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Proteomic identification of the silkworm (*Bombyx mori* L) prothoracic glands during the fifth instar stage

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Synopsis

Although the ecdysteroid of the silkworm had been studied for decades, the proteome of the prothoracic gland, the primary source of ecdysteroid hormones, has not been studied previously. In the present paper, we utilized a proteomic approach to investigate the fifth instar prothoracic gland during the growth and development of the silkworm, *Bombyx mori* L. The two-dimensional electrophoresis results showed that the majority of proteins were acidic proteins, especially concentrated in the area of 25–65 kDa, with pI values of between 4 and 7, and the difference was not distinct. When compared with Qiufeng (Japanese strain), the interspecific distinction was larger than the intraspecific distinction, and 19 particular spots, excized from the third, fifth and ninth days of p50 (Chinese strain) and Qiufeng were subjected to MALDI-TOF–MS (matrix-assisted laser-desorption ionization–time-of-flight MS) analysis. We sorted them into seven catagories: energetics and/or metabolism, storage proteins, protection, lipid metabolism, signal transduction, cell function and unknown function proteins. Of these proteins, arginine methyltransferase is discussed as playing an important role in regulating the activation of ecdysteroidogenesis via transcription or translation.

Key words: arginine methyltransferase, ecdysteroid, matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI-TOF–MS), prothoracic gland, silkworm (*Bombyx mori* L)

INTRODUCTION

The silkworm, *Bombyx mori* L, is one of the economically important insects with a long history of domestication, and its development is regulated by various types of hormones. In general, hormones, such as PTTH (prothoracicotropic hormone), a brain hormone, JH (juvenile hormone) and MH (moulting hormone) play critical roles in the regulation of the silkworm's growth and development.

JH is secreted by the corpora allata and has the function of maintaining larval modality, preventing metamorphosis and development of the adult imaginal disc. In constrast, MH is a type of ecdysteroid hormone secreted by the prothoracic glands which exerts a direct effect on target tissues, regulating growth, moulting and metamorphosis [1].

The prothoracic glands are a pair of semi-transparent saccate cell clusters with conjunct theca, located in the tracheal clusters of the prothorax. The glands mainly synthesize and release MH periodically, which begins to be secreted at metaphase and reaches a peak at anaphase of each larval instar. The activities of the prothoracic glands are regulated by brain hormones, such as PTTH and FMRF amide-related peptide etc. [2–4].

The draft sequence for the genome of the domesticated silkworm (*B. mori*), covering 90.9% of all known silkworm genes and with an estimated gene count of 18510, has been published previously [5], but the research on gene function and regulation which has been performed previously was insufficient. Proteomics is a large-scale study of gene expression at the protein level, which ultimately provides a direct measurement of protein expression levels and insight into the activity of all relevant proteins [6]. So it is possible to gain further understanding of the

Abbreviations used: DTT, dithiothreitol; IEF, isoelectric focusing; IPG, immobilized pH gradient; JH, juvenile hormone; MALDI-TOF–MS, matrix-assisted laser-desorption ionization–time-of-flight MS; MH, moulting hormone; NV, normalization volume; PLU, plutonium; PMF, peptide mass fingerprinting; PRMT, protein arginine N-methyltransferase; PTTH, prothoracicotropic hormone.

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biochemical and physical properties and biological functions of genes via researching the proteome of the silkworm.

In this paper, we utilized a proteomic approach to investigate the proteome of the prothoracic glands during the growth and development of the silkworm, *B. mori*, with the aim of improving the understanding of this important bioprocess and the complex relationship between gene expression and hormone production.

MATERIALS AND METHODS

Animals

Silkworm stains p50 (Dazhao, Chinese strain) and Qiufeng (Japanese strain) were provided by the Silkworm Germplasm Bank at the College of Animal Sciences, Zhejiang University, Zhejiang, People's Republic of China. The silkworms were reared under standard conditions using mulberry leaves and 30 worms were collected from the first day of the fifth instar. The prothoracic glands were removed from the worms and stored at −80°C until use.

Two-dimensional electrophoresis: protein image acquisition and analysis

The sample was mixed with 150 μl of lysis solution containing 20 mM Tris base, 8 M urea, 2 M thiourea, 4% (v/v) CHAPS, 2% (v/v) IPG (immobilized pH gradient) buffer (pH 3–10) and 30 mM DTE (dithioerythritol). The mixture was homogenized fully for 15 min and lysed for nearly 1 h in an ice bath and sonicated for 3 min, then centrifuged twice at 15 000 ***g*** for 15 min at 4°C. The supernatant was mixed with 300 μl of rehydration solution containing 8 M urea, 2% (v/v) CHAPS, 0.5% IPG buffer (pH 3–10), 0.4% DTT (dithiothreitol) and 0.002% Bromophenol Blue before further use.

Two-dimensional electrophoresis was performed following the manufacturer's instructions (Amersham Biosciences). IEF (isoelectric focusing) was carried out using the Ettan IPGphor III platform (Amersham Biosciences) with IPG strips (linear, pH 3–10, 24 cm). The voltage was set to ascende gradually and reached approx. 87 000 V/h for 27 h.

