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ORIGINAL ARTICLE

Shotgun proteomic analysis of wing discs from the domesticated silkworm (*Bombyx mori*) during metamorphosis

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Abstract Proteomic profiles from the wing discs of silkworms at the larval, pupal, and adult moth stages were determined using shotgun proteomics and MS sequencing. We identified 241, 218, and 223 proteins from the larval, pupal, and adult moth stages, respectively, of which 139 were shared by all three stages. In addition, there were 55, 37, and 43 specific proteins identified at the larval, pupal, and adult moth stages, respectively. More metabolic enzymes were identified among the specific proteins expressed in the wing disc of larvae compared with pupae and moths. The identification of FKBP45 and the chitinase-like protein EN03 as two proteins solely expressed at the larval stage indicate these two proteins may be involved in the immunological functions of larvae. The myosin heavy chain was identified in the pupal wing disc, suggesting its involvement in the formation of wing muscle. Some proteins, such as proteasome alpha 3 subunits and ribosomal proteins, specifically identified from the moth stage may be involved in the degradation of old cuticle proteins and new cuticle protein synthesis. Gene ontology analysis of proteins specific to each of these three stages enabled their association with cellular component, molecular function, and biological process categories. The analysis of similarities and differences in these identified proteins will greatly further our understanding of wing disc development in silkworm and other insects.

Keywords *Bombyx mori* · Wing disc · Shotgun · Proteome · Metamorphosis · Gene ontology

Introduction

Insects are the only winged animals among the invertebrates. Insect wings not only aid in flight, but also play an important role in other functions, such as orientation, protection, communication, and courtship. Holometabolous insects undergo drastic morphological changes during metamorphosis. Larval tissues that only function during the larval stage degenerate. Other adult tissues develop through the remodeling of larval tissues or from the imaginal discs (Manabu et al. 2004). In *Bombyx mori*, the wing discs gradually proliferate during the larval stage and undergo extensive proliferation and morphological changes to evaginate from inside the body during pupal ecdysis. Light and electromicroscopic analyses have revealed remodeling of extracellular matrices, cell morphological changes, and cuticle deposition during the wing disc development (Kawasaki et al. 1986; Kawasaki and Iwashita 1987; Nardi et al. 1985). Genes involved in wing disc development include *Acer*, *NEP-L*, and genes encoding cuticle proteins (Quan et al. 2001; Zhao et al. 2001; Noji et al. 2003).

The silkworm (*Bombyx mori*) is an important species economically and a model lepidopteran insect; therefore, it is an ideal candidate for lepidopteran biochemical, molecular genetic, and genomic studies (Nagaraju and

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Goldsmith 2002; Tomita et al. 2003; Goldsmith et al. 2005; Reumer et al. 2008). The whole life cycle of the silkworm can be described as the larval, pupal, and moth stages. Metamorphosis, which begins from larva to pupa and then to the adult moth, initiates after the fifth instar period. The proteomic profiles of wing discs throughout metamorphosis, therefore, allow the identification of key proteins involved in the development of the wing disc and many biological processes.

Following the completion of draft sequences of the genomes of several model organisms, including the silkworm, proteomics has become the focal point in recent entomological research. Shotgun proteomics is a viable method for the large-scale screening of peptides and proteins in a complex biological sample to rapidly generate a global profile of the protein components in cells, tissues, and organs (MacCoss et al. 2002). Liquid chromatography tandem mass spectrometry (LC–MS/MS) is a highly sensitive and high-throughput method based on the shotgun proteomics approach. In a typical LC–MS/MS experiment, proteins in a sample are first digested into peptides, separated by an LC system, and then subjected to MS analysis. The peptides and proteins are identified based on matching their experimentally generated tandem mass spectra to the theoretical best match from a protein database. This approach has been implemented in model insects, such as *Bombyx* (Li et al. 2009a, b), *Drosophila* (Li et al. 2007; Baggerman et al. 2005), and *Anopheles* (Kalume et al. 2005).

In the present study, SDS-PAGE and shotgun LC–MS/MS were employed to identify the proteome of the *B. mori* wing disc, and we utilized bioinformatics analysis to illuminate the differences between proteomic profiles of the wing discs at the larval, pupal, and moth stages of the silkworm and to find valuable clues regarding energy metabolism and signaling mechanisms during metamorphosis of the silkworm.

Materials and methods

Insect rearing and wing disc isolation

The silkworm strain P50 was reared on fresh mulberry leaves under an environment with a 12 h light/12 h dark photoperiod, at 26 ± 1 °C and 60–75 % relative humidity. The developmental stages were synchronized at each molt by collecting new larvae. On the fifth day of the fifth instar, the seventh day of the pupal stage, and the first day of the moth stage, ten larvae were dissected and their wing discs collected. The tissue samples were stored at −70 °C until further use. All experiments were performed in three independent biological repeats and three technical repeats.

