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Analysis of Diapause-Associated Changes in Silkworm Egg Proteins

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The patterns of diapause-associated proteins of silkworm eggs were analyzed by two-dimensional (2-D) gel electrophoresis. Among the hundreds of spots on the 2-D gels, at least two proteins were considered to be associated with diapause. A protein, spot 4, with an approximate molecular weight of 38 kDa and p*I* 6.1 was observed in the HCl-treated, cold-treated, and diapause eggs, respectively. Spot 4 was undetectable in unfertilized eggs and non-diapause eggs at two days after oviposition, suggesting that this protein may be associated with the entrance to diapause. A protein, spot 11, with an approximate molecular weight of 21 kDa and p*I* of 61 was detected in the unfertilized, HCl-treated, and cold-treated eggs, respectively, after oviposition by normal moths. In diapausing eggs, a protein corresponding to spot 11 was observed in 3-, 5-, and 30-day-old eggs, while the protein was not detected one-day-old eggs. The protein corresponding to spot 11 was not detected in unfertilized and non-diapause eggs obtained from subesophabeal ganglion (SG)-extirpated moths either. Spot 11 was also considered to be a diapause specific protein, which occurred at only early embryonic stage under the control of diapause-downregulated gene.

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Introduction

In the silkworm *Bombyx mori*, diapause occurs at the late gastrula stage of embryogenesis. Embryonic diapause in the silkworm is predeterminated by a diapause hormone that is secreted from the subesophageal ganglion (SG) of the maternal pupa. Dorel and Coulon (1988) have analyzed the expression patterns of proteins synthesized in prediapausing eggs of *B. mori* by two-dimensional (2-D) gel electrophoresis of *in vivo* labeled proteins. They identified a protein, No. 30, that is specific for the germ anlage stage (24 hrs after oviposition) and overproduced in the HCl-treated eggs. The biological function of this protein, however, is unknown.

In this study, we investigated two proteins that are associated with the initiation, maintenance, and termination of diapause. In contrast to many previous studies in which newly synthesized, diapause-associated proteins were analyzed, we analyzed the entire complement of egg proteins from crude extracts by 2-D gel electrophoresis. We found two diapause-associated proteins.

Materials and Methods

Animals

A bivoltine hybrid silkworm strain (Jam123 × Jam124)

was used in this study. The maternal larvae were reared on an artificial diet at 25°C. Eggs laid within a 3-hrs window were pooled in order to obtain eggs with synchronous development. Non-diapause eggs were obtained from female moths in which the SG was extirpated immediately following the larval-pupal molt (Fukuda, 1951). In order to terminate diapause, the eggs were incubated at 25°C for 2 days after oviposition, and then incubated at 5°C for 3 months. On the other hand, the eggs were hindered to enter the diapause by treating the eggs at 20 hrs after oviposition with hot HCl (47°C) for 5 min.

Sample preparation

Synchronous eggs (100 mg) were homogenized in 500 μl of PBS buffer contained protease inhibitor cocktail and the soluble protein was isolated by centrifugation at 4°C for 10 min at 14,000 g. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2-D gel electrophoresis and silver staining

The 2-D gel electrophoresis was performed following standard protocols (Amersham Biosciences, 2-D hand-

