Studies on The Proteome of Colleterial Gland and Its *Ng* Mutant of Silkworm (*Bombyx mori*) Using Two-dimensional Electrophoresis and Mass Spectrometry

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Abstract The colleterial gland in the silkworm (*Bombyx mori*) grew gradually until 2 days before emergence, and markedly enlarged due to the accumulation of glue like substances (mainly including 85% water and 11% proteins). However, the *Ng* mutant female moth only secreted a small amount of glue-like substance and laid loose eggs naturally. High-resolution two-dimensional polyacrylamide gel electrophoresis, followed by computer-assisted analysis was used to screen the proteins pattern between normal and *Ng* mutant colleterial gland to find quantitative and qualitative difference in proteins expression. Protein spots were resolved in the secretory region of colleterial gland of silkworm and more than 700 protein spots were resolved and most of the proteins were distributed in the area from 30 ku to 70 ku and pH 4 ~ 8. Through the comparison and analysis, it was found that 3 proteins were only expressed in the later pupae stage and moth stage. However, these proeins have no expression in the *Ng* mutant especially spot No. 2 and 3. These differentially expressed proteins were actin identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The results indicated that the actins participate or regulate the exocytosis of colleterial gland and other differential expressed proteins might be having some relations with the glue-like proteins secretion.

Key words *Bombyx mori*, colleterial gland, *Ng* mutant, two-dimensional electrophoresis, mass spectrometry, proteome

The colleterial gland of silkworm (*Bombyx mori*) grew gradually until day 8 after pupal ecdysis, and markedly enlarged due to the accumulation of glue substances for 2 days before adults emergence[1]. Of silkworm, the glue like protein like other insects was released to the surface of eggs when eggs laying, then the glue-like substance hardened, and fixed the eggs to the surface of plants or other substrata, such that it was not easily detached even in a strong wind or a heavy rain. Therefore, it protected insects during a certain period in its development[2]. In the silkworm gene resource bank, it was found the mutation line, which the female moth only laid loose eggs naturally and no glue-like substance secreted and released to the surface of eggs. However, the mechanism of formation of *Ng* mutant was unknown by now.

Proteomics is the large-scale study of gene expression at the protein level, which will ultimately provide direct measurement of protein expression levels and insight into the activity state of all relevant proteins. The key elements of classical proteomics are the separation of proteins in a sample using two-dimensional gel electrophoresis and their subsequent identification by biological mass spectrometry [3]. The colleterial gland was selected for our proteome analysis for two reasons. First, from the morphology, interestingly, the colleterial gland enlarged and secreted the glue-like proteins markedly in one or two days, however, the *Ng* mutant couldn't secrete the glue-like substance during that development stage. Second, Since proteins were involved in nearly every cellular process, controlled every regulatory mechanism, and provided targets for many fields including development, drug treatment, mutant and so on, it was imperative that we looked to the proteome for better understanding of a complex process. Therefore, we investigated the utility of a proteomic approach to try to improve the understanding of this complex process of *Ng* mutant formation.

In the present experiment, it was analyzed the proteome expression profiles of the secretory region tissue of silkworm variety E981 and its *Ng* mutant from day 11 after pupal ecdysis to adult emergence. We described several distinct patterns of protein expression during the time course of colleterial development and between the normal and mutant colleterial gland. To identity of these differentially expressed protein by in-gel digestion and matrix-assisted laser desorption/ionization-time of flight mass spectrometry, provided some evidence to understand the mechanism of the biology mutant.

1 Materials and methods

1.1 Materials

The experimental silkworm variety, E981 and its

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Ng mutant line, were provided by the Silkworm Genetics and Breeding Laboratory, College of Animal Sciences, Zhejiang University. Silkworm larvaes and pupae were reared and protected under standard technical condition. The secretory region of colleterial gland from the day 11 after pupal ecdysis to the first day after adult emergence were dissected in the ice-cold insect physiological salt solution and stored at minus 78℃ immediately as the original protein extraction sample for further use.

