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Silkworm egg proteins at the germ-band formation stage and a functional analysis of BmEP80 protein

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ABSTRACT

The patterning of embryos in early stages is a critical process for embryo development. In order to understand the molecular mechanism of early embryogenesis in silkworm, 2-DE combined with MALDI-TOF–MS technologies were used to analyze the proteins from diapause-destined eggs at the germ-band formation stage. From over 1000 spots, 93 were selected for analysis and data were obtained from 59 revealing 42 proteins. Gene Ontology annotation showed these proteins were involved in several biological processes at the germ-band formation stage, including cell stress response and protein folding, cell growth and migration, termination of diapause, and nutrition storage. Prominent among them was a new 80 kDa protein, named *Bombyx mori* egg protein 80 (BmEP80). BmEP80 was a component of the eggshell which was secreted by follicle cells during the late vitellogenesis stage to early choriogenesis stage (FCs −5 to +10). It disappears during early embryogenesis and RNAi against it resulted in the collapse of eggs, thus it is likely that BmEP80 is a new component of the silkworm vitelline membrane.

1. Introduction

The silkworm, *Bombyx mori*, is a model species for Lepidoptera, because it has a large size, relatively short and predictable life cycle, high fertility, and adaptation to laboratory culture (Goldsmith and Marec, 2010). It is a holometabolous insect that displays four developmental stages: embryo, larva, pupa, and moth. A female silkworm moth typically produces more than 400 eggs in its short life span and the development of silkworm follicles and eggs is a successive process consisting of previtellogenesis, vitellogenesis, choriogenesis, oviposition, fertilization, and embryogenesis. Previtellogenesis, vitellogenesis and choriogenesis phases are successive developmental stage of follicles.

In general, the embryogenesis of non-diapausing silkworms takes 9.5 days at 25 °C, and early embryogenesis has been divided into five major stages based on morphological criteria (Miya, 1985; Nagy et al., 1994). They are fertilization, blastoderm formation, germ-band formation (GBF), spatula formation, and abdominal appendage formation, referring to the eggs 2 h, 8–10 h, 24 h, 48 h, and 72 h after oviposition at 25 °C, respectively. Just before the GBF stage, the blastoderm differentiates into the primordial germ and the extra-embryonic region (Nagy et al., 1994). A cluster of cells then rapidly thickens and enlarges into the germ-band, which is located in the lateral and ventral regions. At this stage, the peripheral yolk nuclei initiate cellularization, the presumptive serosal cells completely envelope the germ-band and the amnion cells begin to migrate over the germ-band that is ready to develop to the embryo (Nagy et al., 1994). Diapause-destined embryos are arrested at gastrulation that occurs two days after the GBF stage. So GBF stage is a critical period of embryogenesis. However, the information for proteins involved in this physiological process is limited.

In order to understand the molecular mechanism for early embryonic development of silkworm, Mita et al. (2003); Cheng et al. (2004); and Hong et al. (2006) separately constructed cDNA libraries from early embryos (matured eggs, 0 h, 10 h, 24 h, 48 h, 72 h and 96 h old eggs). Hong and his colleagues further studied the mRNA profiles during early embryogenesis stages and found 241 genes were differentially expressed compared to the GBF stage (Hong et al., 2006). Data from microarrays revealed that among these 241 genes, several genes associated with development and cell communication were upregulated. Nevertheless, it is well known that mRNA levels may not correlate with protein levels (Gygi et al., 1999; Nie et al., 2006). Hence, it is necessary to investigate the proteins involved in the germ-band formation (GBF). We are interested in learning which proteins are involved in the organization and progression of embryogenesis at GBF stage. The completion of *Bombyx* genomic project by both Chinese (Xia et al.,

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2004) and Japanese (Mita et al., 2004) groups and proteomic techniques that begins with 2D electrophoresis, 2-DE (Issap and Veenstra, 2008) offers us an opportunity to address this concern.

In the present study, we used 2-DE combined with matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF–MS) to analyze the proteins from the early embryos at GBF stage. As a result, a total of 42 proteins with multiple functions were identified. Prominent among them is a protein we named BmEP80. The first functional analysis of BmEP80 was carried out and resulted in a conclusion that BmEP80 is a putative new component of the silkworm vitelline membrane.

2. Materials and methods

2.1. Insect and protein preparation

The silkworm strain *p50* (*DaZao*) used in this research is maintained at the Institute of Sericulture and System Biology at Southwest University of China and was used for the silkworm Whole Genome Shotgun (WGS) sequences. Eggs were incubated at 25 °C under continuous illumination to make diapause-egg producers. The larvae were reared on fresh mulberry leaves at 25 °C. Both pupation and adult eclosion were gated to assure that synchronized insects were used in the subsequent experiments. Newly merged female moths copulated with male moths, and eggs that were laid for every 1.5 h were pooled. Eggs were collected 24–25.5 h after oviposition for protein extractions. To prevent diapause initiation, about one-day old eggs were treated with HCl (specific gravity 1.075) at 46 °C for 5.5 min. The eggs were incubated under 25 °C with relative humidity of 80%. In some later experiments where we wanted to learn if a particular protein was present in eggshells or egg contents, we used matured eggs. Matured eggs (dissected from virgin moths) were separated by hand into two parts in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to carefully separate the eggshells containing chorion and vitelline membrane from their contents. The residual egg contents (mean oocytes content) were removed from eggshells by washing with PBS buffer.

