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ORIGINAL ARTICLE

Proteomic profiling of the hemolymph at the fifth instar of the silkworm *Bombyx mori*

Jian-Ying Li, Ji-Sheng Li and Bo-Xiong Zhong

College of Animal Sciences, Zhejiang University, Hangzhou, China

Abstract Two-dimensional gel electrophoresis (2-DE) followed by matrix-assisted laser desorption ionization – time-of-flight/time-of-flight mass spectrometry (MS) analysis were used to charaterize the hemolymph proteomic profiles of the silkworm, *Bombyx mori*. At days 4 (V4) and 5 (V5) of the fifth (final) instar, when the larvae were at the fast-growing stage, we found dramatic changes in spots representing proteins having an approximate molecular weight (MW) of 30 kDa. Of these spots, four 30K proteins were highly up-regulated, implying a close association with the growth and development of *B. mori* larvae. To understand the molecular basis and underlying mechanisms involved in development and metamorphosis, the proteome of whole hemolymph at V5 was analyzed using shotgun liquid chromatography tandem mass spectrometry with an LTQ-Orbitrap. A total of 108 proteins were identified without any false discovery hits. These proteins were involved in a variety of cellular functions, including metabolism, development, nutrient transport and reserve, and defense response. Gene ontology analysis showed that 3.4% of these proteins had nutrient reservoir activities and 5.7% were involved in the response to stimulus. Pathway analysis revealed that 22 proteins with common targets were involved in various cellular processes such as immunity, differentiation, proliferation and metamorphosis. These results suggested that some key factors such as the 30K proteins in hemolymph play important roles in *B. mori* growth and development. Moreover, the multiple functions of hemolymph may be operated by a complex biological network.

Key words *Bombyx mori*, gene ontology, hemolymph, LTQ-Orbitrap, pathway, proteomics

Introduction

Insect hemolymph, a complex mixture of proteins, lipids, carbohydrates, nucleic acids, hormones and their degradation products, is primarily responsible for supplying nutrients and transferring metabolic wastes to maintain normal growth and development. Moreover, it is also important for the insect innate immune system. Hemolymph cells perform host defense through phagocytosis and signaling and may employ a form of self–non-self recognition that is independent of microbial patterns (Brennan & Anderson, 2004).

The silkworm, *Bombyx mori*, is a holometabolous Lepidoptera insect. The final (fifth) instar phase of *B. mori* is a critical period for its growth, development, metamorphosis and productivity. Days 4 (V4) and 5 (V5) of the fifth instar are the very stages for *B. mori* fast-growing when the larvae eat a large quantity of mulberry leaves. Two groups of major plasma protein constituents, '30K proteins' and 'storage proteins' (SPs), are synthesized by the fat body cells and released into the hemolymph in a stage-specific manner during the final instar (Sakurai *et al*., 1988; Mori *et al*., 1991; Kishimoto *et al*., 1999). In some insects, the SPs are released in large quantities into the hemolymph during the active feeding period. Then the

Correspondence: Bo-Xiong Zhong, College of Animal Sciences, Zhejiang University, Hangzhou 310029, China. Tel/fax: +86 571 86971302; email: bxzhong@zju.edu.cn

SPs were selectively taken up by the fat body cells during metamorphosis to be stored as protein granules that are required for the development of adult tissues (Haunerland, 1996).

Due to the importance of hemolymph, its components and their functions are being actively researched in various insects. One of the major tools used in this field is proteomics, which has been applied to a wide range of organisms, including *Drosophila* (Levy *et al*., 2004), *Anopheles* (Paskewitz & Shi, 2005), *Bombyx* (Li *et al*., 2006; Zhou *et al*., 2008; Hou *et al*., 2010) and *Apis* (Bogaerts *et al*., 2009). Most of these studies were based on two-dimensional gel electrophoresis (2-DE), which is suitable for comparative proteomics analyses. In the present study, we compared the proteomic profiles of *B. mori* hemolymph at V4 and V5 using 2-DE followed by matrix-assisted laser desorption ionization – time-of-flight (MALDI-TOF)/TOF mass spectrometry (MS) identification. Furthermore, we characterized the proteome of whole hemolymph at V5 using the shotgun proteomics approach, which is based on peptide separation and identification using liquid chromatography tandem mass spectrometry (LC-MS/MS). This method is apposite for the large-scale identification of proteome components, by which we have been able to identify thousands of proteins in *B. mori* endocrine organs, embryos and heads (Li *et al*., 2009a, b; Li *et al*., 2010a, b). The characterized proteome profiles of hemolymph are expected to be useful for a comprehensive understanding of its functions and the underlying mechanisms of development and metamorphosis.

Materials and methods

Silkworm rearing and sample collection

Silkworm strain p50 was reared on fresh mulberry leaves under a 12 h light/12 h dark photoperiod at 26 ± 1°C with 70%–85% relative humidity. The developmental stages were synchronized after the fourth molt by collecting new larvae. The larvae began spinning after 7 days of the fifth instar. Whole hemolymph was collected by cutting the caudal leg of the *B. mori* at V4 and V5. The collected samples were immediately stored at –80°C for subsequent use.

Sample preparation, gel electrophoresis and image analysis

Sample preparation and separation for one-dimensional sodium dodecyl sulfate-polyacrylamide electrophoresis (1D SDS-PAGE) and 2-DE were carried out according to our previously described methods (Zhou *et al*., 2008; Li *et al*., 2009a, b). For the 2-DE, the separation was performed by isoelectric focusing (IEF) with a 24-cm immobilized pH gradient (IPG) strip (pH 3–10, linear) (Amersham Biosciences, Piscataway, NJ, US), followed by protein transfer to a 12.5% sodium dodecyl sulfate – palyacrylamide gel electrophoresis (SDS-PAGE) gel. The electrophoresed 1D and 2D gels were visualized by Coomassie Brilliant Blue R250 (CBB-R250, Sigma, St. Louis, MO, USA) and silver staining, respectively. Triplicate 2-DE replications were performed for each sample. The 2-DE images were analyzed with ImageMaster 2D software, v.6.0 (Amersham Biosciences, Uppsala, Sweden) for spot detection, spot matching and quantitative intensity evaluation. Differentially expressed proteins were regarded to have at least 2-fold changes on the spot intensity ratio.

