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Purification and Partial Characterization of the Storage Protein-like Protein from the 5th Instar Larval Haemolymph of the Chinese Oak Silkworm, *Antheraea pernyi*.

Nam Suk Park, Sang Mong Lee, Jae Yu Moon* and Su Il Seong**

*Miryang National University, Miryang, Kyong Nam, Korea. * College of Agriculture and Life Science, Seoul National University, Suwon, Korea. **The University of Suwon, Suwon, Korea.*

ABSTRACT

The storage protein-like protein has been purified from the 5th instar larval haemolymph of the Chinese oak silkworm, *Antheraea pernyi*, and the preparation was shown to be homogeneous by 7.5% native-PAGE. The molecule was consisted of a single subunit with a molecular weight of 80K, but the number of the subunits was not determined. The protein was defined as glycoprotein by Schiff's reagent staining. Rabbit antibody prepared against the purified protein crossreacted with the 5th instar larval haemolymph proteins of *Antheraea pernyi* and *Antheraea yamamai*, but not with those of *Bombyx mori* and *Bombyx mandarina*.

Key words : Storage protein, *Antheraea pernyi*, 80K protein, Haemolymph protein.

INTRODUCTION

Since the first discovery more than 27 years ago of calliphorin as the major haemolymph protein of the last instar flow fly larvae in *Calliphora erythrocephala* (Munn *et al.*,1967), an increasing number of studies have been concentrated on the characterization of this haemolymph storage protein from numerous species (Munn and Greville, 1969; Munn *et al.*, 1971; Tojo *et al.*, 1978; Tojo *et al.*, 1980; Kramer *et al.*, 1980; Riddiford and Law, 1983; Telfer *et al.*, 1983; Levenbook, 1985; Kanost *et al.*, 1990; Telfer and Kunkel, 1991; Martinez and Wheeler, 1992 : Tojo and Yoshiga, 1993; Martinez and Wheeler, 1994; Wheeler and Buck, 1995; Ancsin and Wyatt, 1996).

The storage proteins are mostly synthesized by the larval fat body and released into the haemolymph, and they are taken up by fat body and accumulated in dense protein granules (Sekeris and Scheller, 1977; Tojo *et al.*, 1978; Haunerland, 1996). A family of insect storage proteins can be classified into three categories: Arylphorin, best-studied storge protein, characterized by an exceptionally high content of aromatic amino acids (18-26%) (Telfer *et al.*, 1983; Palli and Locke, 1987a; Haunerland and Bowers, 1986; Ryan *et al.*, 1986c); Methionine-rich storage protein characterized by a relatively high methionine content (4-8%) and by an increased concentration in females than in males(Tojo *et al.*, 1978; Tojo *et al.*, 1980; Ryan *et al.*,1985b; Bean and Silhacek, 1989); Homohexamer storage proteins, although containing a relatively high content of aromatic amino acids, being immunologically distinct from the arylphorins and/or hexameric flavoproteins, etc. (Roberts, 1983; Mintzas and Reboutsicas, 1984; Telfer and Massey, 1987).

Storage proteins belonged to the above first category have been found in *Bombyx mori* (Tojo *et al.*, 1980), *Manduca sexta* (Ryan *et al.*, 1985), *Locusta migratoria* (Dekort and Koopmanschap, 1987) etc.. However, storage protein in the Chinese oak silkworm, *Antheraea pernyi*, has not been studied. In the present study, we purified and characterized a storage protein-like protein in the haemolymph of the 5th instar larvae of *Antheraea Pernyi*.

MATERIALS AND METHODS

Insects

The Chinese oak silkworms (*Antheraea pernyi*) and the Japanese oak silkworms (*Antheraea yamamai*) were

raised throughout all the stages in the oak tree field established in National Institute of Agricultural Science and Technology, RDA.

The F_1 hybrid silkworm (Jam125×Jam126), *Bombyx mori*, was reared on fresh leaves. The wild mulberry silkworm, *Bombyx mandarina*, was collected from the mulberry field of Miryang National University located in the southern part of Korea.

