韓蠶學誌 41(2), 82~86(1999)
Korean J. Seric. Sci.
<論 文>

Major Hemolymph Proteins and Vitellogenin in Mulberry Longicorn Beetle, *Apriona germari* Hope

Hyung Joo Yoon[1,3], Young Il Mah[1], Kwang Ho Choi[2], Byung Rae Jin[2], Hung Dae Sohn[2] and Jae Yu Moon[3]

[1] *Dept. of Sericulture & Entomology, The National Institute of Agricultural Science & Technology, RDA, Suwon 441-100, Korea*
[2] *College of Natural Resources and Life Science, Dong-A University, Pusan 604-714, Korea*
[3] *College of Agriculture & Life Sciences, Seoul National University, Suwon 441-744, Korea*

ABSTRACT

Hemolymph proteins and vitellogenin from mulberry longicorn beetle, *Apriona germari* Hope, were identified, and their changes were analyzed during the larval-pupal-adult development and in the newly laid eggs. Three major hemolymph proteins were observed in the hemolymph during the larval-pupal-adult development and the intensity of their proteins was clearly observed during the pupal stage. From SDS-polyacrylamide gel electrophoresis analysis, molecular weights of three major hemolymph proteins were approximately 74 kDa, 78 kDa and 85 kDa. Vitellogenin in *A. germari* appeared in the hemolymph of only adult female and is considered to be a product synthesized within 10 days after adult emergence. The molecular weight of vitellogenin was consisted of a heavy subunit (165 kDa) and a light subunit (40 kDa).

Key words : *Apriona germari*, Hemolymph protein, Vitellogenin

INTRODUCTION

The mulberry longicorn beetle, *Apriona germari* Hope, is one of the major pests of mulberry and fig trees (Zhang and Shen, 1980; Hua, 1982). It is very difficult to study ecological and physiological characteristics of the mulberry longicorn beetle, because they have a long life cycle, which takes approximately 2 to 3 years in nature (Yoon and Mah, 1999). *A. germari* larvae bore into the living trunk through egg-laying scars and feed on it (Yoon *et al.*, 1997a; 1997b). Recently, Yoon and Mah (1997; 1999) and Yoon *et al.* (1997c) reported on the artificial rearing and larval developmental charateristics of *A. germari*, but the results dealt only within the scope of ecology.

Insect hemolymph proteins usually show dynamic changes during molting and metamorphosis (Levenbook, 1985; Kanost *et al.*, 1990). Major hemolymph proteins show promise as biochemical marker for growth and development in insects. There are few reports on hemolymph proteins and yolk proteins in coleopteran insects, though much evidence in lepidoptera and diptera has been accumulating (Kanost *et al.*, 1990; Telfer and Kunkel, 1991). In the longicorn beetles, physiological and biochemical aspects are very pooly understood yet. Azuma *et al.* (1993) reported the identification and developmental changes of the major hemolymph proteins in the yellow-spotted longicorn beetle, *Psacothea hilaris*. However, major hemolymph proteins and vitellogenin in *A. germari* are not reported yet. Therefore, this study was conducted to make some physiological foundation of *A. germari*. In this report, we have described the major hemolymph proteins and vitellogenin in *A. germari*.

MATERIALS AND METHODS

Insects

Hatched-larvae or eggs of the mulberry longicorn beetle (*Apriona germari* Hope) were collected from the cultivated mulberry fields (Yoon *et al.*, 1997a; 1997b). The insect have been reared successively on an artificial diet at the insectary. Artificial diet, rearing method and sexual distinction were carried out as described previ-

ously (Yoon *et al.*, 1997c; Yoon and Mah, 1999).

Sample preparation

All hemolymph samples were collected individually. Hemolymph from larva, pupa and adult was collected in a chilled tube with a few phenylthiourea crystal, respectively. Yolk protein from the newly laid eggs was collected by dissection in cold PBS (0.12 M NaCl, 2 mM KCl, 4 mM Na_2HPO_4, 1 mM KH_2PO_4, 0.02% NaN_3, pH 7.3). All hemolymph samples and egg extracts were then centrifuged at 15,000 rpm, 4°C for 10 min to remove hemocytes and debris, and the supernatants were stored at -70°C until use.

Polyacrylamide gel electrophoresis (PAGE)

Non-denatured PAGE was performed in 7.5% gel at 4°C according to the method of Davis (1964). SDS-PAGE was conducted on 10% gel at room temperature at 20 mA current (Laemmli, 1970). Following electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue R-250 for 2 hrs, and destained in 10% methanol and 10% acetic acid for a day.

