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# Purification and Characterization of Storage Protein-1 from *Galleria mellonella*

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**Storage protein-1 (SP-1) of *Galleria mellonella* was identified in hemolymph and fat body by electrophoresis. SP-1 was purified from hemolymph by KBr density gradient ultracentrifugation, DEAE-cellulose (DE52) ion-exchange chromatography, and gel permeation chromatography (Sephadex G-200). Purity of SP-1 was confirmed by Non-SDS PAGE and electron microscope. SP-1 is 9.4 nm in diameter and regular octahedron in shape. SP-1 has isoelectric point of 5.7 and native molecular weight of 365 K dalton and is composed of one type of subunit with molecular weight of 82 K dalton. Triacylglycerol and phospholipid were found to be major lipid components in SP-1.**

**KEY WORDS: Purification, Hemolymph, Storage protein-1, *Galleria mellonella***

Storage proteins were firstly studied in *Calliphora erythrocepala* (Munn *et al.*, 1969) and are known to be present in great amounts in larval hemolymph of holometabolous insects. In *Bombyx mori*, two storage proteins were confirmed in hemolymph and these proteins increase during last larval instar but decrease during metamorphosis by being taken up into the fat body (Tojo *et al.*, 1978, 1980). Storage proteins are synthesized by fat body (Miller and silhacek, 1982b; Kim *et al.*, 1989) and released into hemolymph to account for 80% of hemolymph proteins (Kinnear and Thomson, 1975; Kramer *et al.*, 1980). Storage proteins released into hemolymph are either completely reabsorbed by fat body during prepupal stage (Venkatesch and Chippendale, 1986) or partially absorbed and some remain in hemolymph until the end of pupal stage (Kramer *et al.*, 1980; Bourodopoulou *et al.*, 1981). Storage proteins absorbed by fat body were reported to be used as sources of amino acids necessary for differentiation of adult organ (Munn and Greville, 1969; Levenbook and Bauer, 1984).

Generally, storage proteins have molecular weights of 400 to 500 Kd and are hexamer composed of one or two kinds of subunits with molecular weights of 70 to 80 Kd (Roberts and Brock, 1981). Also some storage proteins contain aromatic amino acids including tyrosine, phenylalanine, and tryptophan in great amounts and are designated arylphorine (Telfer *et al.*, 1983). Storage proteins of *Galleria mellonella* were classified as 4 groups (74 K, 76 K, 81 K, 82 K) (Miller and Sihacek, 1982a). Among four kinds of SP, SP-1 has lowest Rf value and also is major storage protein.

Present work is to purify storage protein-1 from *Galleria mellonella* and their composition was also analyzed as a part of determining the role of storage protein-1 during the formation of adult organs.

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## Materials and Methods

### Insects

Greater wax moth (*Galleria mellonella*) was reared on artificial diet (The Quaker Oats Company) at 27°C with photoperiod of 16L: 8D (Beck, 1960).

### Extraction of hemolymph

Hemolymph was collected by puncturing the abdomen of larvae and the head of pupae with sharp needle and by flushing methods (Chino *et al.*, 1987) in adult. To prevent melanization, a few crystals of phenylthiouea (PTU) were added to the pooled hemolymph, which was then centrifuged at 2,000 g for 10 min at 4°C to remove hemocyte. The supernatant was stored at -70°C until used.

### Extraction of protein from fat body

Fat bodies were dissected from larvae and pupae in cold Ringer's solution (128 mM NaCl, 1. 8 mM CaCl2, 1.3 mM KCl; pH 6.2 in larvae and pH 7.4 in pupae). Fat bodies were homogenized at 4°C and centrifuged at 10,000 g for 10 min and the supernatant was stored at -70°C until sued.

### Electrophoresis

Non-sodium dodecyl sulphate polyacrylamide gel electrophoresis (non-SDS PAGE) was carried out on 6% gel (tube size, 120 × 6 mm ID) at 3 mA per gel in Tris-glycine buffer (pH 8.9) as described by Davis (1964). SDS-PAGE was performed on 8% slab gel (130 × 160 × 1.5 mm) at 25 mA for stacking gel and 35 mA for separating gel according to Laemmli (1970). After electrophoresis, gel was stained in 0.25% Coomassie brilliant blue R 250.