Sample preparation and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Total protein from the wing disc was extracted on ice in 10 ml lysis buffer (containing 2.5 % SDS, 10 % glycerin, 5 % β-mercaptoethanol, and 62.5 mM Tris–HCl pH 6.8) per mg of sample weight with a motor-driven plastic grinder. The homogenate extraction was kept for 10 min at room temperature and then subjected to continued sonication treatment four times in an ice-bath, each time 30 s with a 30-s interval. After centrifugation twice at 20,000*g* and 25 °C for 10 min, the supernatant was aliquoted and stored at −20 °C. The sample was boiled for 2 min and centrifuged at 20,000*g* for 10 min before SDS-PAGE. The sample was separated by SDS-PAGE using a 5 % stacking gel and 12.5 % resolving gel with constant current. The electrophorized gel was stained with Coomassie Brilliant Blue R250 (CBB, Sigma, USA).

In-gel digestion

Each gel lane was manually cut into two bands according to the level of Coomasie staining (Fig. 1), and each band was diced into small pieces (~2 mm^2). The pieces were subjected to an in-gel tryptic digestion process as described by Shevchenko et al. (2006). Briefly, the gel pieces were washed three times using MilliQ water (Millipore, Bedford, MA, USA) and de-stained twice with 25 mM NH_4HCO_3 in 50 % acetonitrile (ACN, Amersham, UK) at 37 °C until the color completely disappeared. The reduction and alkylation of proteins were performed by incubating the gel pieces with 50 mM Tris [2-carboxyethyl] phosphine

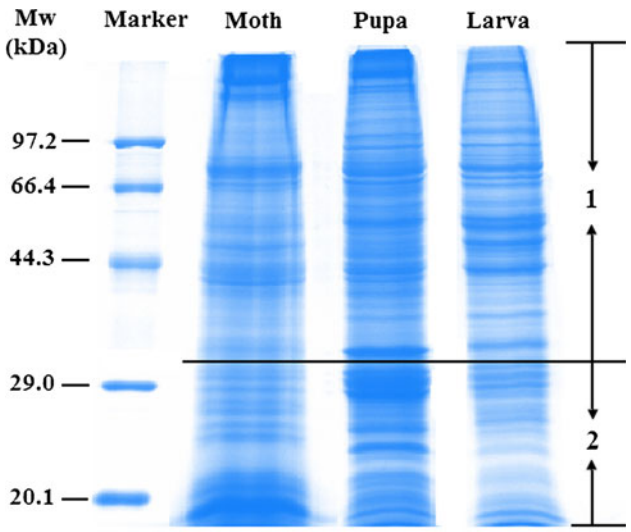

Fig. 1 One-dimensional SDS-PAGE gel separation of wing disc protein samples from the larval, pupal, and moth stage, respectively. The numbers indicate the two bands according to the slicing pattern used for sample fractionation prior to in-gel digestion

(TCEP, Sigma, St Louis, MO, USA) in 25 mM NH_4HCO_3 at 56 °C for 1 h followed by 100 mM iodoacetamide (IAA, Amersham) in 25 mM NH_4HCO_3 for 0.5 h at room temperature in the dark. Gel pieces were washed twice with 25 mM ammonium bicarbonate in 50 % acetonitrile solution, dehydrated twice with 100 % acetonitrile, and dried in a vacuum centrifuge. The proteins were digested with 20 ng/μl modified proteomics grade trypsin (Sigma) overnight at 37 °C. The resulting tryptic peptide mixtures were extracted twice from the gel pieces with 5 % trifluoroacetic acid (TFA, Fluka, Milwaukee, WI, USA) in 50 % ACN solution. The pooled extracts were evaporated in a vacuum centrifuge and resuspended in 0.1 % methanoic acid (Sigma) prior to LC–MS/MS analysis.

Shotgun LC–MS/MS analysis

All digested peptide mixtures were separated by reverse-phase HPLC (RP-HPLC) followed by tandem MS analysis. RP-HPLC was performed on a surveyor LC system (Thermo Finnigan, San Jose, CA). Samples were loaded into a trap column (Zorbax 300SB-C18 peptide traps, 300 μm × 65 mm, Agilent Technologies, Wilmington, DE) at a 3 ml/min flow rate before the split. After flow-splitting down to about 1.5 ml/min, peptides were transferred to the analytical column (RP-C18, 150 μm × 150 mm, Column Technology Inc., Fremont, CA) for separation with a 195-min linear gradient from 96 % buffer A (0.1 % methanoic acid in water) to 50 % buffer B (84 % ACN, 0.1 % methanoic acid in water) at a flow rate of 250 nl/min.

Spectra were acquired by a LTQ linear ion trap mass spectrometer (Thermo Electron Corporation) in data-dependent mode using Xcalibur software. The mass spectrometer was operated in positive ion mode employing collision-induced dissociation (CID) with a source temperature of 160 °C. The spray voltage was 3.0 kV and the normalized collision energy was set at 35.0 % for MS/MS. The MS analysis was performed with one full MS scan (m/z 400–1,800) followed by ten MS/MS scans on the ten most intense ions from the MS spectrum with the dynamic exclusion settings: repeat count 2, repeat duration 30 s, exclusion duration 90 s.

