Korean J. Zool. 33: 346–353, 1990

A Study of Storage Protein in *Lymantria dispar* L.

Jong Youb Won and Hak Ryul Kim

Dept. of Biology, Korea University, Seoul 137-701, Korea

Two storage proteins, storage protein-1 (SP1) and storage protein -2 (SP2) were found in hemolymph and fat body during the development of *Lymantria dispar* L. SP1 has a molecular weight of 440,000 and consists of six identical subunits (MW = 72,000). The pI value of SP1 was 6.2. SP1 shows a similar high concentration during the late larval stage in both male and female. However, SP1 represents a quite different pattern during pupal stage between male and female. SP1 gradually decreases in male but increases in female. SP1 is immunologically identical to yolk protein. Also, SP1 of *L. dispar* shows immunologically partial reactions with storage proteins of *Hyphantria cunea* and *Galleria mellonella*.

KEY WORDS: *Lymantria dispar,* Storage protein

Storage protein is one of major proteins present in hemolymph of holometabolous insects (Roberts and Brock, 1981). Storage proteins are synthesized by fat bodies, released into the hemolymph during the last instar and then selectively taken up by fat bodies during nonfeeding stages (Thomson, 1975; Tojo *et al.*, 1980; Levenbook and Bauer, 1984; Marinotti and Bianchi, 1986). Storage proteins have been also purified and characterized, and their titer determined during development (Kim *et al.*, 1989a, b). However, little information is available on their ultimate fate, functions and their relationship with those of other species.

Present work reports on the characterization, quantitative change during development of strage proteins in Gypsy moth, *Lymantria dispar* L., and then its immunological relationship with those of other species.

Materials and Methods

Insects

Larvae of Gypsy moth, *Lymantria dispar* L. are reared on artificial diet (wheat germ diet premixed with agar, ICN Biochemicals) at 27 ± 1°C and 75 ± 5% relative humidity with the photoperiod of 16h light; 8h dark. Sexes were segregate during larval and pupal stages. *Hyphantria cunea* and *Galleria mellonella* were obtained from the colony in Dept. of Biology, Korea University.

Preparation of Protein Extracts for Electrophoresis and Immunological Analysis.

Hemolymph was collected into a small, chilled test tube after puncturing the abdominal region of larvae and pupae with a fine needle. A few crystals of phenylthiourea were added to the hemolymph to prevent melanization. Collected hemolymph was centrifuged at 10,000 g at 4°C for 10 min to remove hemocytes and cellular debris. The supernatant was stored at −70°C until used. Fat body was dissected from larvae and pupae in cold Ringer's solution (pH 6.2 for larvae, pH 7.4 for pupae) and centrifuged at 10,000 g at 4°C for 10 min, and the supernatant was stored at −70°C until used.

Electrophoresis and Purification

Disc gel electrophoresis was conducted on a 5% polyacrylamide gel at the current of 3 mA per gel according to Davis (1964). Hemolymph

This work was supported by Basic Science Research Support Fund from the Ministry of Education, Republic of Korea in 1989.

(30 mg protein/ml) and fat body extracts (15 mg protein/ml) were each mixed with an equal volume of 0.1 M Tris-glycine buffer (pH 8.3) containing 20% sucrose and 0.006% bromphenol blue.

Slab SDS-PAGE was also performed at room temperature using 8-10% linear gradient polyacrylamide gel containing 0.1% SDS at 30 mA as described by Laemmli (1970). Hemolymph (2.5 μl) and purified SP1 (5 μl, 7 μl) were each mixed with 20 μl of sample buffer (4% SDS, 10% 2-mercaptoethanol, 0.006% bromphenol blue, 20% sucrose, 62.5 mM Tris-glycine, pH 6.8) in microcentrifuge tubes and boiled at 100°C for 3 min. Following electrophoresis, the gel was stained in 0.25% Coomassie brilliant blue and then destained overnight in 50% methanol containing 7% acetic acid, and then in 30% methanol containing 3.5% acetic acid. The gels were fixed in 7.5% acetic caid.

For the purification of storage protein, PAGE was conducted on 5% non-SDS gel. After electrophoresis, the storage protein band was excised with a razor blade and the protein was eluted through electroelution. This process was repeated twice.

