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Characterization and Partial Purification of Storage Protein-1 Receptor in the Fat Body of *Hyphantria cunea*

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***In vitro* tissue culture of fat body of *Hyphantria cunea* in the medium containing [^{35}S]-methionine revealed that storage protein-1 (SP-1) is taken up into fat body of prepupae and 1-day-old pupae. Using Western blotting and ligand binding method, we were able to identify the protein band of the SP-1 receptor protein. For the partial purification, the membrane proteins of fat body cells were solubilized with 1% Triton X-100 and applied to anion exchange chromatography. The results revealed the molecular weight of the receptor protein to be about 80 kDa in SDS-PAGE, and the PI was estimated to be about 6.1. The mobility of the receptor protein in SDS-PAGE was highly dependent on both temperature during electrophoresis and the condition of samples whether they were in reducing or nonreducing.**

KEY WORDS: *Hyphantria cunea*, Fat Body, Uptake, Receptor, Storage Protein-1

Storage protein (SP) is present in large amounts in hemolymph of late larval instar in holometabolous insects (Munn and Greville, 1969). This protein is synthesized by fat body of last instar larvae and released into hemolymph, amounting to 80% of total hemolymph protein in late last instar larvae (Chrysanthis *et al.*, 1981). SP is taken up into fat body starting from prepupal stage and stored as protein granules and used as a source of energy and essential amino acid that is necessary for adult organ differentiation during the pupal stage (Natori, 1986; Marinotti and De Bianchi, 1986; Seo and Kim, 1988).

SP is known to be hexamer composed of approximately 80 kDa subunits (Telfer and Kunkel, 1991). While SP of Lepidoptera is rich in methionine and aromatic amino acids, SP of many dipteran species is low in methionine and aromatic acid (Telfer and Kunkel, 1991).

SP of *Hyphantria cunea* is composed of two kinds of SP designated as storage protein-1 (SP-1) and storage protein-2 (SP-2) (Kim *et al.*, 1989). SP-1 is hexamer composed of 76.7 kDa subunit and present in large amounts in hemolymph of late larval instar and begins to be sequestered by fat body from prepupal stage. SP of *H. cunea* is relatively low in methionine and aromatic acids but rich in valine, characteristics of which are in quite contrast to that of SP in other lepidopteran species. There were a little studies about uptake process of SP into fat body. SP receptor in the range of 120 kDa was found in *Sarcophaga peregrina* and this receptor has Ca^{2+} and pH dependent activity (Ueno and Natori, 1984). SP receptor was also detected in *Calliphora vicina* and their characteristics were examined (Burmester and Scheller, 1992). However, it has been little studied on SP receptor of Lepidoptera except very few examples (Wang and Haunerland,

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1993; Wang and Haunerland, 1994).

In the present work, the receptor protein for SP-1 of *H. cunea* was identified and their characteristics were determined. The effects of temperature and disulfide bond on structural stability were also examined.

Materials and Methods

Insects

Larvae of *Hyphantria cunea* were reared on artificial diet at 26°C and 80% R.H. with a photoperiod of 16L:8D.

Collection and processing of hemolymph and fat body

Hemolymph was collected from last instar larvae by cutting forelegs or from pupae by piercing head with a fine needle. A few crystals of phenylthiourea (PTU) were added to the hemolymph to prevent melanization. The hemolymph was centrifuged at 5,600 g for 10 min to remove hemocytes and cell debris and the supernatant was stored at −70°C until used. Fat body was dissected from day 1 pupae in cold Ringer's solution (128 mM NaCl, 1.8 mM $CaCl_2$, 1.3 mM KCl, pH 7.4) and homogenized. After centrifugation at 15,000 g for 20 min, the supernatant was stored at −70°C until used.