In-gel digestion and MS analysis

The protein spots were excised from 2-DE gels and subjected to in-gel digestion followed by MALDI-TOF/TOF MS analysis according to our previous report (Zhou *et al*., 2008). The gel lane of 1D SDS-PAGE for the hemolymph proteins at V5 was cut into 14 bands according to the deepness of the Coomassie staining. The in-gel tryptic digestion was performed as described previously (Li *et al*., 2009b; Shevchenko *et al*., 2006). The digested peptides were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray tandem mass spectrometry. The Ettan MDLC nanoflow/capillary LC system (GE Healthcare, Pittsburgh, PA, USA) was equipped with a trapping column [PepMap C18, 300-μm i.d. × 5 mm, 3 μm, 100 Å (P/N 160454), Dionex, Sunnyvale, CA, USA] and a nanocolumn [PepMap C18, 75-μm i.d. × 15 cm, 3 μm, 100 Å (P/N 160321), Sunnyvale, CA]. The LC system was connected to an LTQ-Orbitrap mass spectrometer (Thermo Finnigan, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). The peptides were separated in 60-min gradients ranging from 5% to 60% of solvent B (84% acetonitrile, 0.1% methanoic acid in water). Data-dependent acquisition was performed on the mass spectrometer in the positive ion mode with the Xcalibur software version 2.0 (Thermo Electron, San Jose, CA, USA). The MS analysis was performed with one full MS scan (m/z 300–2000) followed by MS/MS scans for the five most intense ions with the following dynamic exclusion settings: repeat count 2, repeat duration 30 s, and exclusion duration 180 s. Collision-induced dissociation (CID) was conducted with a normalized collision energy of 35% for MS/MS.

Database search

The resulting files from MALDI-TOF/TOF MS detection were subjected to the MASCOT search engine (version 2.0; Matrix Science, London, UK) with GPS Explorer software (Version 3.0; Applied Biosystems, Foster City, CA, USA) against the National Center for Biotechnology Information NCBInr nucleotide database for peptide and protein identifications. The in-house database for the query of RAW files from LC-MS/MS was the same as described previously (Li *etal*., 2010a) and comprised predicted *B. mori* protein sequences from the newly assembled silkworm genome (14 623 entries) (International Silkworm Genome Consortium, 2008) as well as the known *Bombyx* (1 510 entries) and *Drosophila* (20 735 entries) proteins from the NCBI Refseq (Reference Sequence) database. The raw data from LC-MS/MS were searched against the in-house database with X!Tandem (http://www.thegpm.org/TANDEM/instructions.html). The mass tolerances of precursor and fragmentation ions were set to 1.2 Da and 0.1 Da, respectively. Two missing cleavage sites were allowed. Static modification on cysteine and variable modifications on methionine were set. The database search results were subjected to the Trans-Proteomic Pipeline (TPP, v4.0 JETSTREAM rev 2) for further validation with a ProteinProphet probability threshold of 0.9 (Keller *et al*., 2005). Moreover, at least two peptides were required to identify a protein. To evaluate the credibility of these identifications, the false discovery rate (FDR) was calculated by searching against the combined forward and reverse database (Li *et al*., 2010a,b; Elias & Gygi, 2007).

Bioinformatics analysis

The identified proteins were classified into Cellular Component, Molecular Function, and Biological Process according to their annotations by searching against the Gene Ontology (GO) database (http://www.geneontology.org) (Gene Ontology Consortium, 2008). To understand the interaction networks related to these proteins and their functions, a pathway analysis was carried out with the Pathway Studio 7.0 software (Ariadne Genomics, Rockville, MD, USA) against the latest attached *Drosophila* database (Nikitin *et al*., 2003).

Results

2-DE patterns of the hemolymph proteomes

The V4 and V5 are the periods of rapid growth for *B. mori* larvae, with the body weight increasing by approximately 50% in one day (data not shown). A comparison of the 2-DE proteome patterns of the hemolymph on these 2 days revealed 155 ± 12 and 148 ± 8 spots with a 74.5% match ratio average. The protein signal intensities around MW 30 kDa were dramatically changed (Fig. 1). Several proteins between p*I* 5–7 were so highly expressed at V5 that they could not be separated well by 2-DE, while the intensities of four protein spots between p*I* 3.5–4.5 were decreased significantly. In contrast, there were no obvious differences in the area of MW > 40 kDa. Four spots of proteins that had a sharp increase in their expression from V4 to V5 were successfully identified by MALDI-TOF/TOF (Fig. 1, Table 1), and all were members of the 30K protein family with different p*I*s and MWs. Based on their relative intensity ratios, the expression levels of these proteins increased over two-fold in 1day.

Whole hemolymph proteome identification

To acquire comprehensive insight into the molecular basis of the hemolymph, the proteome of whole hemolymph at V5 was characterized using shotgun LC-MS/MS analysis. A total of 108 proteins were identified with a minimum ProteinProphet probability of 0.9 and without a false discovery hit. Partial identifications are presented in Table 2. These proteins were involved in a variety of cellular functions, including metabolism, development, transport and reserve, and the defense response. This list also included one of the 30K proteins identified by MALDI-TOF/TOF.