Database search

Database searches were carried out against the in-house database we previously constructed (Li et al. 2009b) which contains a total of 25 325 proteins, including the sequences of the domesticated silkworm (*B. mori*) and wild silkworm (*Bombyx mandarina*). The raw MS/MS spectra were interpreted by Biowork 3.0 (ThermoFinnigan, San Jose, CA, USA) and the database searches performed with the SEQUEST algorithm, which is a module of Biowork 3.0 on a local server. The peptide mass tolerance was 10.0 ppm and the fragment ions tolerance was 1.0. The trypsin enzyme and partial enzymatic cleavage of the amino acid bonds at both ends of the protein were chosen. Two miscleavage sites were allowed. Only b and y fragment ions were taken into account. Fixed modification (Carboxamidomethyl) on cysteine and variable modification (Oxidation) on methionine were set.

Validation processes with the trans-proteomic pipeline (TPP)

Validation of the identified peptides and proteins was carried out according to the TPP software 3.4 manual, which was downloaded from the website (http://tools.proteomecenter.org/TPP.php) and installed with the default options (Keller et al. 2002, 2005; Nesvizhskii et al. 2003). Validation of the identified proteins and peptides was carried using previously described methods (Li et al. 2009b). The protein probability threshold for running Protein-Prophet was set at 0.9.

InterPro annotation and gene ontology (GO) categories

InterProScan software was used to carry out protein sequence searches against the InterPro member databases to identify signatures (Zdobnov and Apweiler 2001). The compiled RAW outputs were subjected to GO category analysis using the Web Gene Ontology Annotation Plot (WEGO) (Ye et al. 2006). The three groups of datasets were simultaneously subjected to online analysis (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) and the *P* values were calculated by the Pearson Chi square test.

Results and discussion

Commonly identified proteins in the wing discs at three developmental stages

We obtained the proteomic profiles of the wing discs at the larval, pupal, and adult moth developmental stages using a shotgun proteomics strategy (based on the proteolytic digestion of complex protein mixtures, LC separation, and tandem MS sequencing) and the in-house database. All proteins were identified by SEQUEST algorithms and further validated by TPP under stringent criteria. We identified 241, 218, and 223 proteins from the larval, pupal, and adult moth stages, respectively (Fig. 2, Supplementary Table 1–3, Supplementary Fig. 1–6). Due to limitations of the silkworm protein database, not all proteins had complete functional annotations. In all, 139 proteins were

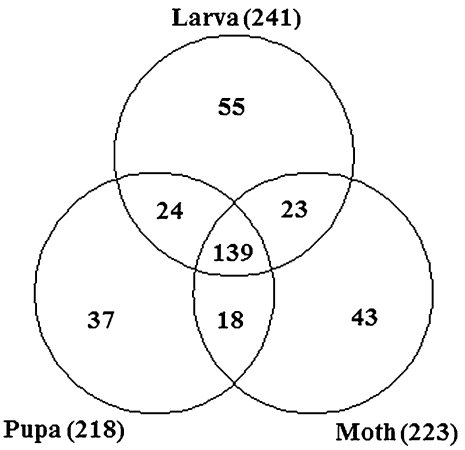

Fig. 2 Venn diagram showing the number of proteins identified in the wing discs from the larval, pupal, and moth stage of silkworms. Each number with no overlap of circles shows the number of proteins uniquely observed in the wing disc sample, while overlapping circles shows the number of identified proteins common to two or three of the analytes

shared by all three developmental stages. Of these, 19 ribosomal proteins (P40, P0, L4, S8, L10A, L13, S3, S5, L7, S7, L14, S24, S9, L18, L5, L17, L19, S4, P1) were identified, and the abundance of these ribosomal proteins in the three stages might be closely related to protein synthesis during the wing disc developmental process. Furthermore, we also identified translation elongation factor 2 and elongation factor 1 subunits shared by all three developmental stages, such as elongation factor 1-alpha (EF-1α), elongation factor 1-gamma (EF-1γ), Ef-1α-like factor isoform 2, and elongation factor 1-beta (EF-1β). EF-1γ belongs to a subunit of silk gland EF-1L (the lighter form) and can facilitate the exchange of EF-1α bound GDP for GTP (Kamiie et al. 2002). EF-2 and EF-1 subunits were identified at all three stages, suggesting many active molecular changes occur during the wing disc developmental process.

Two other important proteins shared by all three stages were disulfide-isomerase like protein ERp57 and calreticulin. ERp57 is a protein disulfide isomerase-related polypeptide and is thought to catalyze the isomerization of non-native disulfide bonds formed in glycoproteins with unstructured disulfide rich domains (Mishra et al. 2005). ERp57 belongs to the endoplasmic reticulum oxidoreductases and its specificity requires accessory factors like calreticulin (Jessop et al. 2009). The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death (Panaretakis et al. 2008). Calreticulin and ERp57 were identified in the wing discs at three developmental stages in this study, suggesting their involvement in the immune function of the silkworm.

Another protein identified at all three stages, the imaginal disc growth factor (IDGF), is a very important protein involved in the growth of wing discs. An insulin signal cascade in wing discs is accelerated by the addition of bombyxin or insulin in culture media (Nijhout and Grunert 2002). The number of wing disc cells increases with the addition of insulin together with IDGF (Kawamura et al. 1999), and this increase is potentially due to IDGF helping insulin bind to its receptor (Varela et al. 2002). Interaction of IDGF with insulin should be clarified in the future.

