

*Full Length Research Paper*

# Soluble proteome analysis of male *Ericerus pela* Chavannes cuticle at the stage of the second instar larva

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Shotgun liquid chromatography tandem mass spectrometry (LC-MS/MS) were employed to character the soluble proteome of male *Ericerus pela* cuticle at the stage of the second instar larva. A total of 278 protein groups (2584 peptides) were identified that involved in a wide spectrum of biological process and molecular function. The proteins were classified into seven categories according to their corresponding functions. Molecular weight (MW) of these identified proteins range from 11 kD to above 110 kD. (isoelectric point) pI were distributed in a range of pH 3.5 to 11.0. Gene ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that the proteins involved in binding, catalytic, cellular process, metabolic process, biological regulation and protein folding, sorting and degradation are highest. Many important functional proteins related to cell motility and cytoskeleton, stress response, DNA transcription translation and signal transduction were identified. Most heat shock proteins were identified in the proteome of *E. pela* cuticle at this stage. Chitin deacetylase, gasp and chemosensory protein (CSP) was also identified. The results were expected to provide a better understanding of the development of *E. pela*.

**Key words:** Shotgun liquid chromatography tandem mass spectrometry, proteome, cuticle, biological process, pathway, metabolism.

## INTRODUCTION

The Chinese white wax scale, *Ericerus pela* Chavannes (Homoptera: Coccidae) is a famous resource insect of China. The wax secreted by the scale insect was widely used in wax printing, engraving print, wax candle production and Chinese medicine about one thousand years ago, and in chemical, pharmaceutical, food and cosmetic industry now (Chen and Feng, 2009). *E. pela* is sexual dimorphism, the female is coated with hard chitin and undergoes hemimetabolism, and the male is coated

with filiform waxy secretion and undergoes holometabolism. The male secretes wax continuously at the early and later stage of the second instar larva, the wax form a thick wax layer that could avoid the sun burning and prevent water evaporation. The larva and pupa that coated with the wax layer does not move until eclosion. The difference between male and female is shown by the cuticle. Cuticle is important to the survival and sexual dimorphism of *E. pela* (Chen et al., 2009).

Insect cuticle consists mainly of chitin and matrix proteins but also containing lipids and phenolic materials (Kalume et al., 2003; Andersen, 2010; Moussian, 2010; Suderman et al., 2010), which accounts for the structural and mechanical support, chemical communication and

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preventing water evaporation (Nguyen et al., 2009; Bogus et al., 2010; Loof et al., 2010; Moussian, 2010). The cuticle of *E. pela* play an important role not only in supporting the body, but also in protecting the insect from high temperature and environmental stresses. The male and female are different in the properties of cuticle because they take different ecological strategy response to adverse environment (Chen et al., 2007). The female is coated with hard chitin cuticle, but the male is coated with soft transparent cuticle and thick wax layer. The wax layer secreted by the male larva is important to the survival of the male *E. pela*. The properties of the cuticle of male *E. pela* not only depend on its chitin, but also on the roles of its protein components. Although insect cuticle proteins have been reported by many researchers (Yatsu and Asano, 2009; Gallot et al., 2010; Papandreou et al., 2010), there is no report on large-scale characterization of the cuticle proteome of *E. pela*. Shotgun proteomics provides an eligible tool for a global profile of the proteins of insect at a special stage (Bandeira, et al., 2007; Dong et al., 2008; Li et al., 2009a, b, 2010). In this study, shotgun liquid chromatography tandem mass spectrometry (LC-MS/MS) was employed to identify the cuticle proteome of male *E. pela* at the stage of the second instar larva. The results were expected to provide a better understanding of the development of *E. pela*. The possible roles of the proteins were also discussed finally.