### Purification of storage protein-1

Storage protein-1 was purified by KBr density gradient ultracentrifugation, DEAE-cellulose (DE-52; Whatman) column chromatography, and gel permeation chromatography (Sephadex G-200; Pharmacia).

For ultracentrifugation, pooled last instar larval hemolymph (approximately 1 ml) was centrifuged at 2,000 g (5°C) for 10 min to remove the hemocytes and diluted with 3.5 ml of Tris-HCl buffer (0.05 M Tris, 1 mM EDTA, pH 8.0) and 1. 98 g of KBr was added, with stirring, to the supernatant to give a final density of 1.31 g/ml. KBr-hemolymph mixture (4 ml) was placed in a 10 ml centrifuge tube and overlayered with 4 ml of 0. 9% NaCl (density = 1.007). The tube was placed in RP65 rotor and centrifuged at 35,000 rpm for 16 hrs at 4°C in a Hitachi Ultracentrifuge (model 70P-72). After centrifugation, sample was fractionated 0.8 ml from the top. Density of each fraction was measured and dialyzed against buffer (0.025 M tris, 1 mM EDTA, pH 8.0) and applied to the gel.

Fractions 12 and 13 containing storage proteins were separated by Anion-exchange chromatography (DE52; Whatman). Unbound proteins were eluted with equilibration buffer (0.05 M tris, 1 mM EDTA, pH 8.0) whereas bound proteins eluted with KCl linear gradient buffer (0 to 0.4 M KCl in equilibration buffer). Absorbance of each fraction was monitored at 280 nm by Shimadzu UV-1201 spectrophotometer. Fractions in each peak were pooled and concentrated using polyethylene glycol #20,000 and applied to Non-SDS PAGE to confirm location of storage proteins.

Fractions in peak containing storage protein-1 obained through EDAE cellulose column chromatography were concentrated to 2 ml and applied to Sephadex G-200 column (600 × 18 mm) equilibrated with Tris-HCl buffer (0.05 M Tris, 1 mM EDTA, pH 8.0). Fractions were monitored at 280 nm and fractions containing purified storage protein-1 as last step were concentrated using Minicon B15 (Amicon Inc.).

### Electron microscopic observation of protein particles

Approximately 10 μl of storage protein-1 (500 μg/ml) concentrated using Minicon B15 was dropped on 10 μl of uranylacetate and mixed together. Copper grid (200 mesh) was smeared with this mixture and then air-dried for 30 min. Dried mixture was observed using JEM 100 CX-II (80 KV).

### Determination of storage protein-1 molecular

**weight**

**1) Native molecular weight**

Native molecular weight of storage protein-1 was determined as described by Andrew (1965) with small modification. Sephadex G-200 was used and void volume determined with blue dextran (2,000,000) and standard molecular weight markers were cytochrome C (Mr = 12, 400), carbonic anhydrase (Mr = 150,000), $\beta$-amylase (Mr = 200,000), apoferritin (Mr = 443, 000), and thyroglobulin (Mr = 669,000).

**2) Subunit molecular weight**

Molecular weight of storage protein-1 subunit was determined on 8% SDS-gle as described by Lambin *et al.* (1976). Standard molecular weight markers were myosin (Mr = 205,000), b-galactosidase (Mr = 116,000), phosphorylase (Mr = 97,400), bovine serum albumine (Mr = 66, 200), and ovalbumin (Mr = 45,000).

**Composition of storage protein-1**

**1) Amino acids**

Five to 10 ng of storage protein-1 was hydrolyzed by addition of HCl. The hydrolysate was derivatized by drivatizing solution and analyzed on HPLC (510 solvent delivery pump × 2ea, 712 WISP automated sample processor, pico tagTM column, 990 photodiode array detector; millipore).

**2) Lipids**

The lipids were extracted from storage protein-1 with chloroform: methanol (2:1, v/v) (Nelson, 1975; Bergelson, 1980). Neutral fats were separated on Silica gel F254 TLC plate with petroleum ether: ethyl ether: acetic acid (60:40:1, v/v) (Chino *et al.*, 1969) and detected by charring after spray with iodine vapor (at 120°C). Standard lipids were tripalmitin, dipalmitin (mixture of 1, 2- and 1, 3-dipalmitin) and monopalmitin (Sigma Co.).