In vitro tissue culture of fat body

Fat body tissues of 7th instar larvae and 1-day-old pupae were washed in Ringer's solution two or three times and preincubated in Grace's insect medium for 10 min. Fat body (approximately 100 mg) was incubated in 100 μl of Grace's insect medium containing 5 μCi of [^{35}S]-methionine for 4 h in a shaking incubator at 37°C as described by Bownes (1982). Following incubation, cultured tissues were homogenized and centrifuged at 10,000 g for 10 min. The supernatants were subjected to immunoprecipitation by anti-SP-1 polyclonal serum and the pellets were dissolved with SDS-PAGE sample buffer. After electrophoresis, gel was dried and exposed to X-ray at −70°C for 1 week according to the procedure of Bonner and Laskey (1974).

Purification of SP-1

SP-1 was purified from the collected hemolymph using KBr ultracentrifugation, anion exchange chromatography (DEAE) and gel filtration (Sephadex G-200) continuously, according to Cheon *et al.* (1993).

Labelling of SP-1 with ^{14}C

Labelling of SP-1 was conducted with *in vitro* reductive methylation using [^{14}C]-formaldehyde according to Jentoft and Dearborn (1979) with some modification. Five hundred μl (1 mg/ml) of purified SP-1 was mixed with 200 μl of ^{14}C-formaldehyde (2 μCi) and 200 μl of 20 mM $NaCNBH_3$ and reacted at 25°C for 15 h with gentle agitation. The sample was dialyzed against 20 mM phosphate buffer (pH 7.0) for 12 h and then against 20 mM Tris buffer (pH 7.0) for additional 12 h and concentrated to 1 ml with PEG.

Identification of SP-1 receptor protein in fat body

1-day-old pupal fat body tissue was homogenized in cold 20 mM Tris buffer (20 mM Tris, 150 mM NaCl, 0.1 mM PMSF, 1 mM EDTA, 0.1 μM aprotinin, pH 7.5) and centrifuged at 15,000 g for 20 min. The precipitate was vortexed in the same buffer and centrifuged again. The final precipitate was homogenized in the same buffer containing Triton X-100 and CHAPS (0.2%, 0.5%, 1%, and 2%, each) and shaked for 2 h at 4°C. The sample was then centrifuged at 15,000 g for 20 min and the supernatant was used for Western blotting, ligand blotting, and fluorography.

Western blotting was conducted as the method of Towbin *et al.* (1979). Ligand blotting was carried out as described by the protocol of Daniel *et al.* (1983) with some modification. Nitrocellulose sheet was incubated with LBB (ligand binding buffer) (20 mM Tris, 150 mM NaCl, 8 mM $CaCl_2$, 0.05% Tween 20, 1% gelatin, pH 7.5) containing SP-1 (100 μg/ml) for 1 h. This sheet was washed with washing buffer (20 mM Tris, 150 mM NaCl, 8 mM $CaCl_2$, pH 7.5) one time violently and shortly and two times at 20 min intervals slowly and then one time again violently

and shortly. Then the sheet was incubated with the antibody against SP-1.

For fluorography, nitrocellulose sheet was washed with LBB after blocking. ^{14}C-labelled SP-1 was incubated in 3 ml of LBB containing 23 μg/ml (2230 cpm/μg) for 80 min and washed twice 5 min each with LBB and dried. After soaking in enhancer, dried nitrocellulose sheet was exposed to X-ray film at −70°C for 7 days.

Partial purification SP-1 receptor protein

Membrane proteins of 1-day-old pupal fat body cells were obtained by the method, including 1% of Triton X-100, mentioned above. And the solution was dialyzed against Tris buffer (20 mM Tris, 1% Triton X-100, pH 6.5) for 16 h and then was applied to DEAE cellulose (DE-52, Whatman) column (1.2 × 10 cm) with Tris buffer at a flow rate of 25 ml/h with 2.5 ml per fraction. Bound protein was eluted with linear gradient (0 - 1 M KCl). Fraction was treated by acetone to remove Triton X-100 and then applied to SDS-PAGE for confirmation of purity.