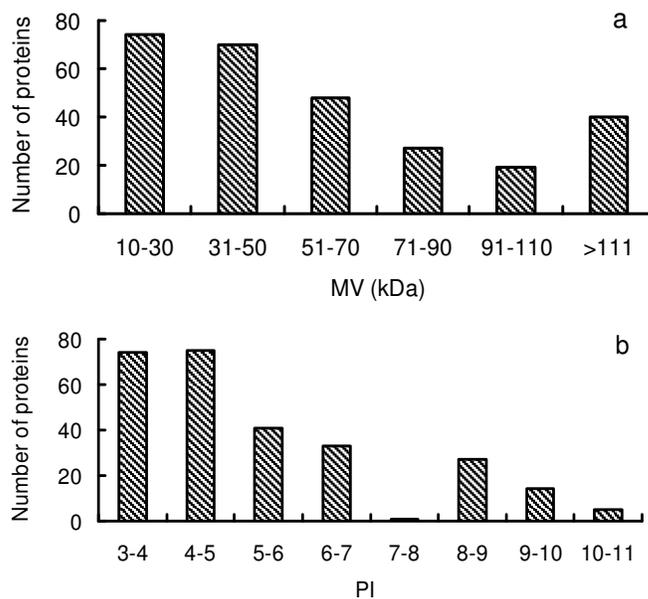
## MATERIALS AND METHODS

### Insect and protein isolation

The male *E. pela* at the stage of the second instar larva were collected from one branch of *Ligustrum lucidum*. The cuticle was detached from the substances in the body and washed in phosphate buffer solution (PBS) for 3 min, repeated three times, then homogenized in lysis buffer (8 M urea, 150 mM Tris, 10 mM DL-Dithiothreitol, 2% protease inhibitor, pH 8.5), centrifuged for 45 min at 15,000 g at 4°C, the supernate was transferred to a new 1.5 ml tube and stored at -80°C for shotgun LC-MS/MS.

### Shotgun LC-MS/MS

The sample of soluble proteins of male *E. pela* cuticle was put in a foam box with enough dry ice and sent to Shanghai Institutes for Biological Sciences. The soluble proteins were digested by trypsin, the digested protein's separation and identification was conducted by a Finnigan LTQ mass spectrometer (ThermoQuest, San Jose, CA, USA) coupled with a Surveyor high performance liquid chromatography (HPLC) system (ThermoQuest). Protein identification using MS/MS raw data was performed with SEQUEST software (University of Washington, licensed to Thermo Finnigan). There was no genomic resources and protein sequences of *E. pela* available, therefore a localization database was constructed from the National Center for Biotechnology Information non-redundancy (NCBI nr) protein database. The peptides and proteins are identified according to the database of proteins of insects, and accession number of these protein sequences were obtained. Protein identification results were filtered with the Xcorr ( $1+ \geq 1.9$ ,  $2+ \geq 2.2$ ,  $3+ \geq 3.75$ ) and DelCn ( $\geq 0.1$ ).



**Figure 1.** Molecular weight (MW) and theoretical isoelectric point (pI) distribution of the identified proteins. (a) distribution of MW, (b) distribution of pI.

## Bioinformatics analysis

The accession numbers of the identified proteins were submitted to UniProtKB (<http://www.uniprot.org>) for function analysis. The proteins were classified into different categories according to their functions. Gene Ontology (GO) classification of the identified proteins was conducted as follows. Accession numbers of these protein sequences were submitted to Blast2GO (<http://blast2go.bioinfo.cipf.es/home>). The corresponding GO numbers were obtained. The GO annotation terms result was conducted using the online GO tool WEGO [Web Gene Ontology Annotation Plot, (<http://wego.genomics.org.cn/>)]. The accession numbers of the identified proteins were subjected to search against Kyoto Encyclopedia of Genes and Genomes (KEGG) reference pathway database (<http://www.genome.jp/kegg>). The pathways in which proteins involved were classified according to the definition of KEGG.

## RESULTS

### Identification and classification of the soluble proteins

A total of 278 protein groups (2584 peptides) were identified. Theoretical molecular weight (MW) of these proteins range from 11 kD to above 110 kD, most of them were below 70 kD (Figure 1a). The theoretical isoelectric point (pI) were distributed in a range of pH 3.5 to 11.0 (Figure 1b). 216 of the 278 identified protein groups have UniProtKB entries, and the proteins were classified into seven categories according to their functions, including

stress resistance (21 proteins), cell motility and cytoskeleton (50 proteins), signal transduction (10 proteins), metabolism (47 proteins), genetic information (47 proteins), transport and protein folding (9 proteins) and others. While the largest set of proteins was assigned to the group of cell motility and cytoskeleton, metabolism and genetic information. We identified more than thirty enzymes involved with intermediary metabolism, signal transduction and muscle contraction, and a lot of proteins associated with different functions, which represented the soluble proteome of cuticle of *E. pela* Chavannes. Part of the proteins was described in Table 1.