1.2 Protein sample preparation

Colleterial gland tissues weighed 20 mg and washed by ice-cold PBS (pH 7.4) buffer twice, and absorbed the PBS buffer using filter paper. Then the samples were put into the Eppendorf tube of sample grinding kit (Amersham Biosciences) added 100 μl lysis solution (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 2% IPG buffer (pH 3 ~ 10), 60 mmol DTT) and completely grinded in the ice. After that, the protein extraction lysis solution was added to 500 μl. The sample solutions were mixed softly and kept in ice about 30 min and sonicated for 30 s every 10 min avoiding any air bubbles appearing, then centrifuged 15 000 r/min for 30 min at 4℃. The resulting solution was transfered into another tube. The protein concentration in the sample solution was determined by the Bradford method[4]. Finally, about 200 μg protein was dealt with the sample cleaning-up kit (Amersham Biosciences) according to the kit's instructions.

1.3 2-D Electrophoresis and gels visualization

The electrophoresis was performed with the sample in-gel application method described by Sanchez *et al.*[5]. The protein pellet was resolved in 450 μl rehydration solution (8.0 mol/L urea, 2% CHAPS, 2.8% DTT, 0.5% IPG buffer pH 3 ~ 10, 0.002% bromophenol blue). Samples were loaded overnight onto 24 cm immobiline dry strips, pH 3 ~ 10 (Amersham Biosciences) at 30 V and resolved by IEF at 8 000 V for 10 h, and the total volt-hours reached 85.0 kVh. Sample strips were immediately equilibrated 2 × 15 min with 50 mmol/L Tris-HCl pH 8.8, 6 mol/L urea, 30% glycerol and 2% SDS. In the first equilibration solution DTT (1%) was included and 2.5% iodoacetamide was added in the second equilibration step to alkylate thiols. Following equilibration, strips were loaded onto 12.5% SDS-polyacrylamide constant gel and resolved in the second dimension 15 W per gels at 15℃ by Ettan DALTsix electrophoresis unit (Amersham Biosciences). Proteins on gels were visualized accorded with the procedures recommended by the handbook of 2-D principles and methods of Amersham Biosciences and ZHAN Xian-Quan's[6] method except that gluteraldehyde was omitted from the fixation step since this cross-linking reagent interfered with extraction of the proteins for identification[7].

1.4 2-D Protein image acquisition and analysis

All the 2D images were scanned by special high-resolution image scanner (Amersham Biosciences) at 400 pixel per inch. The images were analyzed using the imageMaster 2D software supplied by the manufactor.

1.5 Protein in-gel digestion and MALDI-TOF MS identification

The interested protein spots were excised from the silver stained gels. Each sample was washed twice in milli-Q water, then destained by washing with a 1∶1 solution of 30 mmol/L potassium ferricyanide and 100 mmol/L sodium thiosulfate, and equilibrated in 200 mmol/L ammonium bicarbonate for 20 min to pH 8.0. After washing two times in milli-Q water, dehydrated by addition of acetonitrile, then dried in a SpeedVac (Thermo Savant, USA) for 15 min. Subsequently, the spots gel were rehydrated in 10 ~ 20 μl of trypsin (Sigma) solution (20 mg/L in 40 mmol/L NH_4HCO_3 in 9% ACN) and incubated at 37℃ overnight. Peptides were extracted twice by adding 30 μl of the solution including 50% CAN and 5% TFA. Peptides were treated with ZipTips (Millipore, USA) prior to applying on to the sample plate. 1 μl of peptide mixture was mixed with an equal volume 10 g/L α-cyano-4-hydroxycinnamic acid (Sigma) saturated with 50% ACN in 0.05% TFA and analyzed by a Voyager-DE STR MALDI-TOF MS using a delayed ion extraction and ion mirror reflector mass spectrometer (Applied Biosystems, USA). The instrument setting was reflector mode with 160 ns delay extraction time, positive, 60% ~ 65% grid voltage, and 20 kV accelerating voltage. Laser shots at 200 per spectrum were used to acquire the spectra with mass range from 1 000 ~ 4 000 u. External calibration was performed with Peptide Mass Standard kit (Perspective Biosystems), furthermore, the matrix and the autolytic peaks of trypsin served as internal standards for mass calibration.