Characterization of SP-1 receptor protein

Isoelectric focusing was conducted with 5% polyacrylamide gel and 2.4% ampholine (pH 3-10) as determined by Giulian *et al.* (1984).

M.W. of SP-1 receptor protein was determined on 10% SDS gel under denatured and nonreducing condition. Standard molecular weight marker proteins were used : myosin (Mr=200 kDa), β-galactosidase (Mr=116 kDa), phosphorylase b (Mr=97.4 kDa), bovine serum albumin (Mr=66.2 kDa), and ovalbumin (Mr=45 kDa).

For characterization of SP-1 receptor protein on SDS-PAGE, the protein was electrophoresed on 10% gel under reducing condition (+ β-mercaptoethanol) or nonreducing condition (− β-mercaptoethanol) and after heating sample at 96 °C for 2.5 min or not.

Results

Identification of SP-1 receptor protein

Fat body tissues in varying time intervals of early wandering stage to late prepupal stage were subjected to Non-SDS PAGE (Fig. 1). SP-1 begins to appear in fat body from prepupal stage and reaches the peak immediately after pupation. Also, synthetic activity for SP-1 was investigated in last instar larval and day 1 pupal fat bodies, indicating that SP-1 is not synthesized in day 1 pupal fat body (Fig. 2). From this result, we can induce that SP-1 in the fat body of day 1 pupae was taken up during pupation and there may be receptor(s) for SP-1 at the fat body cell membrane.

Fat body cell membrane proteins were dissolved with CHAPS and Triton X-100 and screened on SDS gel (Fig. 3). There are a lot of bands in both panal at almost same sites, but the intensity of each band is somewhat different. After electrophoresis, the sheet was subjected to Western blotting and ligand blotting (Fig. 4). In Western blotting there was one band in all lanes. This is considered to be SP-1. However, in ligand blotting there was one additional band in addition

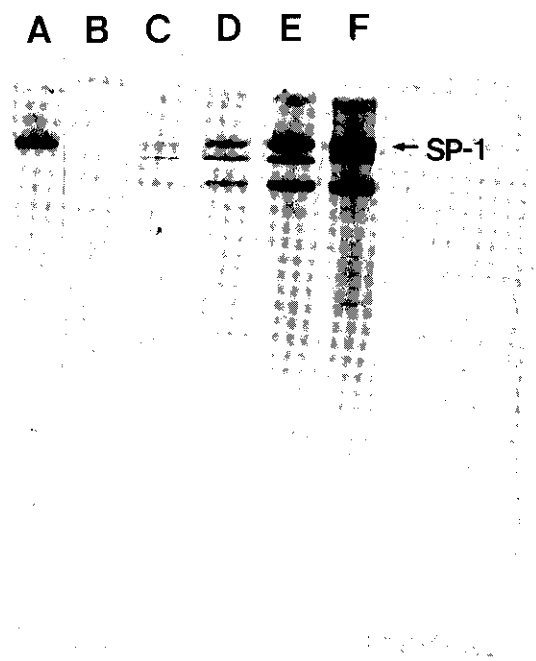

Fig. 1. Non-SDS PAGE of fat body extracts from different stages. (A) Purified SP-1. (B) 7th instar larvae in wandering stage. (C) Prepupae in the beginning. (D) 1-day old prepupae. (E) 2-day old prepupae. (F) Pupae from just pupated to 1-day old.