GO annotation

The GO annotation analysis of the proteins identified in this study contributed to a better understanding of their locations and functions. Of the 108 identified proteins, 95 were classified into the Cellular Component, Molecular Function, and Biological Process categories using the available GO annotations (Fig. 2). Most of the proteins had an intracellular localization, including 29 from organelles and three from the membrane. Approximately 50% and 30% of proteins showed binding and catalytic activities, respectively. In addition, 4.5% and 3.4% of proteins had the transporter and nutrient reservoir activities, respectively. Five proteins were involved in the response to stimulus, including the antimicrobial responses. Furthermore, two proteins were related to the molting cycle.

Biological networks

There were 78 matched entries to the *Drosophila* database included in the Pathway Studio software using the tBLASTn (Basic Local Alignment Search Tool that

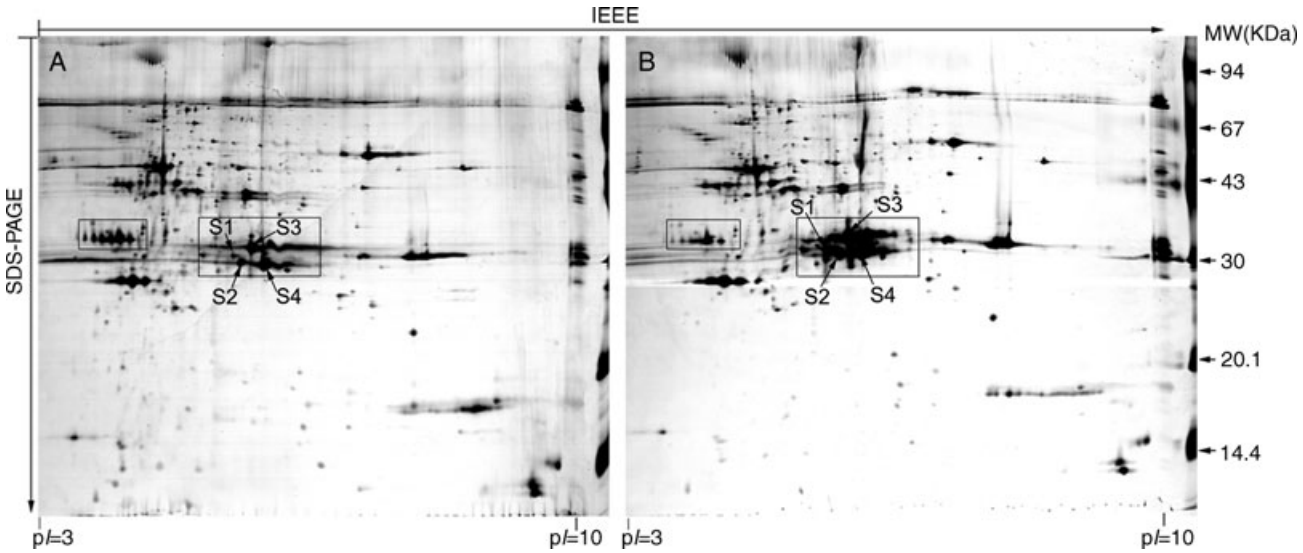

Fig. 1 Proteomic 2-DE patterns of the *Bombyx mori* hemolymph at V4 (a) and V5 (b). Hemolymph proteins were first separated by isoelectric focusing (IEF) with 24-cm immobilized pH gradient (IPG) strips and then transferred to 12.5% sodium dodecyl sulfate – palyacrylamide gel electrophoresis (SDS-PAGE) gels. The protein spots were visualized by silver staining. The four marked proteins were greatly up-regulated at V5. The intensities of the protein spots in the rectangular areas changed dramatically and four of these proteins were successfully identified.

Table 1 Significantly up-regulated proteins at V5 identified with MALDI-TOF/TOF followed by MASCOT database searching.

Spot	Protein name	Accession no.	Score	CI (%)	Peptides matched	Sequence coverage (%)	Theoretical p*I*/MW (kDa)	Intensity ratio (%)	
								V4	V5
S1	Mature 30K lipoprotein	gi|1335608	142	100	12	44.1	6.4/28.4	0.25	0.79
S2	Low molecular 30 kda lipoprotein PBMHP-6 precursor	gi|112984502	118	100	14	70.6	6.1/29.7	0.64	2.01
S3	Low molecular 30 kda lipoprotein PBMHPC-19	gi|156119322	108	100	13	51.7	6.9/29.2	1.21	2.50
S4	Low molecular 30 kda lipoprotein PBMHP-12 precursor	gi|156119320	111	100	11	42.5	6.8/30.0	2.10	4.32

searches translated nucleotide database using a protein query) results. Using the criterion of at least two proteins being involved in the same biological process, 22 proteins were determined to have common targets (Fig. 3). Three proteins, peptidoglycan recognition protein (PGRP-SA), profilin (chic) and plexin A (plexa), localized to the membrane and might play important roles in cell signaling. PGRP-SA and chic are involved in the cell immune response, and plexa and chic are involved in protein migration. Five proteins, chic, alpha-tubulin (alphatub84b), cytosolic malate dehydrogenase (cg5362), abnormal wing disc-like protein (awd), and ribosomal protein L23A (rpl23a), were multifunctional, participating in more than five biological processes. For example, awd is involved in larval development, metamorphosis, maturation, differentiation and other processes (Timmons *et al*., 1993; Dammai *et al*., 2003). In addition, glyceraldehyde-3-phosphate dehydrogenase (gapdh2) was located in mitochondria, and protein disulfide isomerase (pdi) was located in the endoplasmic reticulum. Seven

Table 2 Proteome identifications of the *Bombyx mori* hemolymph at V5.

