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Purification and Characterization of Storage Proteins from the Mulberry Longicorn Beetle, *Apriona germari* Hope

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The storage proteins of the mulberry longicorn beetle, *Apriona germari* Hope, were purified and characterized. Three kinds of storage protein (SP1, SP2 and Sp3) were purified from the last instar larval hemolymph of *A. germari* by the FPLC techniques, anion exchange chromatography and gel permeation chromatography. The SP1, SP2 and SP3 have a native molecular weight of 480, 440 and 420 kDa, respectively. In the SDS-polyacrylamide gel electrophoresis analysis, these storage proteins are composed of a single protein subunit with molecular weight of 90, 85 and 80 kDa, respectively. This result showed that the storage proteins are hexameric protein. The SP1 and SP2 were stained with Schiffs reagent, but SP3 was not stained. It can be assumed that SP1 and SP2 are glycoprotein. Western blot analyses using the each of polyclonal antiserum against purified SP1, SP2 and SP3 showed that the three antibodies reacted with the each of SP bands, respectively. Also, antibodies against SP1 and SP3 cross-reacted with the SP3 and SP1, respectively. However, SP2 was not cross-reacted with these two antibodies. Also, antiserum against SP2 did not cross-reacted with the SP1 and SP3.

Key words : Storage protein, Mulberry longicorn beetle, *Apriona germari*, Hexameric protein

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Introduction

In insects, the storage proteins, larval specific hemolymph proteins, are mostly hexamers with high molecular weights, approximately 500 kDa and are consist of 80 kDa subunits (Haunerland, 1996; Telfer and Kunkel, 1991). They are generally synthesized in the larval fat body, released into the hemolymph, and selectively sequestered into fat body by receptor-mediated process (Levenbook, 1985; Pan and Telfer, 1992). These proteins are generally classified into three groups, arylphorin, methionine-rich storage protein and homohexamer storage proteins (Haunerland, 1996; Shimoda and Saito, 1997). Arylphorin-type storage proteins have a very high aromatic amino acid content (18-26%) and are glycosylated (Haunerland, 1996; Palli and Locke, 1987; Telfer *et al.*, 1983). Methionine-rich storage proteins contain more than 4% methionine and are not glycosylated (Bean and Sihacek, 1989; Haunerland, 1996; Ryan *et al.*, 1985; Tojo *et al.*, 1980). Homohexamer storage proteins, although containing a relatively high content of aromatic amino acids, being immunologically distinct from the arylphorins and/or hexameric flavoproteins, etc (Mintzas and Reboutsicas, 1984; Robert, 1983; Telfer and Massey, 1987).

Storage proteins have been found from diverse insect species. Furthermore, arylphorin-like proteins have been identified in a number of coleopteran species, including the mealworm, *Tenebrio molitor* (Delobel *et al.*, 1992) and the Colorado potato beetle, *Leptinotarsa decemlineata* (DeKort and Koopmanschap, 1994). However, the relevant study of storage protein from the mulberry lon-

gicorn beetle, *Apriona germari* Hope, is not reported yet. Therefore, this study was conducted to obtain primary information of storage proteins of the mulberry longicorn beetle. In this study, we purified and characterized the storage proteins from the larval hemolymph of *A. germari*.

Materials and Methods

Insects

The hatched larvae of the mulberry longicorn beetle, *Apriona germari* Hope, were collected from the cultivated mulberry fields (Yoon *et al.*, 1997a, b). Larvae were reared as previously described (Yoon and Mah, 1999).

Preparation of hemolymph

Hemolymph was collected as previously described (Yoon *et al.*, 1999). Hemolymph was collected in a chilled tube with a few crystals phenylthiourea from last instar larvae. The collected hemolymph was then centrifuged at 13,000 rpm for 20 min at 4°C to remove hemocytes and cell debris. The supernatant was stored -20°C.