Haemolymph collection

The abdominal legs of the above corresponding silkmoth larvae were injured with sharpened scissors and the haemolymph bled from the wound was directlly collected into the precooled test tube with a few crystals of 1-phenyl-2-thiourea to inhibit phenol oxidase. The haemolymph was centrifuged at 5,000g for 15 minutes to remove haemocytes and other devris. The haemolymph prepared was kept at -70°C until protein analysis. In some cases, females and males were used for determination of sex specificity of the haemolymph protein during developmental and metamorphic stages.

Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE was carried out in 7.5% acrylamide slab gels with a buffer system containing 0.5 mM Tris-3.8 mM glycine, pH 8.3. Gels were run at 40 mA constant current for 2 hrs. After electrophoresis, the slab gels were stained overnight with 0.5% Coomasie Brilliant Blue R-250 (Sigma Chemical Co.) in a solution containing methanol, water and acetic acid in a ratio of 5:4:1. SDS-PAGE was performed according to Laemmli (1990) on 11% and 12% polyacrylamide gels containing 0.1% SDS. The proteins were stained with Coomasie Brilliant Blue R-250 (Sigma Chemical Co.). Molecular weights were estimated according to the instruction of high- and low- molecular weight calibration kit (Pharmacia Fine Chemicals). The proteins were stained using Schiff's reagent for sugar (Zacharius,1969) and Sudan Black B for lipid (Swahn, 1953).

Purification of target proteins

Larval and pupal haemolymph proteins of the Chinese oak silkworm were separated on 7.5% nondenaturing polyacrylamide slab gels. The target protein bands on the slab gels recognized by naked eyes using light refraction were cut out without any staining for visualization. The protein bands collected were chopped into eppendorf tubes. The preparation was equilibrated overnight at 4°C against 0.5 mM Tris-3.8 mM glycine buffer with pH 8.3. The preparation was centrifuged at 5000g for 15 min and the supernatant was collected. After filtration of the supernatant using millipore filter with 0.45 μm pore size, the purity was tested on a 7.5% native polyacrylamide gel. In some case, the above process was repeated for better purity of the protein.

Preparation of antiserum and immunological technique

Antiserum against the purified protein was prepared in the New Zealand white male rabbits. One m*l* of emulsified mixture with complete Freund's adjuvant and the purified protein was injected subcutaneously every week. The rabbit was bled from the ear seven days after the fourth injection. The serum collected was clotted after overnight at 4°C. The antiserum was harvested by centrifugation at 6,000g for 15 min at 4 °C and stored at -20°C. Double immunodiffusion test was performed on 1% agarose gel according to Ouchterlony (1968).

RESULTS

Profile of haemolymph proteins during development and metamorphosis.

Many proteins were detected from the larval and pupal stages in the Chinese oak silkworm. The storage protein-like protein was present from the 5th larval instar to pupal stage(Fig. 1). SPL protein represented a major portion of the haemolymph proteins during the period of metamorphosis from larvae to pupae, but only trace amount of the protein could be detected or not in the other developmental stages. Also, this protein did not show sex specificity.

Purification of SPL.

SPL was purified from the haemolymph in the 5th larval stage of the Chinese oak silkworm (Fig. 2). SPL

bands were cut out using light refraction by naked eyes and chopped in pieces, and the protein component was extracted overnight in PBS(Phosphate Buffered Saline), pH 8.6, diffusively. The purity was tested by 7.5% native-PAGE analysis (Fig. 2).

Composition and molecular weight of subunits.

The purified SPL was subjected to 11% SDS-PAGE to determine composition and molecular weight of the subunit(Fig. 3). SPL was consisted of a single subunit, and molecular weight was of about 80,000 dalton.

Detection of sugar and lipid from SPL

SPL in the larval developmental stage (each lane 1 and 3 in CBB, PAS and SBB pannel) was positively stained with PAS for sugars (Caldwell and Pigman, 1965), and not with Sudan Black B for lipid (Fig. 4). This SPL in the larval developmental stages seems to be a kind of glycoprotein. SPL showing identical mobility in the pupal stage (eachlane 2 and 3 in CBB, PAS and SBB pannel), however, was positively stained with PAS and Sudan Black B, indicating that it is glycolipoprotein (Fig. 4).