Preparation of antibody

Egg extract was first subjected to 7.5% PAGE. The vitellogenin bands were cut out and crushed in the PBS buffer by a glass homogenizer. The sample was then centrifuged at 15,000 rpm, 4°C for 10 min to remove debris. The supernatant was mixed an equal volume of Freund's complete adjuvant (Sigma Co.) and injected into the peritoneum of a BALB/c mouse. Freund's incomplete adjuvant (0.1 ml) and vitellogenin (0.1 ml) were thoroughly mixed and used for a booster injection after the first injection. Booster injections were given for three times with interval of 1 week, and the last injection was made 2 weeks after the fourth injection. Blood was collected at the 3rd day after the last injection and centrifuged at 3,000 rpm, 4°C for 10 min. The supernatant was used as antiserum.

Western blot

Following SDS-PAGE, proteins in the gel were electroblotted to a sheet of nitrocellulose membrane (Sigma Co.) at 30 V for 8 hrs (Towbin *et al.*, 1979). After transferring, the nitrocellulose sheet was blocked for 1 hr with TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.2% Tween-20) containing 1% BSA (Bovine Serum Albumin) and then incubated with antiserum for 1 hr. The sheet was washed three times with TBST and incubated with anti-mouse IgG alkaline phosphatase conjugate (1:10,000 v/v, Sigma Co.) in TBST for 1 hr. After incubation, the sheet was again washed three times in TBST vigorously. After washing, substrate solution (0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 5 mM $MgCl_2$) containing BCIP (5-bromo-4-chloro-3-indolyl phos phate) and NBT (nitro blue tetrazolium) (Gibco) was added and the reaction was quenched with distilled water.

RESULTS AND DISCUSSION

To elucidate the changes of the hemolymph proteins during growth and development in *A. germari*, hemolymph was collected from an individual of larva, pupa and adult, and their hemolymph protein profiles were analyzed by non-denatured and SDS-PAGE (Fig. 1, 2, 4). In all stages examined, we could detect major hemolymph protein bands. The intensity of major hemolymph proteins in the larval stages increased according to larval development (Fig. 1). This result revealed that major hemolymph proteins are significantly increased in the last larval development. Actually, most larvae of

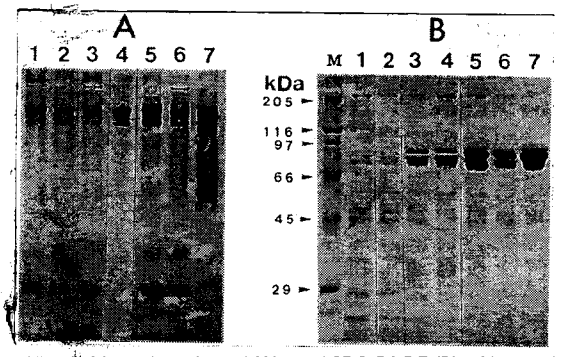

Fig. 1. *Non-denatured (A) and SDS-PAGE (B) of hemolymph proteins during the larval development in A. germari. Lane 1, 4 th instar larva; Lane 2, 5 th instar larva; Lane 3, 6 th instar larva; Lane 4, 7 th instar larva; Lane 5, 8 th instar larva; Lane 6, 9 th instar larva; Lane 7, 10 th instar larva. Molecular weight markers (M) in SDS-PAGE are represented on the left of panel B.*

A. germari pupated at the 8th and 9th instars, but others emerged from the 7 th to the 11th instars (Yoon and Mah, 1999).

Furthermore, the intensity of major hemolymph proteins increased during pupal stage and decreased during adult development (Fig. 2, 4). Although the pattern of major hemolymph proteins in pupal development was more clear than that of adult development, three major proteins were observed in the hemolymph during larval-pupal-adult development. We tentatively named these proteins as major hemolymph proteins I, II and III (Azuma *et al.*, 1993). The revealing pattern of the major hemolymph proteins appear to reflect the changes according to the developmental stages. However, it is not clear how these might affect development of *A. germari*.

Female-specific hemolymph protein in *A. germari* appeared during adult development and considered to be the product synthesized within 10 days after adult emergence (Fig. 2, 4). The sex-limited protein appeared in the female hemolymph and were also detected in eggs with a same electrophoretic mobility (Fig. 3A). Therfore this protein was considered as vitellogenin and/or vitellin (Kunkel and Nordin, 1985). Also the vitellogenin is considered as a dimer (Fig. 3B).

