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RESEARCH REPORT

Proteomic analysis of the silkworm midgut during larval-pupal transition

M Kannan[a], V Suryaaathmanathan[a], M Saravanakumar[b], A Jaleel[b], D Romanelli[c], G Tettamanti[c], M Krishnan[a]

[a]*Insect Molecular Biology Laboratory, Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli - 620 024, Tamil Nadu, India*
[b]*Mass Spectrometry & Proteomics Core Facility, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram - 695 014, Kerala, India*
[c]*Department of Biotechnology and Life Sciences, University of Insubria, Varese - 21100, Italy*

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Abstract

Metamorphosis is a key process in holometabolous insects since specific larval tissues, such as the midgut, the silk gland and the fat body, are remodeled into adult organs, or even disintegrate. However, the role of proteins and factors involved in the remodeling of these organs is still unclear. For this reason we undertook a proteomic study on the larval midgut of *Bombyx mori* to identify proteins whose expression significantly changes during larval-pupal metamorphosis and thus can have a role in this process. 2D-PAGE analysis showed upregulation or new expression of midgut proteins at 45 (pI 5.2), 29.8 (pI 6.11) and 20-kDa (pI 6-7) during the pupal stage. In addition zymogram analysis demonstrated the occurrence of protease activity at 37-kDa at the same developmental stage. By using MALDI-TOF-MS analysis the protein spots from 2D gel were identified as Hemolin, low molecular weight 30-kDa lipoproteins, HSP 20.8 and HSP 20.4, while the proteolytic band was identified as 37-kDa serine protease. The evidence herein presented suggests that the identified proteins could be involved in the immune protection during the remodeling of silkworm midgut that occurs at larval-pupal transition and represents a platform of knowledge necessary for future functional studies.

Key Words: *Bombyx mori*; immune response; midgut remodeling; two-dimensional polyacrylamide gel electrophoresis; zymogram; matrix assisted laser desorption/ionization-mass spectrometry

Introduction

The larval midgut of the silkworm represents the middle part of the alimentary canal and is responsible for the digestion and the assimilation of nutrients that are necessary for the growth and the development of the larva (Dow, 1986; Terra and Ferreira, 1994). The midgut is formed by a monolayered epithelium supported by a basal lamina. On the luminal side, the epithelium is lined by a peritrophic matrix (PM) that plays several roles in insect digestion; moreover PM protects midgut cells from mechanical lesions and acts as a protective physical barrier against ingested pathogens and toxins (Hegedus *et al.*, 2009; Hu *et al*., 2012).

Corresponding author:
Muthukalingan Krishnan
Insect Molecular Biology Laboratory
Department of Environmental Biotechnology
Bharathidasan University
Tiruchirappalli - 620 024, Tamil Nadu, India
E-mail: profmkrish@gmail.com

During metamorphosis, the larval midgut is completely remodeled to form the midgut of the adult insect that, in Lepidoptera, has different food habits or does not eat at all, as in *Bombyx mori*. In particular, the larval epithelium forms a compact mass of cells, called yellow body, which is progressively degraded by autophagic and apoptotic processes (Wu *et al*., 2006; Tettamanti *et al.*, 2007a, b; Parthasarathy and Palli, 2007; Franzetti *et al.*, 2012, 2015) and the products derived from cell digestion is recycled by the newly-forming epithelium that persists until the adult stage. This epithelium originates from the proliferation and differentiation of intestinal stem cells (Franzetti *et al.*, 2015).

In the last few years, several proteomic studies have been performed on this organ to understand the molecular mechanisms responsible for food digestion, nutrient absorption, protein-protein interaction and midgut defense role (Krishnan and Konig, 2011). The works by Kajiwara *et al.* (2005) and Yao *et al.* (2009), performed on *B. mori* fifth

instar larvae, identified dozens of proteins that are involved in the digestion activity, play structural or metabolic roles, contribute to PM formation and belong to signaling pathways. Later on, additional proteomic analyses have focused attention on particular issues: Qin *et al*. (2014) compared protein expression in midgut derived from male and female larvae, while Zhang *et al*. (2011) analyzed midgut proteins in control *B. mori* fifth instar larvae, as well as in larvae undergoing starvation. Finally an accurate study of the proteins that form PM in fifth instar larvae was accomplished by Hu *et al*. (2012). Beside the latter work, which identified ten proteins that participate in defending silkworm from bacteria and viruses (they were accordingly classified as antibacterial and antiviral proteins), none of these studies evaluated proteins that could be involved in midgut tissue remodeling and immunity during pupal stage. This represents a profound gap of knowledge since it has been shown that several enzymes intervene during midgut remodeling along metamorphosis in order to regulate the bacterial content of the alimentary canal. For example Tsuji *et al.* (1998) reported that a 26-kDa serine protease has antimicrobial activity necessary to remove the bacterial flora which normally resides in the larval midgut of *Sarcophaga peregrina* and is released from the decaying epithelium during yellow body formation. This is a prerequisite for its replacement by the adult microbiota. In addition, in *B. mori* (Franzetti *et al.*, 2015), *Heliothis virescens* (Tettamanti *et al.*, 2007b), and *Manduca sexta* (Russell and Dunn, 1991) it has been demonstrated that lysozyme, an hydrolase with potent antibacterial property, is released into the lumen of the newly-forming pupal midgut, during the degradation of the yellow body. This may protect the pupa and the adult from bacterial threats that could originate during the restructuring of the midgut (Franzetti *et al*., 2015).

In the present study, we undertook a proteomic approach to ascertain proteins involved in midgut tissue remodeling and immunity during metamorphosis of *B. mori* with the aim of providing preliminary evidence necessary to undertake functional studies.

Materials and Methods

Insect rearing

Eggs of domesticated silkworm, *Bombyx mori* (crossbreed race, Tamilnadu White X NB4D2) were obtained from the Government Grainage Centre, Tiruchirapalli, India. The eggs were incubated at 27 ± 2 °C and 75 ± 5 % relative humidity for a successful hatch. Newly hatched larvae were fed with chopped, tender mulberry leaves (MR2 variety) until third instar and with coarse leaves up to the last instar (Nirmala *et al*., 1999). After the animals had ecdysed to the last larval stage (fifth instar), they were staged and synchronized according to Franzetti *et al*. (2012).

Sample collection and preparation of protein extracts

Midgut along with PM was dissected from larvae, washed with 0.7 % NaCl solution and stored at -20 ºC. Protein extraction was conducted as previously described by Feng *et al*. (1999) with slight modifications. 20 mg of midgut tissue were homogenized in 100 µl of homogenization buffer (50 mM Tris, 10 mM EDTA, 15 % glycerol, 0.005 % phenylthiourea, pH 7.8) with mortar and pestle. The homogenate was collected into 1.5 ml polypropylene microcentrifuge tubes and centrifuged at 10,000×*g* for 5 min at 4 °C. The supernatant was collected and centrifuged under the same conditions. An aliquot of the supernatant was stored at -80 °C for zymographic analysis. A mixture of 0.07 % of β-mercaptoethanol and 10 % trichloroacetic acid in cold acetone was added to an equal volume of the supernatant, which was then kept at 4 °C for 10 min. The protein extract was centrifuged at 12,000×*g* for 10 min at 4 °C. The pellet was washed in ice-cold acetone with 0.07 % β-mercaptoethanol and centrifuged at 12,000×*g* for 10 min at 4 °C. The washing step was repeated twice. The resultant pellet was re-suspended in 100 µl of lysis buffer containing 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 40 mM Tris-HCl and 1 % (w/v) dithiothreitol (DTT). Then it was centrifuged at 12,000×*g* for 10 min at 4 °C. The total protein concentration was quantified using Bradford's method (Bradford, 1976) and the samples were stored at -80 °C.

