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ORIGINAL ARTICLE

Proteomics of larval hemolymph in *Bombyx mori* reveals various nutrient-storage and immunity-related proteins

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Abstract The silkworm, *Bombyx mori*, is an important economic insect for its production of silk. The larvae of many lepidopteran insects are major agricultural pests and often silkworm is explored as a model organism for other lepidopteran pest species. The hemolymph of caterpillars contains a lot of nutrient and immune components. In this study, we applied liquid chromatography–tandem mass spectrometry to gain a better understanding of the larval hemolymph proteomics in *B. mori*. We identified 752 proteins in hemolymph collected from day-4 fourth instar and day-7 fifth instar. Nearly half the identified proteins (49 %) were predicted to function as binding proteins and 46 % were predicted to have catalytic activities. Apolipophorins, storage proteins, and 30K proteins constituted the most abundant groups of nutrient-storage proteins. Of them, 30K proteins showed large differences between fourth instar larvae and fifth instar larvae. Besides nutrient-storage proteins, protease inhibitors are also expressed very highly in hemolymph. The analysis also revealed lots of immunity-related proteins, including recognition, signaling, effectors and other proteins, comprising multiple immunity pathways in hemolymph. Our data provide an exhaustive research of nutrient-storage proteins and immunity-related proteins in larval hemolymph, and will pave the way for future physiological and pathological studies of caterpillars.

Keywords Hemolymph · *Bombyx mori* · Apolipophorin · Storage protein · 30K protein · Immune

Introduction

The fluid in the circulatory system of insects is termed hemolymph because it corresponds to both the blood and lymph of higher animals (Reiber and McGaw 2009). Hemolymph surrounds all insect organs and cells and therefore, significantly contributes to physiological processes in insects (Gillott 1995). The hemolymph serves important functions including the transport of nutrients, wastes and hormones, and is also involved in the innate immunity response. Hemolymph proteins are synthesized by fat body cells and then secreted into the hemolymph in a time-dependent manner (Tojo et al. 1980; Tomino 1985; Kishimoto et al. 1999). Several abundant hemolymph proteins have been studied to elucidate their role in silkworm development, including lipophorin, vitellogenin, storage proteins and 30K proteins.

Lipophorin is involved in lipid transport in insects (Chino et al. 1981). It has a native molecular weight of 730 kDa and is composed of three proteins: apolipophorin I–III. Apolipophorin-I (250 kDa) and apolipophorin-II (90 kDa) are necessary components of lipophorin from the fifth instar larval to the adult stage (Kim and Kim 1994), whereas apolipophorin-III (19 kDa) can exist in association with lipophorin or free in the hemolymph. Vitellogenin of *B. mori* is a tetramer with molecular weight of 440 kDa, composed of two heavy chains (178 kDa) and two light chains (42 kDa) (Izumi et al. 1980). Vitellogenin in hemolymph increases

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from the spinning stage and until it is taken up by the developing oocytes to form yolk granules during the pupal stage (Yano et al. 1994). The storage protein of *B. mori* is a hexamer (450–500 kDa), consisted of six subunits of 75–85 kDa (Tojo et al. 1980; Telfer and Kunkel 1991; Terwilliger 1999). Storage protein 1 and storage protein 2 showed similar developmental profiles in the hemolymph: increasing from the fifth instar, reaching maximal levels at the time of spinning, and then declining (Tojo et al. 1980). The storage proteins are considered to be absorbed into fat body cells and used as source of amino acids during the pupal stage. *B. mori* 30K proteins contain 24 typical 30K proteins and 12 serine/threonine-rich 30K proteins (Zhang et al. 2012a). They all belong to the Lipoprotein_11 family. 30K protein levels increase progressively in the hemolymph from the fifth instar to the spinning stage, and then they are transported into the oocyte for forming yolk granules (Zhang et al. 2012b).

Based on the *B. mori* genome database (International Silkworm Genome Consortium 2008; Xia et al. 2004), two-dimensional electrophoresis combined with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used to explore the hemolymph proteome (Li et al. 2006, 2012a; Kajiwara et al. 2009; Hou et al. 2010). However, these studies focus on the hemolymph of the final instar larvae or pupae. In the present study, the hemolymph proteome of fourth instar larvae was identified using shotgun liquid chromatography–tandem mass spectrometry (LC–MS/MS), and compared to that of the fifth instar by label-free quantification. Various and abundant nutrient-storage proteins and immunity-related proteins were analyzed, improving our understanding of physiological and pathological functions of hemolymph.

Materials and methods

Hemolymph collection and electrophoresis

Bombyx mori strain p50 (DaZao) were reared on mulberry leaves at 25 °C. The hemolymph was collected from the cuts of larval abdominal legs. The hemolymph was dropped into an ice-cooled Eppendorf tube, which contained a few crystals of phenylthiourea to inhibit melanization. The material was then centrifuged for 20 min at 4,000*g* at 4 °C to remove hemocytes. The supernatant was frozen and stored at −80 °C. Hemolymph was collected from six different development time points (day 2 and 4 of the fourth instar and day 1, 3, 5 and 7 of the fifth instar). Protein concentrations in the supernatant were determined by the Bradford method (Bradford 1976). Equal amounts of hemolymph proteins were separated on 12.5 % (w/v) polyacrylamide gel and visualized by staining with coomassie brilliant blue.