Determination of Molecular Weights

The molecular weight of native storage protein was determined as described by Hedrick and Smith (1968). Standard marker proteins were α-lactalbumin (14,200), carbonic anhydrase (29,000), chicken egg albumin (45,000), bovine serum albumin (monomer, 66,000; dimer, 132,000) and urease (dimer, 240,000; tetramer, 480,000).

In addition, the molecular weights of storage protein subunits were determined on 8-10% linear gradient polyacrylamide gel containing 0.1% SDS according to Lambin *et al.* (1976). Standard molecular weight markers were myosin (205,000), β-galactosidase (116,000) phosphorylase (97,000), albumin bovine plasma (66,000), albumin egg (45,000), carbonicanhydrase (29,000).

Isoelectric Focusing

Isoelectric focusing of storage protein was conducted on 5% polyacrylamide gels using 1% ampholytes (pH 3-10) as described by Wrigley (1968). After polymerization, the empty space at one end of the gel was filled with protective solution (1.5% ampholytes and 10% glycerol) and then connected to an electrophoretic chamber. The upper chamber contained 10 mM H_3PO_4 and the lower chamber 20 mM NaOH. Gels were prerun for 30 min at 200 V and then purified storage protein in 0.2 ml of 1.5% ampholytes and 20% sucrose solution was gently placed at the end of the gel and was run at constant 1 mA per gel up to 450 V. After electrophoresis, one gel was stained and the other gel used for pH determination. The gel was stained with a solution containing 27% isopropanol, 10% acetic acid, 0.05% Coomassie brilliant blue, and 0.5% $CuSO_4$ for 1 h.

Destaining was accomplished using a solution of 12% isopropanol, 7% acetic acid and 0.5% $CuSO_4$. For pH determination, one gel was sliced at 0.3 cm intervals, added to tubes containing 1.0 ml of distilled water, incubated for 24 h, and then the pH measured.

Preparation of the Antiserum and Immunological Analysis

Purified storage protein (100 μg/ml) was mixed with an equal volume of Freund's complete adjuvant (0.5 ml) and injected subcutaneously into a rabbit three times every other day with a fourth injection given 1 week later. Booster injection (0.5 ml protein and 0.5 ml Freund's incomplete adjuvant) was given 2 weeks after the fourth injection. Blood was collected 1 week after the fifth injection, allowed to coagulate at 4°C overnight, and centrifuged at 5000 g for 10 min. The supernatant was used in the immunological tests.

Immunodiffusion was conducted on 1% agarose gel in 10 mM veronal buffer (pH 8.6) containing 0.1% sodium azide for 3 days at room temperature as described by Ouchterlony (1949). Gels were stained in 1% amido black 10 B and destained in 2% acetic acid.

Rocket immunoelectrophoresis was carried out according to Laurell (1966). One percent agarose in 10 mM veronal buffer (pH 8.6) containing

0.1% sodium azide was mixed with an appropriate amount of anti-storage protein to yield 5% anti-storage protein serum. This mixture was coated on a glass plate (6.5 × 6.5 cm). Electrophoresis was conducted in 10 mM veronal buffer (pH 8.6) at 10 V/cm for 2 h. After electrophoresis, the gel was washed in 0.15 M NaCl for 48 h and stained in 1% amido black 10 B.

Tandem-crossed immunoelectrophoresis was carried out as described by Axelson *et al* (1973). Agarose, buffer solution and antibody were the same as described for rocket immunoelectrophoresis. The first dimension was run at 10 V/cm for 2h and the second dimension run at 50 V for 18 h.

Results

Identification of Storage Proteins

Hemolymph and fat body extracts were electrophoresed during development to determine the storage proteins. Two proteins in the hemolymph were present in high concentrations during late larval stage, after which they decreased drastically (Fig. 1). Conversely, the same proteins in fat body are present in small amounts during late larval instar, after which they were accumulated in large amounts (Fig. 2). Upper band on the gel was designated as storage protein-1 (SP1) and lower band called storage protein-2 (SP2). SP1 was purified and characterized, and antibody against it was made to perform immunological analysis.