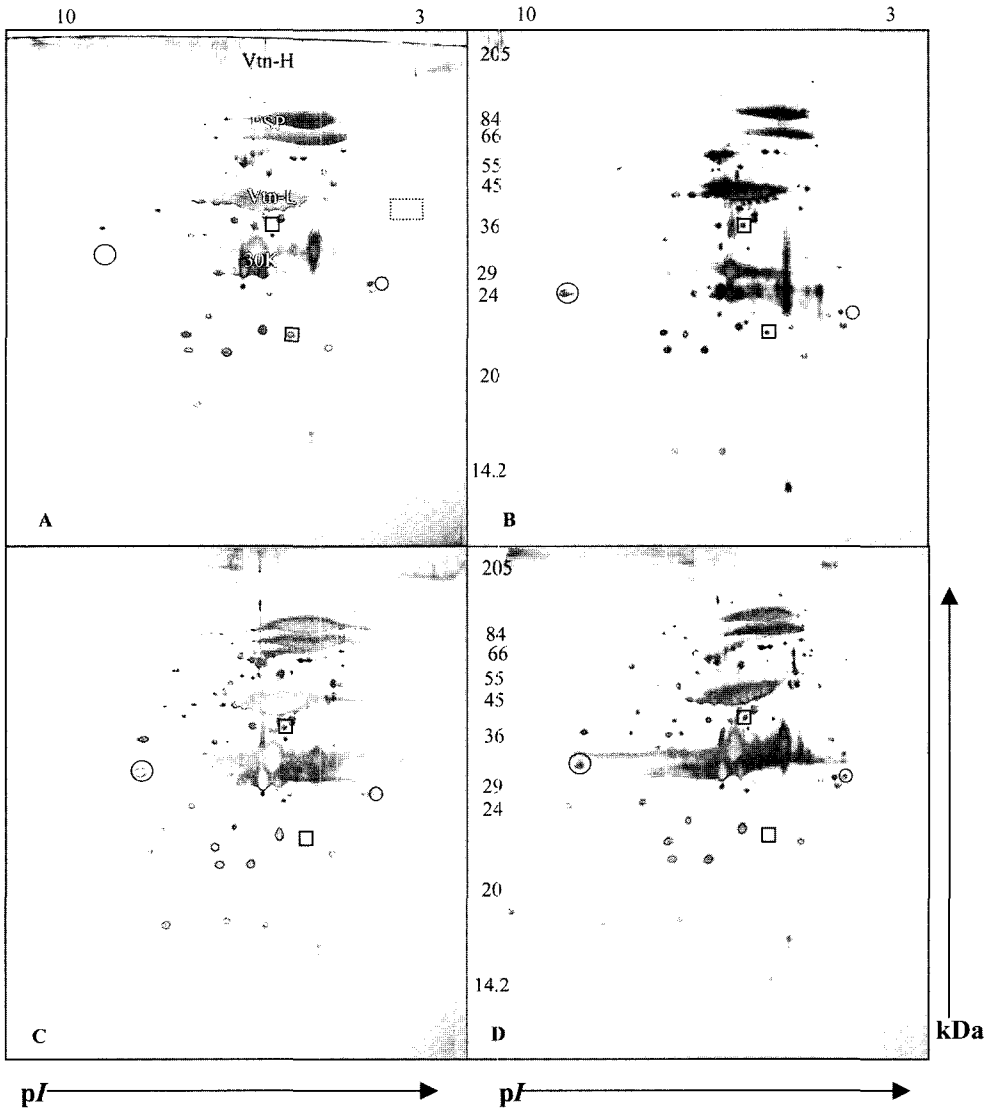

Fig. 1. 2-D gel electrophoresis patterns of proteins from crude extracts of silkworm eggs using 12.5% SDS-polyacrylamide gels. A, Unfertilized eggs; B, Eggs at 1-day after oviposition; C, Eggs at 3-days after oviposition; D, Eggs at 10-days after oviposition. The direction of the pH gradient (10 to 3) and approximate molecular weight of size markers are indicated. Protein spots detected only in unfertilized eggs are shown by the dotted square. Protein spots detected all groups of eggs except the unfertilized eggs are circles. Protein spots that appear to be specifically associated with diapause are indicated by a square and further explained in Figs. 2 and 3. Vtn-H: vitellin heavy chain, Vtn-L: vitellin light chain, ESP: egg specific protein, and 30 K: 30 kDa proteins.

book). Sample containing 150 µg of protein was rehydrated overnight at room temperature in a solution containing 8 M urea, 0.2% (w/v) DTT, 0.5% (v/v) IPG buffer, 0.5% CHAPS and 0.002% bromophenol blue. Separation in first dimension, isoelectric focusing (IEF), was performed with the IPG strips (pH 3 – 10) placed horizontally on a flatbed electrophoresis unit, Multiphor II system (Amersham Biosciences, USA). IEF was performed by gradually increasing the voltage across the IPG strips to 3,500 V and maintaining this voltage for 70 kVh. After IEF, the IPG strips were equilibrated in equilibration solution (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 1% DTT) for 15 min and applied onto vertical 12.5% SDS-polyacrylamide gels. After separation in the second dimension, the gels were fixed in a fixing solution (40% ethanol and 10% acetic acid) prior to silver staining (Amersham Biosciences, USA).