Other important proteins identified at all three stages were heat shock cognate protein, 30K lipoprotein, the proteasome, and some metabolic enzymes, such as transketolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, cytosolic malate dehydrogenase, and peroxiredoxin.

Specific proteins identified in the wing discs at three developmental stages

Our data also revealed many proteins specifically expressed at the larval, pupal, and adult moth stages (55, 37, and 43 proteins, respectively). The proteins specific to larvae are shown in Table 1. The proteins specific to larvae include some metabolic enzymes, such as NADP-dependent oxidoreductase, glyoxylate reductase/hydroxypyruvate reductase, NADPH-specific isocitrate dehydrogenase, aspartate aminotransferase, cystathionine gamma-lyase, fructose-1, 6-bisphosphatase, and 3-hydroxyisobutyrate dehydrogenase. An important immunophilin protein, FKBP45, was also expressed in the larval wing disc. Immunophilins are a large family of proteins that bind the immunosuppressive drugs cyclosporin, rapamycin, and FK506, and possess peptidyl-prolyl cis/trans-isomerase (PPIase) activity (Peattie et al. 1992; Galat 1993; Alnemri et al. 1994; Davies and Sanchez 2005). This enzymatic activity induces the conversion of prolyl-peptide bonds from the trans- to cis-form, a frequent limiting step in protein folding (Galat 1993; Kay 1996; Schiene and Fisher 2000; Davies and Sanchez 2005). In addition, the chitinase-like protein EN03, a member of chitinases in insects, was also identified. Chitinases have widely different functions and are involved in digestion, arthropod molting, defense/immunity, and pathogenicity (Yasuyuki and Subbaratnam 2010). The larval stage is the only stage at which silkworms feed and absorb nutrients from its external environment, and it is, therefore, logical that immunophilin proteins are largely expressed during the larval stage.

Table 2 and 3 shows proteins specifically in the wing disc at the pupal stage and moth stage respectively. Compared with the larval stage, more ribosomal proteins (S10, S23, P2, and S17) were expressed in the pupal wing discs, and the abundance of ribosomal proteins at the pupal stage might be closely related to protein synthesis in the pupal wing discs. The four metabolic enzymes identified in pupa

Table 1 Specific proteins identified in the wing disc of *B. mori* larva

No.	Protein description	Protein accession	No. of peptides	No. of unique Peptides	Cover percent	Mw (kDa)	p*I*
1	Aubergine protein	gil166706856	9	5	6.90	101.34	9.45
2	Y-box protein	gil123301101	7	4	26.47	29.33	11.1
3	RecName: Full = Eukaryotic translation initiation factor 3 subunit H; Short = eIF3 h	gil74848914	9	3	12.17	38.61	5.68
4	Beta-tubulin	gil112983456	8	3	8.53	51.35	4.77
5	NADP-dependent oxidoreductase	gil148298833	5	2	10.75	36.78	6.9
6	Hsc70/Hsp90-organizing protein HOP	gil112983280	4	2	4.81	62.14	6.14
7	45 kDa immunophilin FKBP45	gil112983564	4	2	7.21	44.68	4.75
8	putative CAD trifunctional protein	gil158451125	3	2	4.51	108.75	6.11
9	26S proteasome non-ATPase regulatory subunit 14	gil114052633	3	2	15.43	34.57	5.86
10	Double-stranded RNA-binding zinc finger protein JAZ	gil114051950	2	2	8.14	48.54	5.5
11	NADPH-specific isocitrate dehydrogenase	gil151301209	2	2	7.82	49.00	8.23
12	RecName: Full = Eukaryotic translation initiation factor 3 subunit I; Short = eIF3i	gil121958921	2	2	7.60	36.91	5.71
13	Glyoxylate reductase/hydroxypyruvate reductase	gil114053007	2	2	9.77	37.77	6.67
14	Elongation factor Tu	gil114052967	4	1	3.44	51.03	8.47
15	Chaperonin	gil120444903	3	1	3.30	59.17	5.4
16	RecName: Full = Protein ultraspiracle homolog; AltName: Full = Nuclear receptor subfamily 2 group B member 4; AltName: Full = RXR type hormone receptor CF1	gil1345734	3	1	4.11	52.5	8.4
17	Aspartate aminotransferase	gil114053127	2	1	3.71	47.86	8.79
18	RecName: Full = Eukaryotic translation initiation factor 3 subunit E; Short = eIF3e; AltName: Full = Eukaryotic translation initiation factor 3 subunit 6	gil121996550	2	1	4.27	52.11	5.54
19	Cystathionine gamma-lyase	gil114051239	2	1	5.09	43.03	6.43
20	Fructose-1,6-bisphosphatase	gil114051287	2	1	6.25	36.63	8.4
21	Stathmin	gil114052122	1	1	6.53	33.48	7.74
22	WD repeat domain 61	gil114051355	1	1	6.08	36.25	4.95
23	Nucleosome assembly protein	gil114052018	1	1	4.52	43.05	4.51
24	Juvenile hormone esterase	gil112983178	1	1	6.67	62.80	5.75
25	Replication protein A1	gil112983132	1	1	2.51	66.70	6.88
26	Yellow2	gil126680165	1	1	2.61	52.38	7.11
27	Proteasome 26S non-ATPase subunit 4	gil148298816	1	1	6.08	39.30	4.76
28	Signal transducer and activator of transcription	gil255652895	1	1	3.00	83.99	6.54
29	Heat shock protein hsp20.1	gil112983134	17	6	51.12	20.14	5.46
30	3-hydroxyisobutyrate dehydrogenase	gil195963353	5	3	11.18	34.02	9.14
31	H + transporting ATP synthase delta subunit	gil151301059	5	2	19.25	17.17	5.73
32	Ribosomal protein S2	gil112982669	5	2	11.49	28.07	10.46
33	Small nuclear ribonucleoprotein polypeptide	gil148298654	4	2	15.51	19.65	11.09
34	p23-like protein	gil112983282	3	2	20.12	18.83	4.54
35	larval cuticle protein	gil151301020	2	2	22.58	16.29	6.54
36	chemosensory protein 9	gil112983042	4	1	11.02	14.73	6.29
37	nonclathrin coat protein zeta 1-COP	gil114051996	3	1	10.67	20.61	4.92
38	receptor for activated protein kinase C RACK 1 isoform 1	gil115345341	2	1	3.76	36.04	8.07
39	juvenile hormone binding protein	gil113205954	2	1	4.94	26.64	5.03
40	thioredoxin-like protein	gil148298796	2	1	11.32	11.75	4.93
41	U2 small nuclear ribonucleoprotein A'	gil114052002	2	1	14.52	28.75	9.55
42	eukaryotic translation initiation factor 4H'	gil112983124	1	1	5.90	29.29	9.8