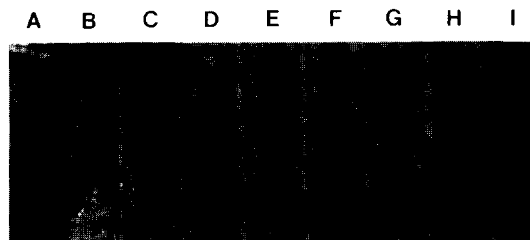

Fig. 1. Polyacrylamide gel electrophoresis of male hemolymph (5 μl each) of *L. dispar* at different stages. A: 5th instar larvae, B: 4-day-old last instar larvae, C: 8-day-old last instar larvae, D: 12-day-old last instar larvae, E: 14-day-old last instar larvae, F: Prepupae, G: 1-day-old pupae H: 3-day-old pupae, I: 5-day-old pupae.

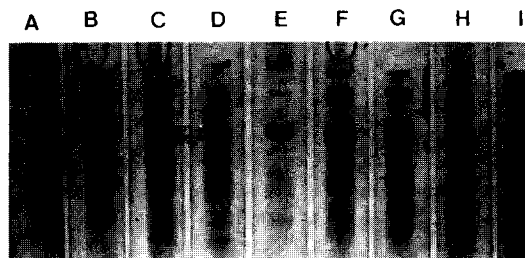

Fig. 2. Polyacrylamide gel electrophoresis of male fat body (15 μl each) of *L. dispar* at different stages. Stages are as shwon in Fig. 1.

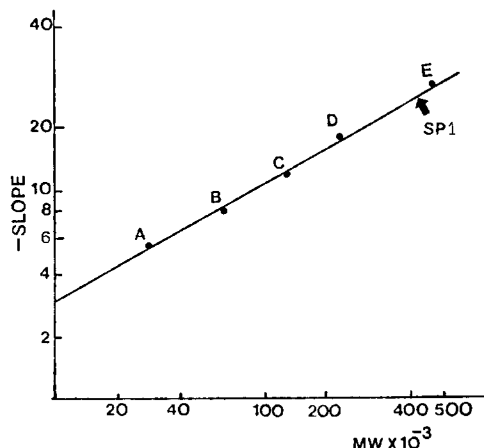

Fig. 3. Determination of M.W. for storage protein-1 (SP1) under native conditions according to the method of Hedrick and Smith (1960) Maker proteins used were (A) carbonic anhydrase, 29,000; (B) Bovine serum, monomer, 66,000; (C) Bovine serum, dimer, 132,000; (D) Urease, dimer, 240,000; (E) Urease, teteramer, 480,000.

Properties of Storage Protein-1

SP1 was estimated to have molecular weight of 440,000 (Fig. 3) and composed of one kind of subunit with molecular weight of 72,000 (Figs. 4, 5). Based on the molecular weights of native storage protein and single subunit, SP1 is thought to be hexamer composed of six molecules of single subunit type. Also, isoelectric point of SP1 was determined to be 6.2 (Fig. 6).

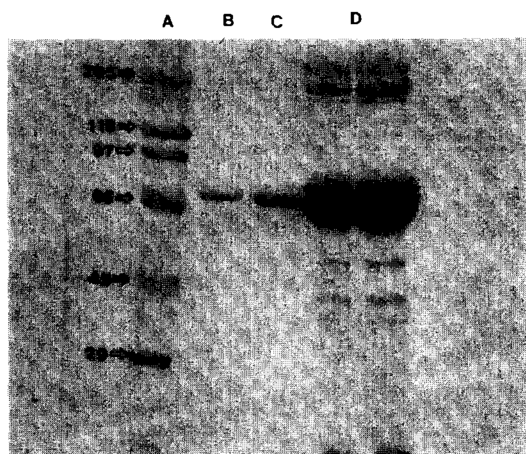

Fig. 4. SDS-PAGE analysis of storage protein-1 (SP1). A. Protein markers. Protein markers used were myosin (205,000) β-galactosidase (116,000), phosphorylase (97,000), albumin bovine plasma (66,000), albumin egg (45,000), carbonic anhydrase (29,000) B. purified storage protein-1 (5 μl) C. purified storage protein-1 (7 μl) D. crude hemolymph (2.5 μl).