### Bioinformatics analysis

The identified proteins were submitted to Blast2GO for GO term annotation. Most proteins have no GO entries or annotation terms because of lack of distinctive features to the known proteins. 83 proteins could be assigned specific cellular component, molecular function or biological process categories. GO annotation identified 9 molecular function and 17 biological process categories, there was no major class dominant in the proteome. The proportions of binding, catalytic, cellular process, metabolic process and biological regulation are highest (Figure 2).

All identified proteins were also been subjected to the KEGG to predict the metabolic pathway. Among the identified proteins, most of them belong to genetic information processing (include protein folding, sorting and degradation, DNA replication and repair, transcription and translation) and metabolism (include energy metabolism, carbohydrate metabolism, amino acid metabolism, nucleotide metabolism). 122 proteins were found to be involved in 11 pathways, and some of them belonging to multiple pathways. Most proteins could not be ascribed any biological function (Figure 3).

### DISCUSSION

Gene expression profiling or transcriptome analysis can predict the level of gene expression and provide more genetic information (Wang et al., 2010; Zhang et al., 2010), but mRNA levels may not correlate with the protein level, so proteome analysis is indispensable for entomological research (Li et al., 2009a). The shotgun proteomics strategy has been widely used in proteome research (Bandeira, et al., 2007; Dong et al., 2008; Li et al., 2009a, b, 2010), and has been employed in the research of model insects, such as *Drosophila melanogaster* (Baggerman et al., 2005), *Anopheles gambiae* (Kalume et al., 2005) and *Bombyx mori* (Li et al., 2009a, b, 2010). Although, there was no fully developed protein database for *E. pela*, genomic resources and protein database of other organisms

provide much reference information. In this report, we characterized the larval cuticle proteome of male *E. pela* using shotgun proteomics approach. 278 protein groups were identified that involved in different biological process. Most pathways in which these proteins involved were housekeeping pathways that supporting cellular activity. Possible contributions of some proteins were discussed thus.

### Cell motility and cytoskeleton

Proteins are key determinants of the insect cuticular physical properties. A lot of proteins related to cell motility and cytoskeleton were identified such as myosin, actin, tropomyosin, tubulin and spectrin. Myosin and actin are important proteins whose functions are related to muscle contraction and motility (Alvite and Esteves, 2009; Lehman et al., 2009; Li et al., 2010; Vikhoreva and Mansson, 2010). Tropomyosin, which interacts with actin in the muscle was also identified. These proteins are represented by multiple isoforms, and are highly conserved and essential within insects (Watabe and Ikeda, 2006; Kim et al., 2009; Creed et al., 2010; Prochniewicz et al., 2010), these proteins are identified abundantly. Although the *E. pela* larva does not move until eclosion, muscle contraction and cell motility are essential for the physiological activity.

Meanwhile, tubulin multigene family was also identified in the *E. pela* cuticular proteome. Tubulin has been described as cytoskeleton proteins, and also plays a key role in the mitotic spindle assembly (Yang et al., 2009). Tubulin is important to cell division and development of *E. pela* larva. Motility and cytoskeleton related proteins were identified abundantly because of their conventional functions.

### Stress response proteins

The cuticle of insects not only supports the insect body, but also protects the insect from environmental stresses (Nguyen et al., 2009). White wax production in China was processed in Hunan and Sichuan province, where the climate is hot and humid. According to wax production and practice, heat stress and high humidity increase wax secretion of *E. pela*, resulting in thick wax layer. Chen et al. (1998, 2007, 2009) further studied links between wax secretion and stress resistance in the *E. pela*, and found that wax secretion is the survival strategy response to heat stress and high humidity. Stress response proteins, which is concurrent with environmental stresses, such as heat shock proteins (HSPs) will protect the cells from damage (Kang et al., 2009; Benoit et al., 2010; Heikkila, 2010; Rungrassamee et al., 2010; Walcott, Heikkila, 2010). In this work, peroxidase, thiol peroxidase and five heat shock protein gene family were detected

