Studies on middle silkgland proteins of cocoon colour sex-limited silkworm (*Bombyx mori* L.) using two-dimensional polyacrylamide gel electrophoresis

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Qualitative and quantitative differences in proteins expressed in the middle silkglands of male and female silkworm larvae that differ in silk colour were investigated by high resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), followed by computer assisted image analysis. About 1000 protein spots were resolved in both the sexes and most proteins were shown to be distributed in the area from 15 kDa to 70 kDa and pH 4–8. It was found that some proteins displayed higher expression in yellow cocoon, while two proteins were only expressed in female silkworm silkgland tissue through the comparison and analysis by two-D software. These proteins especially existed in female silkworm middle silkgland tissue of yellow cocoon. Furthermore, these proteins might be involved in the expression of cocoon colour phenotype.

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1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) (O'Farrell 1975) has a unique capacity for the resolution of complex mixtures of proteins, permitting the simultaneous analysis of hundreds or even thousands of gene products. In 2-D PAGE, proteins are separated according to charge (pI) by isoelectric focusing (IEF) in the first dimension and according to size (Mr) by SDS-PAGE in the second dimension. In proteomic research, 2-D PAGE is an important tool for investigating differential patterns of qualitative and quantitative protein expression. Two-dimensional gel electrophoresis is a powerful tool due to its unrivalled power of being able to separate simultaneously thousands of proteins such that a very large number of proteins can be evaluated in a single experiment (Arnott *et al* 1998; Fountoulakis *et al* 1999). At present, 2-D PAGE is the most widely used method for the analysis of complex protein mixture extracted from cells, tissues, or other biological samples. Because of the complexity of the proteome itself, resolution and reproducibility are the main problems of 2-D PAGE. However, 2-D PAGE based on immobile pH dry strips has higher resolution and reproducibility can separate proteins on a large scale.

Silkworm is a good choice amongst laboratory species for research. More than 400 mutations are recorded in the silkworm gene bank and genetics and some molecular biology aspects have been studied extensively (Nagaraju 2000). However, in the field of silkworm proteome not much is known. Generally, bivoltine silkworm varieties produce a white cocoon. Some varieties, however, can produce coloured cocoons, and are sex-limited (colour cocoons are females and white cocoons are males). The molecular mechanism of cocoon colour form, and the pigments transfer in the silkworm body is not clear. Zeinab and Michel (1996), Tabunoki (2002) and Hiroshi Fujii (1988) observed that pigment-binding protein was in-

Keywords. Middle silkgland tissue; proteome; two-dimensional polyacrylamide gel electrophoresis; yellow cocoon sex-limited variety

Abbreviations used: CHAPS, 3[(cholamidoprophyl) dimethylammonio]-1-propane sulphonate; DTT, dithiothreitol; 2-D PAGE, 2-dimentional polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

volved in the colour of cocoons. The silk was composed of sericin and fibroin that are secreted by middle silkglands and post silkglands, respectively. Hong-sheng (1991) proved that entire pigment in the cocoon was bound to sericin. Pathway of pigment transfer and the pigment position in cocoon, it was very clear that the middle silkgland tissue was one of the main tissues for pigment transfer in the silkworm body. For our experiment, we selected the middle silkgland tissue of cocoon colour sex-limited silkworm variety. Use of this variety can remove the protein spots difference between two varieties that may influence the experiment result. We have also studied the mechanism of cocoon colour and the quantitative and qualitative differences in the proteins of the middle silkgland tissues of limited silkworm variety for cocoon colour using 2-D PAGE. Detailed attempts were also made to compare and analyse proteome maps using 2D image analysis.

2. Materials and methods

2.1 *Chemicals*

The main chemicals used were: IPG buffer (pH 3–10); linear immobilized dry strips pH 3–10 (24 cm length); ultra urea; sodium dodecyl sulphate (SDS); dithiothreitol (DTT); 3[(cholamidopropyl) diemethylammonio]-1-propane sulphonate (CHAPS); N,N,N′,N′-tetramethylethylenediamene (TEMED); Tris, acrylamide, N,N′-methylene bisacrylamide (Bis); glycine; argose; glycerol; low molecular weight calibration kit, sample grinding kit and sample cleaning-up kit were from Amersham Biosciences (UK). Iodoacetamide was from Sigma (USA).