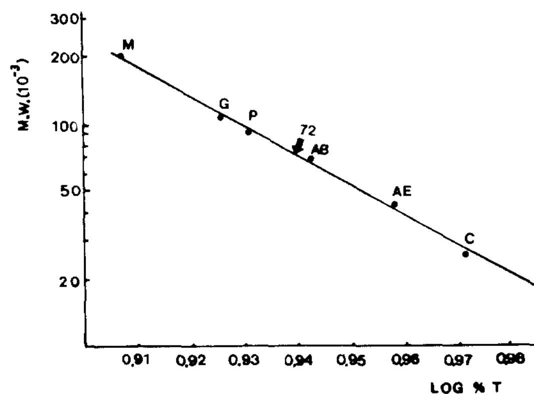

Fig. 5. Determination of molecular weight of storage protein-1 (SP1) subunit by SDS-PAGE (8-10%).

SP1 give positive reactions with PAS and Sudan black B, indicating that SP1 contains carbohydrate and lipid (data not shown).

Quantitative Change of Storage Protein-1 during Development

Quantitative change of SP1 during development in both male and female was determined by rocket immunoelectrophoresis. Some difference was found in quantity of storage protein-1 during development between male and female. As shown in Fig. 7, SP1 of male showed high concentration during last two larval stages while it gradually decreases during pupal stage (day 1-day 5). However, SP1 of female showed similar pattern during last two larval stages but quite different pattern during pupal stage. SP1 of female gradually increases in concentration during pupal stage (Fig. 8).

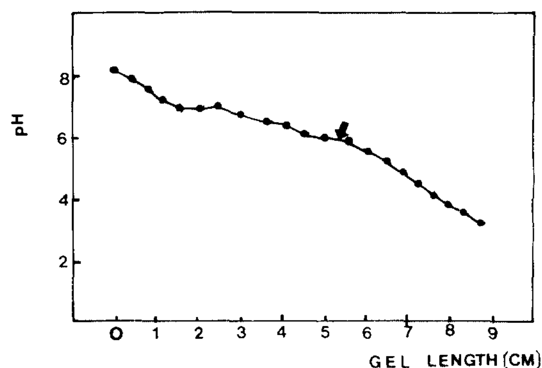

Fig. 6. Determination of pI for storage protein-1 (SP1) by the method of Wrigley (1968).

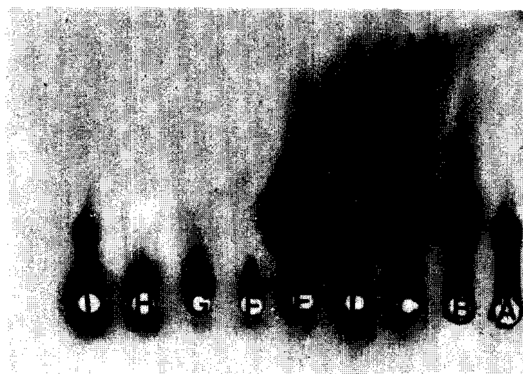

Fig. 7. Rocket immunoelectrophoresis of male hemolymph (10 μl each) at different stages. A: last instar larvae, B: 4-day-old last instar larvae, C: 8-day-old last instar larvae D: 12-day-old last instar larvae, E: 14-day-old last instar larvae F: 1-day-old pupae, G: 3-day-old pupae, H: 5-day-old pupae, I: 7-day-old pupae.

Relationship of Storage Protein-1 with Yolk Protein

Immunodiffusion was carried out to determine relationship between storage protein and yolk

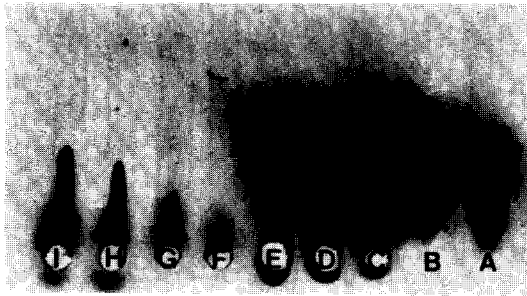

Fig. 8. Rocket immunoelectrophoresis of female haemolymph (15 μl each) at different stages. Stages are as shwon in Fig. 7.