**Table 1.** Classification of the identified proteins.

<b>Protein</b>	<b>Biological process</b>	<b>Molecular function</b>
<b>Cell motility and cytoskeleton</b>		
myo-2		ATP binding
Actin		ATP binding
Tropomyosin 1	Muscle contraction	Actin binding
Paramyosin, long form		Motor activity
Tubulin- $\alpha$ -chain	Protein Polymerization	GTP binding
Tubulin $\beta$ -4 chain	Microtubule-Based Movement	GTP binding
$\alpha$ -Spectrin	Cytoskeleton organization	Actin binding
Collagen $\alpha$ -2(IV) chain		Extracellular matrix structural constituent
Lachesin	Maintenance of epithelial integrity, open tracheal system	Protein binding
Moesin/ezrin/radixin homolog 1	Establishment or maintenance of epithelial cell apical/basal polarity	Actin binding
Calbindin-32		Calcium ion binding
Lectin 4 C-type lectin		Sugar binding
<b>Stress response proteins</b>		
Heat shock protein 20.6	Response to heat	
Heat shock protein 60	Response to stress	ATP binding
Heat shock protein 70 kDa	Response To Stress	ATP binding
Heat shock protein 82	Response To Stress	ATP binding
Heat shock protein 90	Response To Stress	ATP binding
1-cys peroxiredoxin	Cell redox homeostasis	antioxidant activity
Putative peroxidase	Response to oxidative stress	
DnaJ-lik protein	Response to heat	ATP binding
Thiol peroxiredoxin	Response to oxidative stress	
<b>Energy metabolism</b>		
ADP, ATP carrier protein	Transmembrane transport	ATP:ADP antiporter activity
ATP synthase subunit $\alpha$	Plasma Membrane Atp Synthesis Coupled Proton Transport	hydrogenion transporting ATP synthase activity, rotational mechanism
ATP synthase subunit $\beta$	Plasma Membrane Atp Synthesis Coupled Proton Transport	Hydrogenion Transporting Atp Synthase activity, Rotational Mechanism
V-type proton ATPase catalytic subunit A	ATP synthesis coupled proton transport	Hydrogen Ion Transporting Atp Synthase activity, Rotational Mechanism

Table 1. Contd.

lethal (2) 01289	Cell redox homeostasis	Protein disulfide isomerase activity
Transitional endoplasmic reticulum ATPase TER94		ATP binding
<b>Carbohydrate metabolism</b>		
2-oxoglutarate dehydrogenase	Glycolysis	Oxoglutarate dehydrogenase (succinyl-transferring) activity
Aconitase, mitochondrial	Tricarboxylic acid cycle	Aconitate hydratase activity
ATP citrate lyase	Citrate metabolic process;	ATP citrate synthase activity
Pyruvate carboxylase	Pyruvate metabolic process	Pyruvate carboxylase activity
Chitin deacetylase 1	Chitin metabolic process	Chitin deacetylase activity
Gasp	Chitin metabolic process	Chitin binding
Pyruvate dehydrogenase	Metabolic process	Catalytic activity
Pyruvate kinase, muscle, b	Glycolysis	Catalytic activity
Isocitrate dehydrogenase	Tricarboxylic acid cycle	Isocitrate dehydrogenase (NAD <sup>+</sup> ) activity
Probable citrate synthase 2, mitochondrial	Tricarboxylic acid cycle	Citrate (Si)-synthase activity
Enolase	Glycolysis	Phosphopyruvate hydratase activity
Fructose-bisphosphate aldolase	Glycolysis	Fructose-bisphosphate aldolase activity
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	NAD or NADH binding
Phosphoglycerate kinase	Glycolysis	Phosphoglycerate kinase activity
Phosphoglycerate mutase	Glycolysis	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase activity
Triosephosphate isomerase	Metabolic process	Triose-phosphate isomerase activity
Malate dehydrogenase	Tricarboxylic acid cycle	Malic enzyme activity
<b>Nucleic acid metabolism</b>		
Purine biosynthesis protein 6, pur6	Purine nucleotide biosynthetic process	ATP binding
Protein catabolic process		

Table 1. Contd.