Double immunodiffusion test

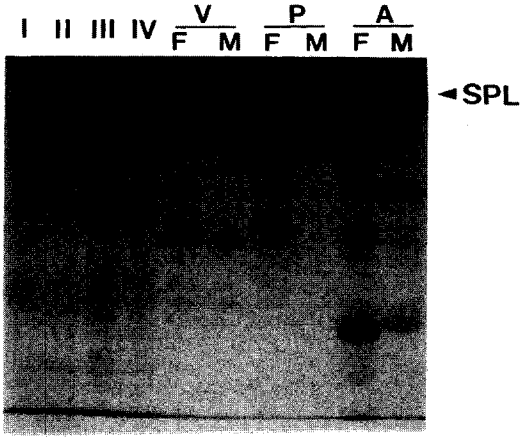

Fig. 1. *Electrophoretic profile of A. pernyi haemolymph proteins during metamorphosis. I, first instar larvae; II, second instar larvae; III, third instar larvae; IV, fourth instar larvae; V, fifth instar larvae; P, pupae; A, adult; F, female; M, male; SPL, storage protein-like protein.*

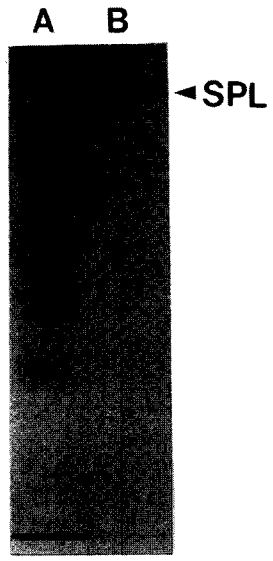

Fig. 2. *Electrophoretic patterns of crude haemolymph(A) and purified storage protein-like protein(B). See fig. 1 for further legend.*

Antiserum was raised against a highly purified SPL that was shown to be homogenous by native-PAGE. Ouchterlony's double diffusion was carried out against crude extracts of haemolymph of the final larval stages in *Bombyx mori, Bombyx mandarina, Antheraea yamamai* and *Antheraea pernyi* (Fig. 5).

From the analysis, the antiserum formed a single

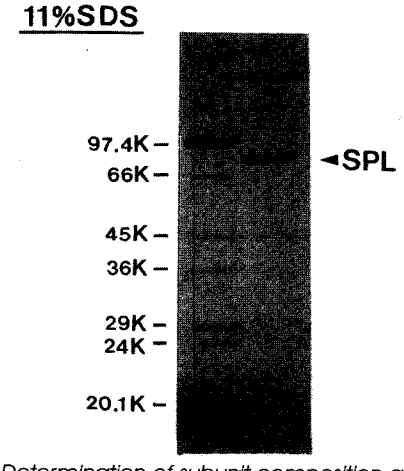

Fig. 3. *Determination of subunit composition and molecular weight on 11% SDS-PAGE. Left lane indicates the M. W. of markers in kilodalton.*

precipitin line with haemolymph proteins of *Antheraea pernyi* and *Antheraea yamamai*, but not with those of *Bombyx mandarina* and *Bombyx mori*.

Discussion

The first step in identifying, comparing and quantitating haemolymph proteins is purification and immunological analysis.

A number of protein purification methods such as high-performance liquid chromatography (HPLC) (Aebersold *et al.*, 1987), polyacrylamide gel electrophresis (PAGE) (Weber and Osborn, 1969 ; Davis, 1964), ammonium sulfate precipitation (Green and Hughes, 1955), density-gradient centrifugation (Chung *et al.*, 1986; Haunerland *et al.*, 1987) were used. In the present study, rapid isolation and purification of storage protein-like protein designated in SPL from the 5th larval haemolymph of the Chinese oak silkworm was achieved by native-PAGE and subsequent diffusion as described in materials and methods. By these two steps, it was possible to obtain SPL protein in homogeneous form.

It is evident that the electrophoretic mobility of SPL on the native PAGE-gel is almost identical to the storage proteins of *Bombyx mori* (Tojo *et al.*,1980), *Bombyx mandarina* and *Antheraea yamamai*(not shown in the data). This result, even though the native M.W. value of SPL was not determined in the present study, indicates that there is a very close resemblance in native M.W. value between SPL and the other silkmoths' storage proteins.