SDS-PAGE

To investigate the protein profile of midgut, protein samples were subjected to 12 % SDS-PAGE (Laemmli, 1970). Approximately 30 µg of proteins were quantified (Bradford, 1976) mixed with an equal volume of 2x sample loading buffer (0.125 M Tris-HCl, pH 6.8, 4 % SDS, 10 % β-mercaptoethanol, 20 % glycerol and 0.02 % bromophenol blue). The mixture was boiled at 95 °C for 5 min and loaded on the gel. SDS-PAGE run was performed until bromophenol blue front reached the end of the gel (50 V and 70 V were used to run stacking and running gel, respectively). The gel was stained with Coomassie brilliant blue R-250 staining solution (0.1 % Coomassie brilliant blue R-250 in 40 % methanol and 10 % glacial acetic acid) for 6 h. The gel was then rinsed with double distilled water and placed in a destaining solution (40 % methanol and 10 % glacial acetic acid) until the appearance of clear bands in the gel. Protein size was estimated according to the standard protein molecular weight marker (PageRuler Plus Unstained Protein Ladder, Thermo Scientific, USA).

Zymogram analysis

To analyze the proteolytic profile of midgut proteases, protein samples (30 µg of protein) were subjected to electrophoresis (80 V at 4 °C) on 12 % SDS-PAGE gels copolymerized with 0.1 % gelatin (Saboia-Vahia *et al.*, 2013). After electrophoresis, the gel was washed twice for 30 min at 4 °C in wash buffer (50 mM Tris base, pH 7.4, 200 mM $CaCl_2$ and 16 mM sodium azide) containing 2.5 % Triton X-100. Then the gel was incubated overnight in the reaction buffer (50 mM Tris base, pH 7.4, 200 mM

$CaCl_2$ and 16 mM sodium azide) at 37 °C. The proteolytic bands were visualized by staining the gel with 0.25 % Coomassie blue R-250 and subsequent destaining with 10 % glacial acetic acid. The molecular mass of the proteases was calculated by comparison with the mobility of protein molecular weight marker. The Coomassie Brilliant Blue R-250 stained protein band from SDS-PAGE, corresponding to the proteolytic band on zymogram gel, was analyzed by MALDI-TOF-MS as described below.

Two-dimensional gel electrophoresis

Midgut proteins were separated by two-dimensional electrophoresis according to the manufacturer's instructions (2DE-DIGE Manual, GE Healthcare, USA). Briefly, 300 µg of proteins were dissolved in 310 µl rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS, 0.28 % DDT, and 1 % IPG buffer, pH 3-10). The protein solution was loaded onto an immobilized pH gradients strip (IPG strip, 18 cm, NL 3-10, Bio-Rad, USA) and kept overnight for passive rehydration of IPG strip. Isoelectric focusing (IEF) was performed using Ettan IPGphor 3 and the profile as follows: 500 V for 30 min (Step), 1000 V for 30 min (Step), 3,000 V for 2 h (Step), and 8,000 V for 3 h (Grad), 8,000 V for 6 h (Step) and a total of 63,000 Vh (voltage multiplied by hours). The current did not exceed 50 mA per strip. After IEF, the strips were equilibrated in Equilibration Buffer I (EB-I, 50 mM Tris-HCl, pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS, and 1 % DTT for 15 min and then in EB II (replaced 1 % DTT with 2.5 % iodoacetamide) for 15 min. The equilibrated gel strips were loaded onto a 12 % SDS-polyacrylamide gel. SDS-PAGE was performed at 15 °C using PROTEAN II Xi Cell unit (Bio-Rad) at constant power of 1 W/gel for 1 h followed by 3 W/gel until the bromophenol blue reached the bottom of the gel. The gel was stained for 2 h or overnight in 0.1 % of Coomassie Brilliant Blue R-250 solution (Sigma) in 40 % methanol and 10 % glacial acetic acid. The gel was rinsed thrice with water and then with a destaining solution containing 10 % glacial acetic acid and 40 % methanol in water until clear protein spots were visible. The gel was stored in 10 % ethanol at 4 °C to avoid microbial contamination. The molecular mass of protein spots was calculated using a protein ladder (PageRuler Plus Prestained Protein Ladder, Thermo Scientific, USA). The analysis was done on duplicates for each sample and a comparison of relative intensity of protein spots between the larval and pupal midgut was performed. The protein gel spots, which showed difference in their intensity, were excised for protein identification by mass spectrometry.

In-gel digestion and MALDI-TOF-MS analysis

Gel spots were subjected to in-gel reduction and alkylation, followed by trypsin digestion, as previously described by Shevchenko *et al*. (2006). Peptides were extracted by using 100 µl of extraction buffer (1:2 (v/v) 5 % formic acid/acetonitrile). Extracted peptides were dried in a SpeedVac to remove organic solvent. Dried samples were dissolved in 10 µl water before mass spectrometry analysis by MALDI-TOF/TOF (UltrafleXtreme, Bruker Daltonics, Germany). The ingel digested and eluted peptides were co-crystallized with α-cyano-4-hydroxycinnamic acid matrix on the target plate (384-well ground steel plate, Bruker Daltonics, Germany) and external peptide mass calibration was applied (Peptide mixture-1, Bruker) according to the manufacturer's instructions. The peptide mass fingerprint (PMF) data were acquired in the mass range of 700-3,500 m/z. The MS/MS fragmentation was carried out for the selected peptide in the LIFT mode of the instrument. In order to confirm the identification, all MS/MS data from LIFT TOF/TOF spectra were combined with the corresponding MS peptide mass (PMF) and mass spectra were imported into the database search engine (BioTools v2.2 connected to MASCOT, version 2.2.04; Matrix Science). MASCOT searches were done by using the database for *B. mori* downloaded from NCBI with the following settings: number of miss cleavages permitted was 1; fixed modifications such as carbamidomethyl on cysteine, variable modification of oxidation on methionine residue; peptide tolerance of 0.7 dalton and fragmentation ion tolerance 1.2 dalton, enzyme used as trypsin and a peptide charge setting as +1.

Quantitative real-time PCR (qRT-PCR)

The midgut was dissected from L5D3 larvae to P5 pupae, immediately frozen in liquid nitrogen, and stored at -80 °C until use. Total RNA was extracted from 20-40 mg of tissue using Trizol reagent (Life Technologies, Carlsbad, USA) according to manufacturer's instructions with slight modifications. RNA was treated with TURBO DNA-free Kit (Life Technologies) to remove any genomic DNA contamination and its integrity was assessed by electrophoresis. RNA was retro-transcribed using M-MLV reverse transcriptase (Life Technologies). qRT-PCR primers were designed for *BmHemolin* (NM_001043623.1), *BmHsp20.8* (NM_001098324.1), *BmHpc6* (NM_001044021.1) and *BmRP49* (NM_001098282.1). The primers used are indicated in Table 1. qRT-PCR was performed with iTaq Universal SYBR Green Supermix (Biorad) using a 96-well CFX Connect Real-Time PCR Detection System (Biorad). $2^{-\Delta\Delta Ct}$ method, with *BmRP49* as housekeeping gene, was used to calculate the relative expression of the genes of interest. The experiment was performed in triplicate. Statistical analysis was performed using ANOVA followed by Tukey HSD test.