Tat-binding protein-1	Protein catabolic process	Endopeptidase activity
Calpain	Proteolysis	Calcium-dependent cysteine-type endopeptidase activity
Ubiquitin carrier protein	Post-translational protein modification	Small conjugating protein ligase activity
<b>Genetic information processing</b>		
Histone H2A	Nucleosome assembly	DNA binding
Histone H2B	Nucleosome assembly	DNA binding
Histone H3	Nucleosome assembly	DNA binding
Histone H4	Nucleosome assembly	DNA binding
Methyltransferase	Methylation	Methyltransferase activity
DNA polymerase	DNA replication	DNA binding
Calmodulin-binding transcription activator (Camta), drome	Regulation of transcription	Transcription regulator activity
Retinoblastoma binding protein 4	Regulation of transcription	
DNA-binding protein smubp-2		DNA binding
40S ribosomal protein SA	Translation	Structural constituent of ribosome
60S ribosomal protein L5	Translation	5S rRNA binding
Ribosomal protein L19	Translation	Structural constituent of ribosome
Elongation factor 1- $\alpha$	Translational elongation	Translation elongation factor activity
Inhibitor of apoptosis protein (AGAP002651-PA)	Post-translational protein modification	Small conjugating protein ligase activity
<b>Signal transduction processing</b>		
1D-myo-inositol-trisphosphate 3-kinase isoform C		Inositol trisphosphate 3-kinase activity
AGAP010767-PA	Regulation of Rho protein signal transduction	Rho guanyl-nucleotide exchange factor activity
Citron ser/thr kinase	Protein amino acid phosphorylation	Protein serine/threonine kinase activity
Integrin- $\alpha$ -1	Integrin-mediated signaling pathway	Receptor activity
GTP binding protein		GTP binding
Putative cAMP-dependent protein kinase regulatory chain type I		Kinase activity

Table 1. Contd.

Calmodulin Protein kinase C, eye isozyme Neuroglian	Adaptation of rhodopsin mediated signaling Axon ensheathment	Calcium Ion Binding Protein binding Calcium ion binding
Cdk5	Protein amino acid phosphorylation	Protein serine/threonine kinase activity
<b>Chemosensory protein CSP3</b> Putative accessory gland protein Transport and protein folding	Protein folding	Unfolded protein binding
ATP-binding cassette sub-family A member 3, putative	ATP binding	ATP-binding cassette sub-family A member 3, putative
Low-density lipoprotein receptor (Ldl)	Receptor activity	Low-density lipoprotein receptor (Ldl)
Calreticulin Chaperonin	Protein folding Protein folding	Calreticulin Chaperonin
Nascent polypeptide associated complex protein alpha subunit	Nascent polypeptide-associated complex	Nascent polypeptide associated complex protein alpha subunit

which were known as stress resistance proteins. HSPs were induced by high temperature and humidity and abundantly identified in the cuticular proteome suggests that *E. pela* possess self-protection mechanisms against environmental stresses though both cuticle protection enhancement and wax secretion. A lot of stress resistance proteins were revealed at the proteomic level could explain the higher tolerance of the male *E. pela* to environmental stress.