**Isoelectric focusing**

Isoelectric focusing of storage protein-1 was conducted on 6% polyacrylamide gel using 2.5% ampholine (pH range 3-10) as described by Wrigley (1968). Electrophoresis was performed at a constant 1 mA per gel up to 450 V with 10 mM $H_3PO_4$ in upper chamber and 20 mM NaOH in lower chamber. After electrophoresis, gels were fixed for 8 hrs in 12.5% TCA solution wich was exchanged eight times at 1 hr intervals and stained in Coomassie brilliant blue R250 for 1 hr and destained using a solution of 12% isopropanol, 7% acetic acid and 0.5% $CuSO_4$.

## Results

**Identification of storage protein-1 (SP-1)**

SP-1 band was identified on 6% gel from hemolymph and fat body extracts during developmental stages. This storage protein-1 in hemolymph begins to appear from early fifth larval instar and maintains high level during pupal stage and then disappears during adult stage (Fig. 1). In the fat body, however, storage protein-1 doesn't appear until late larval instar but is present in high concentration thereafter (Fig. 1).

**Purification of storage protein-1**

Hemolymph of last instar larvae was subjected to KBr density gradient ultacentrifugation and density of each fraction (0.8 ml) from upper end was measured (Fig. 2) and each fraction was electrophoresed with crude hemolymph of last instar lravae. Electopherogram showed that SP-1 (most upper band among 4 SP bands) is present in sizable amounts in fractions 12 and 13 (Fig. 3). Proteins in fractions 12 and 13 were further separated by ion-exchange chromatography on DEAE-cellulose (DE 52) (Fig. 4) and electrophoresed. SP-1 was found to be present in fractions which were collected by washing the column with equilibration buffer (Fig. 5). thus SP-1 appears to be not bound to DEAE-cellulose. SP-1 in peak fractions was further purified by gel permeation chromatography on Sephadex G-200 (Figs. 6, 7).

**Size and shape of storage protein-1**

SP-1 was observed under the elctron microscope (JEM 100 CS-II). SP-1 is 9.4 nm in diameter and regular octahedron in shape (Fig. 8).

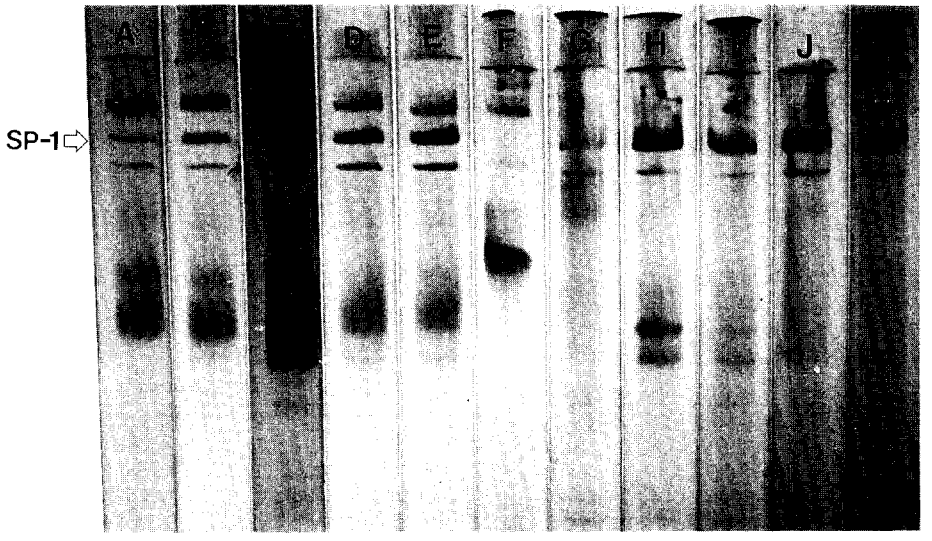


**Fig. 1.** Non-SDS PAGE of hemolymph and fat body extracts from *Galleria mellonella* of different stages. (A) Hemolymph of last instar larvae; (B), (C), (D), (E), and (F) Hemolymph of 5th instar larvae, 6th instar larvae, 1-day-old pupae, 2-day-old pupae and adult, respectively; (G), (H), (I), (J) and (K) Fat body extracts of last instar larvae, prepupae, 0-day-old pupae, 2-day-old pupae and 5-day-old pupae, respectively.