For interpretation of the mass spectra, it was used the Mascot peptide mass fingerprint engine available on the web site (http://www.matrixscience.com) for proteins identification.

2 Results

2.1 Protein extraction rate and 2-DE reproducibility analysis

The proteins of secretory region tissue were extracted by lysis solution three times and protein concentration determined by the Bradford method. Approximately 20 mg tissue was dealt with 500 μl lysis solution and about (127.38 ± 5.39) μg and (114.58 ± 4.73) μg proteins acquired from 1 mg tissue of the E981 and its *Ng* mutant respectively.

The average standard deviation of isoelectric point (p*I*), molecular mass (M_r) of the same protein was 0.21 and 1.11 respectively for 50 protein spots selected randomly. These results showed that the present experiment had high reproducibility.

2.2 The 2D proteins images of normal and *Ng* mutant colleterial gland

The extracted proteins were loaded onto first dimension gel for isoelectric focusing using pH 3 ~ 10 immobilized pH gradient dry strips (24 cm length). For collecting all the information of the gels, it was designed an average gel for spots detection and matching. The image analysis software typically detected approximated 700 spots on each gel following silver staining, and most of the proteins arranged from 30 ku to 70 ku and pH 4 to pH 8. The distribution patterns of general protein spots in these images were almost same. The representative gels were shown in Figure 1.