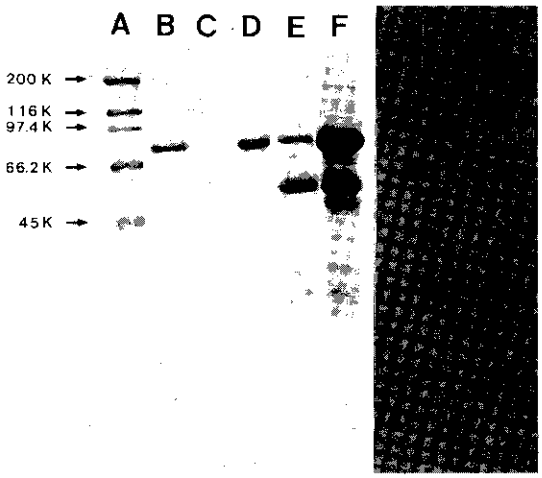

Fig. 2. SDS-PAGE and autoradiogram of fat body extracts. (A) Standard molecular weight markers (Myosin: 200kD, β-galactosidase: 116 kD, Phosphorylase b: 97.4 kD, Bovine Serum Albumin: 66. 2 kD, Ovalbumin: 45 kD). (B) Purified SP-1. (C), (D) Fat body extracts from 7th instar larvae and 1-day old pupae, respectively. (E), (F) Immunoprecipitated proteins of tissue cultured fat body extracts from 7th instar larvae and 1-day old pupae, respectively.(G), (H) Autoradiogram of lane (E), (F) respectively.

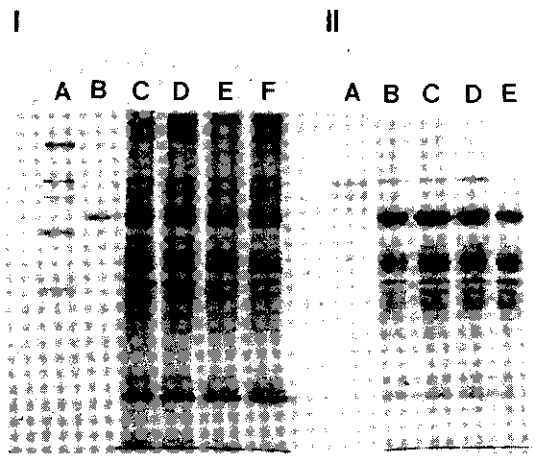

Fig. 3. SDS-PAGE of solubilized proteins of fat body cell membrane for detergent screening. I. (A) Standard molecular weight markers. (B) Purified SP-1 in reducing condition. (C)-(F) Solubilized proteins obtained from the treatment of 0.2%, 0.5%, 1%, 2% CHAPS from left to right, respectively. II. (A) Purified SP-1 in nonreducing condition. (B)-(F) Solubilized proteins obtained from the treatment of 0.2%, 0.5%, 1%, 2% Triton X-100 from left to right, respectively.

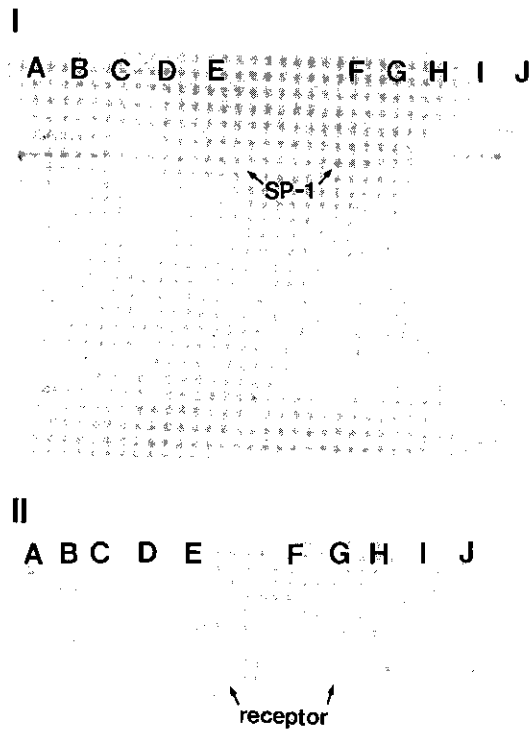

Fig. 4. I. Western blotting of solubilized proteins of fat body cell membrane aganist anti-SP-1 antibody. (A), (F) Purified SP-1 in nonreducing condition. (B)-(E) Solubilized proteins obtained from the treatment of 0.2%, 0.5%, 1%, 2% CHAPS from left to right, respectively. (G)-(J) Solubilized proteins obtained from the treatment of 0.2%, 0.5%, 1%, 2% Triton X-100 from left to right, respectively. II. Ligand blotting of solubilized proteins of fat body cell membrane. (A) Purified SP-1 in reducing condition. (B)-(J) are same with those of I.