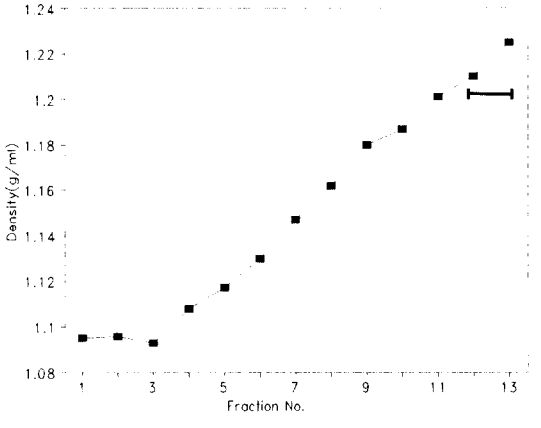


**Fig. 2.** Density profile obtained by KBr density gradient ultra-centrifugation of *Galleria mellonella* hemolymph. Fractions 12 and 13 (> 1.21 g/ml) indicated by a bar were chromatographed on DEAE cellulose (ED-52).

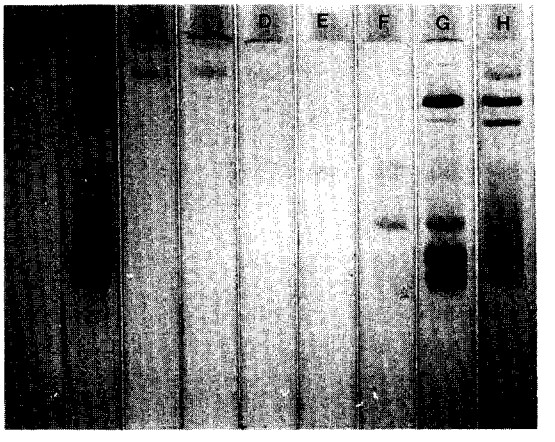


**Fig. 3.** Non-SDS PAGE of fractions from KBr density gradient ultracentrifugation (Fig. 2) and crude hemolymph of last instar larvae. (A) and (H) crude hemolymph of last instar larvae; (B), (C), (D), (E) and (F) fractions of 3rd, 4th, 6th, 8th and sum of 10th and 11th, respectively. (G) fractions of 12th and 13th. Fractions 12 and 13 were chromatographed on DE-52.

## Molecular weight and isoelectric focusing of storage protein-1

native molecular weight of SP-1 was estimated to be 365 K dalton (Fig. 9) and SP-1 consists of single subunit with molecular weight of 82 K dalto (Fig. 10).

Purified SP-1 was isoelectric focused on 6% polyacrylamide gel. Isoelectric point of SP-1 was estimated to be 5.7 (Data not shown).

## Lipid component and amino acid composition of storage protein-1

SP-1 contains triacylglycerol, 1, 2-diacylglycerol, monoacylglycerol, and phospholipid. Especially triacylglycerol and phospholipid are present in

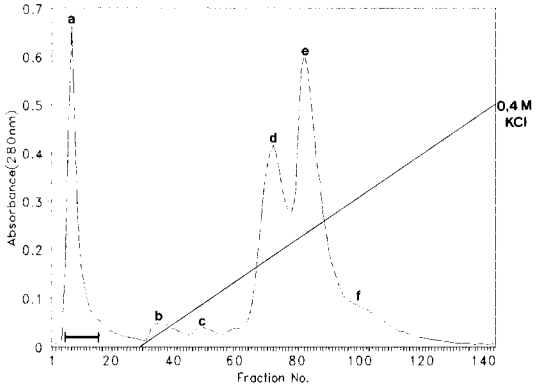


**Fig. 4.** Elution profile obtained by DE-52 column chromatography of fractions 12 and 13 of ultracentrifugation (Figs. 2, 3). Elution buffer was equilibration buffer (0.05 M Tris, 1 mM EDTA, pH 8.0). Linear gradient elution was performed from 0 to 0.4 M KCl in equilibration buffer. The eluate in the area indicated by a bar (peak a) was chromatographed on Sephadex G-200.

