript levels 24 h post-inoculation at a time when the bacterium surmounts the midgut epithelium, suggesting an immune response in the hemocoel rather than in the gut itself. We have challenged bee larvae by injection of buffer and bacteria, respectively, and analyzed the peptide pattern by gel electrophoresis of hemolymph samples collected 24 and 48 h post-injection. Our results reveal the induction of three immune peptides after septic injury of fourth instar larvae, i.e., hymenoptaecin, defensin1 and abaecin (Figs. 2 and 3). None of these AMPs was detected in hemolymph samples collected from noninfected larvae. In accordance with this observation, no antimicrobial activity was measured in control samples by the zone-inhibition assay, suggesting that no or very low amounts of AMPs are constitutively produced.

Hymenoptaecin is by far the most prominent immune peptide induced in larvae (Fig. 2) and also in young adult bees (Fig. 3) after challenge with viable *E. coli* bacteria. It is also transiently expressed in larvae (but not in adults) after aseptic wounding, possibly because the soft cuticle of larvae is more vulnerable to injury and consequently to microbial invasions. Detailed analysis of the peptide composition of hymenoptaecin

Fig. 3. Comparison of the immune response of larvae and adult bees. **A:** Gel electrophoretic analysis of large hemolymph proteins. Freshly emerged worker bees (1–2 days old) and fourth instar larvae were either mock-infected with PBS or challenged with 10^4 and 10^3 , respectively, *E. coli* 682 bacteria. Hemolymph samples were collected 24 h post-injection from a total of 8 individual larvae or adults per group followed by separate gel analysis for each replicate. Aliquots of 1 μ l were mixed with dissociation buffer and one representative hemolymph sample of non-infected (n.i.) or mock-infected (PBS) and two samples of bacteria-challenged individuals were applied on 10% polyacrylamide/0.1% SDS gels. Electrophoresis was according to Laemmli (1970). **B:** Gel electrophoretic analysis of small hemolymph proteins. Aliquots (1.5 μ l) of the same samples as analysed above were applied onto 15% polyacrylamide/0.1% SDS gels. Electrophoresis was according to Schagger and von Jagow (1987). Gels were stained with coomassie brilliant blue G250. Differentially expressed immune peptides/proteins are indicated by arrow heads. Major proteins identified in the hemolymph of larvae and young adult bees are marked by arrows. The identification of gel-excised proteins was done by nano-RP-HPLC and tandem-mass spectrometry: ApoLp, apolipoprotein (retinoid- and fatty-acid binding protein); ASP-3c, antennal-specific protein 3c; CE, carboxylesterase; FABP, fatty acid binding protein-like protein; HP30, hypothetical protein (MW ~30 kDa); IDGF-4, imaginal disc growth factor 4; MRJP, major royal jelly protein; PGRP-S2, peptidoglycan recognition protein-S2; PO, phenoloxidase; SDR, short-chain dehydrogenase/reductase; VHDL, very high-density lipoprotein (gi/110762106). To facilitate the identification of defensin1, the protein pattern of royal jelly (RJ) that constitutively contains defensin1 (= royalisin) is shown as control.

indicated major differences of this bee-specific immune peptide from all other known classes of insect AMPs. Under physiological conditions, hymenoptaecin inhibited the growth of Gram-negative and Gram-positive bacteria and affected permeabilization of the outer and inner membrane of *E. coli* (Casteels et al., 1993). The expression of hymenoptaecin and abaecin is apparently regulated by the Imd signaling pathway as shown recently by RNA interference studies. The activation of Relish transcription factor, a component of the Imd pathway, was significantly reduced by RNAi and as a consequence, the transcription of both hymenoptaecin and abaecin was simultaneously suppressed to the same extent as demonstrated by quantitative RT-PCR (Schlüns and Crozier, 2007). Thus, the ample synthesis of hymenoptaecin induced in fourth instar bee larvae upon aseptic and septic injury indicates the activation of the humoral immune response system at the late larval stage.

Adult Worker Bees Use a Broader Spectrum of Defense Strategies Than Larvae to Eliminate Microbial Infections

At least seven proteins appeared consistently in the hemolymph of young adult hive bees after septic wounding in all series of experiments carried out during two summer seasons (Fig. 3). Three of them, hymenoptaecin, defensin1, and abaecin, were also induced in bee larvae (Fig. 2) and are effector molecules expressed at the end of the humoral immune response cascade. The other four proteins, phenoloxidase, peptidoglycan recognition protein-S2, carboxylesterase, and HP30, are induced specifically in adult bees (Table 1). The overall pattern of hemolymph proteins from individual worker bees (up to 10 individuals per group) was rather similar in separate gel analyses. This feature facilitated the detection of novel immune-related proteins.