Results and Discussion

Although in recent years proteomic approaches have been successfully used in various research fields related to silkworm biology, more information on the proteins that are expressed during the remodeling of silkworm midgut along metamorphosis is needed. In the present study the total protein was extracted from midgut of larvae (L5D4) up to pupae (P2) and subjected to SDS-PAGE, zymogram analysis and 2D-PAGE.

Table 1 Primers used for qRT-PCR analysis in the present study

Gene	Genbank number	Primer sequence
BmHPC-6	NM_001044021.1	F: TATGGACAAAGGATGGAAAGG R: AACTTGAGGGCGTGATGGT
BmHemolin	NM_001043623.1	F: ACTGGTCATCAAGGAGGTTTG R: GCGTTCGTTTCTCTGGTTTAG
BmHsp20.8	NM_001098324.1	F: TCTCTTCTACCATTCGTGTTGG R: ACTTGGAACTTGTCCTTGTCG
BmRp49	NM_001098282.1	F: AGGCATCAATCGGATCGCTATG R: TTGTGAACTAGGACCTTACGGAATC

Upregulated proteins and active proteases were identified using MALDI-TOF-MS analysis. qRT-PCR analysis was used to validate data on the upregulated proteins.

SDS-PAGE and zymogram analysis of midgut proteins

The electrophoretic protein profile of midgut showed a dramatic change in protein expression in the range of 10 to 250-kDa during the transition from larva to pupa. In particular, prepupal-pupal stage (PP to P2) showed major expression of bands at 20, 29, and 45-kDa (see arrows in Fig. 1A). On the other hand, zymogram analysis evidenced a single proteolytic band at the pupal stage (Fig. 1B). These data demonstrate the co-occurrence of protease activity and upregulated proteins in silkworm midgut tissues since prepupal stage.

Two-dimensional electrophoresis of midgut proteins

To visualize the differential expression of midgut proteins between larvae and pupae, proteins from both stages (L5D5 and P2) were subjected to 2D-PAGE. The distribution of protein spots in the 2D gel was observed in the range 4-8 pI (Fig. 2). Spots at 45, 29 and 20-kDa, observed in the pI range of 5-6, 6-7 and 6-8, respectively, were seen to be upregulated in samples at the pupal stage. The expression level of each protein spot in larvae and pupae is indicated in Table 2.

Identification of upregulated proteins and active protease using MALDI-TOF-MS

Upregulated proteins (Fig. 2) and the proteolytic band found after the comparison between zymogram and SDS-PAGE (Fig. 1) were identified through mass spectrometry. The resulting MS fragments for proteolytic band and each 2DE protein spots are reported in Supplementary Figures 1 - 7. For upregulated proteins Uniprot ID, pI/MW, protein score and biological function are presented in Table 2. The 2D gel results for these proteins showed a good correlation (pI and MW) with NCBI database.

37-kDa serine protease

The proteolytic band was identified as 37-kDa serine protease. This serine protease is synthesized in silkworm as a zymogen at late spinning stage and activated upon pupation, as demonstrated by western blot analysis (Kaji *et al*., 2009). Since increasing evidence is implicating serine proteases within apoptotic processing, particularly in the generation of nuclear features such as condensation, fragmentation and DNA degradation observed in late-stage apoptosis (Muffitt *et al.*, 2007), this enzyme could indeed be involved in the remodeling of midgut tissues during larva to pupa transition by degrading larval midgut structures, as previously postulated (Kaji *et al*., 2009; Krishnan and Kannan, 2014). Alternatively, since the degradation of larval cells mainly occurs through the intervention of autophagy, which requires the action of lysosomal enzymes (Franzetti *et al*., 2012), we hypothesize that this digestive enzyme could instead participate to the final digestion of the cytoplasmic components derived from these cells, thus contributing to generate nutrients that can be readily absorbed by the newly-forming pupal-adult midgut (Franzetti *et al.*, 2015).

Hemolin (spots 1, 2, 3)

Hemolin is a member of immunoglobulin superfamily which is composed of four immunoglobulin domains (Sun *et al.*, 1990). Although the biological function of Hemolin is not completely clear, its ability to bind hemocytes and bacteria, as well as the similarity of Hemolin to neural cell adhesion molecules, suggest a possible role for Hemolin in regulating the adhesion of hemocytes to foreign cells (Faye and Kanost, 1998). In particular, Hemolin has been isolated from the hemolymph of two insects, *Hyalophora cecropia* (Sun *et al*., 1990) and *M. sexta* (Ladendorff and Kanost, 1991) after immune challenge.

In the present study, the upregulation of protein spots 1 and 2, and the new expression of spot 3 in the pupal midgut were detected in 2D-PAGE analysis. The protein spots were identified as Hemolin of *B. mori*. The three spots are located at approximately 45 kDa in the pI range of 5-6. This is the first report on the existence of different Hemolin isoforms in the silkworm midgut. Also in spinning larvae of *M. sexta* three Hemolin isoforms were isolated (Yu and Kanost, 1999). Lindstrom-Dinnetz *et al.* (2005) reported that Hemolin can generate different isoforms based on the existence of additional genes or due to alternative splicing.

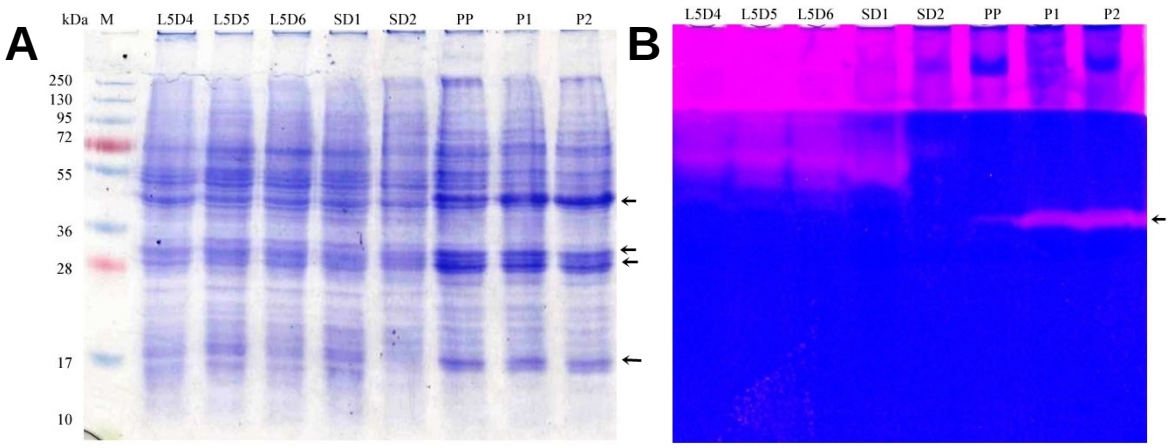

Fig. 1 SDS-PAGE (A) and zymogram profile (B) for midgut protein during larval to pupal transition in the silkworm. L5D4-L5D6: Fifth instar larvae day 4 to day 6, SD1-SD2: Spinning stage day 1 and 2, PP: prepupal stage, P1-P2: Pupal stage day 1 and 2,M: Molecular weight marker. Arrows in SDS-PAGE and zymogram gel indicates the upregulated proteins and protease activity, respectively.