After IEF separation, the sample strips were immediately equilibrated twice for 15 min in equilibration buffer [50 mM Tris base, 6 M urea, 30% (v/v) glycerol, 2% (v/v) SDS and 0.002% Bromophenol Blue]. Into the first equilibration solution, 1% DTT was included and 2.5% iodoacetamide was added into the second buffer.

After equilibration, the strips were loaded on to SDS polyacrylamide gels (12.5% gels) (240 × 200 × 1 mm) and sealed with 0.5% agarose. Separation was then performed on the Ettan DALsix vertical electrophoresis system (Amersham Biosciences), and the separated proteins were visualized by silver staining which is compatible with MALDI-TOF–MS (matrix-assisted laser-desorption ionization–time-of-flight MS).

After silver staining, the image was scanned and analysed using Image Master 2D™ software (Version 2002, 01, Amersham Biosciences).

In-gel digestion

The protein spots of interest were excized manually from the gels with a clean scalpel blade and then digested in the gel using a method described previously [7].

MALDI-TOF–MS analysis

The peptide was dissolved in 1.2 μl of 0.1% TFA (trifluoroacetic acid) and then 0.3 μl of this mixture was mixed with an equal volume of 10 mg/ml CHCA (R-cyano-4-hydroxycinnamic acid), and analysed by MALDI-TOF–MS (4700 proteomics analyser, Applied Biosystems).

The instrument settings used were: reflector mode, positive-ion mode and 20 kV accelerating voltage. Laser shots (1000 per spectrum) were used to acquire the spectra with a mass range from 800 to 4000 Da, and internal calibration was performed.

Protein identification and database searching

Protein identification using PMF (peptide mass fingerprinting) was performed by the MASCOT search engine (http://www.matrixscience.com) against the NCBI (National Center for Biotechnology Information) protein sequence database.

RESULTS

Protein expression of prothoracic glands in the silkworm *B. mori*

Figure 1 shows that as many as 512 protein spots were expressed on the third day of the fifth instar, and approx. 82.12% of spots were concentrated between pI 4 and 7, with molecular masses of 29–66 kDa. Proteins with a mass of under 25 kDa and a pI of above 9.0 were rare. These results imply that the majority of proteins in the prothoracic glands are acidic and neutral, and few low-molecular-mass and alkaline proteins were expressed.

Comparison of protein spots during development of the fifth instar

The protein spots of the first day were shown to be similar to those of the third day of the fifth instar, with molecular masses concentrated between 25 and 65 kDa, and were mostly acidic or neutral proteins. Subsequently, 31 protein spots of interest were screened in the first day compared with the third day of the fifth instar (Figure 2). Meanwhile, 88 newly expressed proteins were identified as being present on the third day compared with the first day of the fifth instar, with pI values of between 3.9 and 6.0.

Figure 3 and Table 1 show the comparison of protein spots isolated from the prothoracic glands of the fifth and ninth days with the third day of the fifth instar. When larvae reached the fifth day (the total fifth instar duration is nine days), 61 newly