Table 1 continued

No.	Protein description	Protein accession	No. of peptides	No. of unique Peptides	Cover percent	Mw (kDa)	p*I*
43	hypothetical protein LOC778505	gil148298707	1	1	15.87	13.51	8.6
44	mobility group protein 1B	gil151301198	1	1	14.29	13.40	8.6
45	short-chain dehydrogenease/reductase-like	gil114050773	1	1	10.81	27.64	7.63
46	RecName: Full = Eukaryotic translation initiation factor 3 subunit G; Short = eIF3 g; AltName: Full = Eukaryotic translation initiation factor 3 subunit 4	gil122119431	1	1	10.95	30.27	8.2
47	small nuclear ribonucleoprotein E	gil114051397	1	1	16.84	10.99	9.57
48	ML-domain containing secreted protein	gil114052116	1	1	12.34	16.97	6.28
49	cleavage and polyadenylation specific factor 5	gil114051528	1	1	12.78	26.00	8.81
50	ubiquitin-conjugating enzyme E2	gil114050835	1	1	10.06	18.04	8.64
51	Arp2/3 complex subunit	gil115292423	1	1	12.36	20.48	8.3
52	odorant binding protein LOC100301497	gil237648976	1	1	8.45	15.83	5.08
53	TPA: putative cuticle protein	gil223671346	1	1	5.88	27.04	6.3
54	TPA: putative cuticle protein	gil223671242	3	2	18.56	19.94	5.46
55	RecName: Full = Chitinase-like protein EN03; AltName: Full = Imaginal disc growth factor-like protein; Flags: Precursor	gil74848915	1	1	6.24	47.92	7.64

Table 2 Specific proteins identified in the wing disc of *B. mori* pupa

No.	Protein description	Protein accession	No. of peptides	No. of unique Peptides	Cover percent	Mw (kDa)	p*I*
1	Molting fluid carboxypeptidase A	gil112983046	5	2	6.47	53,979.56	5.76
2	Carboxylesterase	gil114051680	4	2	7.41	61,415.99	5.22
3	Beta-N-acetylglucosaminidase 1	gil145651816	3	2	5.07	69,655.34	5.77
4	hemolin	gil112982982	3	2	9.51	44,820.95	5.12
5	hypothetical protein LOC778506	gil148298789	3	2	16.54	28,434.35	4.25
6	sterol carrier protein x	gil112983786	2	1	2.05	57,879.03	6.54
7	signal recognition particle receptor alpha subunit	gil148298802	1	1	4.59	67,380.1	7.58
8	ARP1 actin-related protein 1-like protein A	gil114053021	1	1	4.52	42,674.16	6.62
9	carboxylesterase CarE-12	gil189181680	1	1	3.33	64,158.56	6.6
10	TPA: putative cuticle protein	gil223671088	1	1	2.52	57,380.61	5.87
11	serpin 10	gil226342882	10	4	12.87	57,750.42	4.97
12	TPA: putative cuticle protein	gil223671372	8	4	32.58	27,716.94	8.42
13	electron-transfer-flavoprotein beta polypeptide	gil114053151	5	3	13.44	27,239.61	8.46
14	TPA: putative cuticle protein	gil223671292	5	3	18.97	24,542.27	6.45
15	TPA: putative cuticle protein	gil223671198	5	2	14.14	34,663.53	6.26
16	diapause bioclock protein	gil192293810	5	2	17.44	18,285.3	6.12
17	unnamed protein product	gil227262754	3	2	19.50	17,985.11	10.96
18	translation initiation factor 5A	gil112982832	3	2	16.25	17,524.78	5.16
19	ribosomal protein S10	gil112983505	3	2	10.62	18,192.01	9.91
20	juvenile hormone binding protein	gil255977208	3	2	7.76	27,319.31	6.39
21	RAB6A, member RAS oncogene family	gil114052691	2	2	11.00	23,628.66	5.53
22	chemosensory protein 7	gil112983910	2	2	20.17	13,357.56	9.04
23	serpin-27	gil195972056	2	1	2.05	10,3952.87	4.83
24	thioredoxin peroxidase	gil114052210	2	1	5.73	25,283.89	8.33
25	ribosomal protein S23	gil112983954	2	1	7.69	15,994.64	10.57