Purification of storage proteins

Storage proteins (SP1, SP2 and SP3) were purified from last larval hemolymph of *A. germari* using FPLC (Pharmacia LKB) techniques. Hemolymph was suspended in 50 ml buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM PMSF) containing ammonium sulfate at a consentration of 50% saturation. After centrifugation at 13,000 rpm for 30 min at 4°C, the precipitate was suspended in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF. After centrifugation at 13,000 rpm for 20 min at 4°C, the supernatant was subjected to gel permeation chromatography on a Superdex 200 HR 10/30 column (Pharmacia LKB) in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF with a flow rate of 0.5 ml/min. The storage proteins-enriched fractions, identified by SDS-PAGE, were applied to anion exchange chromatography on a Mono Q HR 5/5 column (Pharmacia LKB) in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF with a flow rate of 1 ml/min. Proteins were eluted in a linear segment sodium chloride gradient (0-0.5 M). Fractions containing purified storage proteins were identified by SDS-PAGE analysis.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on 10% gel at room temperature according to the method of Laemmli (1970). After electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue R-250, Schiffs reagent for sugar (Zacharius, 1969) and Sudan Black B for lipid (Swahn, 1953), respectively. Molecular weight markers [myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), egg albumin (45,000) and carbonic anhydrase (31,000); Bio-Rad] were used as standards.

Preparation of polyclonal antibody

The purified SP1, SP2 and SP3 were mixed with equal volume of Freunds complete adjuvant (a total of 200 μl) and injected into Balb/c mice, respectively. Three successive injections were performed with one-week interval beginning a week after the first injection with antigens mixed with equal volume of Freunds incomplete adjuvant (a total of 200 μl). Bloods were collected 3 days after the last injection and centrifuged at 13,000 rpm for 5 min. The supernatant antibodies were stored at -70 until use.

Western blot analysis

For Western blot analysis, SDS-PAGE was carried out as described above. Proteins were blotted to a sheet of nitrocellulose membrane (Sigma, 0.45 μm of pore size) (Towbin *et al.*, 1979). The blotting was performed in transfer buffer (25 mM Tris and 192 mM glycine in 20% methanol) at 30 volts overnight at 4°C. After blotting, the membrane was blocked by incubation in 1% BSA solution for 2 hr at room temperature. The blocked membrane was incubated with antiserum solution (1:1,000 v/v) for 1 hr at room temperature and washed in TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Tween 20). Subsequently, the membrane was incubated with goat anti-mouse IgG alkaline phosphatase conjugate (1:10,000 v/v, Sigma) for 30 min at room temperature. After repeated washing, substrate solution (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$) containing NBT (nitro-blue tetrazolium) and BCIP (5-bromo-4-chloroindolyl phosphate) was added. The reaction was quenched with distilled water.

Determination of molecular weight

The native molecular weights of storage protein were determined by gel filtration chromatography. Standard proteins [thyroglobulin (669,000), ferritin (440,000), catalase (232,000), aldolase (158,000) bovine serum albumin (67,000); Pharmacia LKB] were separately layered onto a Superdex 200 HR 10/30 column (Pharmacia LKB) and eluted in 20 mM Tris-HCl buffer (pH 8.0) with a flow rate of 0.5 ml/min.