2.2 *Material*

The cocoon colour sex-limited silkworm variety CY was provided by the Silkworm Genetics and Breeding Laboratory, College of Animal Sciences, Zhejiang University, Hangzhou (PRC). The silkworm was reared under standard conditions. The middle silkgland tissue of the fourth day in fifth instar was dissected in the ice-cold 0·75% insect physiological salt solution and stored at – 78°C immediately as the original protein extraction sample for further use.

All the electrophoresis systems and 2-D images analysis software were from Amersham Biosciences. The high-speed centrifuge CF 15R was the product of Hitachi, Japan.

2.3 *Sample preparation*

About 20 mg middle silkgland tissue was washed in ice-cold PBS (pH 7·4) buffer twice, the PBS buffer was absorbed using filter paper. Then the sample was collected into the Eppendorf tube of sample grinding kit and 100 μl lysis solution was added (8 mol/l urea, 4% CHAPS, 2% IPG buffer, pH 3–10, 60 mmol DTT) and ground thoroughly for about 1 min in the ice. Later, the protein extraction lysis solution was added to 500 μl. The sample solutions were mixed gently and kept in ice for about 30 min and sonicated for 30 s every 10 min avoiding any air bubbles and centrifuged at 15,000 rpm for 20 min at 4°C. The resulting solution was removed into another tube. The protein concentration in the sample solution was determined by the Bradford method (Bradford 1976). Furthermore, about 350 μg protein was subjected to clean up, using the sample cleaning-up kit and a small protein pellet was obtained for electrophoresis analysis.

2.4 *The first dimensional isoelectric focusing electrophoresis with immobilized pH gradient dry strips*

Electrophoresis was performed with the sample in-gel application method described by Sanchez *et al* (1997). The protein pellet obtained above was rehydrated with 450 μl (for 24 cm length dry stripe) rehydration solution (including 8·0 mol/l urea, 2% CHAPS, 2·8% DTT, 0·5% IPG buffer, pH 3–10, 0·002% bromophenol blue) to resolve the protein pellet. The IPG strips were then positioned with the gel side down in the strip holder without any bubbles and the strips were covered with the special covering fluid and the holder lid immediately. The holder was moved onto the IPGphor unit platform, and all the electrophoresis steps were carried out at 20°C. The rehydration step was carried out at low voltage 30 V for 12 h, and the highest voltage in IEF was 8000 V. The total IEF volt-hours was about 68000 kVh. After IEF separation the 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 (2%) was included and 2·5% iodoacetamide was added in the second equilibration step to alkylate thiols.

2.5 *The second dimensional vertical SDS-PAGE and silver stain*

SDS-PAGE was performed using 1 mm thick 12·5% SDS polyacrylamide constant gel. The strips were held in place with 0·5% agarose dissolved in SDS-Tris running buffer and the electrophoresis was carried out at 5 W per gel at first for about 30 min and then adjusted to 15 W. After elecatrophaoresis gels were stained with silver nitrate, the second dimensional electrophoresis was carried out at 15°C controlled by MultiTemp®. The stain methods mainly accorded with the procedures recommended by

the handbook of 2-D principles and methods of Amersham Biosciences and Zhan's (2001) method.

2.6 *2-D protein spots scan and image analysis*

All 2-D images were scanned by a high-resolution image scanner at 600 pixel per inch. The images were analysed using the ImageMaster 2D software (version 2002·1) including image background subtraction, spot detection, spots matching, spots volume normalization, proteins molecular weight and isoelectric point analysis.

3. Results

3.1 *The 2-D protein maps of middle silkgland of yellow cocoon (female) and white cocoon (male) silkworm*

All the extracted proteins from the middle silkgland were loaded onto first dimension gel for isoelectric focusing using pH 3–10 immobilized pH gradient dry strips (24 cm length). Furthermore, all the steps from sample preparation to the 2-D PAGE were done more than two times for testing the reproducibility. Results showed that the image pattern had high reproducibility that was sufficient for 2-D analysis. Two representative gels are shown in figure 1. The general protein spots distribution pattern in the two images were almost same. The image analysis software typically detected approximately 1000 spots on each gel following silver staining, and most of the proteins were rangeing from 15 kDa to 70 kDa in molecular mass, and pH 4 to pH 8 in isoelectric point.