Protein best name	Protein description	Protein prob.	# Pep	AA coverage (%)	Biological process	Molecular function
Metabolism[§]						
BGIBMGA000672-PA	Knockdown, isoform A[†]	0.995	2	5.2	Cellular carbohydrate metabolic process	Citrate (si)-synthase activity
BGIBMGA000715-PA	Cg12262[†]	0.988	3	7.8	Cardiac muscle cell differentiation	Acyl-coA dehydrogenase activity
BGIBMGA001876-PA	Amylase proximal[†]	1.000	7	6.1	Lipid metabolic process	Phospholipase A1 activity
BGIBMGA002493-PA	CG10576, isoform A[†]	0.999	4	11.3	Cellular process	Aminopeptidase activity
BGIBMGA004965-PA	Ugt86De[†]	1.000	6	8.2	Metabolic process	Glucuronosyltransferase activity
BGIBMGA007558-PA		1.000	19	12.2	Hyaluronan metabolic process	Peptidase inhibitor activity
BGIBMGA012309-PA	Cg6287[†]	1.000	5	7.9	L-serine biosynthetic process	NAD or NADH binding
BGIBMGA013131-PA		1.000	16	31.9	Glycolysis	Fructose-bisphosphate aldolase activity
BGIBMGA014181-PA	Thiolase[†]	1.000	4	6.7	Metabolic process	Binding
BGIBMGA014204-PA		0.969	2	4.5	CTP biosynthetic process	ATP binding
gi|112983816	Glyceraldehyde-3-phosphate dehydrogenase	1.000	7	15.4	Glycolysis	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity
gi|114050855	Triacylglycerol lipase	0.999	3	7.5	Ethanol oxidation	Binding
gi|114051770	26S proteasome non-ATPase regulatory subunit 13	1.000	8	9.6	Proteolysis	Endopeptidase activity
gi|114051866	Isocitrate dehydrogenase	1.000	6	9.6	Glyoxylate cycle	Isocitrate dehydrogenase ($NADP^+$) activity
gi|114052072	H+ transporting ATP synthase beta subunit isoform 1	1.000	5	11.1	ATP synthesis coupled proton transport	Hydrogen ion transporting ATP synthase activity, rotational mechanism
gi|114052278	ATP synthase	0.997	3	4.3	Chitin catabolic process	Cation binding
gi|114052488	Alcohol dehydrogenase	0.905	2	2.4	Fatty acid beta-oxidation	Acetyl-CoA C-acyltransferase activity
gi|114052561	Cytosolic malate dehydrogenase	1.000	6	11.5	Cellular carbohydrate metabolic process	Binding
gi|114052607	SUMO-1 activating enzyme	0.953	2	4.4	Pentose-phosphate shunt	Transaldolase activity
gi|114052613	Transaldolase	1.000	4	9.3	One-carbon metabolic process	Adenosylhomocysteinase activity
gi|114052677	Phosphoserine aminotransferase	0.988	4	5.5	L-serine biosynthetic process	Catalytic activity
gi|114053191	Glycyl-trna synthetase	0.931	3	1.3	Carbohydrate metabolic process	Alpha-amylase activity
gi|114053313	GTP binding protein	1.000	6	11.8		GTP binding
gi|115345328	H+ transporting ATP synthase beta subunit isoform 2	1.000	5	11.1	ATP synthesis coupled proton transport	Hydrogen ion transporting ATP synthase activity, rotational mechanism

to be continued.

Table 2 Continued.

Protein best name	Protein description	Protein prob.	# Pep	AA coverage (%)	Biological process	Molecular function
gi|153791817	S-adenosyl-L-homocysteine hydrolase	0.929	2	3.3	Fatty acid beta-oxidation	Acetyl-CoA C-acyltransferase activity
gi|24648160	CG5237 ‡	0.928	3	0.8		Protein binding
Development§						
BGIBMGA002175-PA		1.000	10	12.1		
BGIBMGA003608-PA	Elongation factor 1alpha48d, isoform A†	1.000	15	14.3	Determination of adult lifespan	GTP binding
gi|112982792	Y-box protein	1.000	4	12.7	Dorsal/ventral axis specification, ovarian follicular epithelium	mRNA 3'-utr binding
gi|112982865	Profilin	1.000	22	27.3	Mesoderm development	
gi|112982880	Translationally controlled tumor protein	1.000	7	12.1	Positive regulation of cell size	Guanyl-nucleotide exchange factor activity
gi|112983696	Bmsqd-1	1.000	5	8.4	Wing disc dorsal/ventral pattern formation	Actin binding
gi|112983920	Imaginal disk growth factor	0.978	14	4.1	ATP synthesis coupled proton transport	Hydrogen ion transporting ATP synthase activity, rotational mechanism
gi|112983958	Annexin IX ioform A	1.000	3	12.2	Chitin-based larval cuticle pattern formation	GTP binding
gi|112984370	Ribosomal protein L13A	1.000	4	19.8	Bristle morphogenesis	Structural constituent of ribosome
gi|112984502	Low molecular lipoprotein 30K precursor	1.000	11	7.4	Embryonic development ending in seed dormancy	
gi|112984526	Promoting protein	0.999	3	6.5	Actin filament organization	Actin binding
gi|114051710	Mitochondrial prohibitin complex protein 2	0.998	3	9.0		
gi|148298685	Fructose 1,6-bisphosphate aldolase	0.999	3	8.4	Oogenesis	DNA binding
gi|153791847	Abnormal wing disc-like protein	0.999	3	14.4	Glycyl-tRNA aminoacylation	ATP binding
gi|162952015	Annexin IX isoform B	0.999	3	6.5	Wing disc dorsal/ventral pattern formation	Actin binding
gi|162952017	Annexin IX isoform C	0.999	3	6.5	Wing disc dorsal/ventral pattern formation	Actin binding

to be continued.