### Metabolism

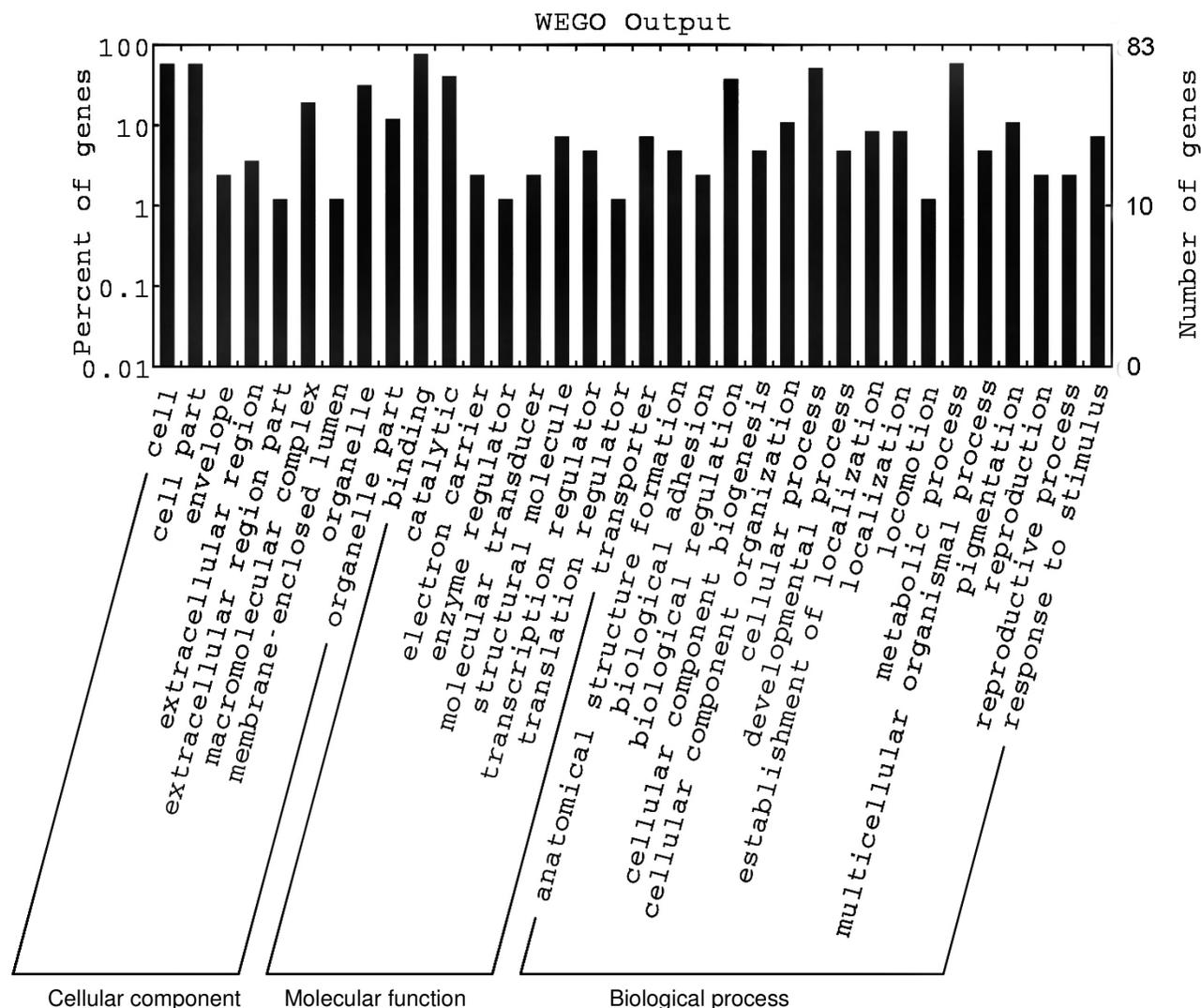
Among the proteins found during this proteomic study, many proteins were involved in metabolism,

while most of them were involved in carbohydrate metabolism. These proteins cover a wide spectrum of biological functions, such as oxidative phosphorylation, transport, electron carrier activity, ATP binding and catalytic activity.

Energy metabolism is necessary to provide energy for insect physiological activity. ADP, ATP carrier protein and ATP synthase were identified in the proteome of *E. pela* cuticle. ATP synthase plays a crucial role in oxidative phosphorylation (Willers and Cuezva, 2010). These housekeeping proteins were also observed in survey of proteome of *Culex pipiens quinquefasciatus* and *B. mori* (Ribeiro et al., 2004; Hou et al., 2007), which were important to energy metabolism. We also identified a lot of proteins involved in

carbohydrate metabolism (including citrate cycle, pyruvate metabolism, glycolysis), which account for the housekeeping pathways supporting cellular activity. We identified 2 proteins (Chitin deacetylase and Gasp) known to be involved in chitin metabolic process, which is important to insect cuticle (Shrestha et al., 2004; Cai et al., 2006; Nisole et al., 2010). Proteins involved in purine nucleotide biosynthetic process and protein catabolic process were identified. As a whole, these results illustrate that, abundant proteins involved in metabolism were important to cuticle physiological activity.