SPL is composed of a single subunit with M.W. value of approximately 80,000 daltons. This value shows good agreement with 72-83 KDa subunits of the best-studied storage protein class arylphorin. Several lepidopteran [such as *Bombyx mori* (Tojo *et al.*, 1980), *Galleria mellonella* (Kramer *et al.*, 1980) and Manduca sexta (Ryan *et al.*, 1985] and dictyopteran [*Biatta orientalis* (Duhamel and Kunkel, 1983)] arylphorins possess different types of subunits that are distinguishable by SDS-PAGE, but it is recognized that this SPL in the study has a single subunit.

In amino acid composition, SPL is rich in tyrosine and phenylalanine (not shown in the present study). Therefore, SPL is distinct from *Bombyx* storage protein containing an exceptionally high content of methionine (Tojo *et al.*, 1980). Higher tyrosine in this SPL can belong to same class of tyrosine rich protein of weevils, *Sitophilas oryzae* and *Rhynchophorus palmarum* (Colo-

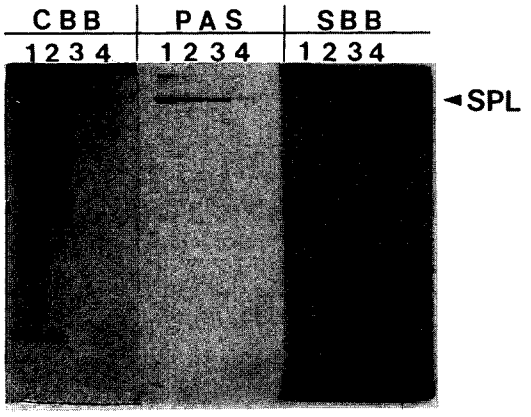

Fig. 4. *Detection of sugar and lipid on the purified protein. The proteins were stained with Coomasie Brilliant Blue R-250 (CBB), with Sudan Black B (SBB) for lipids and with PAS reagent for carbohydrate. 1, crude haemolymph at larval stage; 2, crude haemolymph at pupal stage; 3, purified SPL protein at larval stage; 4, purified SPL protein at pupal stage.*

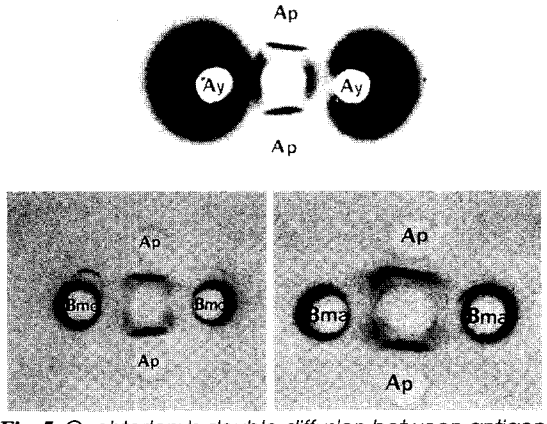

Fig. 5. *Ouchterlony's double diffusion between antigen haemolymphs of several silkmoths. Antiserum to the protein purified from Antheraea pernyi larval haemolymph is located in the center wells, and the four antigens are arranged in the peripharal wells. Ap, Antheraea pernyi; Ay, Antheraea yamamai; Bmo, Bombyx mori; Bma, Bombyx mandarina.*

ptera: Curculionidae), but is different from them in M.W. values (Rahbe' *et al.*, 1990)

Antiserum prepared against purified storge protein-like protein (SPL) was used for immunological comparison and identification among lepidopteran silkmoths, *Antheraea yamamai, Bombyx mori* and *Bombyx mandarina*. From this analysis it is evident that SPL in *Antheraea pernyi* and *Antheraea yamamai* are immunologically identical. The antiserum, however, did not form a precipition line on Ouchterlony's immunodiffusion test agarose gel with *Bombyx mori* and *Bombyx mandarina*. This results suggest that *A. pernyi* has a phylogenetically higher relationship with *A. yamamai*, but not with *B. mori* and *B. mandarina*.

SPL did not show a sex specificity as characterized by native-PAGE, SDS-PAGE. Vitellogenin with female specificity (ono *et al.*, 1975) and male specific serum proteins with male specificity in fruit fly (Katsoris *et al.*, 1990) are reported. It is considered that, however, SPL has no sex-specific role in physiological metabolism because the protein bands are detected on the polyacrylamide gel both in qemale and in male haemolymph at the final larval stage.

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