Table 2 continued

No.	Protein description	Protein accession	No. of peptides	No. of unique Peptides	Cover percent	Mw (kDa)	p*I*
26	small GTP-binding protein Rab10	gil148298847	2	1	5.91	23,125.54	8.3
27	FK506-binding protein	gil169234934	2	1	6.94	23,880.86	4.77
28	myosin heavy chain	gil197322818	2	1	2.15	96,020.13	5.09
29	heat shock protein hsp 19.9	gil112983420	2	1	7.34	19,890.56	6.53
30	pyridoxine 5′-phosphate oxidase	gil112984194	1	1	7.93	29,865.32	8.76
31	mRNA cap-binding protein eIF4E	gil114051309	1	1	9.52	24,445.6	5.77
32	S-phase kinase-associated protein	gil114052370	1	1	11.73	18,535.89	4.4
33	ribosomal protein P2	gil112984336	1	1	16.07	11,538.04	4.68
34	cyclic AMP-regulated protein	gil114051842	1	1	6.75	18,577.87	5.02
35	ribosomal protein S17	gil112984008	1	1	16.54	15,421.8	9.71
36	juvenile hormone resistence protein I	gil169234673	1	1	1.17	59,260.58	8.83
37	RecName: Full = Putative defense protein; Flags: Precursor	gil121950233	1	1	19.30	18,482.23	8.72

Table 3 Specific proteins identified in the wing disc of *B. mori* moth

No.	Protein description	Protein accession	No. of peptides	No. of unique Peptides	Cover percent	Mw (kDa)	p*I*
1	Antitrypsin isoform 1	gil253809709	13	7	28.83	43,428.4	5.41
2	TPA: putative cuticle protein	gil223671248	9	4	49.32	15,426.59	5.85
3	Tropomyosin isoform 2	gil114052272	7	3	13.73	32,580.61	4.73
4	Putative dopa decarboxylase protein	gil158451399	5	2	8.94	49,250.8	6.38
5	Very low-density lipoprotein receptor isoform 2	gil160333140	4	2	5.21	60,851.78	5.35
6	Low molecular lipoprotein 30 K precursor	gil156119322	4	2	9.06	29,242.32	6.9
7	Pupal cuticle protein	gil187423907	4	2	11.86	26,316.66	5.95
8	Serpin 25	gil226342910	2	1	2.76	44,958.38	8.01
9	Carboxylesterase	gil209171176	2	1	4.07	61,378.97	5.28
10	Ubiquinol-cytochrome c reductase core protein II	gil163838684	2	1	6.41	46,129.47	9.2
11	TPA: putative cuticle protein	gil223671244	2	1	13.30	19,509.2	6.51
12	TPA: putative cuticle protein	gil223671121	2	1	10.32	31,692.67	6.32
13	Phosphoserine aminotransferase 1	gil114052677	2	1	4.12	40,199.08	6.97
14	Malate dehydrogenase	gil153792270	1	1	2.45	67,258.39	6.12
15	Olfactory receptor	gil238623699	1	1	3.44	43,410.13	8.8
16	Vacuolar ATP synthase subunit H	gil114052663	1	1	3.37	55,130.49	6.15
17	NADPH cytochrome P450 reductase	gil162462676	1	1	1.60	77,689.24	5.5
18	Carotin binding protein	gil12743947	1	1	3.65	36,764.46	8.61
19	SUMO-1 activating enzyme	gil114052607	1	1	4.42	38,355.8	5.78
20	Eukaryotic initiation factor 5C	gil148298697	1	1	3.34	47,962.42	6.11
21	Histone H3	gil114051013	1	1	23.53	15,388.03	11.27
22	Mitochondrial prohibitin complex protein 2	gil114051710	1	1	4.35	33,176.41	9.7
23	Thioredoxin	gil114052058	1	1	4.18	31,877.99	5.11
24	ORF1	gil1549143	1	1	3.70	52,180.59	9.29
25	TPA: putative cuticle protein	gil223670966	1	1	21.38	14,815.82	9.27
26	Aspartic protease	gil90992734	1	1	5.19	41,448.37	6.05
27	Pupal cuticle protein	gil187423907	12	5	26.48	26,316.66	5.95