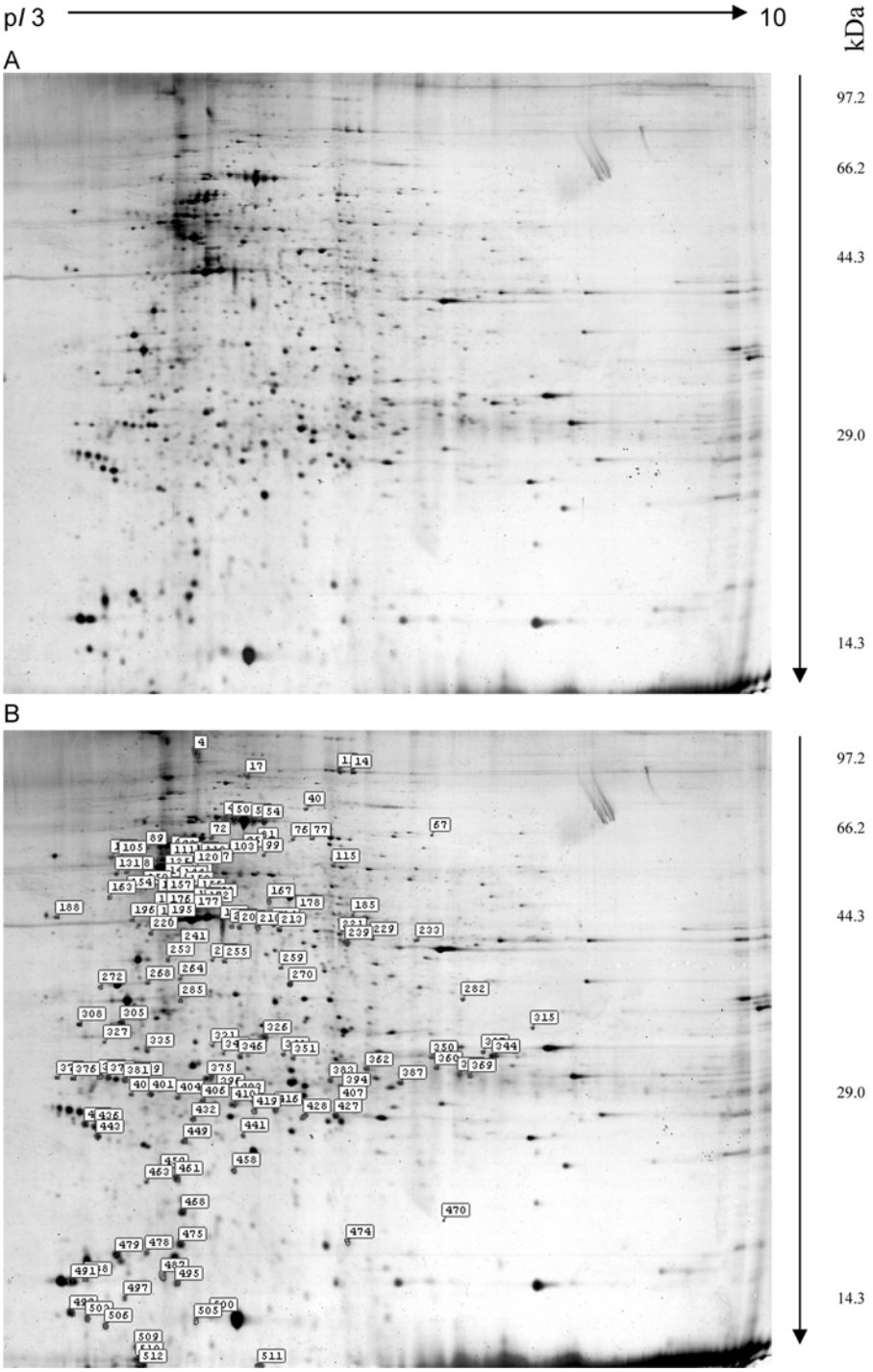

Figure 1 **Two-dimensional electrophoresis image of the prothoracic glands of the silkworm**
Prothoracic glands from the Chinese strain p50 were used for analysis. (A) Gel from the third day of the fifth instar. (B) Gel showing spots of interest from the third day of the fifth instar compared with the first, fifth, seven and ninth days (numbered spots).

expressed proteins were visualized through two-dimensional electrophoresis compared with the third day. Approx. 60% of these newly expressed proteins have pI values of between pI 5 and 7.

Compared with the third day of the fifth instar, the general protein distribution pattern in these images was almost the same on the ninth day, and 64 protein spots were newly expressed on the ninth day.

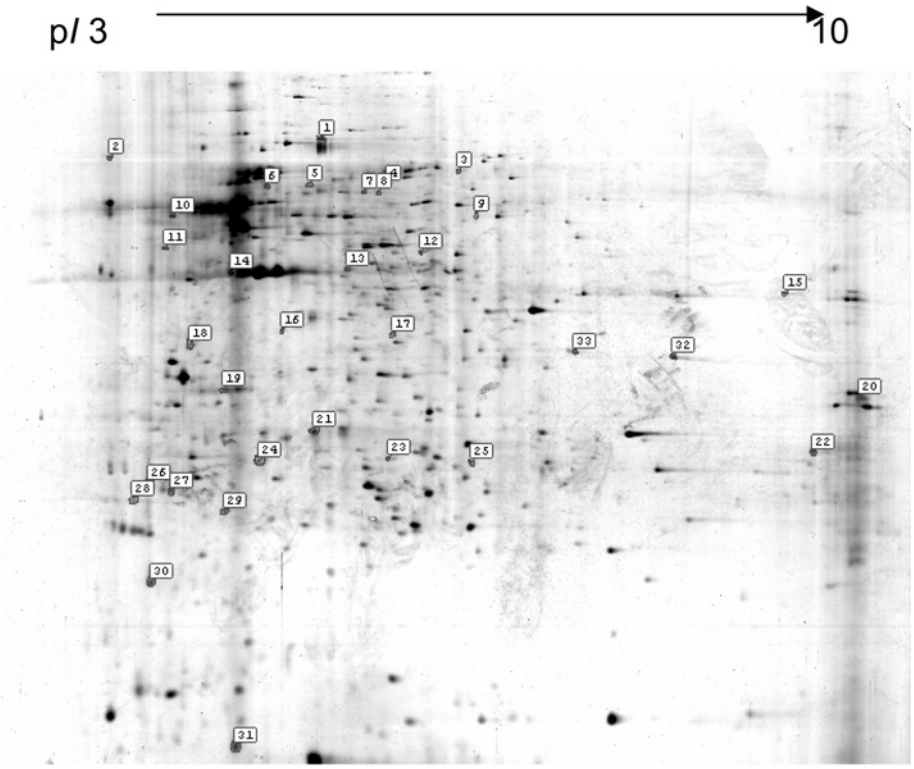

Figure 2 Two-dimensional electrophoresis image of the prothoracic glands during the development of the fifth instar
The spots of interest present in the first day compared with the third day of the fifth instar are indicated.