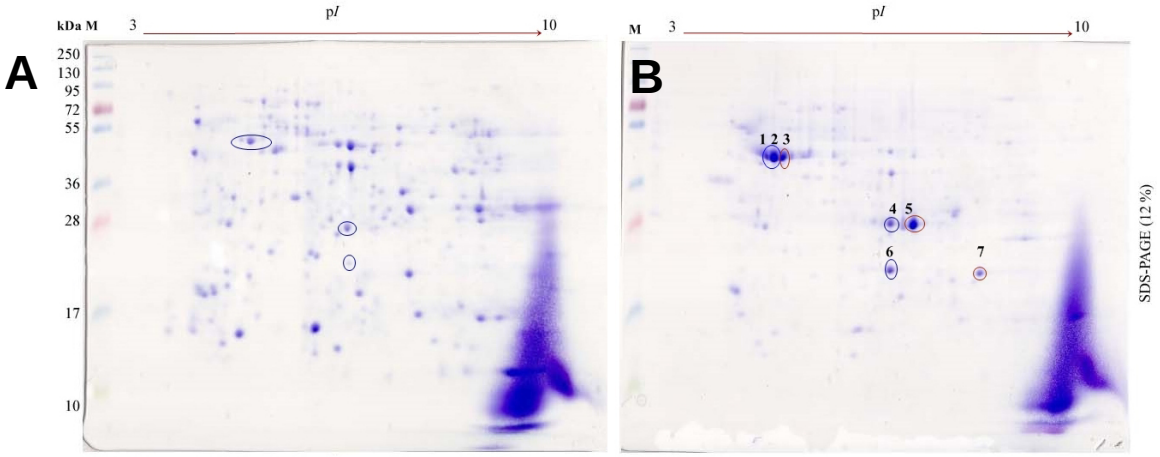

Fig. 2 Two-dimensional electrophoresis of larval (A) and pupal (B) migut protein. Approximately 300 µg of protein from larval (L5D4) and pupal (P2) midgut samples were used for IEF (17 cm IPG strips, Bio-Rad) and separated on 12 % SDS-PAGE.

Hou *et al*. (2010) reported that presence of two forms of Hemolin in the hemolymph of *B. mori* at late pupal stage. Further, they suggested that isoforms of proteins with unique pI values might occur because of post-translation modifications, such as phosphorylation. Interestingly, the present study found three forms of Hemolin from the pupal midgut of *B. mori*. In our study qRT-PCR showed that the transcript levels of Hemolin were strikingly upregulated since SD1 (Fig. 3A) compared to larval stage. Similarly Kim *et al.* (2005), by using Northern blot analysis, showed that mRNA levels for Hemolin increased significantly during spinning to pupal transition. It is worthy to note that the loss in *Hemolin* gene function causes susceptibility to microbial infection, as well as depletion of phenoloxidase activity, in different lepidopterans (Eleftherianos *et al*., 2006; Terenius *et al*., 2007, 2008). Aye *et al.* (2008) reported that Hemolin may have a role in the rearrangement of tissues during larval-pupal transition. These data, together with the increased expression of Hemolin, both at transcriptional and translational level, in silkworm midgut suggest that it has crucial roles in midgut remodeling and immune response in this tissue during metamorphosis.

Table 2 List of midgut proteins identified by MALDI-TOF-MS analysis

Band/ Spots	Uniprot Accession number	Protein description	Unique peptide hits	Score	Theoric pI/Mw (Da)	Biological process	Larval stage	Pupal stage
Proteolytic band	B3Y578	37-kDa protease (Serine protease)	10	76	6.46/37212	Serine type endopeptidase activity	NIL	★
1, 2 and 3	Q6R3M0	Hemolin	17	170	5.12/45278	Innate immunity	○	●
		Hemolin	22	236			○	●
		Hemolin	15	141			NIL	★
4	LP1_BOMMO	Low molecular weight 30 kDa lipoprotein PBMHP-6	17	82	6.11/29829	Anti-apoptotic function	○	●
5		Low molecular weight 30 kDa lipoprotein PBMHP-6	3	205			NIL	★
6	Q9GN07	Heat Shock protein 20.8	8	82	5.98 / 20804	Stress response	○	●
7	Q9GSB6	Heat Shock protein 20.4	8	79	6.54 / 20415		NIL	★

White circle denotes normal expression, black circle denotes upregulation, star denotes new expression, and NIL denotes the absence of protein spot.

Low molecular weight 30-kDa lipoprotein (spots 4, 5)

The 29-kDa protein is a member of the 30-kDa lipoprotein (isoform) family which is among the most abundant proteins in the hemolymph of fifth instar larvae of *B. mori*. The accumulation of these proteins into the hemolymph during development is regulated at the transcriptional level in a stage-dependent manner (Pakkianathan *et al*., 2012, 2014). Furthermore they reported that these proteins can be observed as isoforms at 28, 29 and 30-kDa. These authors also reported that 30-kDa proteins are synthesized in the peripheral fat body, thereafter secreted to the hemolymph, and finally translocated into the perivisceral fat body where they are used during metamorphosis (Pakkianathan *et al*., 2012).

In the current study, we observed an upregulation of protein spot 4, while spot 5, identified as a 29-kDa low molecular weight lipoprotein, is newly expressed in the midgut at prepupa-pupa stage (PP-P2). The mass spectrum with three of the predicted tryptic fragments for 29-kDa low molecular weight lipoprotein (spot 5) is shown in Figure 4. Maki and Yamashita (2001) reported the degradation of 30-kDa family protein during pupation by 30-kDa proteases; however in the present study we observed a sudden expression of 29-kDa lipoprotein in the pupal gut of *B. mori*. In addition, qRT-PCR analysis for low molecular weight 30-kDa lipoprotein (*BmHpc6*) showed significant higher transcription levels from P1 stage (Fig. 3B). Earlier reports stated that 30-kDa lipoprotein has anti-apoptotic properties and it was able to prevent apoptosis in insect or mammalian cells (Kim *et al*., 2001; 2003; 2004; Park *et al*., 2012). The present study confirms that 29-kDa low molecular weight lipoprotein is highly expressed during metamorphosis: its possible role as an anti-apoptotic factor could be necessary to secure the newly-forming pupal midgut from unwanted programmed cell death (Franzetti *et al.*, 2015).

Heat shock protein (spots 6, 7)

Small heat shock proteins (sHSPs) are molecular chaperones associated with nuclei, cytoskeleton and membranes. They bind to partially denatured proteins, thereby preventing irreversible protein aggregation during any kind of stress (Sun and MacRae, 2005). In addition, they play a vital role in embryonic development, cell differentiation, cell cycle and hormonal stimulation (Schlesinger, 1990; Currie and Tufts, 1997; Lewis *et al*., 1999). Xu et al. (2012) reported that upregulated transcription of sHSPs (HSP 19.9, HSP 20.4, HSP 22.6 and HSP 25.4) could protect cells during stress by offering resistance to apoptosis, cytosketon modulation, thermotolerance, protection against oxidative stress, cell growth and regulation of apoptosis.