Protein digestion and liquid chromatography–tandem mass spectrometry

Hemolymph proteins were digested as follows according to the Filter-Aided Sample Preparation (FASP) method (Wisniewski et al. 2009). The cell-free hemolymph was diluted 5 times with 20 mM PBS (pH 7.4). 500 μg hemolymph proteins in PBS were place in an ultrafiltration tube (MWCO 3,000, Millipore, USA), and then reduced with 10 mM dithiotreitol (DTT) for 150 min at 37 °C, and then alkylated with 50 mM iodoacetamide (IAA) for 40 min in the dark. After washing twice with 8 M urea and then twice with 50 mM NH_4HCO_3 in the ultrafiltration tube, proteins were incubated with trypsin (1/50 μg protein) overnight at 37 °C in 150 μL 50 mM NH_4HCO_3. Tryptic peptides were collected by centrifugation and concentrated to dry. Dried peptides were resuspend in 0.1 % formic acid, and then separated using the Thermo Fisher Scientific EASY-nLC 1000 system and EASY-Spray column (C18, 2 μm, 100 Å, 75 μm × 50 cm) with a 2–100 % acetonitrile gradient in 0.1 % formic acid over 180 min at a flow rate of 250 nL/min. The separated peptides were analyzed using a Thermo Scientific Q Exactive mass spectrometer operating in data-dependent mode. Up to 20 of the most abundant isotope patterns with charge ≥2 from an initial survey scan were automatically selected for fragmentation by higher energy collisional dissociation with normalized collision energies of 27 %. The maximum ion injection times for the survey scan and the MS/MS scans were 20 and 60 ms, respectively, and the ion target value for both scan modes was set to 1E6. A dynamic exclusion of ions sequenced previously within 18 s was applied.

Protein identification and quantification

Raw MS files were converted into Mascot generic peak lists by MaxQuant version 1.3.0.1 (Cox and Mann 2008). Proteins were identified by searching peak lists containing MS/MS spectra using the Andromeda search engine against an integrated silkworm proteome database, containing 22,117 protein sequences from NCBI and silkDB (Xia et al. 2004; Duan et al. 2010; Cox et al. 2011). The search parameters for protein identification specified the initial precursor and fragment mass tolerances of 6 and 20 ppm, respectively. The first search tolerance was set to 20 ppm, followed by a main search tolerance of 6 ppm. HCD fragment ion mass tolerance was set to 20 ppm. Carbamidomethylation of cysteine was set as fixed modification and N-terminal protein acetylation and methionine oxidation as variable modifications. The minimal peptide length was set to six amino acids and up to two miscleavages were allowed. The false discovery rate (FDR) was set to 0.01 for peptides and proteins. Protein table was filtered to

eliminate the identifications from the reverse database and common contaminants. A minimum of one unique peptide was required for protein identification.

To compare the abundances of different proteins within a sample, we used the intensity-based absolute quantification (iBAQ) algorithm, which normalizes the summed peptides intensities identifying a protein by the number of theoretical peptides (Schwanhausser et al. 2011). In contrast, to compare protein abundances across different samples, we used label-free quantification (LFQ) algorithm, which compares the intensities of the same peptides detected in different samples (Luber et al. 2010). A minimum of two ratio counts and unique + razor peptides was set for LFQ.

Protein annotation

Protein annotation was based on predictions from Blast2GO (Conesa et al. 2005). BLASTp searches were done against the non-redundant database with an expectation value maximum of 1E3. The systematical names of serine proteases, serine protease inhibitors, 30K proteins and ENF peptides' binding proteins were annotated according previous reports (Zhao et al. 2010, 2012; Zhang et al. 2012a).

Results

Identification of larval hemolymph proteins

The developmental profile of hemolymph proteins in the fourth and fifth instars showed significant differences by SDS-PAGE (Fig. 1a). The intensely stained bands were annotated according to previous reports (Tsuchida et al. 2010; Hou et al. 2010; Zhang et al. 2012b). Apolipophorin-I was abundant at the end of the fourth larval instar and at the start of the fifth larval instar. The storage proteins showed higher abundance in the late days of each instar than in the early days. The 30K proteins were abundant only after day-4 fifth instar. These results indicated that substantial differences in the hemolymph were occurring between the fourth and fifth larval instars.

Liquid chromatography–tandem mass spectrometry was employed to reveal the detailed differences in the proteome of hemolymph proteins between the fourth instar and the fifth instar. Analysis was performed in duplicate on hemolymph proteins from day-4 of the fourth instar (IV-4) and day-7 of the fifth instar (V-7). In total, 5,992 tryptic peptides, which assembled to 752 proteins, were identified (Supplementary Tables 1 and 2). The majority of proteins (477 proteins, 63 %) identified were expressed in both day-4 fourth instar and day-7 fifth instar. There were fewer unique proteins in hemolymph from day-7 fifth instar (50 proteins) than from day-4 fourth instar (225 proteins) (Fig. 1b).