Results and Discussion

The egg samples were divided into five groups; unfertilized eggs, diapause eggs, non-diapause eggs, HCl-treated eggs, and cold-treated eggs. The protein migration patterns of unfertilized and fertilized eggs were generally very similar (Fig. 1). Protein spots that were detected in both unfertilized and fertilized eggs composed the largest group of spots (Fig. 1). Spots that were detected in unfertilized but not in fertilized eggs as well as spots that were detected in fertilized but not in unfertilized eggs are indicated by squares and circles, respectively, in Fig. 1. By 2D-gel electrophoresis, Yara *et al.* (1994) compared the protein pattern of the fertilized eggs at an early embryonic stage and unfertilized eggs after *in vitro* translation of mRNAs. They reported that there were roughly 10 – 35% more countable protein spots generated from unfertilized eggs in comparison to fertilized eggs. On the basis of their findings, they suggested that the integrity of the major classes of maternal mRNAs are substantially conserved (*i.e.,* they remain translatable) for at least 10 hrs after oviposition.

Egg proteins with isoelectric points between 8.0 and 5.5 and molecular weights between 45 and 30 kDa are shown in Fig. 2. A protein, spot 4, with an approximate molecular weight of 38 kDa and p*I* of 6.1 was observed in HCl-treated, cold-treated, and diapause eggs (Fig. 2). Spot, 4 was undetectable in unfertilized eggs and non-diapause

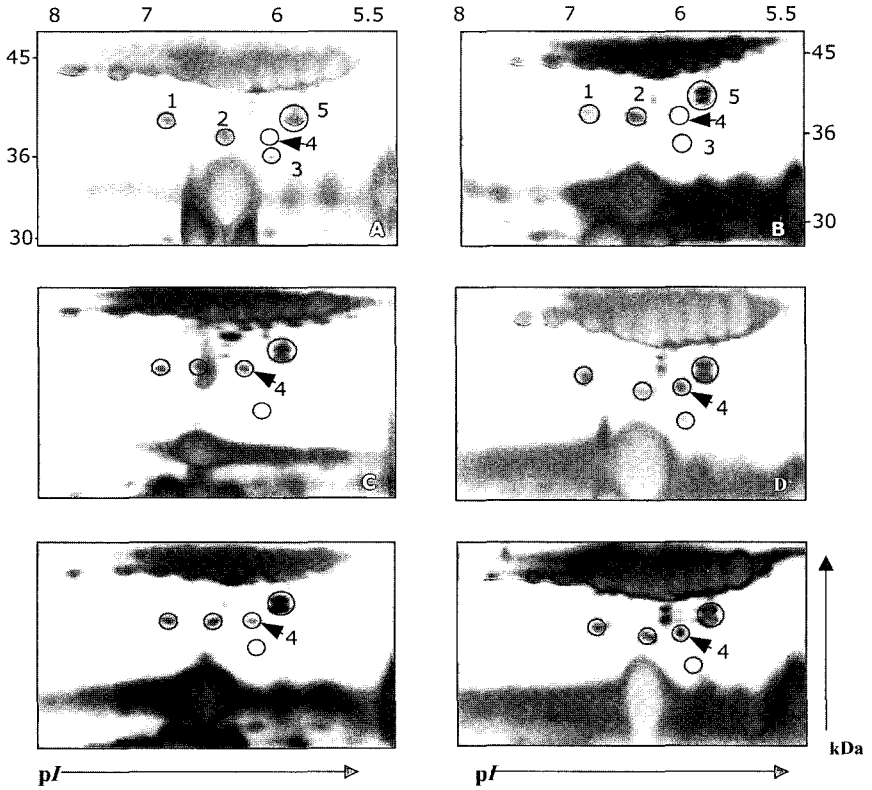

Fig. 2. 2-D gel electrophoresis patterns of proteins within the pH 8.0 to 5.5 and 45 to 30 kDa region of the gel. A, Unfertilized eggs; B, 1-day-old non-diapause eggs; C, Eggs at 1-day after oviposition; D, Eggs at 5-days after oviposition; E, 1-day-old eggs after HCl-treatment; F, 10-day-old eggs after cold-treatment.

eggs at two days after oviposition, suggesting that it is associated with the entrance to diapause. The protein expression of spot 4 is expected to coincide with fertilization in diapause-programmed eggs. The biological role, if any, of this protein is still unclear. Sonobe and Odake (1986) have identified a putative *pnd*$^+$ gene-specific protein (22 kDa) by 2-D gel electrophoresis observed in diapause and HCl-treated diapause eggs, but not non-diapause eggs. This *pnd*$^+$ gene-specific protein is involved in the process of diapause determination, which acts after fertilization (Suzuki *et al.*, 1999). The spot 4 protein may play a same role as the *pnd*$^+$ gene-specific protein.