It is regretful that there was no identified protein similar to wax genes of plant, such as wax synthase and CER genes of *Arabidopsis thaliana*



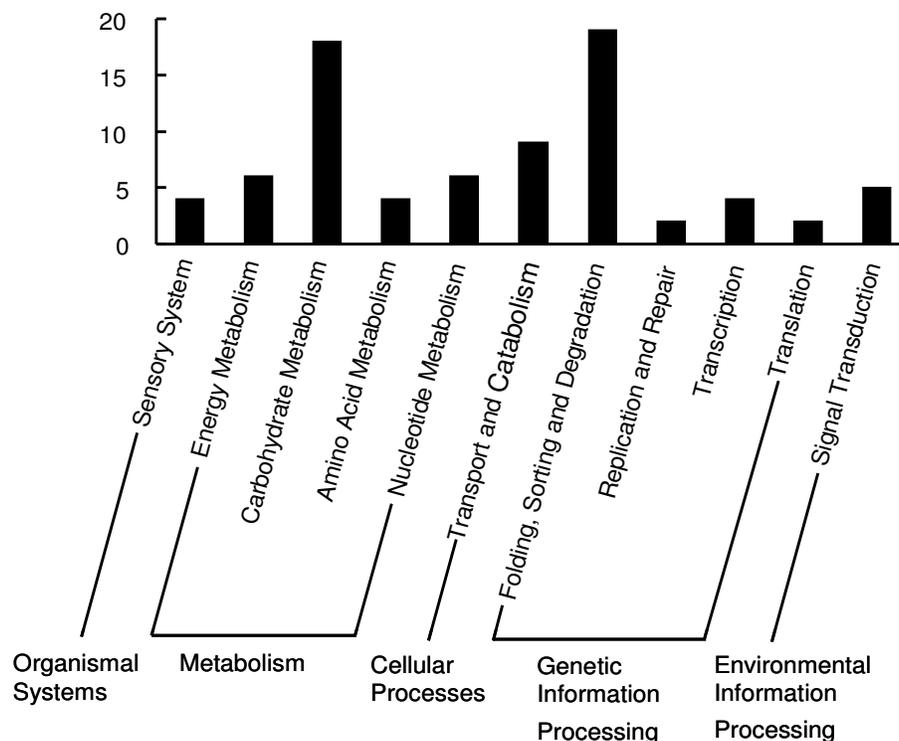
**Figure 2.** Gene ontology (GO) categories of the identified proteins.

(Rowland et al., 2007; Klypina, Hanson, 2008; Holmes, 2010). This maybe because proteins of *E. pella* share lower homology with wax genes of plant, no homologous proteins were identified in the cuticle proteome of *E. pella*. Although, silk gland proteins from the silkworm has been studied (Zhang et al., 2006), there was no reference sequence of silkworm because of the different metabolism pathways of *E. pella*, which result in different component of wax to silk. Progress in understanding genes involved in wax metabolism was hindered by difficulty in accurately describing wax gene product functions based on their amino acid sequence homologies with known proteins (Goodwin et al., 2005). This would explain why no proteins involved in wax metabolism was identified in the proteome research of *E. pella*.

### Proteins associated with genetic information processing and signal transduction

We identified 47 proteins known to be involved in genetic information processing. These proteins are histone, DNA polymerase, ribosomal proteins, elongation factor 1-alpha, chaperonin, methyltransferase and so on. These results confirmed high expression activities at this stage.

Ten proteins are associated with signal transduction pathways including GTP binding protein, cAMP-dependent protein kinase, protein kinase C and Cdk5. GTP binding protein have been suggested to play an important role in many signal transduction pathways that involved in receptor phosphorylation, down-regulation and pathway switching (Gomperts et al., 2009). Chemosensory protein (CSP) was identified in the cuticle



**Figure 3.** Classification of pathways according to the definition in KEGG.

proteome. CSPs has been found to play an important role in recognition of different ant colony by combining with cuticular hydrocarbons (Ozaki et al., 2005). Based on the research of *Camponotus japonicus* and *Apis mellifera ligustica* (Ozaki et al., 2005; Dani et al., 2005), CSP play a crucial role in swarming of *E. pela*.

The *E. pela* cuticle proteome is a complex mixture of proteins, in this proteomic study, we found many housekeeping proteins and insect-specific proteins. because of the bottleneck for many shotgun proteomics experiments, there were some proteins not identified, and we did not found proteins related to the interesting property of scale insect, such as wax secretion. Because the cuticle proteins were insoluble, it is difficult to isolate the cuticle proteins, they were not identified in this soluble proteome research.

The results of this study are based on large-scale characterization of the cuticular proteome of *E. pela*, and thus can be considered as fully representative. The pathway analysis and protein function classification at the proteomic level provided many information of physiological activity of *E. pela* at the stage of the second instar larva. The function of some important proteins warrant further studies.

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