Table 2 Continued.

Protein best name	Protein description	Protein prob.	# Pep	AA coverage (%)	Biological process	Molecular function
gi|21355813	Sar1 CG7073-PB, isoform B‡	0.996	3	6.9	Determination of adult lifespan	Binding
gi|45553457	Sar1 CG7073-PE, isoform E‡	0.999	3	8.4	Chitin-based larval cuticle pattern formation	GTP binding
Transport/reserve§						
BGIBMGA009027-PA	Larval serum protein 1 gamma†	1.000	10	5.6	Lipid transport	Lipid transporter activity
BGIBMGA009599-PA	CG31150†	1.000	5	3.3	Lipid transport	Lipid binding
BGIBMGA011424-PA	Transferrin 1†	1.000	19	16.3	Cellular iron ion homeostasis	Ferric iron binding
BGIBMGA013341-PA		1.000	17	10.0	Transport	Nutrient reservoir activity
BGIBMGA013342-PA	Retinoid- and fatty acid-binding glycoprotein†	1.000	192	27.1	Lipid transport	Fatty acid binding
BGIBMGA013893-PA	CG15828, isoform B†	1.000	8	3.4	Lipid transport	Fatty acid binding
BGIBMGA013894-PA	CG15828, isoform B†	1.000	11	3.2	Lipid transport	Lipid binding
gi|112982932	Ferritin	1.000	4	11.6	Cellular iron ion homeostasis	Ferric iron binding
gi|124430725	Arylphorin	1.000	34	16.1	Transport	Nutrient reservoir activity
gi|169234936	Sex-specific storage-protein SP1	1.000	61	24.4	Transport	Nutrient reservoir activity
Defense response§						
BGIBMGA002907-PA	CG9027, isoform C†	1.000	4	17.8	Defense response to bacterium	Superoxide dismutase activity
gi|112983481	Alpha-tubulin	0.987	2	5.1	Antimicrobial humoral response	Lysozyme activity
gi|112983994	Peptidoglycan recognition protein	1.000	9	14.3	Defense response	Muramyl dipeptide binding
gi|112984208	Lysozyme	1.000	10	21.2	Response to heat	Protein binding
gi|169646838	Heat shock protein 25.4	0.984	2	8.5	Antimicrobial humoral response	GTP binding
Antioxidation§						
BGIBMGA001351-PA	CG10863†	0.999	8	6.2	Oxidation reduction	Aldehyde reductase activity
gi|112982996	Thiol peroxiredoxin	0.999	5	11.3	Cell redox homeostasis	Antioxidant activity
gi|114052210	Thioredoxin peroxidase	0.978	2	5.7	Cell redox homeostasis	Antioxidant activity
Protein translation/ modification/folding§						
BGIBMGA000074-PA	Fibrillarin†	1.000	2	4.4	RNA processing	Methyltransferase activity
gi|112982735	Ribosomal protein P0	1.000	11	24.4	DNA repair	DNA-(apurinic or apyrimidinic site) lyase activity
gi|112983010	Translation elongation factor 2	0.997	3	2.8	Mitotic spindle elongation	GTP binding
gi|112983090	Eukaryotic translation initiation factor 3 subunit 1	0.930	2	10.8	Translational initiation	Translation initiation factor activity

to be continued.

Table 2 Continued.

Protein best name	Protein description	Protein prob.	# Pep	AA coverage (%)	Biological process	Molecular function
gi|112983276	Ribosomal protein L5	1.000	22	38.1	Translation	5S rRNA binding
gi|112983495	Ribosomal protein L9	1.000	6	14.7	Mitotic spindle elongation	rRNA binding
gi|112983898	Elongation factor 1 gamma	1.000	14	11.3	Translation	Translation elongation factor activity
gi|112983926	Arginine kinase	0.999	4	7.3	Phosphorylation	arginine kinase activity
gi|112984022	Ribosomal protein S9	1.000	7	15.5	Mitotic spindle elongation	rRNA binding
gi|112984058	Ribosomal protein S7	1.000	10	15.3	Translation	Structural constituent of ribosome
gi|112984112	Ribosomal protein S3	0.965	4	5.3	Translation	RNA binding
gi|112984266	Ribosomal protein L23A	1.000	6	8.0	Biological process	Molecular function
gi|112984318	Ribosomal protein L18	1.000	5	14.8	Mitotic spindle elongation	Structural constituent of ribosome
gi|112984422	Ribosomal protein L17	0.997	4	5.3	Mitotic spindle elongation	Structural constituent of ribosome
gi|112984454	Protein disulfide isomerase	1.000	10	12.6	Cell redox homeostasis	Protein disulfide isomerase activity
gi|114052472	Peptidylprolyl isomerase B	1.000	6	17.0	Protein folding	Isomerase activity
gi|162952033	Ribosome-associated protein P40	1.000	3	7.8	Mitotic spindle elongation	Structural constituent of ribosome
Miscellaneous proteins[§]						
BGIBMGA000903-PA	Transport and golgi organization 7[†]	0.978	2	5.4	Golgi organization	Catalytic activity
BGIBMGA002288-PA		0.9782	3	5.4		
BGIBMGA003276-PA		1.000	4	21.1		
BGIBMGA004395-PA		0.999	4	12.2		
BGIBMGA004397-PA		1.000	8	20.3		
BGIBMGA004398-PA		1.000	14	13.5		
BGIBMGA004399-PA		1.000	4	18.8		
BGIBMGA006022-PA	La autoantigen-like, isoform A[†]	0.985	2	3.1	RNA processing	5S rRNA primary transcript binding
BGIBMGA006405-PA	Heterogeneous nuclear ribonucleoprotein at 98DE, isoform D[†]	0.964	2	4.2	Alternative nuclear mRNA splicing, via spliceosome	mRNA binding
BGIBMGA007286-PA		1.000	10	17.2		
BGIBMGA008179-PA		1.000	8	10.2		
BGIBMGA008238-PA	Plexin A, isoform A[†]	1.000	4	1.3	Axon guidance	Axon guidance receptor activity
BGIBMGA008847-PA		1.000	4	19.3		
BGIBMGA010036-PA		0.999	3	10.1		
BGIBMGA010048-PA		0.999	3	10.1		
BGIBMGA010979-PA		1.000	7	17.0		

to be continued.