Egg proteins with isoelectric points between 8.0 and 5.0 and molecular weights between 29 and 20 kDa are shown in Fig. 3. A protein, spot 11, with an approximate molecular weight of 21 kDa and p*I* of 6.3 was detected in unfertilized, HCl-treated, cold-treated, and one-day-old eggs after oviposition by normal moth. A spot corresponding to spot 11, however, was not detected in unfertilized and non-diapause eggs obtained from moths from which the SG was extirpated. A spot corresponding to this protein was also not observed in 3-, 5-, 10- and 30-day-old diapause eggs kept at 25°C. In these eggs, mitotic activities of the embryo are down regulated and cell division finally stops by 3 days after oviposition. Thus, one-day-old eggs are diapause-programmed eggs but not diapause eggs. Because eggs at one day after oviposition are not in diapause, spot 11 may be detectable at this time, but disappears at 3 days after oviposition because of diapause entrance. It is possible that the protein corresponding to spot 11 is downregulated during diapause and subsequently activated in response to physical stress such as cold or hot-HCl treatment. In non-diapause-programmed eggs, it is thought that proteins corresponding to spot 11 are not detected due to a maternal effect (*e.g.*, diapause-inducing factor) of the SG-extirpated mother moth.

There are four major patterns of diapause-related gene expression (Delinger, 2002): 1) genes uninfluenced by diapause, 2) diapause-downregulated genes, 3) diapause-upregulated genes, and 4) genes expressed intermittently during diapause. Diapause-upregulated genes are further subdivided into three categories: genes that are upregulated throughout diapause, through early-diapause, and through late-diapause. In this study, the protein corre-