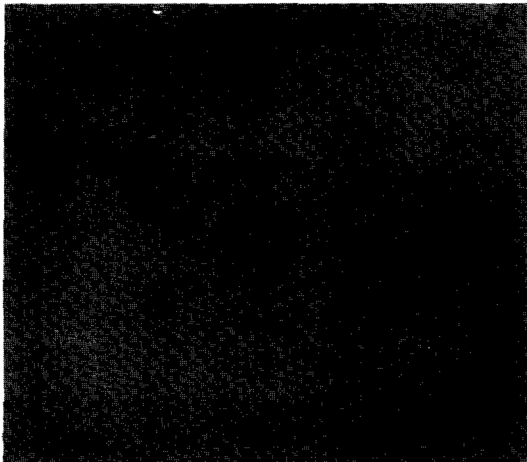

Fig. 9. Immunodiffusion pattern with anti-SP1. A: anti-SP1 (15 μl), B: purified SP1 (15 μl), C: Ovary extracts (15 μl).

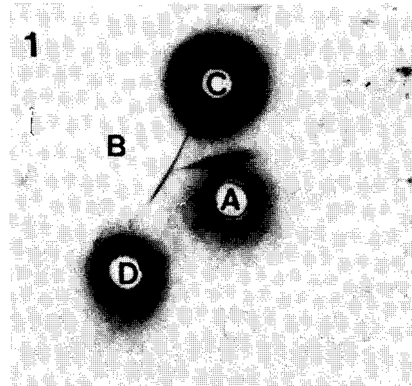

Fig. 10. Immunodiffusion pattern with anti-SP1. A: anti-SP1 (15 μl), B: purified SP1 (15 μl), C: hemolymph of *H. cunea* (10 μl), D: hemolymph of *G. mellonella* (10 μl).

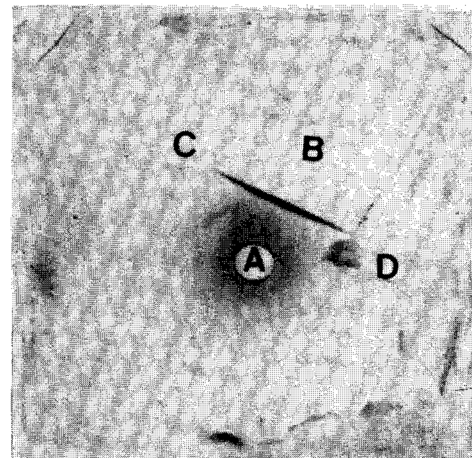

Fig. 11. Immunodiffusion pattern with anti-SP1. A: anti-SP1 (15 μl), B: purified SP1 (15 μl), C: purified SP of *G. mellonella* (15 μl), D: purified SP1 of *H. cunea* (15 μl).

protein. SP1 was immunologically identical to yolk protein (Fig. 9).

Relationship between SP1 of *Lymantria dispar* and those of other species

Immunological relationship between storage protein of *L. dispar* and those of *G. mellonella* and *H. cunea* was investigated using immunodiffusion. Hemolymph of *H. cunea* contains protein immunologically similar to SP1 of *L. dispar* (Fig. 10). To investigate whether this protein is SP1 of *H. cunea*, immunodiffusion and Tandem-crossed immunoelectrophoresis was performed using purified SP1 of *H. cunea* (Figs. 11, 12, 13). The results showed all negative reactions, indicating that SP1 of *L. dispar* is not immunologically related to SP1 of *H. cunea*. Also, SP1 of *L. dispar* showed a weak, partial reactions with the hemolymph and storage protein of *G. mellonella* (Figs. 10, 11). These results indicate that some partial relationship is immunologically present among storage proteins of these moths although it is not demonstrated that immunologically related hemolymph protein of *H. cunea* is other storage protein (possibly storage protein-2).

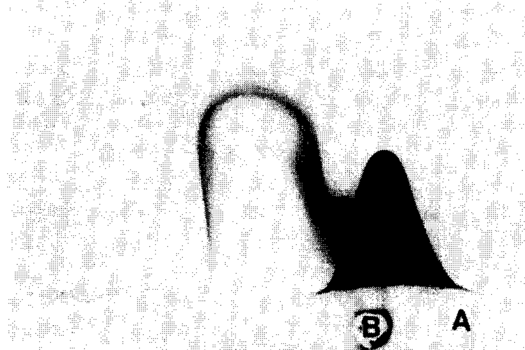

Fig. 12. Tandem-crossed immunoelectrophoresis of purified SP1 and larval hemolymph of *H. cunea* with anti-SP1. A: purified SP1 (15 μl). B: last larval hemolymph of *H. cunea* (15 μl).