Table 2 Continued.

Protein best name	Protein description	Protein prob.	# Pep	AA coverage (%)	Biological process	Molecular function
BGIBMGA011092-PA	CG7993†	0.901	2	4.1		
BGIBMGA011549-PA	CG14661†	1.000	13	17.3		
BGIBMGA012030-PA		1.000	4	2.8		
BGIBMGA012642-PA	CG10424†	1.000	6	12.4		
BGIBMGA012645-PA		1.000	18	31.0		
BGIBMGA013781-PA		0.999	7	34.2		
BGIBMGA014578-PA	Histone H1†	0.984	2	6.8	Chromatin assembly or disassembly	DNA binding
gi|112983052	Chemosensory protein 11	1.000	7	28.7	Determination of anterior/posterior axis, embryo	Diacylglycerol binding
gi|112983548	p27k	1.000	33	31.3		
gi|148298667	Signal sequence receptor	1.000	5	5.4		Signal sequence binding
gi|153792009	Heterogeneous nuclear ribonucleoprotein A1	0.960	1	5.6	Regulation of alternative nuclear mRNA splicing, via spliceosome	mRNA binding

†The *Drosophila melanogaster* proteins in NCBInr database retrieved by BLASTP (E-value<e-15) with predicted *Bombyx mori* protein sequences.

‡The *Drosophila melanogaster* proteins in the composite database searching against for the LC-MS/MS data.

§The identified proteins were classified according their GO annotations.

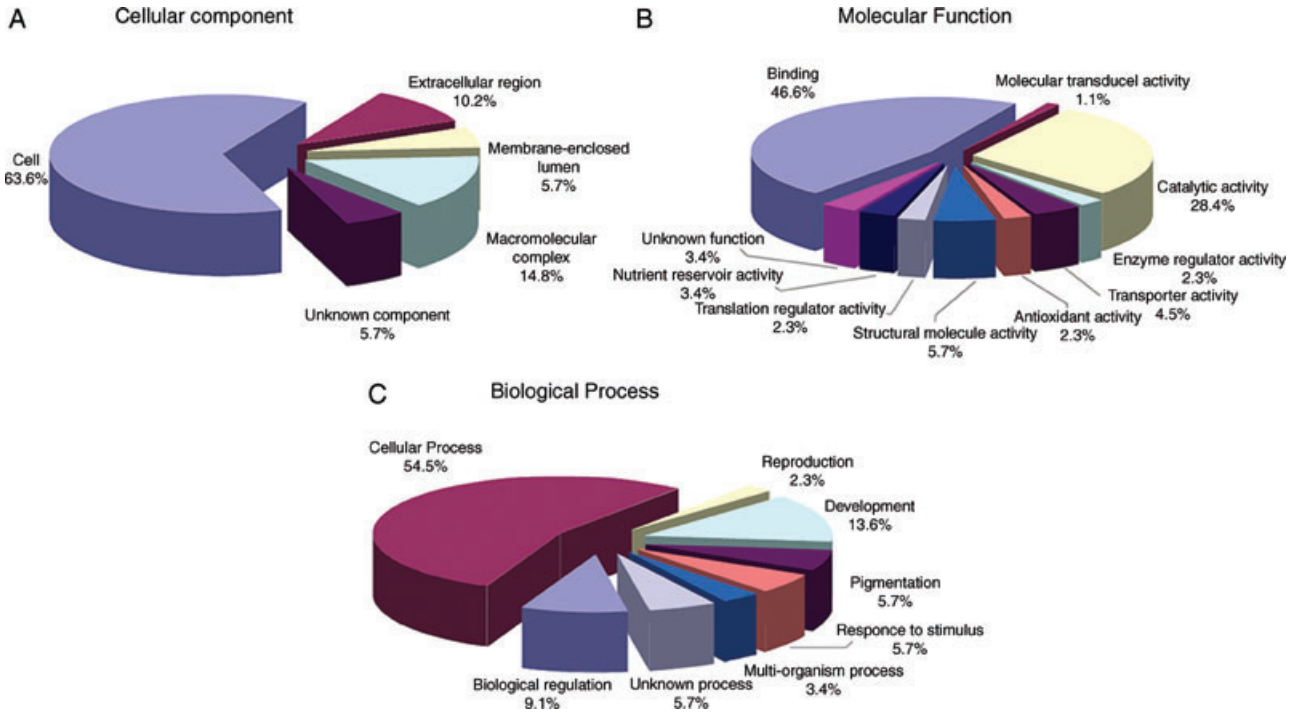

Fig. 2 Gene ontology (GO) categories of the identified proteins from the *Bombyx mori* hemolymph at V5. The annotations for the identified proteins were retrieved by searching against the GO database with their sequences. The proteins were classified into Cellular Component (a), Molecular Function (b) and Biological Process (c) according to their GO annotations.

proteins, including awd, rpl23a, Y-box protein (yps), ribosomal protein S3 (rps3), heterogeneous nuclear ribonucleoprotein at 87F, isoform A(Hrb87F), heterogeneous nuclear ribonucleoprotein at 98DE, isoform (hrb98de) and La autoantigen-like (la), were found in the nucleus. Other proteins, such as alphatub84b, imaginable disk growth factor (idgf4), thiol peroxiredoxin (jafrac1), knockdown protein, isoform A (kdn), cg5362, Bmsqd-1 (sqd), ribosomal protein P0 (rplp0), elongation factor 1alpha48D, isoform A (ef1alpha48d), fructose 1,6-bisphosphate aldolase (ald) and translation elongation factor 2 (ef2b) were distributed in the cytoplasm. yps and sqd are both transcription factors involved in RNA localization and implicated in oogenesis, mRNA splicing, and larval development, respectively. All these components composed a complex network that regulated biological processes.