Each 3 g of matured/oviposited eggs and the same amount of clean eggshells were pulverized in liquid nitrogen with a mortar and pestle. The proteins were extracted from these tissue powders by vigorously shaking with 1.5 ml lysis buffer (8 M urea, 4% (m/v) 3 [(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS, Sangon, Shanghai, China), 4% (m/v) dithiothreitol (DDT, BBI, Canada)) at 4 °C for 40 min. The extractions were centrifuged (4 °C, 12,000 × *g*, 15 min) twice and the supernatants were saved at −20 °C until use. The proteins from egg content were resuspended in PBS buffer and then lysed in the above lysis buffer. The concentration of proteins was determined by Bradford kit as described by manufacturer's instructions (TIANGEN, Beijing, China).

2.2. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) was carried out as described by manufacturer's instructions (GE Healthcare, USA). In brief, a total of 250 μg proteins were dissolved in rehydration solution (8 M urea, 2% CHAPS, 0.28% DDT, and 1% IPG buffer, pH 3–10) and loaded onto an immobilized pH gradients strip (IPG strip) (18 cm, line, pH 3–10, GE Healthcare, Sweden). Isoelectric focusing (IEF) was ran in Ettan IPGphor 3 (GE Healthcare, Sweden) at 50 V for 12 h, 100 V for 1 h, 300 V for 1 h, 500 V for 1 h, 1000 V for 0.5 h, 3000 V for 0.5 h, 5000 V for 0.5 h, 8000 V for 100,000 V h (voltage multiplied by hours), and 500 V for holding. After IEF, the IPG strips were immediately equilibrated for two steps. One percent DDT was added in the first equilibration solution (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) and replaced with 2.5% iodoacetamide (GE Healthcare, England) in the second step. For each equilibration step, the incubation time was 15 min. The strips were then subjected to second-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing condition. Proteins were separated in a 12.5% polyacrylamide gels (length: 250 mm, height: 200 mm, thickness: 1 mm). Electrophoresis was performed in an Ettan DALTsix vertical electrophoresis system (Amersham Biosciences, Sweden) with 40 mA per gel at 10 °C. The 2-DE gels were stained by silver nitrate and scanned by an Image Scanner III (GE Healthcare, USA) with LabScan™ 6.0 software (GE Healthcare, USA). The images were obtained at 300 dpi resolution at OLD calibration transparent mode. All images were analyzed by ImageMaster™ 2D platinum 6.0 software (GE Healthcare, USA), and the parameters for spots detecting were set to smooth: 2–5; minarea: 30–50; and saliency: 5–15.

2.3. Proteins identified by MALDI-TOF–MS and the construction of local database

From the 1000 spots on the gel, we selected 93 of the most abundant. These spots were excised, cut into small pieces (about 1 mm^3), and transferred into 0.5 ml Eppendorf tubes. In-gel tryptic digestion was carried out according to previously described methods (Hou et al., 2007). In brief, 100 μl destaining solution (15 mM $K_3[Fe(CN)_6]$, 50 mM $Na_2S_2O_3$) was added to each tube for 10 min destaining. The pieces were washed with 100 μl milli-Q water for 10 min for three times and dehydrated with 100 μl acetonitrile (ACN) for 5 min. Fifty microgram trypsin (10 mg/mL in 25 mM NH_4HCO_3, pH 8.0, Sigma, USA) was added to each tube and incubated at 37 °C for a 20 h digestion. The peptides obtained from the digestion were extracted twice from the gel pieces with 50% ACN/5% trifluoroacetic acid (TFA, Sigma, USA). The supernatants were pooled and concentrated on a Centrivap Cold Trap (LABCONCO, USA). The resultant peptides were resolved in 3 μl of 50% ACN/0.5% TFA and equally mixed with saturated α-cyano-4-hydroxycinnamic acid (CHCA, Sigma, USA). The mass spectra of the peptides were recorded on a Voyager DE PRO MALDI-TOF–MS (Applied Biosystems, USA) between 800 Da and 2500 Da with an accelerating voltage of 20 kV, grid voltage of 75–80%, and delay time of 100–120 ns. Standards kit (Sequazyme TM peptide mass standards Kit, Applied Biosystems, USA) and trypsin auto-digestion fragments were used as the external and internal calibration, respectively.

The peptide mass fingerprintings (PMF) were processed by Data Explorer TM software (Applied Biosystems, USA). For creating the peak list, parameters were set as follow: chose "default or noise filter (0.7 for correlation)" for noise filter and chose "peak deisotoping" for peptides. The peak list was searched against the local database (see below) by the General Protein/Mass Analysis for Windows software (GPMAW, version 6.10, http://www.gpmaw.com/) (Peri et al., 2001). In all searches, parameters were set according to the previous work (Zhang et al., 2006b; Li et al., 2006) as ±1.0 Da peptide tolerance, maximum 1 miss cleavage, minimum 5 hits, and carbamidomethylation on cysteine residues. The local database was constructed with 21,265 FASTA protein sequences, which included 6642 sequences downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) with the keyword of silkworm and 14,623 sequences generated by the annotation of silkworm genomic sequences (http://silkworm.swu.edu.cn/silkdb/doc/download.html). The database was sent to GPMAW software for making the digest database, and the parameters were set to carbamidomethylation on cysteine residues, and the trypsin digestion.