Quantification of expression of proteins expressed during the development of the fifth instar

Figure 4 shows the spot number and NV (normalization volume) comparison of the first, fifth and ninth days with the third day. We found that the newly expressed or disappeared spots all had NVs of less than 30. Compared with the first day, the newly expressed NV of the third day in the fifth instar was 6.345%, and increased to 29.658 in the ninth day compared with the third day of the fifth instar. Normally, the silkworm will finish the larval stage and metamorphose into pupae after the ninth day. Therefore the proteomic change of the prothoracic gland fifth larval instar is not distinct. Correspondingly, an NV of 22.733% for the newly expressed spots on the third day of the fifth instar was high.

Identification of protein spots isolated from the prothoracic glands of the fifth instar

The protein spots of interest were excized manually from the silver-stained gels using a clean scalpel blade. The gel was rehydrated in 10–20 μl of trypsin solution [20 ng/μl in 40 mM ammonium bicarbonate in 9% (v/v) acetonitrile] and incubated at 37°C overnight for digestion. The digested peptide mixture was analysed by MALDI-TOF–MS using a Voyager-DE STR mass spectrometer (Applied Biosystems) using delayed ion extraction and an ion mirror reflector. Protein identification using PMF was performed by the MASCOT search engine against the NCBI protein sequence database. Figure 5 shows spot 38 of the fifth day compared with the third day and its PMF graph. The PMF database-searching results are shown in Table 2.

The matched peptides for spot 38 in the prothoracic glands of the fifth day are shown in bold font as follows: M[1]PK**VVYHYFACK**ALGESGR**MLLAYGGQDFEDHR**VLSADWPDFKPKTPFGQTPVLVIDGK**QYAQSTAICR**YLGR**KYGLAGANDEEAFEIDQNVEFLHDIR**AKA**AAVYYEADEELKAK**KHEDFSKNVYPDMLKKLNSIVEANKGHIAAGKLTWGDFVFTSMFDYLK**TMLQIPDLEVQYPAFK**KVLQSVLTQPKVK**AFLDLGRPYEFEF**[206]

The protein was identified as GST2 (glutathione transferase 2) (EC 2.5.1.8). This is a family of isoenzymes with a broad substrate specificity and appears to be of significance in the detoxification and metabolism of many xenobiotics and organic peroxides, which is important to insect ecdysis or MH synthesis.

A total of 19 specific spots which were expressed in the prothoracic glands of the fifth instar were analysed by MALDI-TOF–MS (see Table 3). We categorized these newly expressed or disappeared proteins into seven groups: energy and/or metabolism, signal transduction, storage proteins, protection, lipid metabolism, cell function and unknown function.

The protein PLU (plutonium), a protein of varying size of between 165 and 171 amino acids, is a specialized cell-cycle