Discussion

2-DE analysis combined with MALDI-TOF MS and shotgun LC-MS/MS are two complementary approaches for proteomic research. Bogaerts *et al*. (2009) analyzed the proteome of honeybee hemolymph with the two methods and only found about one-quarter of common identifications. The proteome profiles of *B. mori* hemolymph during different developmental stages were analyzed with 2-DE followed by MALDI-TOF MS, and dozens of identified differential expression proteins were predicted to be involved in the metamorphosis, programmed cell death and biosynthesis of silk protein (Li *et al*., 2006; Hou *et al*., 2010). In the present study, we employed the two methods to characterize the proteome profiles of *B. mori* larval hemolymph. Four 30K proteins up-regulated sharply during the active feeding period were identified by MALDI-TOF MS. These proteins, including PBMHP-6 precursor, PBMHP-12 precursor and PBMHPC-19, were also validated by Hou *et al*. (2010). The obvious up-regulation of these proteins seems the result of disappearance of juvenile hormone in the hemolymph which leads to the sharp increase of their mRNAs in the fat body (Ogawa *et al*., 2005). These results suggest that these 30K proteins may be the key factors promoting the growth and development of *B. mori* during these late larval stages. In addition, the shotgun proteomic analysis revealed a complex protein aggregation involved in metabolism, development, transport and reserve, and immunity.

The 30K proteins of *B. mori* are synthesized in the fat body and released into the hemolymph from the third day

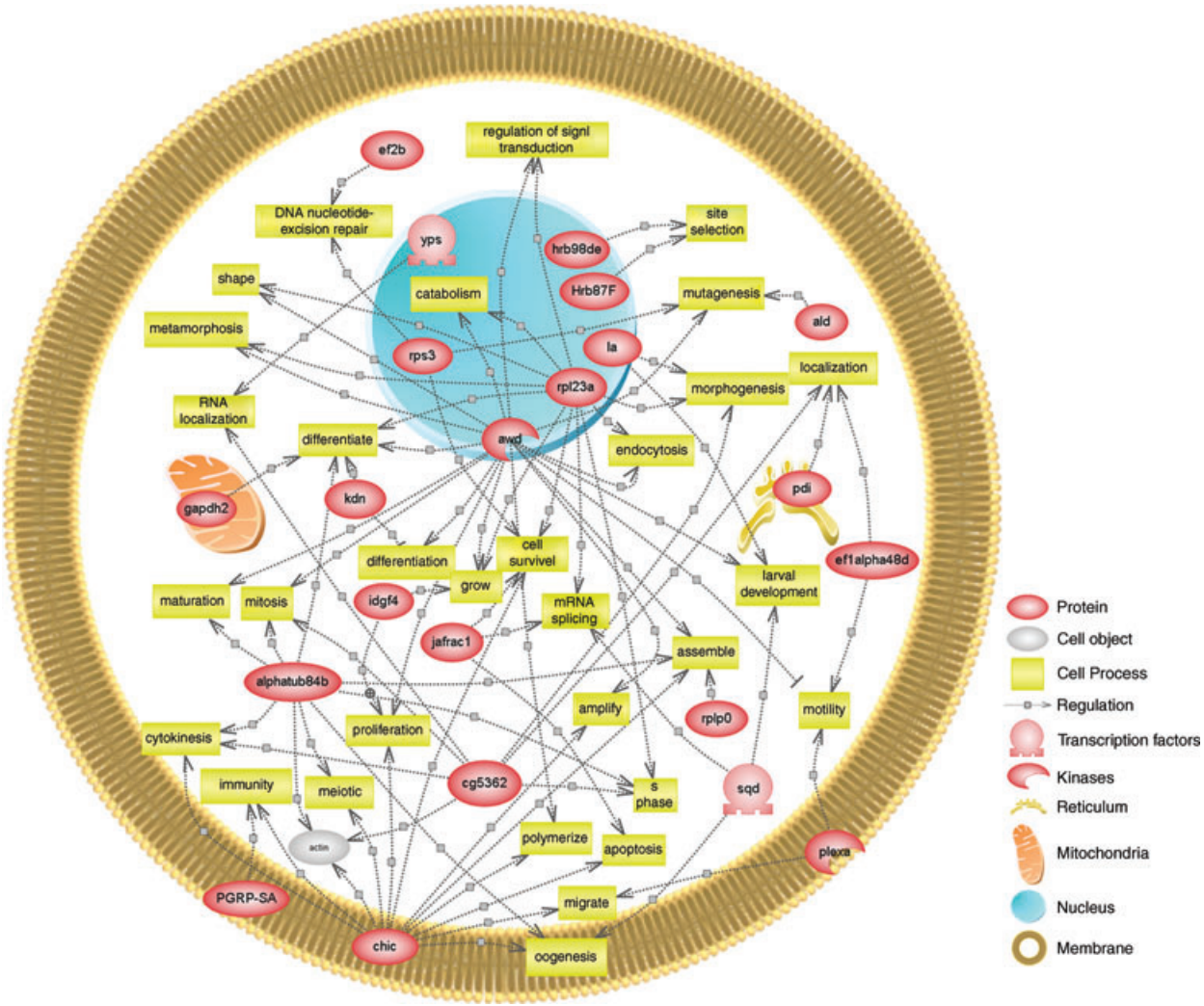

Fig. 3 Network of the proteins identified in *Bombyx mori* hemolymph with common targets. The proteins (red) in the network are the *Drosophila* homolog of our identifications. These proteins take part in various cell processes (yellow) with positive or negative effects. At least two identified proteins were involved in each biological process. Pathway Studio software was used to map the networks of the identified proteins based on characterized *Drosophila* pathways. The subcellular localization of the proteins was also predicted based on the descriptions in the database.