Table 3 continued

No.	Protein description	Protein accession	No. of peptides	No. of unique Peptides	Cover percent	Mw (kDa)	p*I*
28	TPA: putative cuticle protein	gil223671220	6	4	42.88	20,901.59	7.01
29	Lectin 4 C-type lectin	gil114052520	9	3	16.14	25,749.06	6.08
30	Mitochondrial cytochrome c	gil194303591	6	3	24.07	11,752.36	9.63
31	CPFL family cuticle protein 1	gil255652877	5	5	7.36	25,332.44	6.39
32	Thymosin isoform 2	gil221579752	2	2	20.45	14,737.44	4.83
33	Proteasome alpha 3 subunit	gil114051245	2	1	4.71	28,258.04	5.27
34	Ribosomal protein L7Ae	gil114052793	2	1	9.23	14,208.57	7.69
35	Ribosomal protein L9	gil112983495	2	1	6.32	21,377.03	9.94
36	Time interval measuring enzyme TIME	gil115529203	2	1	11.05	18,251.28	6.12
37	Hypothetical protein	gil229002332	1	1	16.36	12,313.46	8.95
38	Ribosomal protein L31	gil148298875	1	1	11.29	14,313.53	10.69
39	Signal peptidase complex subunit 2	gil114052092	1	1	12.50	20,592.75	8.33
40	Bm kettin	gil168823429	1	1	0.23	54,4514.58	5.93
41	Eukaryotic translation initiation factor 6	gil114052170	1	1	9.80	26,345.77	4.7
42	Signal peptidase 18 kDa subunit	gil114052797	1	1	8.43	20,254.83	9.18
43	Fumarylacetoacetase	gil160333413	1	1	8.48	36,202.93	8.34

were carboxylesterase, beta-N-acetylglucosaminidase, CarE-12, and pyridoxine 5′-phosphate oxidase. A component of the myofibril thick filament, myosin is composed of two heavy chains and many light chains. The identification of the myosin heavy chain in the wing disc during metamorphosis in the pupal-moth stage indicates its involvement in the formation of wing muscle.

In the wing discs at the moth stage, seven putative cuticle proteins were identified. Insect cuticle proteins are major components of an exoskeleton. When insects molt, the exoskeleton is renewed via biosynthesis of new cuticle proteins and degradation of old ones. The insect cuticle is constructed from many cuticle proteins with different temporal and spatial patterns (Willis 1996). Some proteins specifically identified at the moth stage, such as mitochondrial prohibitin complex protein 2 and proteasome alpha 3 subunits, may be involved in the degradation of old cuticle proteins. In addition, the identification of ribosomal proteins (L7Ae, L9, L31) suggests they might be closely related to new cuticle protein synthesis during the developmental process.

Gene ontology (GO) analysis of the functional categories

To further understand the function of the identified proteins, they were functionally categorized based on universal GO annotation terms (Ashburner et al. 2000) using the online GO tool WEGO [Web Gene Ontology Annotation Plot, (http://wego.genomics.org.cn/)]. GO analysis of the 55, 37, and 43 specific proteins identified in wing discs at the larval, pupal, and adult moth stage, respectively, linked these proteins to the "cellular component", "molecular function", and "biological process" categories (Fig. 3). Most of these proteins were categorized into the same functional classes.

"Cellular component" was the most common functional category shared by all three stages.

The differences in functional classes between the three groups of proteins indicate functions unique to a developmental stage. Only one class, "transcription regulator" was unique to the larval stage, and the class unique to the pupal stage was "antioxidant". Proteins only identified at the moth stage were classified in "extracellular region" and "protein-DNA complex process."

The 24, 18, and 23 proteins shared by the larval and pupal, pupal and moth, and moth and larval stages, respectively, were also analyzed by GO (Fig. 4). Most of the categories related to the three groups of proteins were the same. The special functional classes involving common proteins between the larval and pupal stage were "electron carrier", "translation regulator", "transporter", and "cellular component organization". Only one class, "death", involved a common larval and moth protein. The special functional classes involving common proteins between the pupal and moth stages were "endomembrane system", "membrane-bounded organelle", "organelle membrane", and "vesicle and cellular component biogenesis".