3.2 *Differential proteins analysis*

All the steps from protein sample preparation to 2-D PAGE electrophoresis were carried out simultaneously and the operating condition were almost the same. The protein spot distribution pattern was nearly same in the two images (figure 1). Analysed by the software (Image Master 2D, Amersham Biosciences), one of the gels was designated as the reference gel, and was matched with the other gel. The result showed that the spot matching rate of the two gels was very high and that most of the main proteins expression volume in the two gels was almost the same. However, some proteins which the expression intensity was higher in the middle of silkgland of the yellow cocoon (female) silkworm were higher than the corresponding spots in the white cocoon (male), and several proteins expression volume differences reached as almost 10 times. The spot volume value was calculated by the software based on the protein spot colour intensity and area, which was just a relative value. The background subtraction and protein spots volume normalization that influence the volume value were done before the spot volume calculation. Besides, there were two proteins which were expressed only in the middle silkgland of the female larvae (yellow cocoon.). Figure 2 shows the main representative differential proteins, while table 1 lists proteins isoelectric points, molecular weight and relative expression volume. For further identification, whether these proteins were actually different and also increase the protein spots separation resolution, pH 4–7 dry strips (24 cm length) were used for a repeat experiment. The result was identical. This indicates that the proteins may be involved in the occurrence of cocoon colour (female).

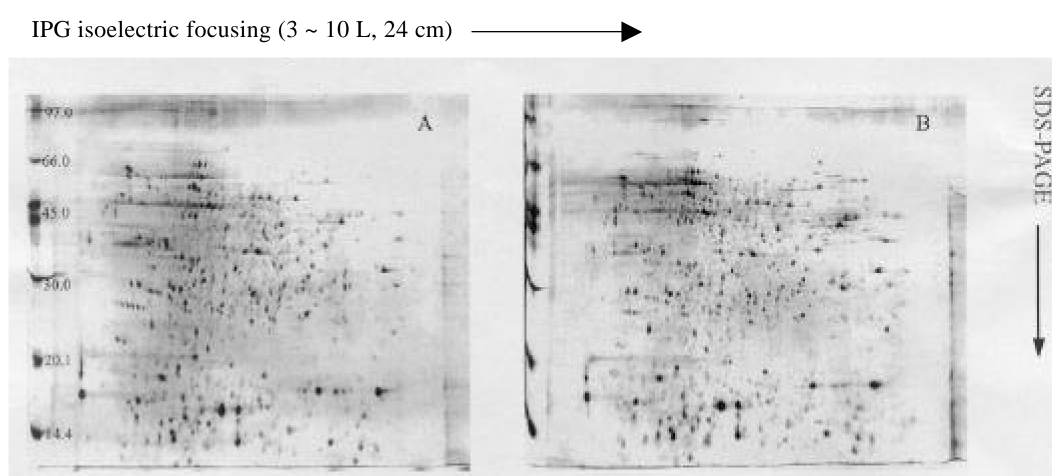

Figure 1. 2-D-protein map of middle silkgland of yellow cocoon (female) (**A**) and white cocoon (male) (**B**) silkworm, *B. mori*.