to SP-1 band in all lanes. This additional band is regarded as receptor protein band. Any nonspecific binding between SP-1 and a band on the sheet hardly can withstand successfully to the end during this procedure. However to confirm the result, ^{14}C labelled SP-1 was subjected to fluorography, showing that band shown in ligand blotting appeared in the same level (Fig. 5). Because fluorography originally removes the

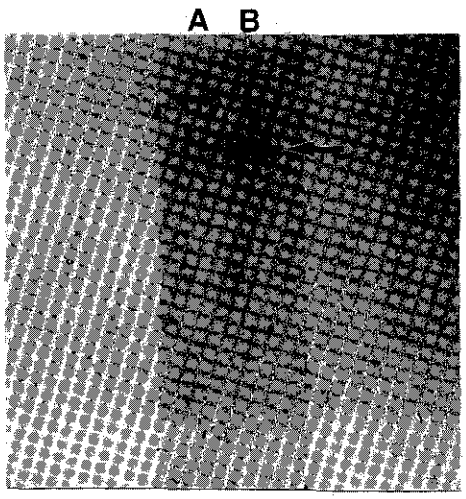

Fig. 5. Fluorogram of nitrocellulose sheet incubated with [^{14}C]-labelled SP-1 in fluorography. (A) [^{14}C]-labelled purified SP-1. (B) Fat body cell membrane proteins solubilized with the treatment of 2% Triton X-100.

possibility of nonspecific binding of anti-SP-1 polyclonal serum to other site, we can confirm that receptor protein is present at fat body cell membrane.

Partial purification of SP-1 receptor protein

Samples were applied to ion exchange chromatography, showing one unbound peak and three bound peaks (Fig. 6). Fractions in each peak were grouped, dialyzed against Tris buffer (20 mM Tris, 1% Triton X-100) and treated with acetone to remove Triton X-100. The samples were subjected to SDS-PAGE, indicating that receptor protein was contaminated with a protein which appeared faintly below the receptor band (Fig. 7). Although we didn't examine the activity of the pooled solutions, it was possible to think that the receptor protein was solubilized in peak c fractions because there was no band around 80 kDa in lane C and D, while in lane E we could find a clear band of that size. And this about 80 kDa protein band coincides well with previous data (Fig. 4 and 5) in molecular weight.

Property of SP-1 receptor protein

Molecular weight of SP-1 receptor protein was

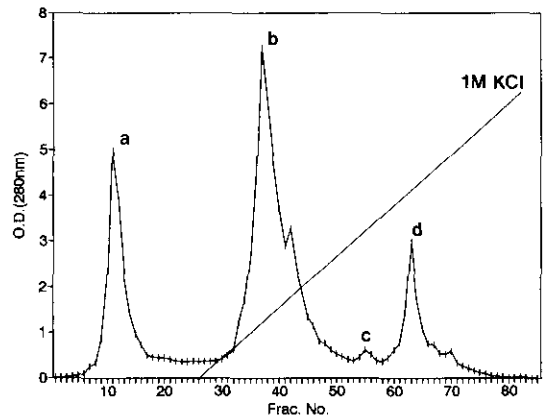

Fig. 6. Elution profile of DEAE ion exchange chromatography of the solubilized fat body cell membrane proteins. Equilibration buffer (20 mM Tris, 1% Triton X-100, pH 6.2) was used as elution buffer. Linear gradient was performed from 0 to 1 M KCl in elution buffer.