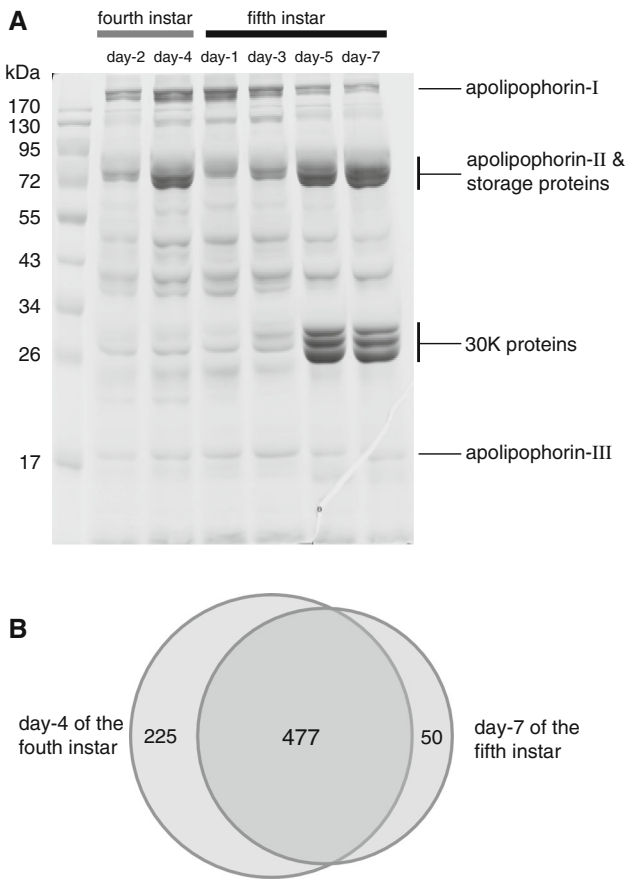

Fig. 1 Electrophoresis and identification of hemolymph protein. Hemolymph protein was collected from six different development stages, including day-2, day-4 fourth instar and day-1, -3, -5, -7 fifth instar. Equal amounts of hemolymph proteins were separated on 12.5 % (w/v) polyacrylamide gel and visualized by staining with coomassie brilliant blue (**a**). High-abundance proteins are indicated by *lines*, including apolipophorins, storage proteins, and 30K proteins. 702 proteins and 502 hemolymph proteins were identified in hemolymph from day-4 fourth instar and day-7 fifth instar, respectively (**b**). 477 proteins were shared by both day-4 fourth instar and day-7 fifth instar

Gene ontology of larval hemolymph proteins

The Blast2GO analysis tool was used to predict the function of the identified hemolymph proteins. The majority of the sequences (671 proteins, 89 %) were assigned at least one GO term. Of these, 587 (78 % of the total) proteins could be predicted to be associated with molecular functions. In both day-4 fourth instar and day-7 fifth instar proteomes, binding (GO: 0005488) and catalytic activity (GO: 0003824) were the two major molecular function categories predicted (Fig. 2a). Predicted binding activity included protein binding (GO: 0005515), ion binding (GO: 0043167), small molecule binding (GO: 0036094), nucleic acid binding (GO: 0003676), carbohydrate binding (GO: 0030246), and lipid binding (GO: 0008289) (Fig. 2b). Predicted catalytic activity included hydrolase activity (GO: 0016787, EC3),

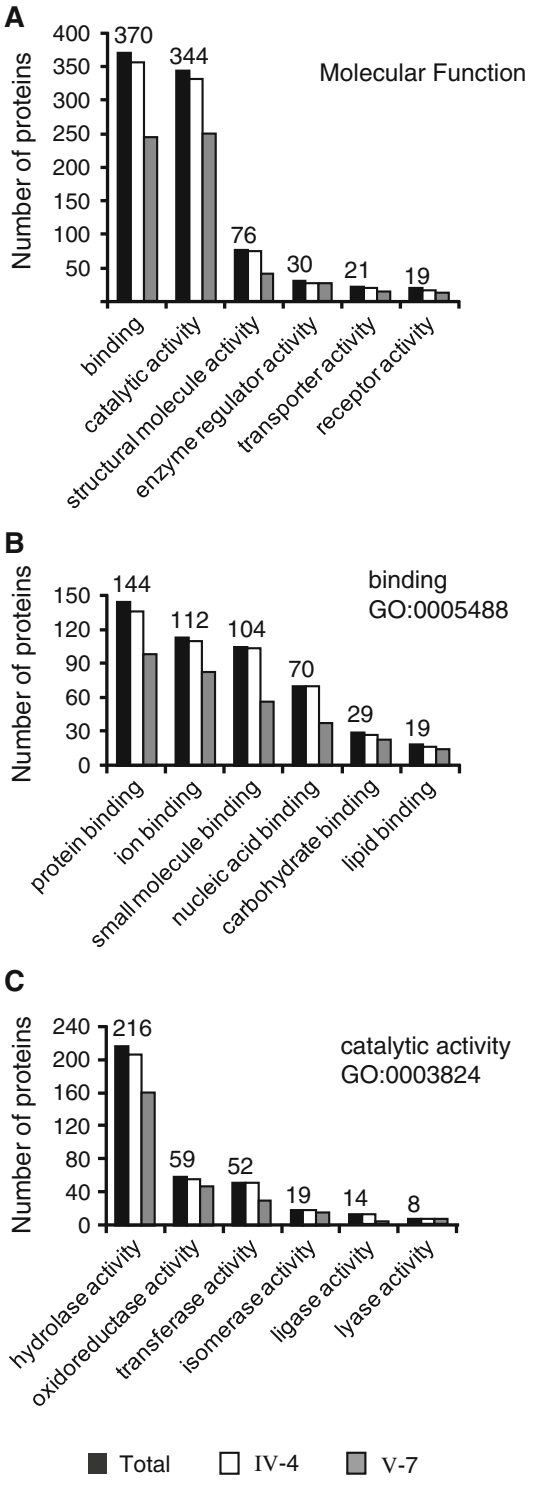

Fig. 2 Protein categorization by gene ontology. Six largest terms from third level of molecular function are listed (**a**). Binding (**b**) and catalytic activity (**c**) sub-terms from fourth level of Molecular Function are respectively unfolded. *Bars* represent the number of proteins in each term. The total proteins, proteins from day-4 fourth instar, and day-7 fifth instar were respectively indicated by *black* (with number of proteins for that term), white, and *gray bars*

oxidoreductase activity (GO: 0016491, EC1), transferase activity (GO: 0016740, EC2), isomerase activity (GO: 0016853, EC5), ligase activity (GO: 0016874, EC6), and lyase activity (GO: 0016829, EC4) (Fig. 2c).