Results

The mulberry longicorn beetle, *A. germari*, storage pro-

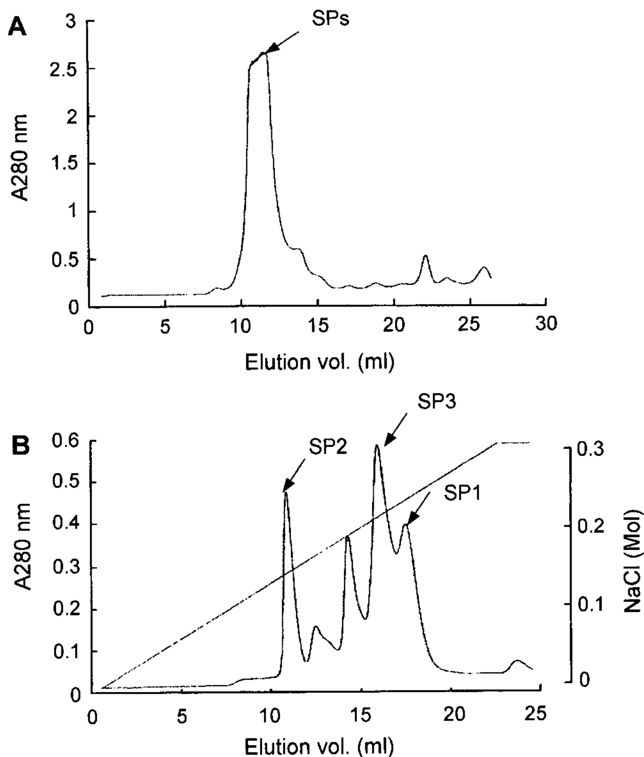

Fig. 1. Purification of the storage proteins (SPs) from the larval hemolymph of *A. germari* by two-step FPLC techniques, gel permeation chromatography and anion exchange chromatography. Crude larval hemplymph of *A. germari* was applied to Superdex 200 HR gel permeation chromatography (A). The SPs-enriched fractions were pooled, and then applied to Mono Q HR anion exchange chromatography coumn (B). The protein concentration was monitored by absorbance at 280 nm.

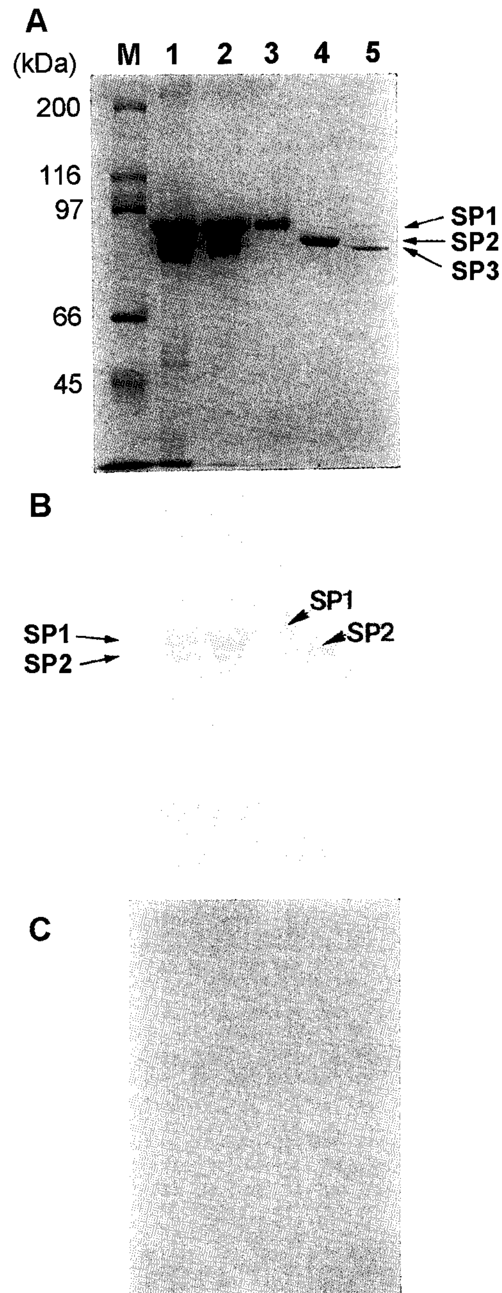

Fig. 2. Detection of sugar and lipid on the storage proteins (SPs) from the larval hemolymph of *A. germari*. After SDS-PAGE, the proteins were stained with Coomassie Brilliant Blue (A), PAS reagent for carbohydrate (B) and Sudan Black B for lipids (C). Lane 1, crude hemolymph at larval stage; lane 2, Superdex 200 HR fraction; lane 3, purified SP1; lane 4, purified SP2; lane 5, purified SP3. M indicates molecular weight markers. The SP1, SP2 and SP3 are marked on the panel.

teins (SPs) were purified and characterized by electrophoresis and FPLC. After ammonium sulfate precipitation, the storage proteins were purified by two-step column chromatography (Fig. 1). The first step was gel permeation chromatography on a Superdex 200 HR column (Fig. 1A). The SPs, identified by SDS-PAGE (data not shown), was eluted as a single peak. In the next step, the SPs-enriched fraction was applied to Mono Q HR anion-exchange chromatography column (Fig. 1B). The SPs were eluted by a linear NaCl gradient from 0 to 0.3 M in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF. SP1 was eluted as a single peak at 0.24 M NaCl. Also, SP2 and SP3 were eluted as a single peak at 0.14 M and 0.21 M NaCl, respectively. For determination of molecular weights of SPs, fractions containing purified SP1, SP2 and SP3 were applied to Superdex 200 HR gel permeation chromatography column, respectively (data not shown).