In the present study, an upregulated protein spot 6 and the newly-expressed protein spot 7 from pupal midgut were identified as sHSP 20.8 and 20.4, respectively. qRT-PCR analysis showed that the transcript levels of *BmHSP* 20.8 were significantly upregulated since W stage (Fig. 3C). This sHSP helps proteins to fold properly and allows the newly-formed proteins to translocate across cell membranes (MacRae, 2000; Sejerkilde *et al*., 2003).

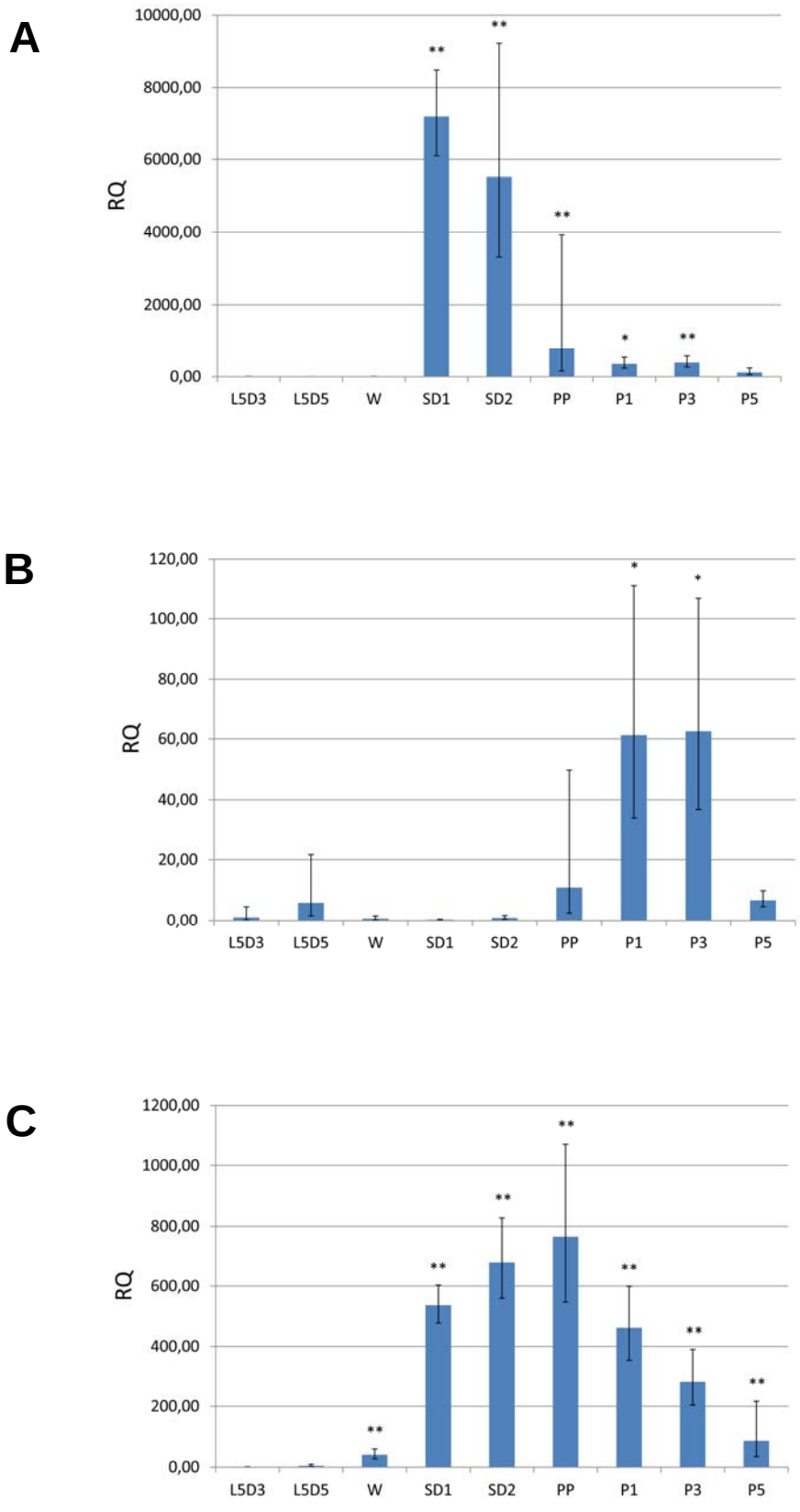

Fig. 3 qRT-PCR analysis of *BmHemolin* (A), *BmHpc6* (B) and *BmHsp20.8* (C) genes expression during larval-pupal transition. L5D3-L5D5: Fifth instar larvae day 3 to day 5, W: wandering stage, SD1-SD2: Spinning stage day 1 and 2; PP: prepupal stage, P1-P5: Pupal stage day 1 to day 5, RQ: Relative Quantitation. Values represent mean ± SE (*, $p < 0.05$; **, $p < 0.01$ compared to L5D3 for *BmHemolin* and *BmHsp20.8* and SD1 for *BmHpc6*).

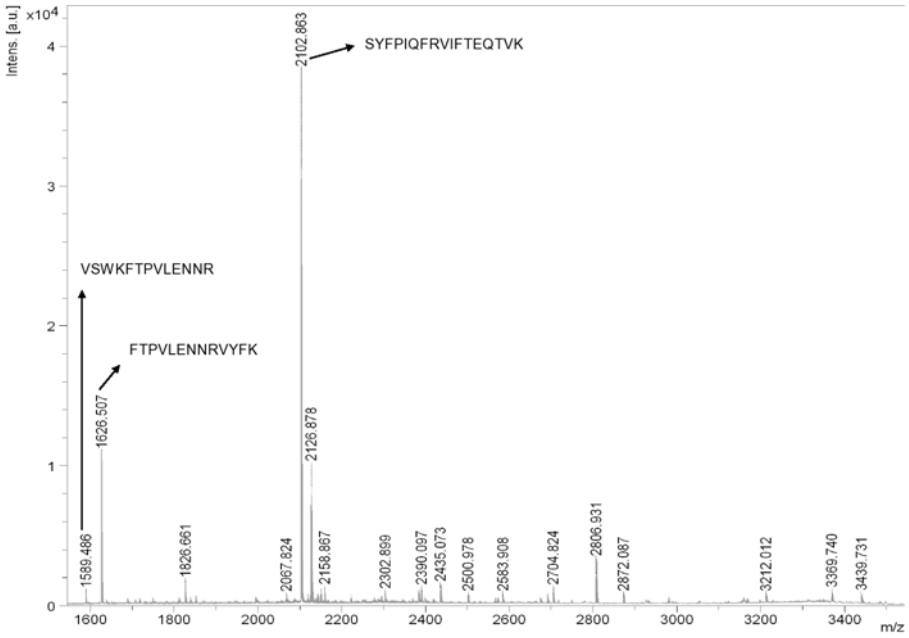

Fig. 4 MALDI-TOF-MS analysis of tryptic fragments of spot 5. The 2DE spot was cut into small pieces, subjected to overnight in-gel trypsin digestion, and then prepared for analysis by MALDI-TOF-MS. Peaks at 1589.486, 1626.507 and 2102.863 corresponded to the predicted tryptic fragments of 29 kDa low molecular weight lipoprotein.