Highly expressed larval hemolymph proteins

To estimate the abundance of proteins within a proteome, we used the iBAQ algorithm, which normalizes the summed peptide intensities by the number of theoretically observable peptides of the protein (Schwanhausser et al. 2011). The 20 most highly expressed hemolymph proteins from day-4 fourth instar included 6 nutrient-storage proteins (apolipophorins, storage proteins and 30K proteins) and 6 protease inhibitors (from families of TIL, serpin, kunitz, kazal, ITI, and *L. obliqua* protease inhibitor) (Fig. 3a). The 20 most highly expressed proteins from day-7 fifth instar included 8 nutrient-storage proteins (apolipophorins, storage proteins and 30K proteins) and 6 protease inhibitors (from families of TIL, serpin, kunitz, kazal, and amfpi) (Fig. 3b). Other abundant proteins included imaginal disc growth factor, lysozyme, aldo-keto reductase, cuticular protein, chemosensory protein, transferrin, fibroin p25, and some proteins with, as yet, unknown function.

Quantitative differences of nutrient-storage proteins in larval hemolymph

Electrophoretic separation of hemolymph proteins revealed some striking differences in the levels of the nutrient-storage proteins between the fourth and fifth instar (Fig. 1a). To more rigorously quantify these differences in protein abundance, we used label-free quantification. For this analysis, the MS signals of the same peptide detected in different samples were quantified relative to each other (Fig. 4). The results suggested that there was no significant difference in abundance of apolipophorin-I, -II, -III and storage protein-1, -2, -3 between day-4 fourth instar and day-7 fifth instar. Storage protein-4 and -5 were, respectively, IV-4-specific and V-5-specific proteins, but their abundances were much lower than that of storage protein -1, -2 and -3. The three storage proteins, -3, -4 and -5, were first identified and named by this study. This study identified 23 30K proteins in hemolymph: 14 from day-4 fourth instar and 16 from day-7 fifth instar. Nine of the 30K proteins were unique to the day-7 fifth instar (Typical 30K proteins, BmLP-1, -2, -3, -4, -5, -6, -7, -9, and -14), whereas seven were unique to day-4 fourth instar (include six serine/threonine-rich 30K proteins BmLP-26, -28, -30, -32, -35, and -36). The abundance of 30K proteins was about five fold higher in day-7 fifth instar than in day-4 fourth instar (Supplementary Table 2).