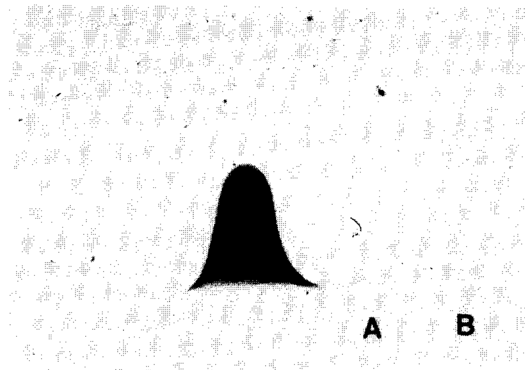

Fig. 13. Tandem-crossed immunoelectrophoresis of purified SP1 and purified SP1 of *H. cunea*. A: purified SP1 (15 μl). B: purified SP1 of *H. cunea* (15 μl).

Discussion

Storage proteins of *Lymantria dispar* appears to be to bands on the gel during the late pupal stage. However, concentrations of storage proteins in the hemolymph during late larval stage and in fat body during early pupal stage are extremely high, so that it is impossible to confirm the number of storage protein bands on gel during these stages.

Storage proteins are generally present in high concentration in hemolymph during last larval stage but in low concentration during pupal stage (Tojo *et al.*, 1980; Kim *et al.*, 1989a, b). In *L. dispar*, however, storage proteins are present in sizable amounts in hemolymph during late pupal stage (day 7). This fact indicates that storage proteins might be present during early larval stages. Also, some difference between male and female was found in quantity of storage proteins during pupal stage. SP1 of male shows gradual decrease but that of female represents gradual increase during pupal stages. Based on the immunological similarity between SP1 and yolk protein, difference in concentration of storage protein between male and female is thought to be due to the development of ovary in female. Infact, storage proteins were reported to be involved in the formation of yolk protein in other insects (Ogawa and Tojo, 1981; Kim *et al.*, 1989a).

In both male and female storage proteins show high concentration during late pupal stage. It is thought that storage proteins are released again into hemolymph to provide proteins or amino acids necessary for the formation of each organ. Storage proteins of several insects were reported to be used for the differentiation of various organs (Munn and Graville, 1969; Levenbook and Bauer, 1984).

Molecular weights of SP1 and it's subunit of *L. dispar* were determined to be 440,000 and 72,000 respectively. Based on their molecular weights, SP1 is considered to be hexamer made up of six molecules of single subunit type. Storage proteins in most insects tested have the molecular weights in the range of 500,000 and are hexamer composed of one type of subunit (Tojo *et al.*, 1978, 1980) or two types of subunit (Kramer *et al.*, 1980; Bianchi and Marinotti, 1984; Kim *et al.*, 1989a, b).

Storage proteins of several insects were reported to be immunologically identical (Bianchi and Marinotti, 1984). Immunological relationship among storage proteins of *L. dispar, H. cunea* and *G. mellonella* was investigated using immunodiffusion and tandem-crossed immunoelectrophoresis. SP1 of *L. dispar* shows partial reaction with storage protein of *G. mellonella*. However, SP1 of *L. dispar* represents partial reaction with hemolymph, but not with SP1 of *H. cunea*. This indicates that some immunologi-

cally related protein (possibly SP2) is present in hemolymph. Above results demonstrate that some immunological similarity is present among storage proteins of *L. dispar, H. cunea,* and *G. mellonella*. Also, based on the intensity of precipitin line, SP1 of *L. dispar* is immunologically more close to hemolymph protein (possibly SP2) of *H. cunea* rather than storage protein of *G. mellonella*.

References

Axelson, N. H., J. Kroll, and B. Weeks, 1973. A Manual of Quantitative Immunoelectrophoresis: Methods and Applications, universitesforlaget, oslo.