Small HSPs contribute to the folding and assembling of polypeptides by minimizing the aggregation of non-native proteins and targeting non-native or aggregated proteins for degradation and removal from the cells (Schlesinger, 1990; Feder and Hofmann, 1999). According to what previously reported, the function of this protein in silkworm midgut could be related to the regulation of apoptotic and autophagic process, although this relationship still needs to be confirmed (Gu *et al.*, 2012). It is worthy to underline that the expression of sHSP 20.8 has been seen to increase in the midgut of *Antheraea pernyi* as a response to biotic stress (Zhang *et al.*, 2015), thus it could have a cytoprotective action in midgut cells during metamorphosis if bacterial or viral infection occurred.

Conclusions

The overall study emphasizes the potential role of midgut proteins in anti-microbial, apoptotic and stress response in *B. mori* larva during metamorphosis. It acquires additional importance if we consider that, during metamorphosis, the animal is devoid of the PM that normally acts as a protective barrier by isolating bacteria that are present in the gut lumen, thus a comprehension of the mechanisms that contribute to the protection of the insect during this critical developmental phase is fundamental. Furthermore, our results also contribute to the characterization of the proteins that have a role in the remodeling of lepidopteran midgut tissues.

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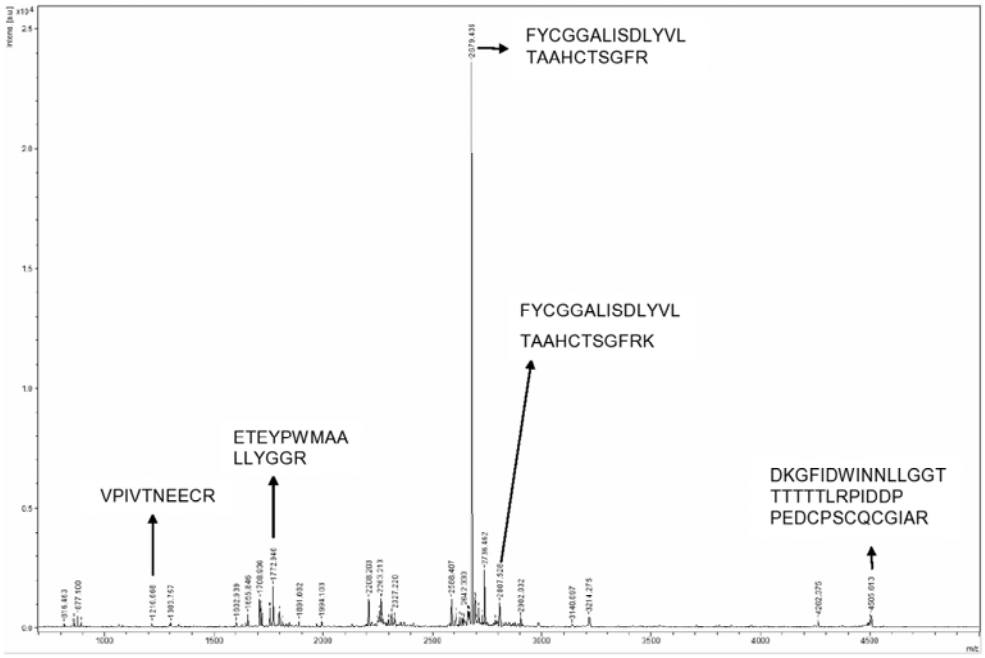
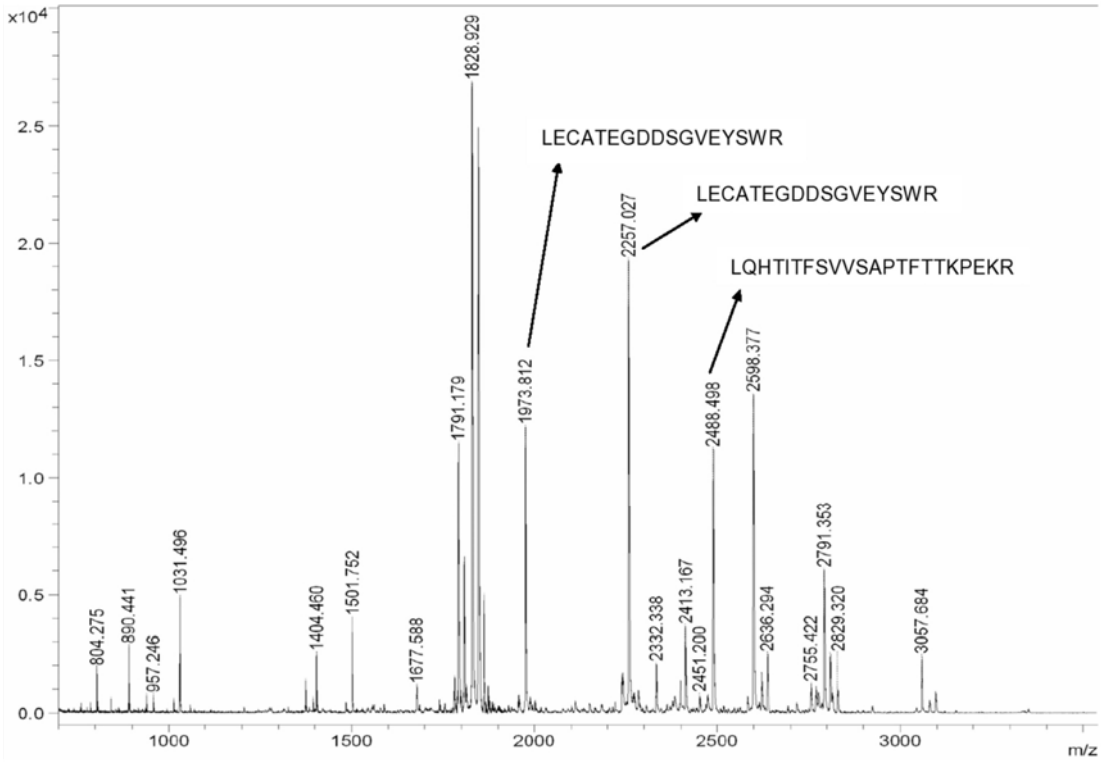
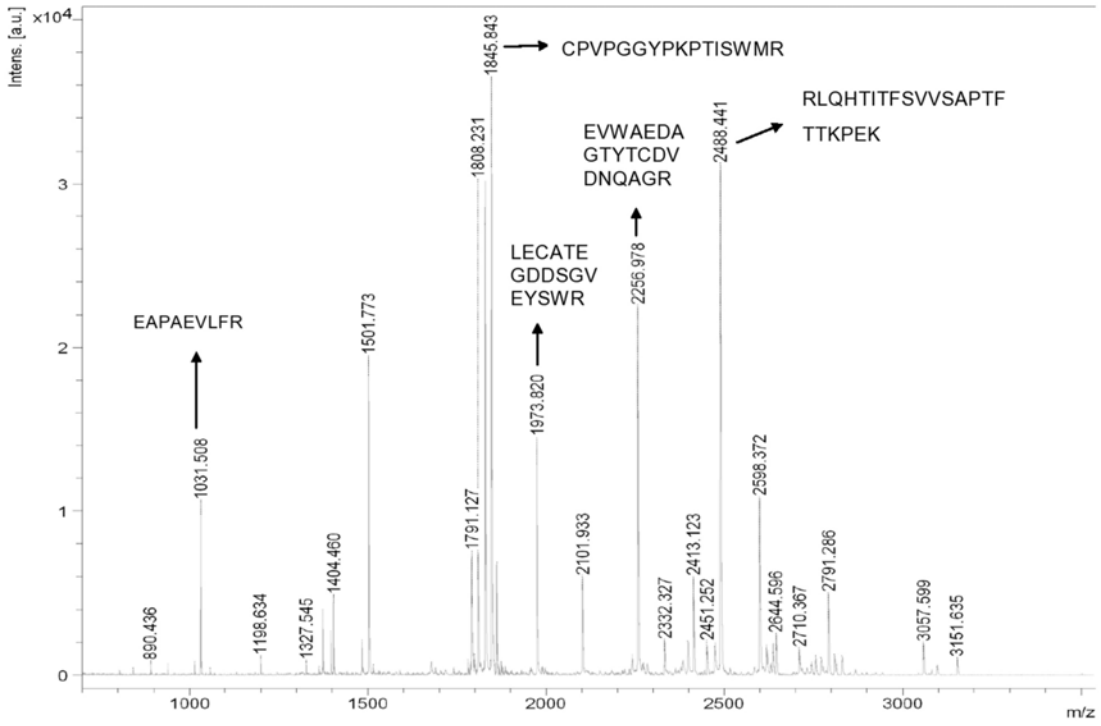