In the SDS-polyacrylamide gel electrophoresis analysis, these purified storage proteins were composed of a single protein subunit, respectively (Fig. 2A). To confirm glycosylation of the *A. gemari* storage proteins (SPs), PAS staining was performed. The SP1 and SP2 were stained

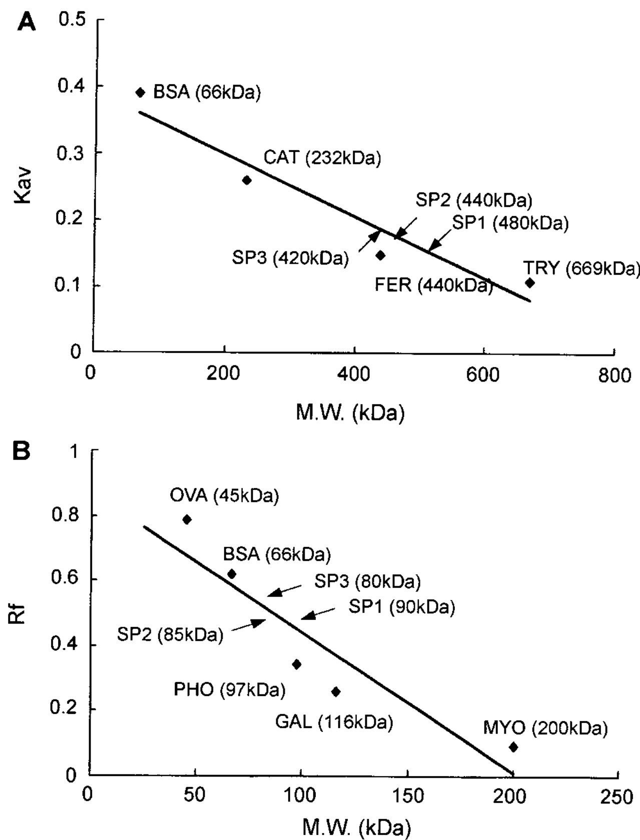

Fig. 3. Molecular weight determination of the *A. germari* storage proteins (SPs). Molecular weights of native storage proteins were determined by Superdex 200 HR gel permeation chromatography column (A) and molecular weights of storage protein subunits were determined by SDS-PAGE (B). Molecular weight standards: TRY, thyroglobulin (669,000); FER, ferritin (440,000); CAT, catalase (232,000); MYO, myosin (200,000); GAL, β-galactosidase (116,000); PHO. Phosphorylase b (97,000); BSA, bovine serum albumin (67,000); OVA, ovalbumon (45,000).

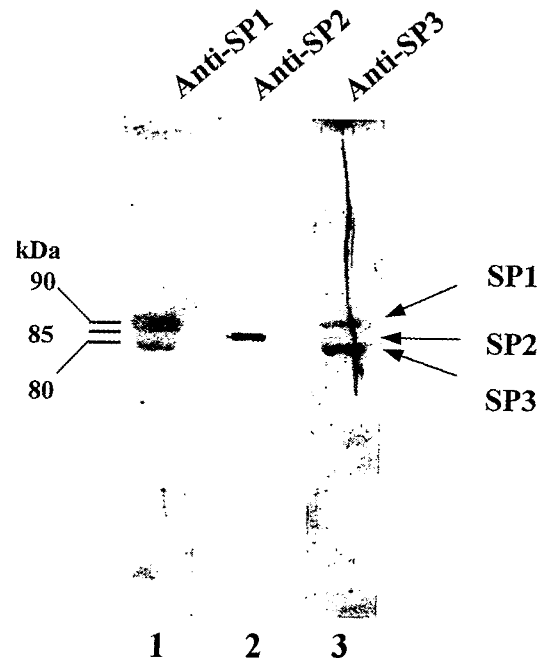

Fig. 4. Western blot analyses of the larval hemolymph of *A. germari* using the each of polyclonal antiserum against purified SP1, SP2 and SP3. Lane 1, polyclonal antiserum against purified SP1; lane 2, polyclonal antiserum against purified SP2; lane 3, polyclonal antiserum against purified SP3. Molecular weights and signals of SP1, SP2 and SP3 are indicated by arrow.

with Schiffs reagent, but SP3 was not stained (Fig. 2B). In the Sudan Black B staining for lipid, three storage proteins were not stained (Fig. 2C).