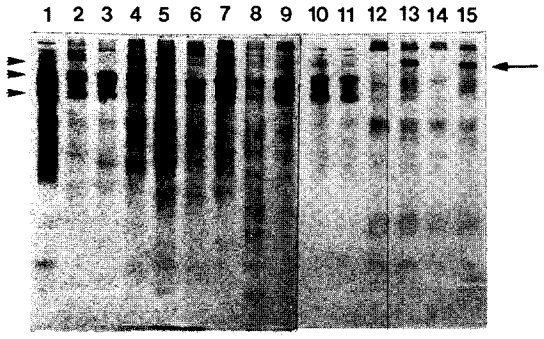

Fig. 2. *Non-denatured PAGE of hemolymph proteins during the pupal-adult development in A. germari. Lane 1, 10th instar larva; Lane 2, the day of pupation, male; Lane 3, the day of pupation, female; Lane 4, 5 days after pupation, male; Lane 5, 5 days after pupation, female; Lane 6, 10 days after pupation, male; Lane 7, 10 days after pupation, female; Lane 8, 15 days after pupation, male; Lane 9, 15 days after pupation, female; Lane 10, the day adult emergence, male; Lane 11, the day adult emergence, female; Lane 12, 10 days after adult emergence, male; Lane 13, 10 days after adult emergence, female; Lane 14, 30 days after adult emergence, male; Lane 15, 30 days after adult emergence, female. Three arrow heads indicate the stage-specific existence of major hemolymph proteins. Arrow indicates female-specific protein (vitellogenin).*

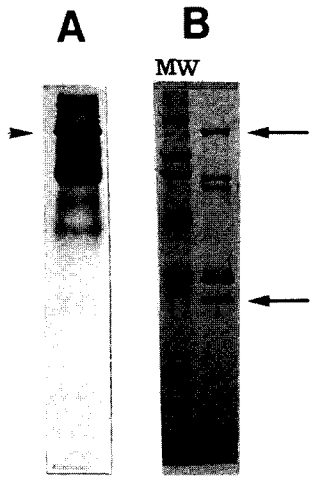

Fig. 3. *Non-denatured (A) and SDS-PAGE (B) of yolk protein of newly laid egg in A. germari. Arrows indicate native molecule (arrow head) and subunits (two arrows) of vitellogenin.*

To verify composition of subunits of vitellogenin in *A. germari*, we have prepared vitellogenin band from eggs by extracting from non-denatured PAGE gels (Fig. 3A) and produced vitellogenin antibody from mouse.

In SDS-PAGE analysis, pattern and intensity of major hemolymph proteins were also similar to those of non-denatured PAGE (Fig. 4A). The subunit of three major hemolymph proteins checked by non-denatured PAGE was determined by SDS-PAGE and their subunit molecular weight was approximately 74 kDa, 78 kDa and 85 kDa. However, it is difficult to explain the relationships between these major hemolymph proteins in non-denatured PAGE and SDS-PAGE in this study.

Female-specific protein considered to be vitellogenin was detected as two bands in hemolymph of the adult development (Fig. 4A). Thus, the result indicates that vitellogenin is a dimer and consisted of a heavy subunit and a light subunit. Furthermore, Western blot analysis with antibody confirmed the result obtained from SDS-PAGE analysis (Fig. 4B). The molecular weight of two

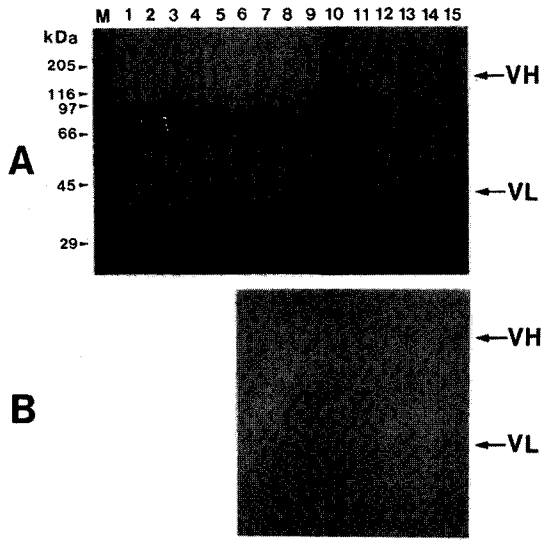

Fig. 4. *SDS-PAGE (A) and Western blot analysis (B) of hemolymph proteins during the pupal-adult development in A. germari. Lane 1, 10th instar larva; Lane 2, the day of pupation, male; Lane 3, the day of pupation, female; Lane 4, 5 days after pupation, male; Lane 5, 5 days after pupation, female; Lane 6, 10 days after pupation, male; Lane 7, 10 days after pupation, female; Lane 8, 15 days after pupation, male; Lane 9, 15 days after pupation, female; Lane 10, the day adult emergence, male; Lane 11, the day adult emergence, female; Lane 12, 10 days after adult emergence, male; Lane 13, 10 days after adult emergence, female; Lane 14, 30 days after adult emergence, male; Lane 15, 30 days after adult emergence, female. Molecular weight markers (M) are represented on the left of panel. Three asterisks indicate subinits of major hemolymph proteins. Two arrows indicate heavy subunit (VH) and light subunit (VL) of vitellogenin.*