Bianchi, A. G. and O. Marinotti, 1984. A storage protein in *Rhynchosciara americana* (Diptera: Sciaridae). *Insect Biochem.* **14**:453-461.

Davis, B. J., 1964. Disc electrophoresis, II. Methods and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**:404-427.

Hedrick, J. L. and A. J. Smith, 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Archs. Biochem. Biophys.* **126**:155-164.

Kim Hak Ryul, C. S. Kang, and Richard T. Mayer, 1989a. Storage proteins of the fall webworm, *Hyphantria cunea* Drury., *Arch. Insect Biochem. Physiol.* **10**:115-130.

Kim Hak Ryul, Sook J. Seo, and Richard T. Mayer, 1989b. Properties, synthesis, and accumulation of storage proteins in *Pieris rapae* L., *Arch. Insect Biochem. Physiol.* **10**:215-228.

Kramer, S. J., E. C. Mundal, and J. H. Law, 1980. Purification and properties of manducin, an amino acid storage protein of the hemolymph of larval and pupal *Manduca sexta*. *Insect Biochem.* **10**:279-288.

Laemmli, U. K., 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T_4. *Nature, L.* **277**:680-685.

Lambin, P., D. Rochu, and J. M. Fine, 1976. A new method for determination of molecular weights of proteins by electrophoresis across a sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel. *Analyt. Biochem.* **74**:567-575.

Laurell, C. B., 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analyt. Biochem.* **15**:45-52.

Levenbook, L. and A. C. Bauer, 1984. The fate of the larval storage protein calliphorin during adult development of *Calliphora vicina*. *Insect Biochem.* **14**:77-86.

Marinotti, O. and A. G. De Bianchi, 1986. Uptake of storage protein by *Musca domestica* fat body. *J. Insect physiol.* **32(9)**:819-825.

Munn, E. A. and G. D. Greville, 1969. The soluble proteins and glycoproteins in larvae of the greater wax moth, *Galleria mellonella*. *Insect Biochem.* **12**:301-309.

Ogawa, K. and S. Tojo, 1981. Quantitative changes of storage proteins and vitellogenin during the pupal adult development in the silkworm, *Bombyx mori* (Lepidoptera: bombicidae). *Appl. Ent. Zool.* **16**:288-296.

Ouchterlony, O., 1949. Antigen-antibody reactions in gels. *Acta. Path. Microbiol. Scand.* **26**:507-515.

Roberts, D. B. and H. W. Brock, 1981. The major serum proteins of Dipteran larvae. *Experientia* **37**:103-110.

Thomson, J. A., 1975. Major patterns of gene activity during development in holometabolous insects. *Adv. Insect physiol.* **11**:321-398.

Tojo, S., T. Butchaku, V. J. Ziccardi, and G. R. Wyatt, 1978. Fat body protein granules and storage proteins in the silkmoth, *Hyalophora cecropia*. *J. Cell Biol.* **78**:823-838.

Tojo, S., M. Nakata, and M. Kobayashi, 1980. Storage proteins in the silkworm, *Bombyx mori*. *Insect Biochem.* **10**:289-303.

Wrigley, C. W., 1968. Gel electrofocusing: A technique for analyzing multiple protein samples by isoelectric focusing. *Sci. Tools.* **15**:17-23.

(Accepted April 15, 1990)

매미나방(*Lymantria dispar* L.)의 貯藏蛋白質에 관한 연구

원종엽 · 김학열(고려대학교 이과대학 생물학과)

두 종류의 저당단백질(SP1, SP2)이 매미나방의 발생기동안 혈림프와 지방체에서 발견되었다. SP1은 분자량이 440,000이며 6개의 동일한 subunit(분자량 72,000)으로 구성되어 있다. 또한 SP1은 당과 지질을 함유한 복합단백질이며 그 등전점은 6.2이었다. SP1은 암수 모두 종령기동안 비슷한 고농도를 보여 주었지만 번데기시기에는 암수가 상이한 패턴을 보여 주었다. 즉 SP1은 수컷에서 점진적 감소를 보여준 반면 암컷에서는 점진적 증가를 보여주었다. SP1은 면역학적으로 난황단백질과 동일성을 보여 주었고 미국흰불나방과 꿀벌부채나방의 저장단백질과 부분적인 동질성을 보여주었다.