The molecular weight of native SP1, SP2 and SP3 was estimated to approximately 480, 440 and 420 kDa by gel permeation chromatography, respectively (Fig. 3A). The molecular weight of subunits of SP1, SP2 and SP3 was determined to approximately 90, 85 and 80 kDa, respectively (Fig. 3B). These results indicated that SP1, SP2 and SP3 are hexameric proteins composed of six molecules of a single subunit each weighting 90, 85 and 80 kDa.

Western blot analyses using the each of polyclonal antiserum against purified SP1, SP2 and SP3 showed that the three antibodies reacted with the each of SP bands, respectively (Fig. 4). Also, antibodies against SP1 and SP3 cross-reacted with the SP3 and SP1, respectively. However, SP2 was not cross-reacted with these two antibodies, SP1 and SP3 antisera. In addition, antiserum against SP2 did not cross-reacted with the SP1 and SP3.

Discussion

We have purified and characterized the storage proteins from the larval hemolymph of *A. germari.* The storage proteins were purified by two-step FPLC techniques, gel permeation chromatography and anion-exchange chromatography. The purified proteins are respectively divided into SP1, SP2 and SP3 according to molecular weight of subunit. While different names have been used in the previous reports for this protein, e.g. major hemolymph protein I, II and III (Azuma *et al.*, 1993; Yoon *et al.*, 1999), it is called storage protein in this study.

In the SDS-PAGE, these purified storage proteins were composed of a single protein subunit, respectively. The SP1 and SP2 were stained with Schiffs reagent, but SP3 was not stained. In general, the storage proteins are known to be glycosylated except methionin-rich storage protein (Haunerland, 1996). Therefore, we assumed that SP1 and SP2 are glycosylated storage proteins and SP3 is methio-

nin-rich storage protein. In the Sudan Black B staining for lipid, three storage proteins were not stained. It can be assumed that SPs were not contained lipid.

The molecular weight of native SP1, SP2 and SP3 was approximately 480, 440 and 420 kDa with 90, 85 and 80 kDa subunits, respectively. These results indicated that SPs are hexameric proteins composed of six molecules of a single subunit. In most insects, the storage proteins are generally hexamers or heterohexamers with native molecular weights of about 500 kDa (Haunerland, 1996; Shimoda and Saito, 1997; Telfer and Kunkel, 1991). The storage proteins of some lepodopteran species, *Calpodes ethlius* (Palli and Locke, 1987), *Hyphantria cunea* (Song *et al.*, 1997), and the hymenopteran, *Apis mellifera* (Ryan *et al.*, 1984) are composed of a single subunit type. In the other lepidopteran species, *Bombyx mori* (Tojo *et al.*, 1980), *Manduca sexta* (Ryan *et al.*, 1985), *Agrius convolvuli* (Shimoda and Saito, 1997), and the dictyopteran, *Blatta orientalis* (Duhamel and Kunkel, 1983), arylphorin-type storage proteins are possessed two types of subunits.

Western blot analysis was carried out using polyclonal antiserum against purified SP1, SP2 and SP3. The results showed that antibodies against SP1 and SP3 cross-reacted with the SP3 and SP1, but SP2 was not cross-reacted. Although arylphorin and homohexamer storage proteins are glycosylated, these proteins are immunologically distinct from each other (Haunerland, 1996; Mintzas and Reboutsicas, 1984; Roberts, 1983; Telfer and Massey, 1987). In this point of view, this result suggests that SP2 is arylphorin in *A. germari*.

In conclusion, these results have shown the storage proteins profiles in *A. germari*. Further studies are needed to determine the gene structure and evaluate the role of these proteins in relation to the amino acid conservation.

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