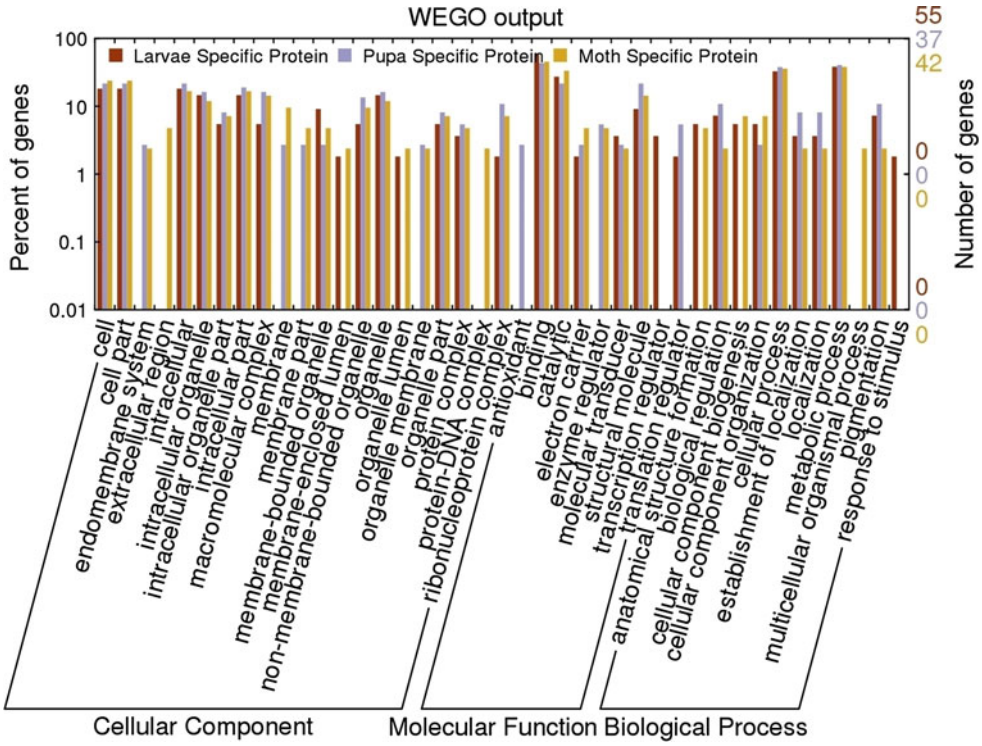

Fig. 3 Gene ontology categories for proteins specific to the wing discs of larva and pupa, and moth. The identified proteins were classified into "cellular component", "molecular function", and "biological process" by WEGO according to their GO signatures. The percentage of genes is proportional to total genes. The number of genes indicates the amount of genes with available GO terms in each group

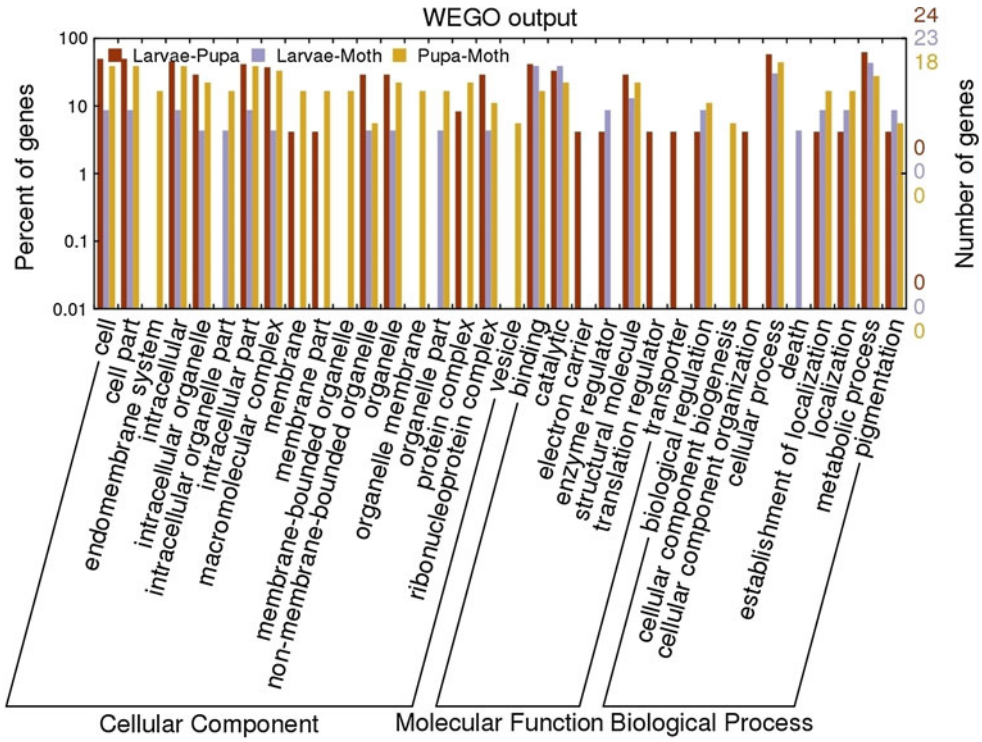

Fig. 4 Gene ontology categories for the proteins in wing discs shared by larva and pupa, pupa and moth, and moth and larva. The identified proteins were classified into "cellular component", "molecular function", and "biological process" by WEGO according to their GO signatures. The percentage of genes is proportional to total genes. The number of genes indicates the amount of genes with available GO terms in each group

Conclusion

Shotgun proteomics based on LC–MS/MS provides a highly sensitive and high-throughput method for the large-scale characterization of protein components in cells, tissues, and organs. However, bioinformatics analysis is indispensable for accurately interpreting MS data and providing a better understanding of the basic relationships

between proteome components. With the development of genomics and bioinformatics, shotgun LC–MS/MS will be a promising strategy in proteomics research.

In this study, shotgun LC–MS/MS provided a large amount of information on the proteomic profiles of wing discs at the larval, pupal, and moth stages during metamorphosis of the silkworm. Specific proteins identified at the three stages and common proteins shared by each pair of stages were identified and analyzed. Interesting proteins with different functions were identified and GO analysis of these proteins provided a global view of their functions. Functionally important proteins were involved in "cellular component", "molecular function", and "biological process" categories. Our findings are in line with shotgun analysis results reported by Huijuan Yang and Jin-e Chen (Yang et al. 2010; Chen et al. 2013). An analysis of the similarities and differences of these identified proteins will further our understanding of wing disc development in the silkworm and other insects.

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

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