of the fifth instar (Izumi *et al*., 1981). These proteins are also considered to be essential for normal development of the *B. mori* embryo (Zhong *et al*., 2005). Taken together, these observations indicate that the highly expressed proteins are the 30K proteins that are released from the fat body cells, which reach peak levels in the hemolymph at V5. Furthermore, their high expression levels were accompanied by the rapid growth and development of the larvae, implying an association between these proteins and rapid growth.

LC-MS/MS analysis showed eight proteins were related to nutrient transport, including five for lipid transport (Table 2). For example, ferritin and the predicted protein BGIBMGA011424-PA, a homolog of *Drosophila* transferrin 1, are involved in the storage and transportation of iron ions. During the late larval stage, holometabolous insects need to reserve a large amount of energy and materials for the development of adult tissues during metamorphosis. Therefore, abundant SPs are usually expressed to store amino acids for the production of adult proteins (Haunerland, 1996; Wheeler *et al.*, 2000). In *B. mori*, SPs occur in two forms, SP1 and SP2, and accumulate in a stage-dependent fashion in the larval hemolymph (Sakurai *et al*., 1988; Kishimoto *et al*., 1999). Many of

the peptides in this analysis were used to identify the sex-specific storage-protein SP1 and arylphorin (SP2). Because these proteins had an MW of approximately 85 kDa, equal to that of the subunits of native SP (Tojo *et al.*, 1980), there seemed no obvious up-regulation at V5 seen from the 2-DE gels. Although the SPs are accumulated in large quantities in the hemolymph during the active feeding period in some insects (Haunerland, 1996), these data indicate that *B. mori* released the accumulation of SPs at the fast-growing stages.

Another major role of hemolymph is metabolism, which is carried out with multifarious enzymes. We identified 28 (25.9%) proteins with different enzyme activities that were related to metabolism. Of these enzymes, the alcohol dehydrogenase was proven to be specially expressed at the late stage of the fifth instar by 2-DE analysis (Li *et al*., 2006). Other enzymes were involved in glycolysis, lipid metabolism and (adenosine triphosphate) synthesis. These results implied the multiple functions of hemolymph and the important roles of these enzymes in maintaining normal life processes.

The growth and development of *B. mori* have close relationships with the dynamics of hemolymph components. The most well-known components involved are the juvenile hormone and ecdysones. However, for the protein components, several with important roles were identified in the present study. The Bmsqd-1, homolog of *Drosophila* SqdA which performs important roles in Gurken localization during oogenesis (Norvell *et al*., 1999; Zha *et al*., 2005), may be related to development of the reproductive system. The imaginal disk growth factor, which was considered to be secreted from fat body (Kawamura *et al*., 1999), is likely to be involved in the development of insect embryo and larva. The homolog of *Drosophila* imaginal disk growth factor has been purified from *Bombyx* hemolymph and was proposed that its expression in hemolymph was regulated by the fat body in response to nutritional conditions (Wang *et al*., 2009).

Insect innate immunity is based on the recognition of microbial molecules, such as peptidoglycans and lipopolysaccharides, by specific receptors and the subsequent activation of cellular responses. Peptidoglycan fragments that have some definite structure are recognized as signal molecules and elicit the synthesis of antibacterial proteins in the fat body (Iketani *et al*., 1999). PGRP-SA, which is found in the *B. mori* hemolymph and was identified in this study, has an affinity for peptidoglycan and the ability to trigger the prophenoloxidase cascade upon binding to peptidoglycan (Yoshida *et al*., 1996). The PGRP-SA together with lysozyme, alpha-tubulin and the predicted protein BGIBMGA002907-PA in hemolymph, compose part of the immune system that responds to bacterial challenges.

Chemosensory protein 11 is a member of the chemosensory protein (CSP) family, but its functions are still not clear. It has been identified in *B. mori* larval endocrine organs, embryos and head (Li *et al*., 2009a,b; Li *et al*., 2010 a, b). The expression levels of chemosensory protein 11 and some immunity-related proteins such as the PGRP-SA were higher in the hemolymph of *B. mori* reared on an artificial diet compared with those reared on fresh mulberry leaves (Zhou *et al*., 2008). Interestingly, in addition to PGRP-SA, a unique odorant-binding protein (OBP), antennal binding protein 7, was also up-regulated in *B. mori* plasma after bacterial infection (Song *et al*., 2006). These results suggest that some chemoreception molecules might be involved in insect innate immunity, although GO annotation showed that antennal binding protein 7 was related to embryonic development (Table 1).

Multi-functional proteins always participate in multiple biological processes, while each pathway may involve a lot of molecules. The bioinformatic analysis can help us to classify and localize the proteins and uncover the interactions with other members. Bioinformatic analysis of the hemolymph proteome components revealed a complex biological network that controlled differentiation, apoptosis, metamorphosis and immunity. This network is likely involved in maintaining the normal development of *B. mori*.

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Disclosure

The authors declare that they have no conflict of interest.

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