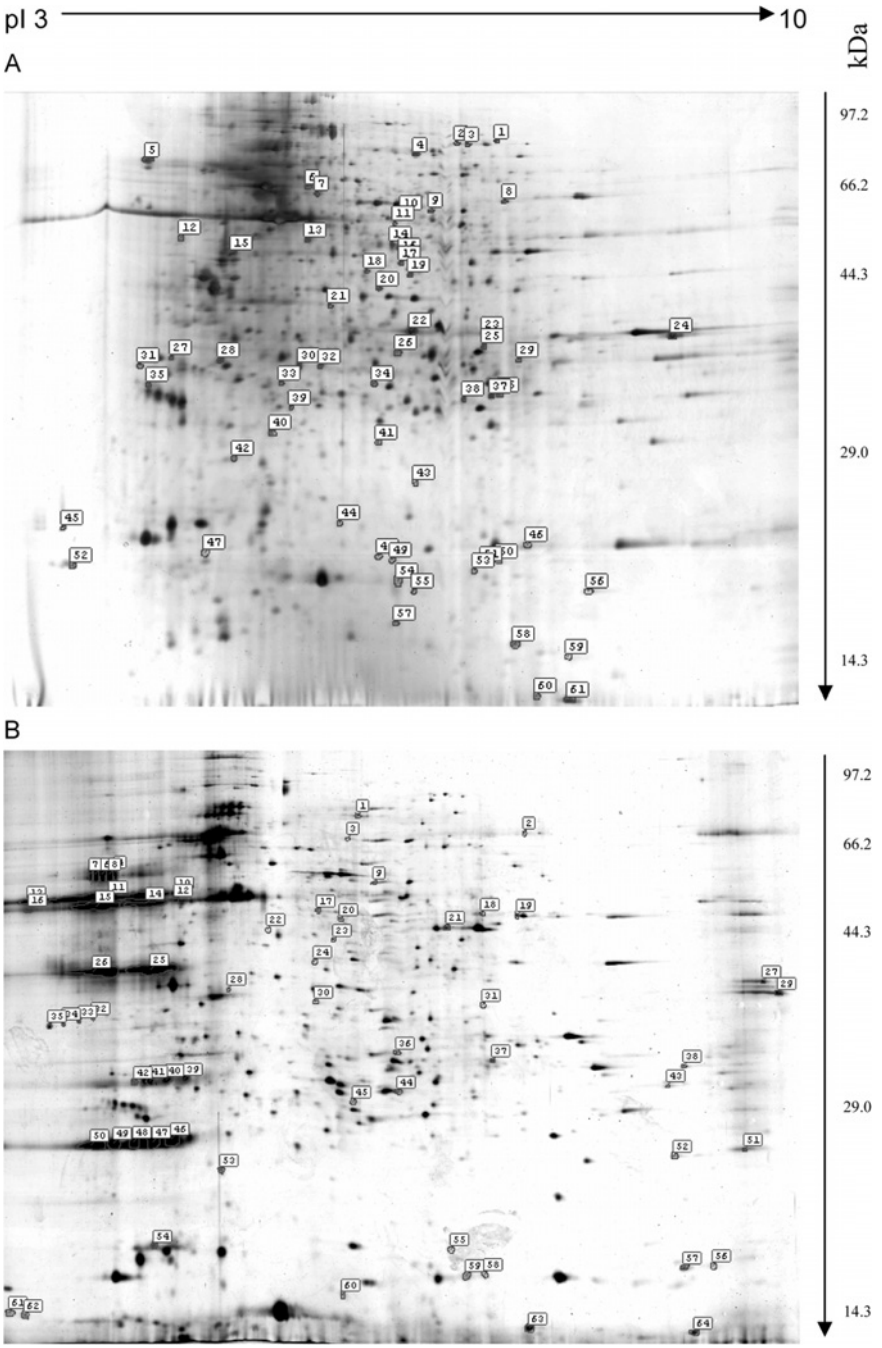

Figure 3 Comparison of protein spots in the prothoracic glands of the fifth and ninth days with the third day of the fifth instar

Prothoracic glands from the Chinese strain p50 were used for analysis. (A) The marked spots are the proteins which were expressed specifically in the fifth day compared with the third day of the fifth instar. (B) The marked spots indicate the particular proteins expressed in the ninth day compared with the third day of the fifth instar.

regulator required to repress DNA replication following completion of meiosis, and to establish cleavage mitoses in the zygote [8]. Therefore the discrepancy between the theoretical and experimental molecular masses of PLU in Table 3 may reflect the variation in residue number.

Comparison of protein spots from the prothoracic glands of the Chinese strain p50 with the Japanese strain Qiufeng

The activities of the prothoracic glands between strains are quite different. This affects the quantity and time course of MH

Table 1 Expression of specific spots of the first, fifth and ninth days compared with the third day of the fifth instar
The Chinese strain p50 (Dazhao) was used.

Day of fifth instar	Newly expressed spots	Mainly disappeared spots
First	1–31	4, 11, 14, 17, 40, 49, 50, 54, 72, 76, 96, 104, 105, 111, 117, 125, 128, 131, 141, 155, 167, 171, 172, 177, 199, 208, 210, 233, 241, 253, 254, 255, 259, 264, 268, 270, 272, 285, 315, 326, 331, 335, 337, 344, 346, 350, 351, 360, 370, 374, 381, 387, 394, 400, 401, 403, 404, 406, 407, 410, 416, 419, 428, 432, 435, 436, 441, 443, 449, 458, 459, 461, 463, 468, 475, 479, 487, 488, 491, 497, 500, 503, 505, 506, 509, 510, 511, 512
Fifth	1–61	4, 40, 49, 50, 53, 54, 72, 76, 77, 81, 95, 96, 98, 99, 103, 104, 105, 107, 110, 111, 117, 125, 131, 141, 144, 152, 167, 178, 185, 195, 198, 208, 210, 213, 221, 229, 233, 239, 241, 253, 264, 270, 282, 285, 305, 327, 335, 341, 350, 360, 362, 368, 369, 370, 373, 376, 381, 383, 394, 400, 403, 404, 407, 432, 441, 443, 487, 500, 509, 510, 511, 512
Ninth	1–64	4, 11, 14, 40, 49, 50, 54, 72, 76, 77, 81, 95, 96, 98, 99, 103, 104, 105, 107, 110, 117, 125, 131, 141, 144, 152, 155, 167, 171, 172, 178, 185, 195, 198, 204, 208, 210, 213, 221, 229, 233, 239, 241, 253, 264, 268, 270, 272, 285, 305, 308, 327, 335, 340, 341, 346, 350, 360, 362, 368, 369, 370, 376, 379, 381, 387, 394, 400, 403, 404, 407, 419, 432, 441, 443, 458, 459, 461, 463, 468, 475, 487, 495, 497, 499, 500, 503, 505, 506, 509, 510, 511, 512