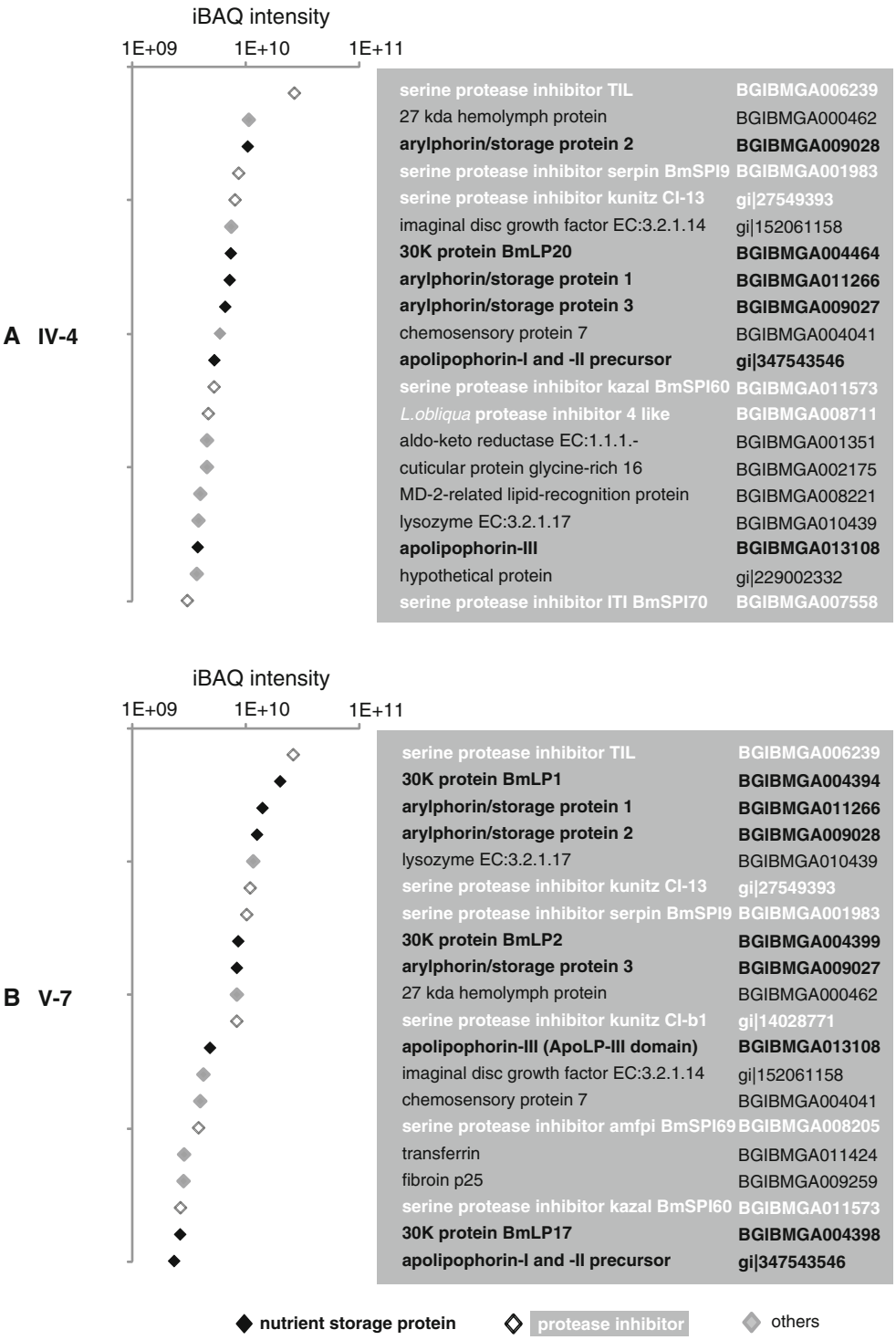

Fig. 3 Twenty most abundant proteins in *B. mori* hemolymph from day-4 fourth instar (**a**) and day-7 fifth instar (**b**). The abundance value of each protein was estimated as iBAQ intensity. *Colors* show the protein category: nutrient-storage protein, *black*; protease inhibitor, *white*; other protein, *gray*. "BGIBMGA" are nomenclature of *B. mori* proteins deposited at website: http://silkworm.genomics.org.cn/, and "gi" are accession number of *B. mori* proteins deposited in Genebank

Identification and quantitation of immunity-related proteins in larval hemolymph

Beside nutrient-storage proteins, protease inhibitors are also expressed very highly in hemolymph, and play important roles in immune defense. To reveal the overall functional network of the immune components in hemolymph, a proteome-wide identification of immunity-related proteins was performed based on previous reports (Tanaka et al. 2008; Tanaka and Yamakawa 2011; Wan et al. 2013). Totally, 85 proteins were identified, including recognition (18), signaling (22), effectors (16) and other immune molecules (29)

nutrient storage proteins

1	apolipophorin I and II precursor	gi|347543546
2	apolipophorin-III	BGIBMGA013108
3	arylphorin/storage protein 1	BGIBMGA011266
4	arylphorin/storage protein 2	BGIBMGA009028
5	arylphorin/storage protein 3	BGIBMGA009027
6	arylphorin/storage protein 4	BGIBMGA008860
7	arylphorin/storage protein 5	BGIBMGA008859
8	30K protein BmLP1	BGIBMGA004394
9	30K protein BmLP2	BGIBMGA004399
10	30K protein BmLP3	BGIBMGA004396
11	30K protein BmLP4	BGIBMGA004395
12	30K protein BmLP5	BGIBMGA004456
13	30K protein BmLP6	BGIBMGA004457
14	30K protein BmLP7	BGIBMGA004397
15	30K protein BmLP9	BGIBMGA004400
16	30K protein BmLP12	BGIBMGA004405
17	30K protein BmLP13	BGIBMGA004454
18	30K protein BmLP14	BGIBMGA004404
19	30K protein BmLP17	BGIBMGA004398
20	30K protein BmLP19	BGIBMGA004463
21	30K protein BmLP20	BGIBMGA004464
22	30K protein BmLP21	BGIBMGA004465
23	30K protein BmLP23	BGIBMGA004467
24	30K protein BmLP24	BGIBMGA004468
25	30K protein BmLP26	BGIBMGA004473
26	30K protein BmLP28	BGIBMGA004474
27	30K protein BmLP30	BGIBMGA004385
28	30K protein BmLP32	BGIBMGA004476
29	30K protein BmLP35	BGIBMGA014098
30	30K protein BmLP36	BGIBMGA014171

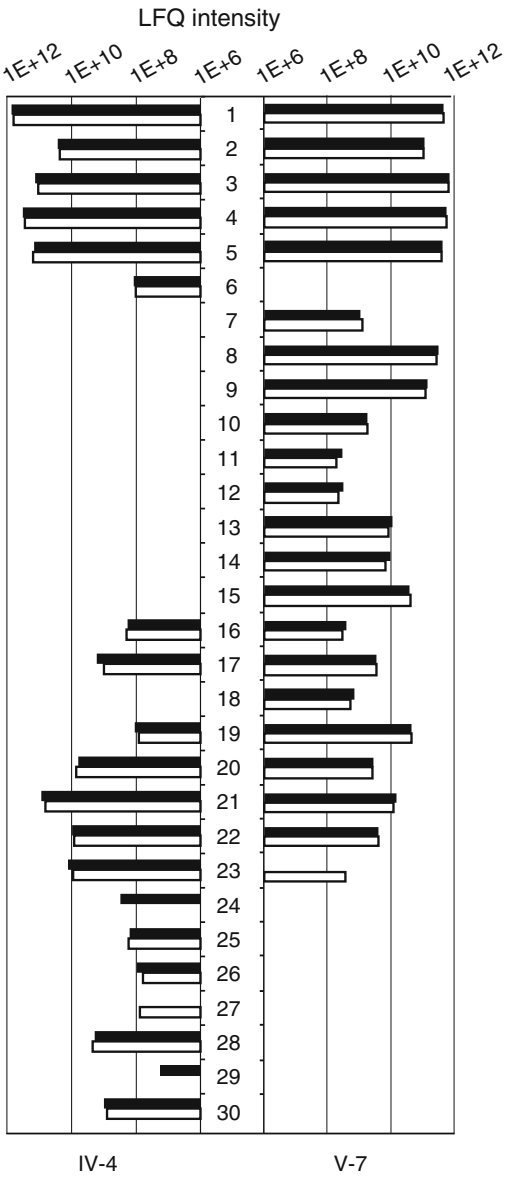

Fig. 4 Identification and quantitation of nutrient-storage proteins in hemolymph from day-4 fourth instar (IV-4) and day-7 fifth instar (V-7). Label-free quantification (LFQ) intensity was used to show the protein abundance. Nutrient-storage proteins include apolipophorins, storage proteins and 30K proteins. Duplicates are shown in *black* and *white bars*