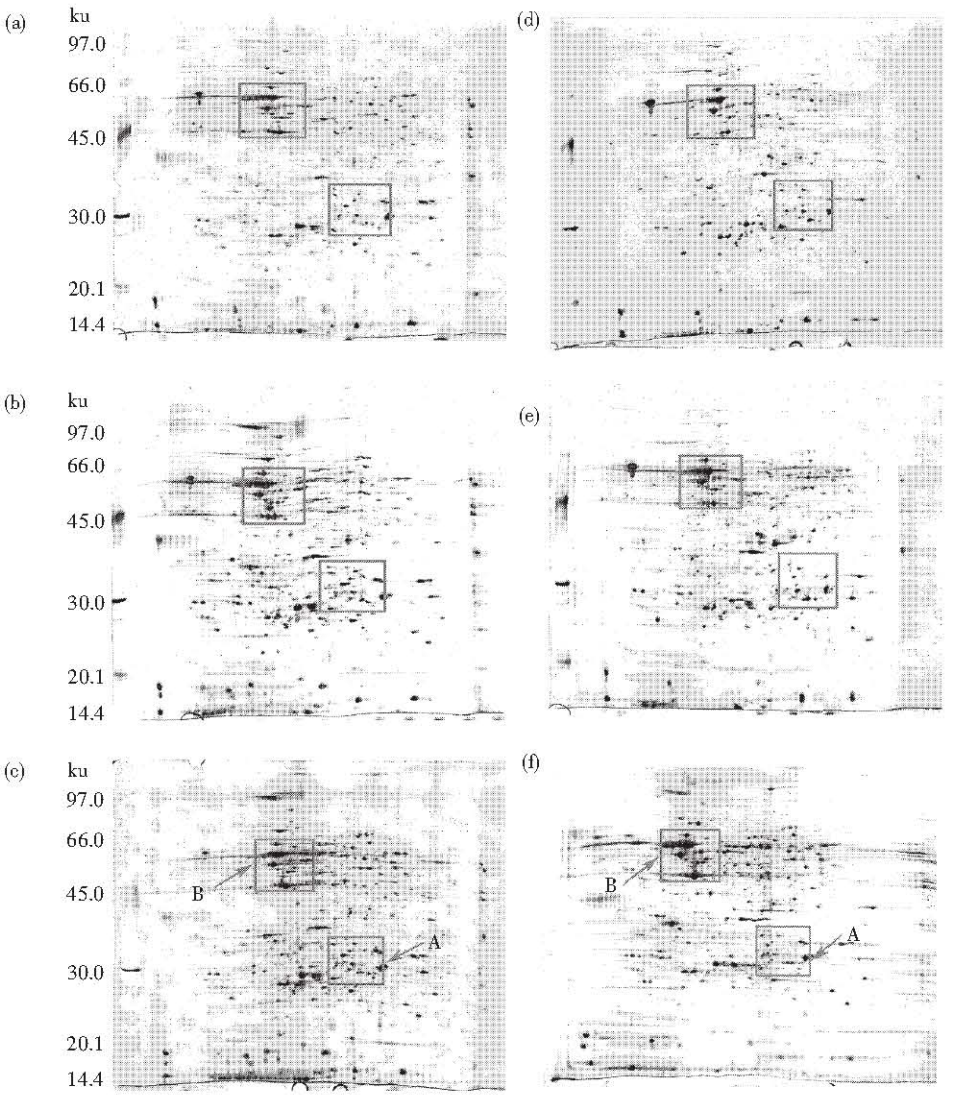

Fig. 1 Representative 2-D electrophoresis protein profiles resolved from colleterial glands of the silkworm variety E981 and its *Ng* mutant during the different development

Proteins were solubilized from day 11 after pupation to day 1 after emergence and separated in the first dimension by IEF using immobiline dry stripes, pH 3 ~ 10. Separation in the second dimension was performed using 12.5% constant gels followed by silver staining. Differentially expressed proteins were excised from gels and identified by MS. (a) E981: Day 11 after pupation; (b) E981: Day 12 after pupation; (c) E981: Day 1 after emergence; (d) *Ng* mutant: Day 11 after pupation; (e) *Ng* mutant: Day 12 after pupation; (f) *Ng* mutant: Day 1 after emergence.

2.3 Differential proteins analysis

Because this was the first reported application of proteome analysis on silkworm colleterial gland, the experiment technologies were developed and optimized. All the steps from protein sample preparation to 2-D PAGE electrophoresis were carried out simultaneously

and the operating condition were almost same. From the Figure 1, it was easily found that the protein spots distribution patterns were nearly same in these images. Under the help of the software, some proteins' expression volume in different development days were no significant difference, however, there were 3 proteins (spots 1, 2 and 3) expressed in the last one or two days of pupation and moth emergence stage in normal colleterial gland, whereas, it was found that the spot 1 only appeared in the day of emergence in the *Ng* mutant, and the spot 2 and 3, which appeared in the normal colleterial gland of E981, but never expressed in the *Ng* mutant and these proteins were shown in Figure 2. The three days was the important period for glue like substance secretion. All these differential proteins were excised for identification by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The spot 1 was unknown protein in the silkworm and the highest identity protein was found in fruit fly. The spot 2 and 3 were the same protein, cytosolic actin A3. The representative peptide mass fingerprint profile of spot 3 was shown in Figure 3. These proteins were grouped according to their patterns of expression and listed in Table 1.