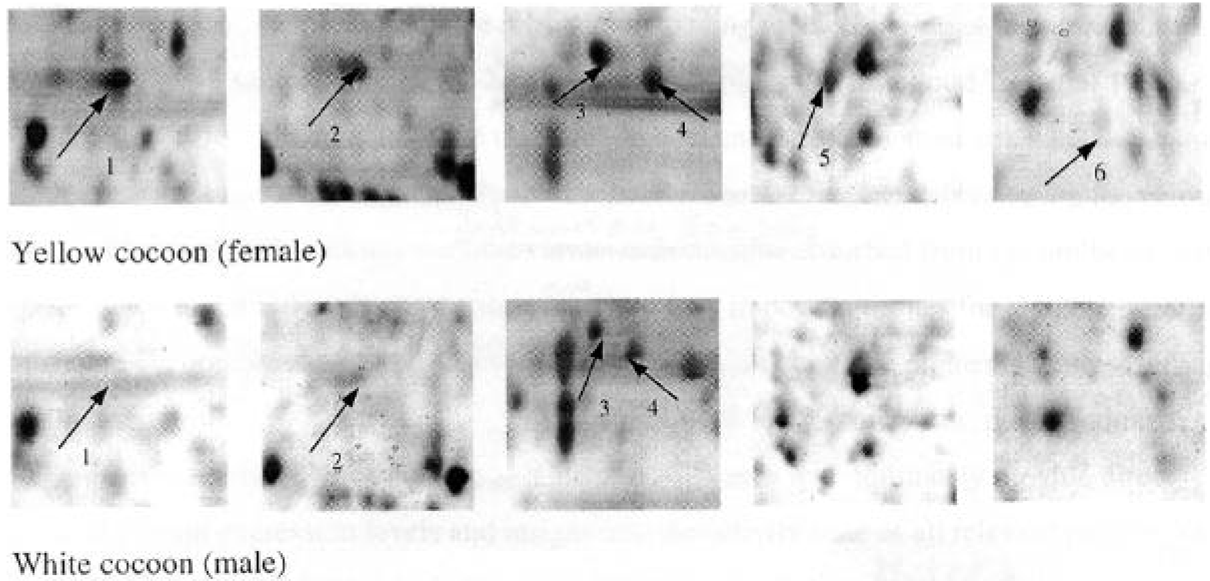

Figure 2. The main representative differential proteins in the middle silkgland presented between the yellow cocoon (female) and white cocoon (male).

Table 1. The main parameters of the differential proteins as depicted in figure 2.

Spot	p*I*	Mr/kDa	Relative spots normalization volume		Index
			Yellow cocoon	White cocoon	
1	4·85	18·51	0·508	0·057	11·2
2	4·38	35·02	0·267	0·048	17·9
3	3·79	53·30	0·367	0·082	22·3
4	3·94	51·88	0·194	0·101	52·1
5	6·74	44·12	0·249	–	–
6	6·49	25·04	0·034	–	–

Index = Volume in white cocoon/Volume in yellow cocoon = X 100.

4. Discussion

We used the update 2-D electrophoresis system manufactured by Amersham Biosciences. This system utilizes the IPGphor for IEF in the first dimension, and the highest voltage reaches up to 8000 V. The rehydration and IEF steps were carried out in the same holder made up of special ceramic material. These IPG holders have a special surface treatment to minimize protein adsorption. The technique of sample in-gel application can avoid the protein precipitate in the holder caused by sample cups loading method, therefore, the protein sample rehydration loading method can provide higher resolution, better reproducibility and more sample volume for 2-D PAGE (Rabilloud *et al* 1994).

The pigments transfer from the midgut to the middle silkgland and yield coloured silk. Although carotenoids are responsible for yellow cocoon, the carotenoids are not synthesized in the silkworm, instead are largely absorbed from the mulberry leaves only. Midgut tissue and middle silkgland tissue are two very important tissues for pigment transfer from digestive canal to cocoon. Lu (1991) has shown that the pigments that got through the two tissues are controlled by different genes. Soren *et al* (2001) showed that proteomics, post-genomic research, will ultimately provide direct measurement of protein expression levels and insight into the activity state of all relevant proteins. Genomic constitution perhaps the same in the middle silkgland of yellow cocoon and white cocoon silkworm, but the protein expression might be different due to the difference in activation of functional genes resulting in different phenotypes. The main purpose of present study was to separate the proteins from the male and female larvae with sex-limited colour cocoon, using 2-D gel electrophoresis, and to analyse the difference of these proteins.

Thus our group attempted to study the middle silk gland protein pattern to find some different proteins and study their expression responsible for the colour formation of the cocoon. The results suggested that two differential proteins existed, and that in the yellow cocoon the expression volume of some proteins was much higher. The test material in the present investigation showed the difference between female and male individuals only through phenotypical differences in the cocoon colour. However, there were no distinct differential proteins between the silkgland tissue of female and male larvae within the same variety of no sex-limited character. Therefore, the differential proteins found in the present experiment may be attributed to the cocoon colour. However, further detailed studies with regard to the structure, N-end sequence and the main function of these proteins in the middle silk gland are required.

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