(Fig. 5a, b). Recognition proteins include beta-1, 3-glucan recognition protein, peptidoglycan recognition protein, C-type lectin, hemocytin, lipopolysaccharide-binding protein and thioester-containing protein. Signaling proteins include clip serine protease, serpin-type serine protease inhibitor, ENF peptides and ENF peptides-binding proteins. Effectors consisted of prophenoloxidase, lysozyme, and different antimicrobial peptides (attacin, cecropin, defensin, gloverin and lebocin). Other immune-related proteins mainly include antioxidant enzymes (catalase, peroxidase, peroxiredoxin, superoxide dismutase) and protease inhibitors (TIL-type, kazal-type, amfpi-type, ITI-type, WAP-type, and kunitz-type serine protease inhibitors, cysteine proteinase inhibitor, carboxypeptidase inhibitor and *L. obliqua* protease inhibitor-like protein). Label-free quantification result suggested that the abundance of recognition, signaling, effectors and other immune molecules in day-7 fifth instar hemolymph were respectively 1.3-, 2.2-, 2.7- and 1.7-fold higher than in day-4 fourth instar hemolymph. Thirty-one immunity-related proteins were stage-specific proteins, either expressed only in day-4 fourth instar or expressed only in day-7 fifth instar. However, 87 % of them (27) were of low abundance (<1E + 09), and 77 % of them (24) lacked repetition (Fig. 5), indicating that proteins of low abundance are more challenging to quantify accurately by label-free MS analysis.

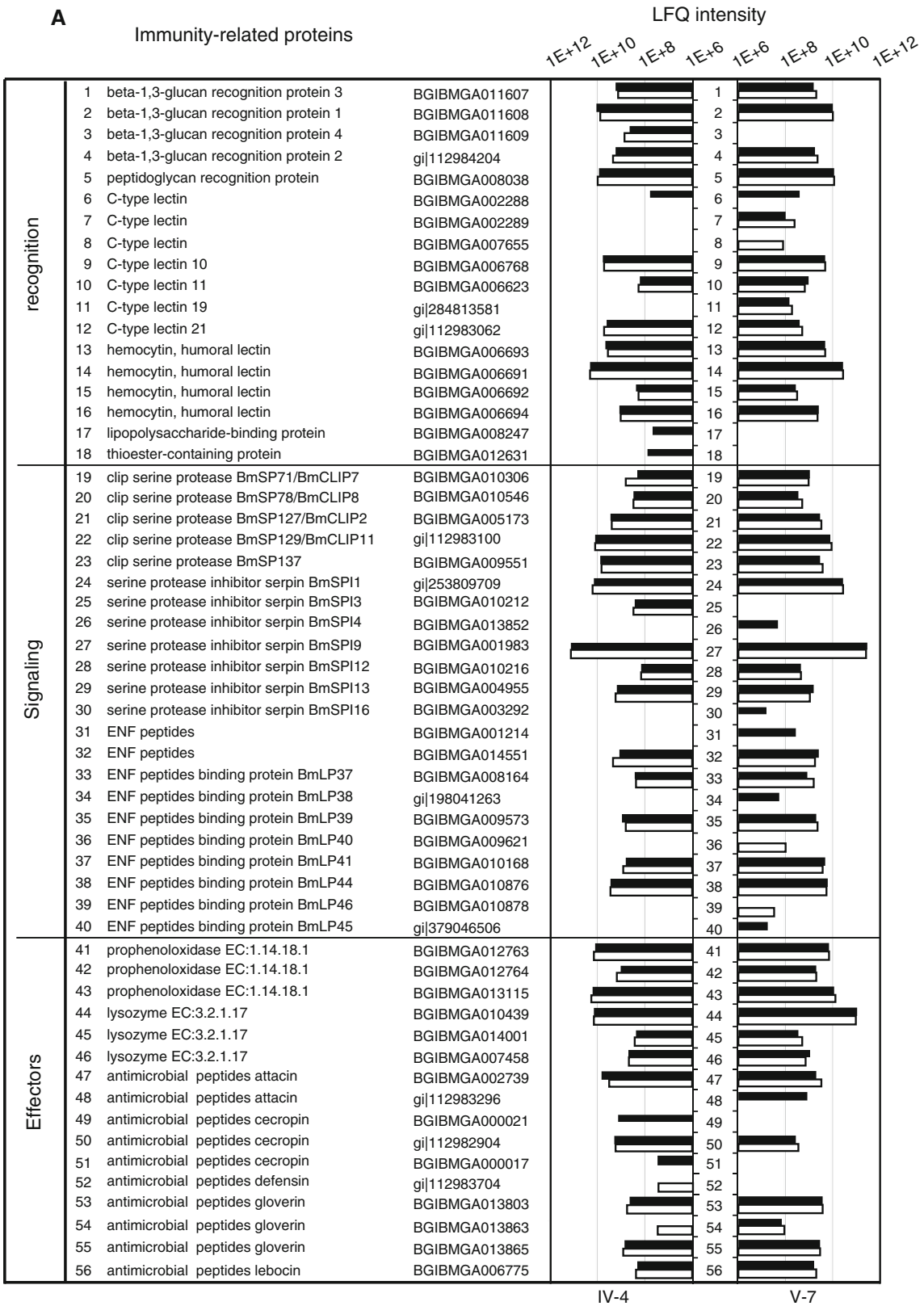

Fig. 5 Identification and quantitation of immunity-related proteins in hemolymph from day-4 fourth instar (IV-4) and day-7 fifth instar (V-7). Label-free quantification (LFQ) intensity was used to show the protein abundance. Identified immunity-related proteins include recognition protein, signaling proteins, effectors (**a**), and other proteins (**b**). Duplicates are shown in *black* and *white bars*