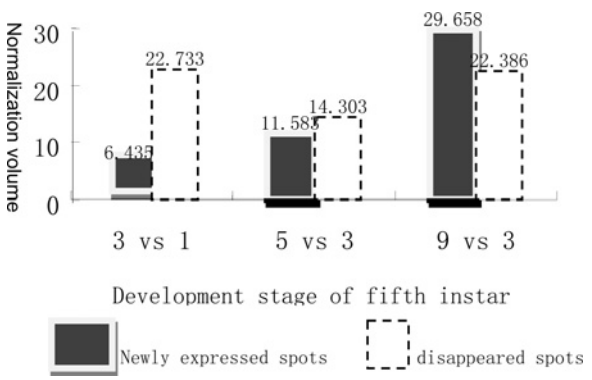

Figure 4 Quantification of some of the proteins expressed during the development of the fifth instar
The NV was used to quantify the expression of the protein spots of interest in the first, fifth and ninth days with that of the third day of the fifth instar. The prothoracic glands used were dissected from the Chinese strain p50.

biosynthesis and excretion. We compared the protein spots from the prothoracic glands of the Chinese strain p50 with the Japanese strain Qiufeng in order to understand the differences between these strains.

When compared with the two-dimensional image of the fifth day proteins from p50 prothoracic glands, more of the Qiufeng protein spots were present at a pI of between 3 and 7. In this pI range, 103 newly expressed and 115 disappeared spots were screened respectively.

As shown in Figure 6(B), the differences in the spots from the prothoracic glands of the Qiufeng and p50 strains, including newly expressed and disappeared spots, were much more than the discrepancy in the innerspecific strain p50. On the fifth day of Qiufeng, there were 151 newly expressed spots and 123 disappeared spots compared with the p50 strain.

DISCUSSION

The silkworm, *B. mori*, is a large-size insect that has been continuously domesticated for several thousand years. Its life cycle is divided into larva, pupa and adult, via moulting of the cuticle to accomplish metamorphosis.

Moulting is one of the physiological characterics of the silkworm that has a strong relationship with a reduction in cuticle extension and is regulated by hormones, especially MH [9–11].

Generally speaking, the silkworm moults six times in its life cycle, and the first four moultings are larva to larva and the final two moultings cause metamorphosis from larva to pupa and pupa to adult. The type of moulting is determined by the relative titre of JH and MH [12,13]. As the prothoracic glands are the primary source of MH, they been investigated previously over the past decades. As a result, MH is one of the best-studied insect hormones.

The activity of the prothoracic gland undergoes specific developmental changes during the larval instars. In the present study, a proteomic approach was used to study prothoracic glands during development. Newly expressed spots which appeared at the ninth day had an NV max of 29.658% compared with the third day of the fifth instar. When larvae grow to the fifth day (total fifth instar duration was 9 days), 61 newly expressed spots were visualized through two-dimensional electrophoresis, compared with that of the third day. Compared with the third day of the fifth instar, the general protein spot distribution pattern in the image

Table 2 PMF database searching results for protein spot 38 from the fifth day of the fifth instar (5–38)

Spot number 38 from the fifth day of the fifth instar was identified as being glutathione transferase 2 (NCBI accession number gi 31559115), with a molecular mass of 23481.9 Da and a pI of 5.98. Δ, difference between the observed and calculated molecular masses.

Amino acid residue		Molecular mass (Da)			Sequence
Start	End	Calculated	Observed	Δ	
182	191	1112.6674	1112.651	−0.0164	VLQSVLTQPK
4	12	1186.5714	1186.5355	−0.0359	VVYHYFACK
60	69	1197.5681	1197.5452	−0.0229	QYAQSTAICR
102	114	1471.6951	1471.6707	−0.0244	AAAVYYEADEELK
194	206	1603.7791	1603.7517	−0.0274	AFLDLGRPYEFEF
194	206	1603.7791	1603.7517	−0.0274	AFLDLGRPYEFEF
20	33	1667.7482	1667.7074	−0.0408	MLLAYGGQDFEDHR
20	33	1667.7482	1667.7074	−0.0408	MLLAYGGQDFEDHR
102	116	1670.8271	1670.7728	−0.0543	AAAVYYEADEELKAK
165	180	1908.9775	1908.9298	−0.0477	TMLQIPDLEVQYPAFK
75	99	2865.3325	2865.27	−0.0625	YGLAGANDEEAFEIDQNVEFLHDIR
75	99	2865.3325	2865.27	−0.0625	YGLAGANDEEAFEIDQNVEFLHDIR
74	99	2993.4275	2993.3652	−0.0623	KYGLAGANDEEAFEIDQNVEFLHDIR