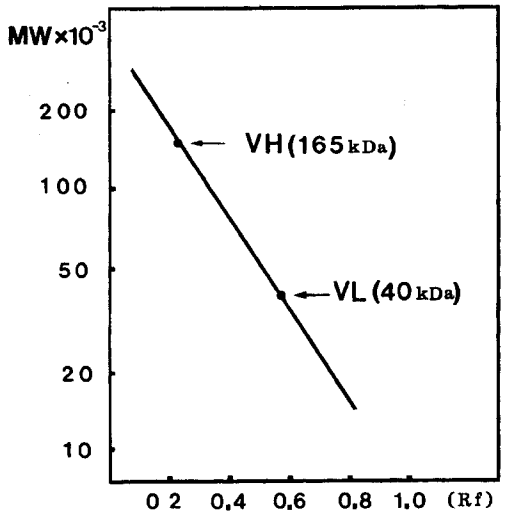

Fig. 5. *Molecular weight determination of vitellogenin subunits by SDS-PAGE. Arrows indicate 165 kDa heavy subunit (VH) and 40 kDa light subunit (VL) of vitellogenin.*

different subunits of vitellogenin was approximately 165 kDa (heavy subunit) and 40 kDa (light subunit), respectively (Fig. 5). This result suggests that pattern and molecular weight of vitellogenin of *A. germari* are very similar to those of *P. hilaris* (Azuma *et al.*, 1993). They are classified into the class I vitellogenin, which is very common in the insect vitellogenin (Kunkel and Nordin, 1985).

In conclusion, our results have shown the hemolymph protein profiles and vitellogenin in *A. germari*. A detailed study of the hemolymph proteins and vitellogenin of *A. germari* would provide a further information for the physiological study.

REFERENCES

Azuma, M. Y. Ohta, H. Kanazawa, H. Kai and R. Kobara (1993) Identification and developmental changes in plasma proteins of the yellow-spotted longicorn beetle, *Psacothea hilaris* (Pascoe) (Coleoptera: Cerambycidae). *Appl. Entomol. Zool.* **28** : 141-149.

Davis, B. J. (1964) Disc electrophoresis-II. Method and application to human serum proteins. *Ann. New York Acad. Sci.* **121** : 404-427.

Hua, L. (1982) A check list of the longicorn beetles of China Coleoptera: Cerambycidae. pp. 67. China Zhongsham University, Guangzhou.

Kanost, M. R., J. K. Kawooya, J. H. Law, R. O. Ryan, M. C. van Heusden and R. Ziegler (1990) Insect hemolymph proteins. *Adv. Insect Physiol.* **22** : 299-396.

Kunkel, J. G. and J. H. Nordin (1985) Yolk proteins. In: Comprehensive Insect physiology, biochemistry and pharmacology. (Vol. 8, G. A. Kerrurt and L. I. Gilbert, eds.) pp. 83-111, Pergamon Press, Oxford.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** : 680-685.

Levenbook, L. (1985) Insect storage proteins. In: Comprehensive Insect physiology, biochemistry and pharmacology. (Vol. 8, G. A. Kerrurt and L. I. Gilbert, eds.) pp. 307-346, Pergamon Press, Oxford.

Telfer, W. H. and J. G. Kunkel (1991) The function and evolution of insect storage hexamers. *Annu. Rev. En-*

tomol. **36** : 205-228.

Towbin, H., T. Staehe and J. Gordon (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76** : 4350-4354.

Yoon, H. J. and Y. I. Mah (1997) The estimate of larval growth of mulberry longicorn beetle, *Apriona germari* Hope, on the basis of the larval head capsule width, larval weight and length. *Korean J. Seric. Sci.* **39** : 174-179.

Yoon, H. J. and Y. I. Mah (1999) Life cycle of the mulberry longicorn beetle, *Apriona germari* Hope, on an artificial diet. *J. Asia-Pacific Entomol.* 2(2): 169~173.

Yoon, H. J., Y. I. Mah, I. G. Park, S. B. Lee and S. Y. Yang (1997a) The mode of hibernation of mulberry longicorn beetle, *Apriona germari* Hope in Korea. *J. Seric. Sci. Jpn.* **66(2)** : 128-131.

Yoon, H. J., I. G. Park, Y. I. Mah, S. B. Lee and S. Y. Yang (1997b) Ecological characteristics of mulberry longicorn beetle, *Apriona germari* Hope, at the hibernation stage in mulberry fields. Korean *J. Appl. Entomol.* **36** : 67-72.

Yoon, H. J., I. G. Park, Y. I. Mah and K. Y. Seol (1997c) Larval development of mulberry longicorn beetle, *Apriona germari* Hope, on the artificial diet. *Korean J. Appl. Entomol.* **36** : 317-122.

Zhang, S. and Y. W. Shen (1980) Forest insects of China. (G. Xiao, ed.) pp. 461-463, China Forestry Publishing House, Beijing.