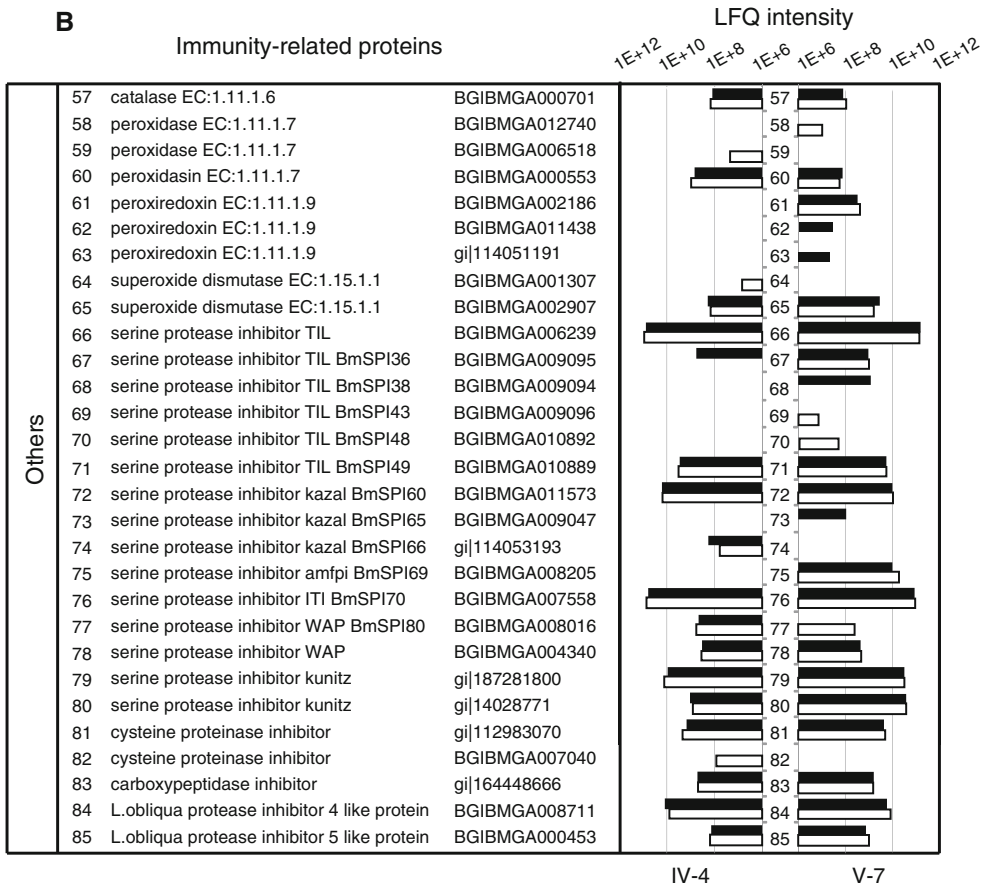

Fig. 5 continued

Discussion

Bombyx mori is the model insect of lepidoptera for molecular studies. Larvae of the lepidopteran insects are called caterpillars. Many caterpillars are considered to be pests, because of the damage they inflict on crop plants and agricultural produce. Caterpillars are voracious feeders being required to eat a lot to store nutrients for metamorphosis and adult life. This study describes, for the first time, the proteome of hemolymph from fourth instar silkworm and compares this with the hemolymph proteome from the final larval instar. The comparison reveals various proteins associated with nutrient storage in caterpillar hemolymph. In addition, multiple humoral and cellular immunity pathways were identified, providing a global understanding of the defense system in caterpillar hemolymph.

Nutrient-storage and transport proteins in larval hemolymph of *B. mori*

Previous studies found four kinds of nutrient-storage proteins in silkworm, including apolipophorin, vitellogenin, storage proteins and 30K proteins. This study suggested that different nutrient-storage proteins were needed to meet nutrient requirement in different developmental stages: apolipophorin-I, -II, -III and storage protein -1, -2, -3 are produced with similar amount in day-4 fourth instar and day-7 fifth instar larvae; vitellogenin was not detected in caterpillar hemolymph in this study, indicating that it may emerge after the wandering stage (Lin et al. 2013); 30K proteins are at low abundance in the fourth instar, but their levels significantly increase in the fifth instar, consistent with previous microarray results (Zhang et al. 2012a). The 16 30K proteins identified in the fifth instar belong to the typical 30K protein subfamily, while the 6 30K proteins expressed exclusively in the fourth instar are from the serine/threonine-rich subfamily (Zhang et al. 2012a).