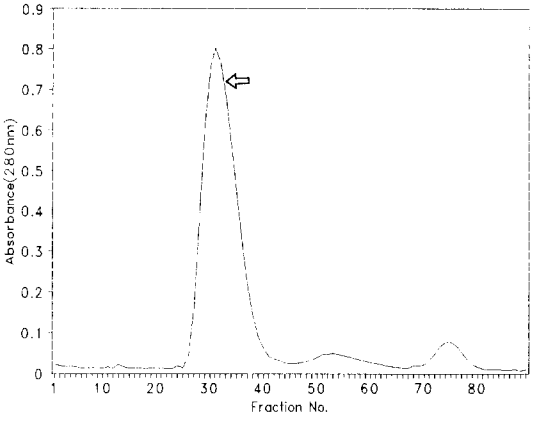


**Fig. 6.** Elution profile obtained by Sephadex G-200 column chromatography of eluate indicated by a bar (peak a) in DE-52 column (Figs. 4, 5). Bed volume was 165 ml, flow rate 24 ml/hr and volume of each fraction was 2 ml. The open arrow indicates peak of storage protein-1.

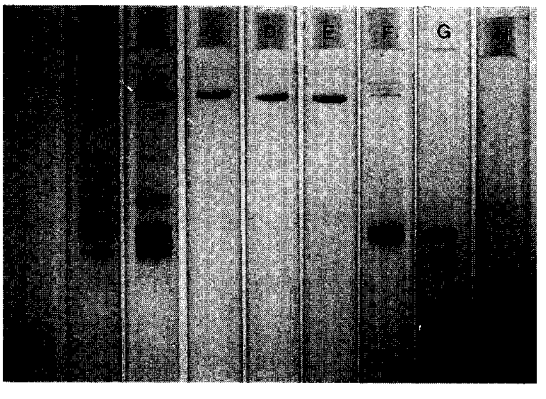


**Fig. 5.** Non-SDS PAGE of fractions of each peak in DE-52 column (fig. 4). (A) crude hemolymph; (B) fractions 12 and 13 from ultracentrifugation (Fig. 3); (C), (D), (E), (F), (G), and (H). eluate of peaks a, b, c, d, e and f in DE-52 column (Fig. 4).

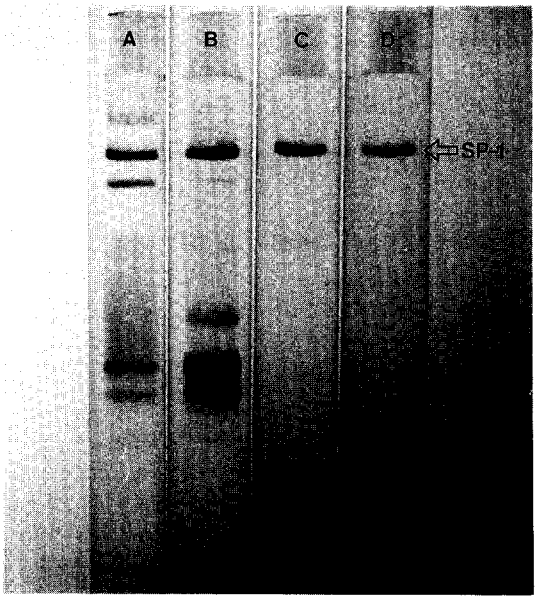


**Fig. 7.** Steps in the purification of the storage protein-1 from the hemolymph of the wax moth (*Galleria mellonella*) monitored using electrophoresis on 6% polyacylamide gels. (A) crude hemolymph; (B) KBr density gradient ultracentrifugation; (C) ion exchange chromaxtography on DEAE-cellulose (DE-52); (D) gel permeation chromatography on Sephadex G-200.

large amounts (approximately 80%).

Amino acids were analyzed from SP-1. Aromatic amino acids including tryptophan, phenylalanine and tyrosine account for 16.71 mol% (Table 1).