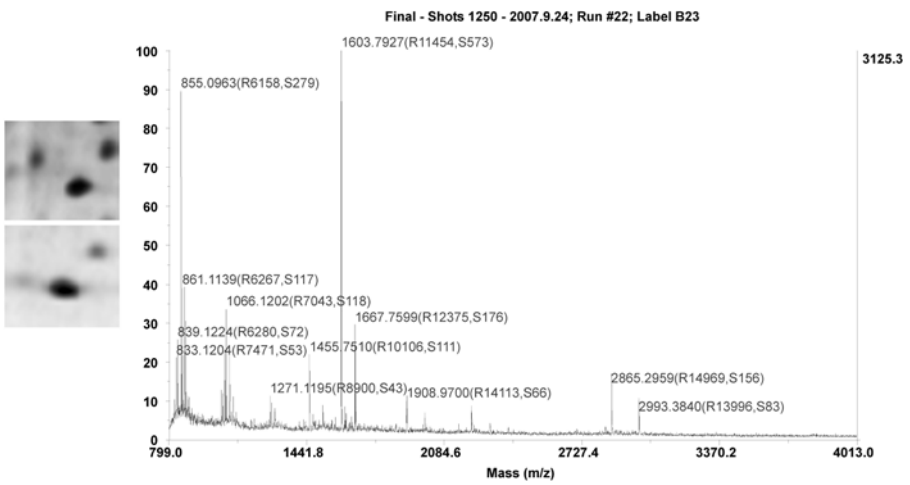

Figure 5 Spot number 38 of the fifth day of the fifth instar and its PMF graph

The protein spot was excized manually from the silver-stained gels and rehydrated in trypsin solution. The digested peptide mixture was analysed by MALDI-TOF–MS using a Voyager-DE STR mass spectrometer using delayed ion extraction and an ion mirror reflector.

was almost the same in the ninth day, but 64 protein spots were newly expressed in the ninth day. A total of 19 particular protein spots which were expressed in prothoracic glands of the fifth instar were analysed by MALDI-TOF–MS. We categorized the newly expressed or disappeared proteins into seven groups: energetics and/or metabolism, signal transduction, storage proteins, protection, lipid metabolism, cell function and unknown function (Table 3).

Pyruvate kinase, a protein from the category of energy and/or metabolism, is one of the enzymes which regulates the glycolytic pathway when the oxygen supply is low or when the body cannot meet energy requirements [14–16]. Pyruvate kinase was not present in the first and ninth days. This implies that this enzyme is required to activate ecdysteroidogenesis during the middle of the fifth intsar (from the third to the fifth day).

Post-translational methylation of arginine is one of the widespread epigenetic modifications in eukaryotes that is catalysed by PRMTs (protein arginine N-methyltransferases). PRMTs have been implicated in a variety of biological processes, such as the regulation of transcription, translation, signal transduction, DNA repair and apoptosis etc. [17]. Arginine methylation has a milder effect on proteins than other post-translational modifications, modulating certain processes rather than acting as an on/off switch.

Table 3 Mass spectrometric details of spots expressed in prothoracic glands of fifth instar
(5/3), third day of fifth instar; (5/5), fifth day of fifth instar; (5/9), ninth day of fifth instar.

Category	Spot number	Protein name	Accession number	Coverage (%)	Molecular mass (kDa)/pI	
					Theoretical	Experimental
Energy and/or metabolism	221 (5/3)	Ser/Thr kinase	gi|3047011	14	57.951/8.79	57.590/6.257
Energy and/or metabolism	233 (5/3)	Pyruvate kinase CG7070-PB, isoform B	gi|24648964	21	55.024/7.97	56.746/6.952
	259 (5/3)	α-Amylase	gi|413895	27	53.692/5.64	53.169/5.724
Storage proteins	24 (5/5)	Haemolymph 30K protein precursor (clone 19): silkworm	gi|84793	14	29.224/6.9	27.008/6.946
Protection	458 (5/3)	Glutathione transferase E1 CG5164-PA	gi|19922526	20	24.944/5.59	26.765/5.294
	38 (5/5)	Glutathione transferase 2 [*B. mori*]	gi|31559115	54	23.482/5.98	23.827/5.844
Lipid metabolism	341 (5/3)	Glucose 6-phosphate dehydrogenase	gi|38156654	16	41.116/5.83	41.876/5.744
Signal transduction	95 (5/3)	Heat shock protein 60 related CG2830-PA	gi|17864606	15	68.593/5.49	68.401/5.39
	335 (5/3)	Arginine methyltransferase 1 CG6554-PA	gi|21356361	28	42.778/4.99	42.398/4.513
	51 (5/9)	Signal recognition particle 19 kDa protein	gi|1016766	39	18.562/9.97	17.590/9.552
	57 (5/9)	PLU	gi|27652009	30	19.339/8.26	22.990/8.716
Cell function	475 (5/3)	Mod (mdg4) protein	gi|17026304	23	17.874/5.07	17.160/4.814
	31 (5/5)	Kettin	gi|4454135	23	26.142/ 5.85	26.387/6.081
Unknown function	59 (5/3)	GA22130-PA	gi|125774907	25	36.386/5.30	36.370/5.342
	229 (5/3)	GA12872-PA	gi|125982096	24	56.848/6.66	56.947/6.547
	379 (5/3)	CG31216-PA	gi|24648247	54	40.092/4.83	38.701/4.418
	59 (5/5)	CG32824-PA	gi|24643443	43	16.143/8.72	15.227/8.265
	43 (5/9)	CG5708-PA, isoform A	gi|24583264	34	26.556/8.57	26.459/8.543
	52 (5/9)	GA12157-PA	gi|125986742	38	22.024/8.39	24.608/8.366