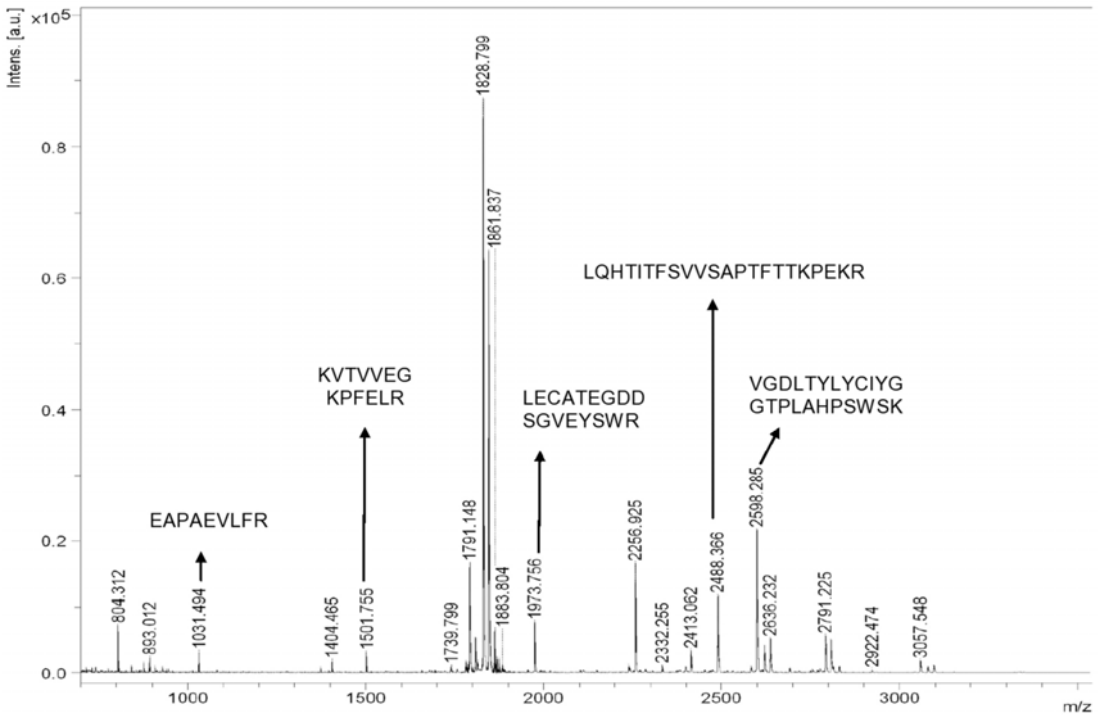
Suppl. Fig. 1 MALDI-TOF-MS analysis of tryptic fragments of proteolytic band. The 2DE spot was cut into small pieces, subjected to overnight in-gel trypsin digestion, and then prepared for analysis by MALDI-TOF-MS. Peaks at 1216.668, 1772.946, 2679.436, 2807.528 and 4505.613 corresponded to the predicted tryptic fragments of 37-kDa serine protease.



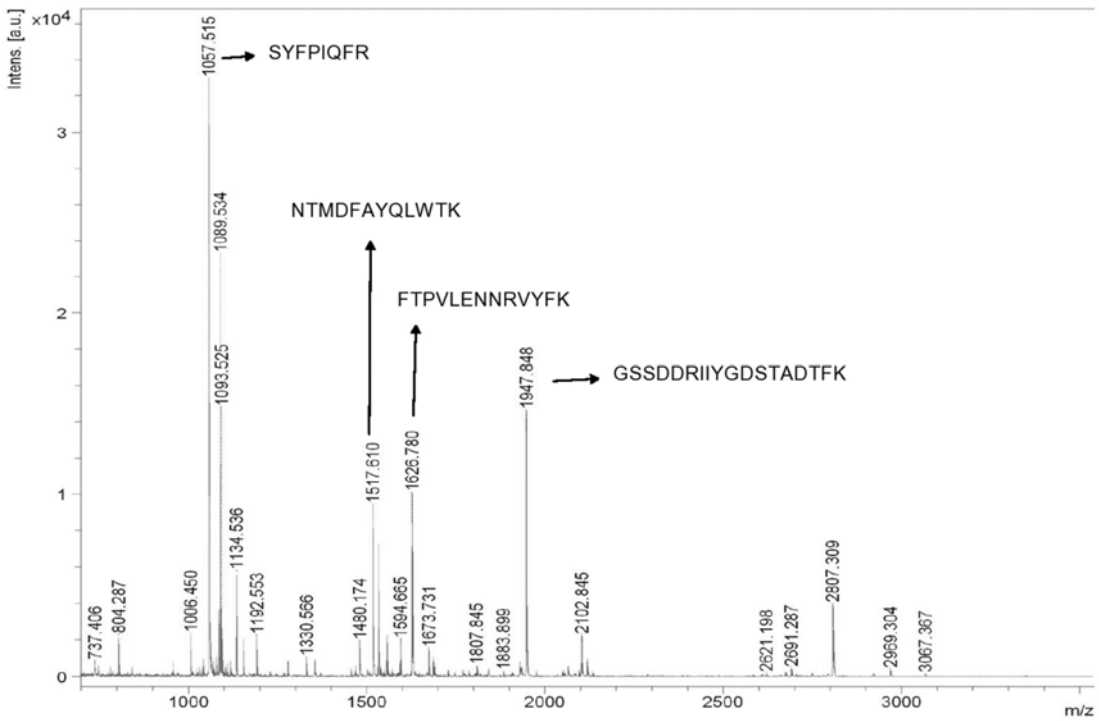
Suppl. Fig. 2 MALDI-TOF-MS analysis of tryptic fragments of spot 1. The 2DE spot was cut into small pieces, subjected to overnight in-gel trypsin digestion, and then prepared for analysis by MALDI-TOF-MS. Peaks at 1973.812, 2257.027 and 2488.498 corresponded to the predicted tryptic fragments of Hemolin.