## Discussion

Hemolymph and fat body extracts of *Galleria mellonella* were electrophoresed during developmental stages to determine storage

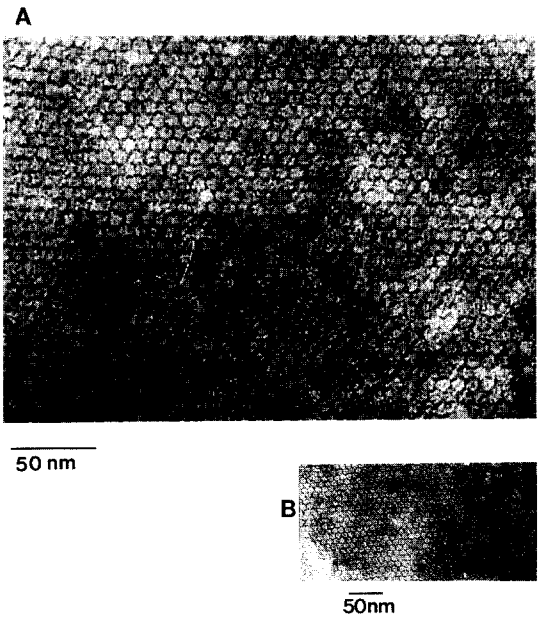


**Fig. 8.** Electron micrograph of purified SP-1 negatively stained with uranylacetate.

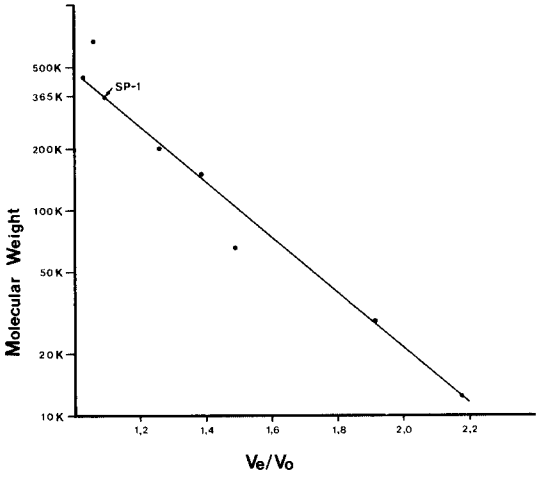


**Fig. 9.** Gel filtration on Sephadex G-200 of the purified SP-1 showing the native molecular weight. Standard molecular weight markers were cytochrome C (12.4 K), carbonic anhydase (29 K), BSA (66 K), alcohol dehydrogenase (150 K), β-amylase (200 K), apofferitin (443 K) and thyroglobulin (669 K).

proteins. Four storage protein bands were identified and one (SP-1) of them which has lowest Rf value and is major band was purified and characterized.

In most insects SP released into hemolymph

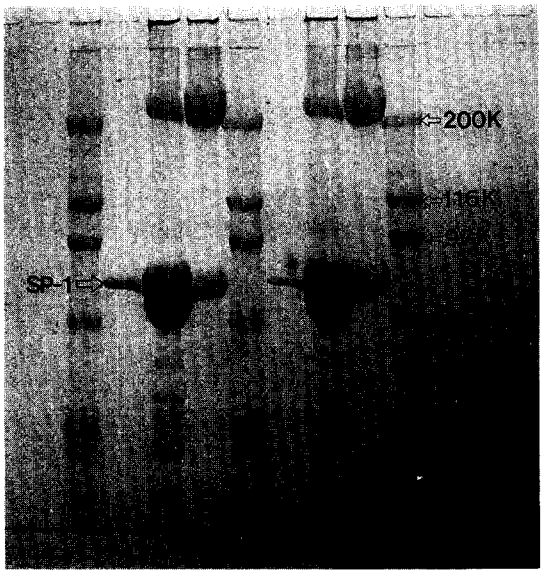


**Fig. 10.** SDS PAGE of purified SP-1. (B) and (F) purified SP-1; (C) and (G) crude hemolymph of last instar larvae; (D) and (H) purified lipophorin of the wax moth, *Galleria mellonella;* (A), (E) and (I) M.W. standard markers for SDS [myosin (200 K), β-galactosidase (116 K), phosphorylase (97.4 K), BSA (66.2 K), ovalbumin (45 K)].

was reabsorbed by fat body during prepupal stage (Natori, 1986; Venkatesch and Chippendale, 1986). In present work with *Galleria mellonella* SP-1 was not completely absorbed by fat body along with pupation but present in high concentration in hemolymph until late pupal stage. This SP-1 is a little different in absorbing time from SP generally known but shows characteristics similar to SP of *Dacus oleae* (Bourdopoulou *et al.*, 1981) and Manducin of *Manduca sexta* (Kramer *et al.*, 1980).