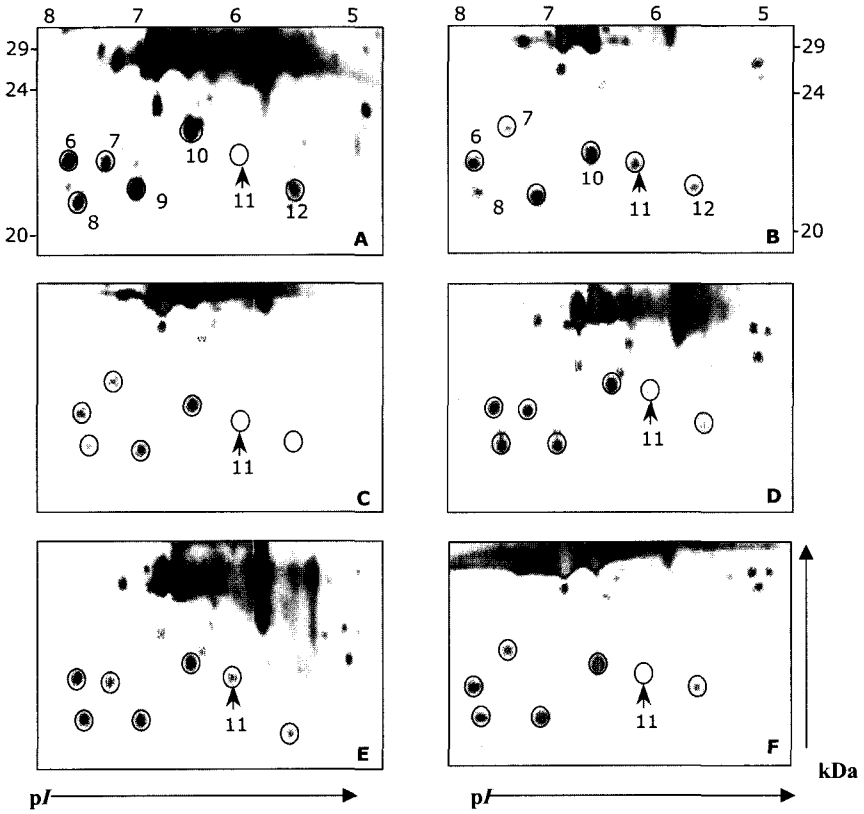

Fig. 3. 2-D gel electrophoresis patterns of proteins within the pH 8.0 to 5.0 and 29 to 20 kDa region of the gel. A, Unfertilized eggs obtained from an SG-extirpated female moth; B, Unfertilized eggs normal moths; C, Non-diapause eggs at 1 day after oviposition by SG-extirpated moths; D, 10-day-old eggs after cold-treatment; E, Eggs at 1-day after oviposition; F, Eggs at 10-days after oviposition.

sponding to spot 11 appears to follow the second gene expression pattern. However, the correlation between the level of gene and protein expression is still unclear.

An excessive amount of yolk was a major problem for the 2-D gel electrophoresis. In insect eggs (oocytes) the euplasm usually constitutes much less than 10% of total egg content. The remaining 90% or more is yolk, consisting largely of lipid and proteins. Lipids often comprise about 40% of the dry weight of the terminal oocyte and most of the lipid is stored as triglycerol. The protein content of the yolk is usually approximately equal to the lipid content, and 60 – 90% of yolk proteins are derived from vitellogenins (Chapman, 1998). In *B. mori*, egg specific protein and 30 K proteins are the major protein in the yolk (Tomino, 1985). Because even the best 2-D gels can routinely resolve no more than 1,000 proteins, only the most abundant proteins can be visualized by gel electrophoresis if a crude protein mixture is separated (Pandey and Mann, 2000). Thus, the majority of the spots in our study was putatively derived from the major yolk proteins. As a result, the intensity of the minor spots was relatively lower and was difficult to analyze. Our analysis may be more accurate if an effective method is developed for the removal of the three major yolk proteins.

In the future, proteins corresponding to spot 4 and 11 as well as other selected spots will be analyzed by mass spectrometric techniques and N-terminal sequencing. Through comparative database and sequence homology searches with other organisms, we may be able to determine the amino acid sequence and putative biological function of the interesting proteins. A more complete protein sequence database of *B. mori* will be essential in this endeavour.

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References

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Chapman, R. F. (1998) The insects: Structure and Function, 4th ed, Cambridge University Press, pp. 303.

Denlinger, D. L. (2002) Regulation of diapause. *Annu. Rev. Entomol.* **47**, 93-122.

Denlinger, D. L., K. H. Joplin and G. D. Yocum (1990) Diapause specific proteins expressed by the brain during the pupal diapause of the flesh fly, *Sarcophaga crassiplapis. J. Insect Phisyol.* **36**, 775-783.

Dorel, C. and M. Coulon (1988) Regulation of gene expression in prediapausing embryos of the silkworm, *Bombyx mori*: pattern of protein synthesis. *Cell Differ.* **23**, 87-92.

Fukuda, S. (1951) The production of the diapause eggs by transplanting the suboesophageal ganglion in the silkworm. *Proc. Japan Acad.* **27**, 672-677.

Pandey, A. and M. Mann (2000) Proteomics to study genes and genomes. *Nature* **405**, 837-846.

Suzuki, M. G., T. Terada, M. Kobayashi and T. Shimada (1999) Diapause-associated transcription of BmEts, a gene encoding an ETS transcription factor homolog in *Bombyx mori. Insect Biochem. Mol. Biol.* **29**, 339-347.

Tomino, S. (1985) Major plasma proteins of *Bombyx mori. Zool. Sci.* **2**, 293-303.

Yamashita, O., T. Yaginuma, H. Iwasaki, M. Takahashi and T. Niimi (1997) Cloning the cDNAs encoding *Bombyx* homologues of Cdc2 and Cdc2-related kinase from eggs. *Insect Mol. Biol.* **6**, 131-141.

Yara, M., E. Kosegawa, Y. Sugimoto and K. Kaga (1994) Comparison of translation products of mRNA from unfertilized eggs of *Bombyx mori. J. Seric. Sci. Jpn* **63**, 336-340.