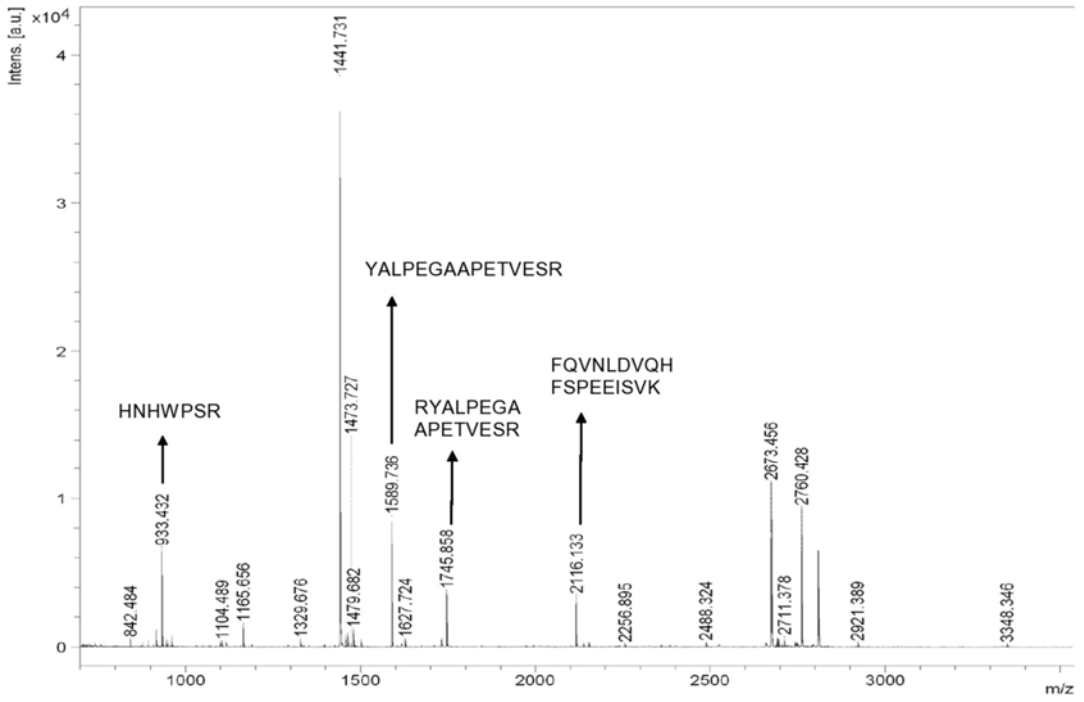
Suppl. Fig. 3 MALDI-TOF-MS analysis of tryptic fragments of spot 2. The 2DE spot was cut into small pieces, subjected to overnight in-gel trypsin digestion, and then prepared for analysis by MALDI-TOF-MS. Peaks at 1031.508, 1845.843, 1973.820, 2256.978 and 2488.441 corresponded to the predicted tryptic fragments of Hemolin.



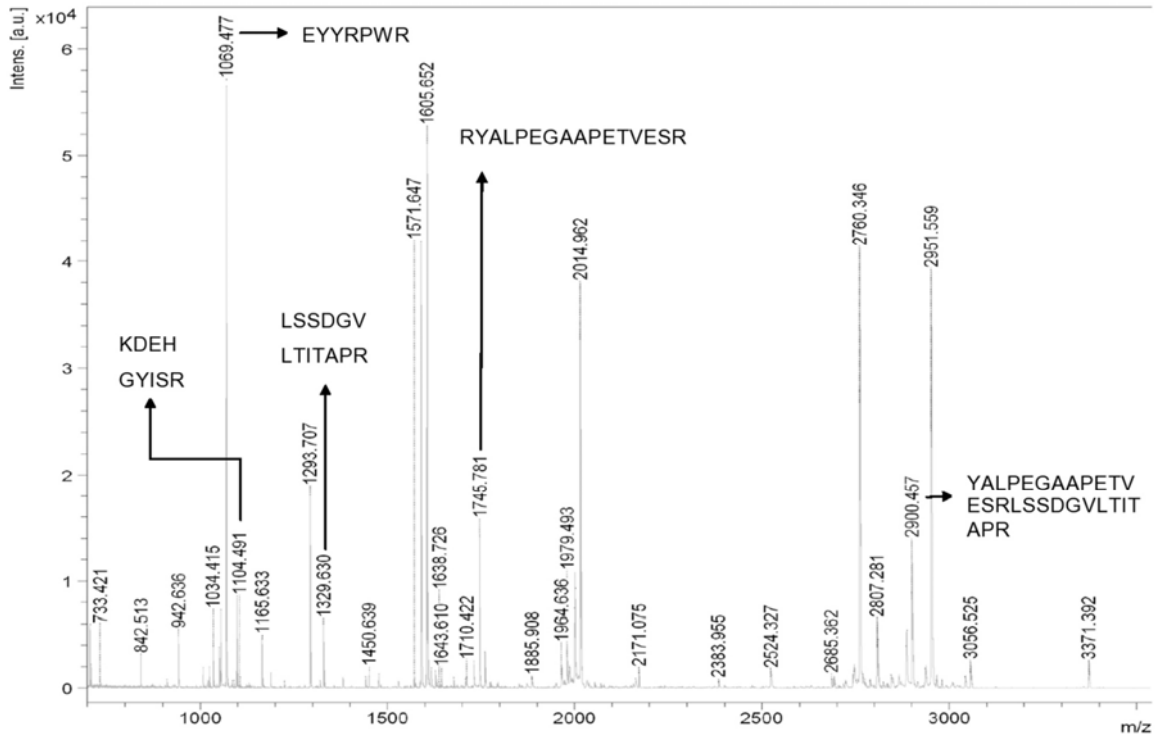
Suppl. Fig. 4 MALDI-TOF-MS analysis of tryptic fragments of spot 3**.** The 2DE spot was cut into small pieces, subjected to overnight in-gel trypsin digestion, and then prepared for analysis by MALDI-TOF-MS. Peaks at 1031.494, 1501.755, 1973.756, 2488.366 and 2598.285 corresponded to the predicted tryptic fragments of Hemolin.



Suppl. Fig. 5 MALDI-TOF-MS analysis of tryptic fragments of spot 4. The 2DE spot was cut into small pieces, subjected to overnight in-gel trypsin digestion, and then prepared for analysis by MALDI-TOF-MS. Peaks at 1057.515, 1517.610, 1626.780 and 1947.848 corresponded to the predicted tryptic fragments of 29 kDa low molecular weight lipoprotein.



Suppl. Fig. 6 MALDI-TOF-MS analysis of tryptic fragments of spot 6. The 2DE spot was cut into small pieces, subjected to overnight in-gel trypsin digestion, and then prepared for analysis by MALDI-TOF-MS. Peaks at 933.432, 1589.738, 1745.858, 2116.133 corresponded to the predicted tryptic fragments of HSP 20.8.



Suppl. Fig. 7 MALDI-TOF-MS analysis of tryptic fragments of spot 7. The 2DE spot was cut into small pieces, subjected to overnight in-gel trypsin digestion, and then prepared for analysis by MALDI-TOF-MS. Peaks at 1104.491, 1069.477, 1329.630, 1745.781, 2900.457 corresponded to the predicted tryptic fragments of HSP 20.4.

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