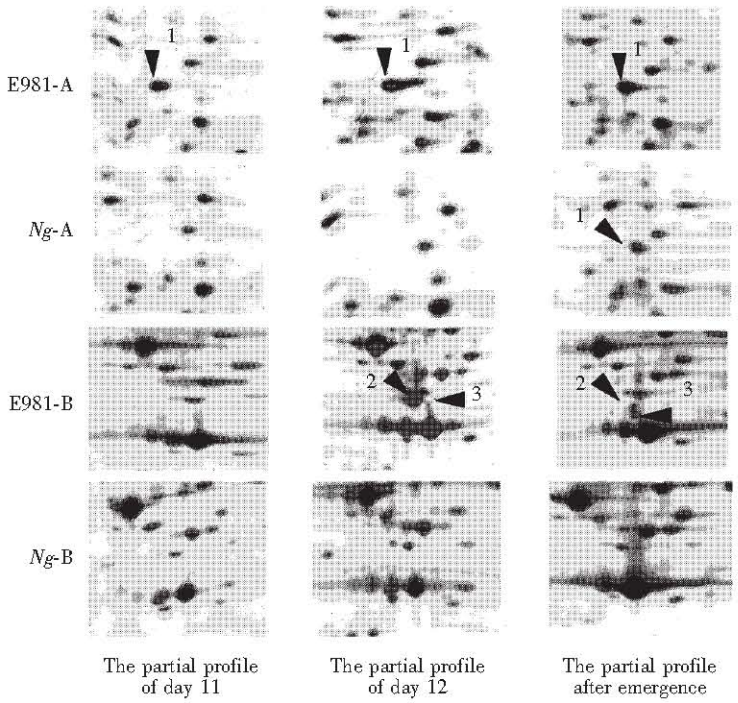

Fig. 2 The special proteins expressed in the colleterial gland between E981 and its *Ng* mutant

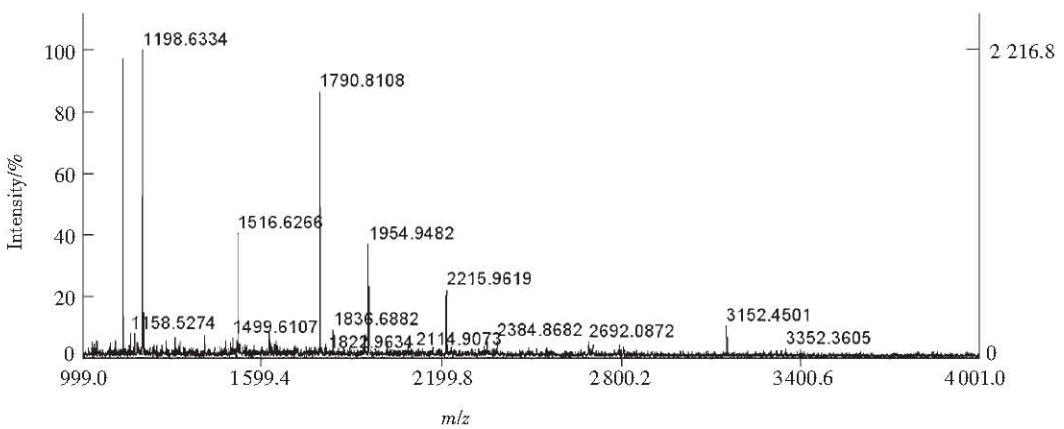

Fig. 3 Peptide mass fingerprinting of protein spot No. 3

Sliver-stained spot No. 3 was excised and destained, followed by enzymatic digestion. Peptides were analyzed with a Voyager-DE STR MALDI-TOF MS using a delayed ion extraction and ion mirror reflector mass spectrometer. After baseline correction, peak deisotoping and peak detection, 39 peaks were used for database searches in Mascot which identified the protein as cytosolic actin A3 (*Bombyx mori*) with 42% protein sequences coverage.

Table 1 Proteins identified by mass spectrometry

Spot No.	Identified protein	Gene No.	M_r	pI	Sequence coverage/%
1	CG15710-PA- *Drosophila melanogaster*	Gi 24654144	30 436	8.58	43
2, 3	actin A3, cytosolic-silkworm	Gi 84751	41 905	5.39	42

3 Discussion

To date, the mainstay of proteomics is two-dimensional electrophoresis, which separates proteins based on their inherent isoelectric point (pI) and molecular mass (M_r), and the separated proteins are subsequently identified by mass spectrometric methods. Applied the immobile dry strips and sample in-gel application technique in present experiment acquired higher resolution, better reproducibility and more sample volume for 2-D PAGE electrophoresis suggested by Rabilloud *et al.*[8].