In the process of purifying SP-1 through column chromatography, Shapiro method (1984) purifying lipophorin was employed to remove lipophorin and low density hemolymph proteins.

Palli and Locke (1987) observed structure of SP under the electron microscope. SP was observed to be approximately 10 nm in diameter and pentahedral prism, and regular octahedron. In the present work with *G. mellonella* SP-1 is 9.4 nm in diameter and regular octahedron in shape. Also, SP-1 has molecular weight of 365 K dalton and is composed of one type of subunit with molecular

**Table 1.** Amino acid composition of SP-1 from *Galleria mellonella*.

| Amino acid | mol % |
|---|---|
| Cys; C | 2.54 |
| Asx* | 10.27 |
| Glx* | 10.52 |
| Ser; S | 5.40 |
| Gly; G | 6.17 |
| His; H | 1.22 |
| Arg; R | 4.48 |
| Thr; T | 3.96 |
| Ala; A | 6.18 |
| Pro; P | 3.75 |
| Tyr; Y | 2.43 |
| Val; V | 7.10 |
| Met; M | 1.25 |
| Ile; I | 4.91 |
| Leu; L | 8.59 |
| Phe; F | 6.24 |
| Trp; W | 8.03 |
| Lys; K | 6.94 |
| Total | 100.00 |

Axz*, Glx* mean the sum of Asparagine & Aspartic Acid and Glutamine & Glutamic Acid, respectively.

weight of 82 Kd, indicating that native molucule is tetramer. This result was different from hexamer type generally known so far (Roberts and Brock, 1981), indicating diversity of storage protein structure with different insect species.

Lipid component of SP-1 was not much investigated but reported to contain mostly 1, 2-DG and phospholipid. SP-1 of *G. mellonella* was found to contain TG and phospholipid in greatest amounts. SP-1 of *G. mellonella* has high amounts of aromatic amino acid including tryptophan but contents of tyrosine and phenylalanine were relatively low. This result indicates that SP-1 of *G. mellonella* is non-arylphorine storage protein (Telfer *et al.*, 1983).

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## 꿀벌부채명나방(***Galleria mellonella*** L.)의 저장단백질-1(storage protein-1)의 정제 및 물리화학적 연구

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꿀벌부채명나방(*Galleria mellonella* L.)의 저장단백질-1(SP-1)의 존재를 혈림프와 지방체로부터 전기영동으로 확인하였고, 혈림프를 KBr 밀도구배초원심분리하여 형성된 밀도에서 1.21 g/ml 이상의 fraction 12와 13으로부터 DEAE-cellulose (DE 52) ion-exchange chromatography와 gel permeation chromatography (Sephadex G-200)에 의해서 순수분리하였다. 분리된 저장단백질-1의 순수도는 Non-SDS PAGE와 전자현미경으로 확인한 결과, Non-SDS PAGE에서는 단일밴드로 나타났고, 전자현미경조사에서는 단백질입자가 동일한 모양과 형태로 존재하고 있는 것으로 나타났다. 전자현미경 관찰결과 저장단백질-1의 직경은 9.4 nm이고 정팔면체의 구조로 밝혀졌다. 또한, 저장단백질-1의 등전점은 5.7이었고, 순수분리된 저장단백질-1의 native molecular weight를 gel permeation chromatography로 조사한 결과 365 KD로 나타났으며, 구성소단위는 SDS-PAGE로 조사한 결과 82 KD의 분자량을 가지는 한종류의 구성소단위로 되어 있음을 밝혔다. 위의 결과로 보아 꿀벌부채명나방의 저장단백질-1은 한종류의 구성소단위 4개로 구성된 tetramer로 존재함을 알 수 있었다. 또한, 저장단백질-1에 존재하는 지질을 TLC(thin-layer chromatography)로 조사한 결과, 다량의 triacylglycerol과 phospholipids가 존재함을 밝혔다.