Some of nutrient-storage proteins are predicted to transport carbohydrates and lipids. Lipophorins could carry lipids from sites of synthesis to sites of storage and utilization (Chino et al. 1981). Storage proteins contain 4.8–6.9 % (w/w) of carbohydrate (Kim et al. 2003). Vitellogenins are lipoglycoproteins with 7–16 % lipid and 1–14 % carbohydrate (Urich 1994). 30K proteins contain an N-terminal lipid-binding domain and a C-terminal sugar-binding domain (Yang et al. 2011). These nutrient-

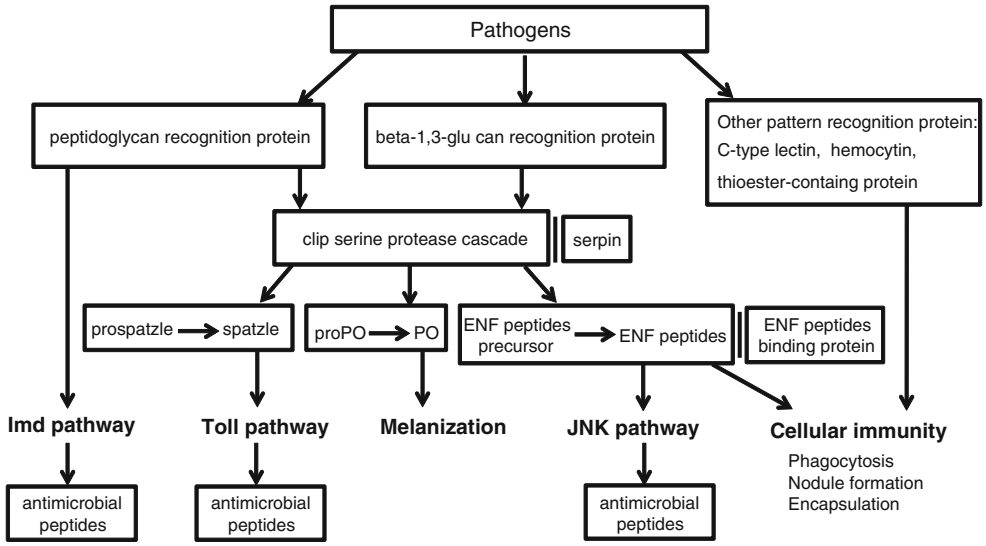

Fig. 6 Pathways of immune responses in *B. mori* hemolymph. This figure presents both humoral immunity pathways (Imd pathway, Toll pathway, Melanization, JNK pathway) and cellular immunity pathways in hemolymph. PO represents phenoloxidase. Serpins act as regulators of Clip serine proteases, and ENF peptides-binding proteins act as regulators of ENF peptides

storage proteins may not only provide amino acids source, but also transport essential carbohydrates and lipids to sites as required for development and metamorphosis.

Immunity pathways in larval hemolymph of *B. mori*

This study presents both humoral and cellular immunity pathways in hemolymph (Fig. 6). It is well known that the activation of the Toll pathway is dependent on the proteolytic cleavage of the spaetzle (Jang et al. 2006), and the JNK pathway requires activated ENF peptides (Tsuzuki et al. 2012), while the melanization pathway relies on activated prophenoloxidase (Cerenius and Soderhall 2004) (Fig. 6). All these activations are based on the extracellular cascade reaction of clip serine protease, which could rapidly amplify the immune signal after pathogens are recognized by peptidoglycan recognition protein (PGRP) and beta-1,3-glucan recognition protein (βGRP) (Tanaka and Yamakawa 2011) (Fig. 6). This study identified five clip serine proteases in the larval hemolymph, along with seven serpins that are potential inhibitors of them (Fig. 5a). These activated humoral responses finally lead to melanization (deposition of melanin onto pathogens to kill them) and the activation of intracellular signaling pathways that produce antimicrobial peptides (Fig. 6). Many antimicrobial peptides (attacin, cecropin, defensin, gloverin, and lebocin) are found at low concentrations in the normal hemolymph (Fig. 5a). The levels of these peptides are significantly induced to reach high concentration after being infected with pathogens.

Some pattern recognition proteins that elicit cellular immunity reactions have been identified in *B. mori*, including C-type lectins, hemocytins, and thioester-containing protein (Figs. 5a, 6). C-type lectins are involved in hemocyte nodule formation and opsonization (Koizumi et al. 1999). Hemocytin could stimulate aggregation of hemocytes (Jomori and Natori 1992). Thioester-containing protein could promote phagocytosis by hemocytes (Levashina et al. 2001). ENF peptides are also involved in cellular immunity, stimulating hemocytes to spread on foreign surfaces, and activating hemocytic phagocytosis (Ishii et al. 2010). Eight ENF peptides-binding proteins were identified in this study, which were considered to act as regulators of ENF peptides.

Previous researches had found at least 16 serine protease inhibitors in *B. mori* hemolymph. They are named CI 1–13, because they show inhibitory activity against chymotrypsin (Zhao et al. 2012). This study identified 27 protease inhibitors, 22 of which are serine protease inhibitors. Serine protease inhibitors from the serpin family regulate immune response via inhibiting endogenous serine proteases, while other serine protease inhibitors are cysteine-rich proteins and show inhibitory activities against proteases from pathogens (Zhao et al. 2012), and may prevent the proliferation of pathogens (Li et al. 2012b).

Conclusions

The study revealed the proteome of *B. mori* hemolymph from day-4 fourth instar and day-7 fifth instar. Nutrient-storage proteins showed high abundance in the hemolymph, while immune-related proteins were numerous in the hemolymph. Comparative proteomics suggested that abundance of 30K proteins in the fifth instar was significantly higher than in the fourth instar, for meeting nutrient

requirement of pupa and moth. Various immune-related proteins comprise multiple humoral and cellular immunity pathways in the hemolymph.

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Conflict of interest The authors declare that they have no conflict of interest.

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