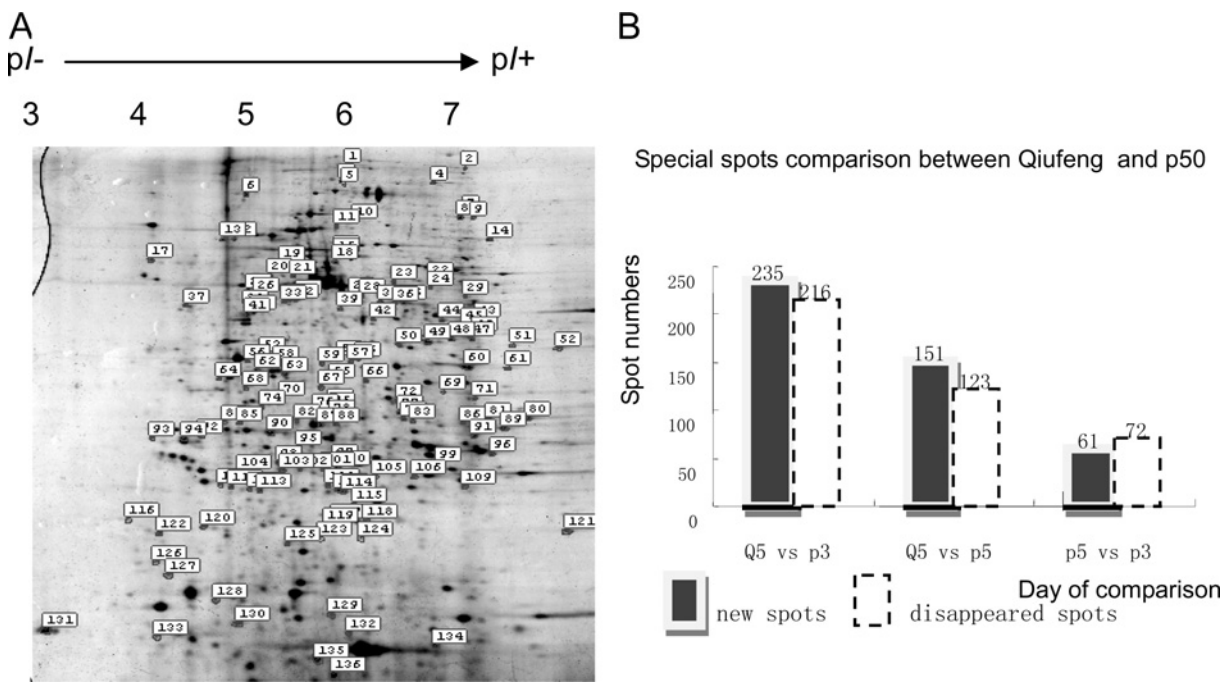

Figure 6 Comparison of protein spots isolated from prothoracic glands from the Chinese strain p50 and the Japanese strain Qiufeng
(A) Two-dimensional electrophoresis image of the prothoracic gland in the fifth day of the fifth instar (Qiufeng). (B) Comparison of the newly expressed and disappeared protein spots between the Qiufeng and p50 strains during the development of the fifth instar. p3, p50 prothoracic gland from the third day; p5, p50 prothoracic gland from the fifth day; Q5, Qiufeng prothoracic gland from the fifth day.

Arginine methyltransferase was detected both in p50 and Qiufeng strains, and the difference was that it appeared on the third day of the fifth instar of p50 and was visible in the two-dimensional electrophoresis of the proteins extracted from Qiufeng 2 days later. We suggest that arginine methyltransferase can also play a role in regulating the activation of ecdysteroidogenesis by affecting transcription or translation. We propose the following: ecdysteroidogenesis is triggered by PTTH first, when arginine methyltransferase is not expressed, resulting in inefficient transcription or translation. Therefore MH is synthesized and excreted at a lower level, resulting in a deferral in the critical titre of MH being reached and a delay in moulting.

p50 is a polyvoltine strain of silkworm and Qiufeng is a bivoltine strain. In our research, arginine methyltransferase appeared on the fifth day of Qiufeng, but was present by the third day of p50. We suggest that the expression of arginine methyltransferase at different time points may reflect a difference in length of the fifth instar. In other words, arginine methyltransferase may be one of the important enzymes in different voltines.

In conclusion, proteomic analysis of the prothoracic glands of the silkworm showed a difference in the newly expressed or disappeared protein spots during the development of the fifth instar and between strains. The results of the present study imply that these proteins are related to the biosynthesis and secretion of MH and metabolic activities in the prothoracic glands.

FUNDING

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