The silkworm's colleterial gland could secret glue like substance that is very important for insect life circulation. It was reported that the secretions of the colleterial glands consisted of a viscous fluid which contained about 85% water and 11% proteins with a small amount of free amino acids and carbohydrates[1], and an average value of the tensile-shear-strength of the substance showed 115.7 N/cm^2 at plateau level measured by the method of Japan Industrial Standard K6850[9]. The glue-like substance appeared to be an interesting material that might be very useful in examining the mechanism of bio-adhesion, and for utilization as a biomaterial in medicine and food or other fields in future. However, the colleterial gland of genetic *Ng* mutation line silkworm developed normally, but produced only a small amount of glue-like substance in the corresponding period so as to lay the loose eggs naturally.

In our proteome analysis of the colleterial gland of silkworm, it was identified some proteins that changed in expression level between normal and *Ng* mutant colleterial gland. The spot No. 2 and No. 3 were cytosolic actin identified by MS, and its expression volume was relative high. Whereas, actin was a highly conserved protein of the contractile system, presented in the cytoskeleton of all eucaryotic cells. Actins were involved in a variety of cellular events such as motion, chromosome segregation, transport of macromolecules and endo- and exocytosis[10]. Interestingly, in *Bombyx mori* silk gland cells, cytoplasmic actin was an abundant component of apical bundles of microfilaments, which participated in exocytosis of silk proteins[11]. The actins only expressed in the two days that the glue-like proteins were secreted markedly. Furthermore, there were no actins expressed in the *Ng* mutant which almost couldn't secret the glue-like substances during the all development stage especially in one or two days before emergence. The main content of secretions from both colleterial gland and silk gland were proteins, it indicated that actins were important functional proteins, which participated or regulated the secretion of colleterial gland, might be having the same function in silk gland. In view of spot No. 1, which expressed in the last three days in E981 and only expressed in the emergence day in *Ng* mutation, it was indicated that this protein was a very important functional protein in the colleterial gland although it was an unknown protein in silkworm, and the highest identity protein was found in fruit fly. It was necessary to study with regard to the main function of the protein in the colleterial gland in further.

In this paper we have demonstrated that the differential proteins expressed between the normal and mutant colleterial gland by 2-D gels. Protein amounts are sufficient to identified individual proteins by trypsin digestion and MS. Two interesting proteins were found using proteomics method. Additionally, the formation of *Ng* mutant may be caused by some gene mutations that lead to some functional proteins especially the actin couldn't be expressed in colleterial gland, that may be the reason why the *Ng* mutant couldn't secret the glue-like proteins.

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家蚕雌性附腺及其 *Ng* 突变体的蛋白质组差异研究

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摘要 家蚕雌蛾性附腺在化蛾前2到3天开始大量分泌胶状粘性蛋白，其贮存部迅速地膨大，而其 *Ng* 突变体的雌蛾性附腺不能正常分泌胶状粘性物质. 分别对家蚕（*Bombyx mori*）的正常及 *Ng* 突变体雌蛾性附腺分泌部组织的蛋白质进行提取，并采用双向凝胶电泳和计算机辅助分析方法，对提取的蛋白质混合物进行分离和比较分析，并对主要差异表达的蛋白质用质谱鉴定. 实验结果表明，用银染法，平均每张电泳图谱可以分离约 700 个蛋白质点，其中大部分的蛋白质点分布在 pH 4～8 范围内，其分子质量主要集中在 30～70 ku 区域. 比较分析发现一些差异表达蛋白，其中 No2，3 蛋白质点经质谱鉴定为肌动蛋白 A3，该蛋白质只在化蛹后期正常雌性附腺组织中特异表达，而 *Ng* 突变体中肌动蛋白 A3 的缺失，暗示了肌动蛋白 A3 可能与家蚕雌性附腺的胶状粘性物质的胞外分泌有关.

关键词 家蚕，雌性附腺，*Ng* 突变，双向电泳，质谱，蛋白质组
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