A second line of defense reactions in the innate immune system of insects is the activation of the prophenoloxidase (proPO) cascade that leads to a local increase of cytotoxic quinones and ultimately to melanin synthesis, which plays a fundamental

role in cuticle pigmentation, wound healing, and encapsulation of microbes and parasites. Phenoloxidase is mainly synthesized by free circulating hemocytes as inactive prophenoloxidase. Activation of proPO is triggered by pattern-recognition proteins that bind peptidoglycans, β -1,3 glucan, lipopolysaccharides, or other compounds and successively initiate a cascade of serine proteases. A final consequence of this process is the removal of an inhibitory amino-terminal peptide from the proPO molecule that leads to an active PO (Cerenius and Söderhäll, 2004).

The genome of *A. mellifera* contains only one proPO gene copy (Honey Bee Genome Sequencing Consortium, 2006). The mature bee PO apparently has a molecular mass of about 75 kDa, as estimated by gel filtration and SDS-PAGE (Zufelato et al., 2004). Although proPO is constitutively expressed during honey bee development, its amount in larval and early pupal stages is low and increases steadily at the end of the pupal stage and further on in newly emerged bees (Lourenço et al., 2005). No conclusion can be made about the presence of proPO or its activation by wounding in larvae because the bulk of hexamerins with molecular masses in the range of 80 kDa conceals its detection by SDS-PAGE, but the apparent low amounts of proPO present in all larval stages (Lourenço et al., 2005) makes it unlikely that proPO activation plays a major role in combating invasion of microorganisms by bee larvae. However, adult bees clearly make use of this defense strategy. Activation of proPO is induced by wounding and septic injury (and occasionally also in control individuals) as seen by the occurrence of a 75-kDa PO polypeptide (Fig. 3A). Previous studies demonstrated that the dynamics of PO activation levels has caste-specific characteristics. In workers, PO activity increases with age and reaches a plateau within the first week of adult life whereas the number of hemocytes steadily decreases (Schmid et al., 2008).

Another polypeptide whose synthesis is up-regulated in adult bees after injection of viable *E. coli* bacteria is the peptidoglycan recognition protein (PGRP)-S2 (Fig. 3). Consistently, Evans et al. (2006) have observed a strong increase in the transcript

abundance of the PGRP-S2 gene in adult workers. PGRPs are major components of pathogen recognition. They may either function solely as recognition proteins for pathogen-associated molecular patterns (PAMPs) or may exert amidase activity and then act as scavengers that degrade bacterial cell wall components (Steiner, 2004; Kaneko and Silverman, 2005). There are four PGRPs encoded in the honey bee genome, compared to thirteen and seven in *Drosophila* and *Anopheles*, respectively (Evans et al., 2006). Of these four, named PGRP-S1, PGRP-S2, PGRP-S3, and PGRP-LC, the latter is a membrane bound protein, whereas the other three types are presumably circulating proteins.

Among the polypeptides induced specifically in adult bees challenged with *E. coli* but not after aseptic wounding are two novel proteins that hitherto have not been detected in bees or other insects in connection with an immune response (Table 1). The first one is carboxylesterase (CE), the other one is a protein annotated as hypothetical protein (HP30). The CE belongs to a large family of type B esterases and lipases that act on carboxylic esters; it has a conserved catalytic triad composed of serine (S209), glutamate (E344), and histidine (H467). Insect CEs are likely components of hemocytes that kill and degrade pathogens together with other hydrolytic enzymes such as acid phosphatases and proteases (Trenczek, 1998).

In addition to CE, a protein with a calculated mass of about 30 kDa (HP30) is specifically induced in adult bees after microbial challenge (Fig. 3). HP30 appears to be unique for the genus *Apis*. This assumption is supported by our preliminary PCR analyses indicating that genomic DNAs derived from *A. florea*, *A. dorsata*, and *A. cerana* encode HP30 (not shown). Interestingly, no close HP30 homologues were detected in the genomic sequences of other insects, including the nonsocial hymenopteran wasp *Nasonia vitripennis*. According to sequence motif analysis (<http://npsa-pbil.ibcp.fr>), the HP30 protein expresses some interesting features: it contains a signal peptide at its N-terminal and a Ser, Arg, Ile (SRI) sequence at the C-terminal end (the latter could serve as a target signal for peroxisomes), two

potential N-glycosylation sites that might explain its slower migration in SDS-PAGE gels (Fig. 3). Most notably, the HP30 polypeptide, as well as the CE, contain classical leucine zipper motifs that could function as a domain interacting with other proteins.

ACKNOWLEDGMENTS

The authors thank Dr. U. Rdest (Institute of Microbiology, Würzburg) and Ina Vöth-Rasmussen (BEEgroup, Würzburg) for help in the initial work with the in vitro culture of bee larvae. We are indebted to D. Ahrens-Lagast (BEEgroup, Würzburg) for management of the bee colonies and Dr. S. Fuchs (Institut für Bienenkunde, Oberursel), Dr. I. Illies and Dr. S. Berg (Fachzentrum Bienen, Bayerische Landesanstalt für Weinbau und Gartenbau, Veitshöchheim) for providing a flight room during winter season. We are grateful to Professor H.J. Gross for his stimulating interest during the course of this work and for critical reading of the manuscript.

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