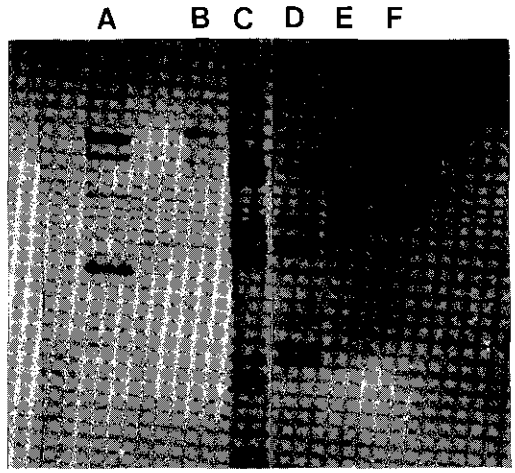

Fig. 7. SDS-PAGE of several parts among the DEAE column eluates. (A) Standard molecular weight markers. (B) Purified SP-1 in nonreducing condition. (C) Fat body cell membrane proteins solubilized with the treatment of 1% Triton X-100. (D)-(F) Eluates of peak a, b and c respectively, in the DEAE column. Arrow indicates the receptor band.

estimated to be approximately 80 kDa as determined by SDS-PAGE under nonreducing condition (Fig. 8). Also, isoelectric point is estimated to be 6.1 (Fig. 9), but there was a

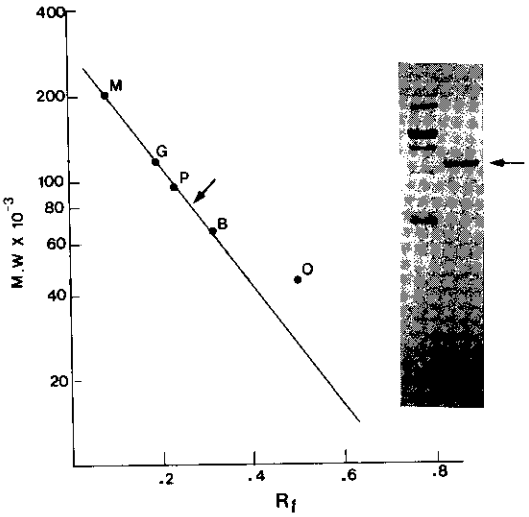

Fig. 8. Estimation of moleculat weight for SP-1 receptor in SDS-PAGE. Used standard proteins were (M) Myosin (200 kD), (G) β-galactosidase (116 kD), (P) Phosphorylase b (97.4 kD), (B) Bovine Serum Albumin (66.2 kD), (O) Ovalbumin (45 kD).

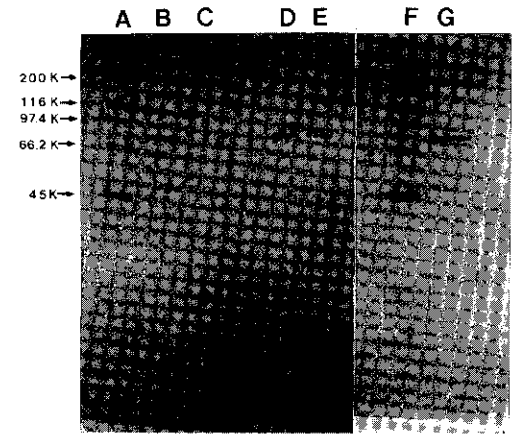

Fig. 10. SDS-PAGE of partially purified SP-1 receptor solubilized from fat body cell membrane. SDS-PAGE was performed at 4°C except (F), (G) which was performed at room temperture. (A), (F) Standard molecular weight markers. In reducing condition, (B) Boiled at 96 °C for 2 min 30 sec, (C), (F), (G) Incubated at room temperture. In nonreducing condition, (D) Boiled at 96°C for 2min 30sec, (E) Incubated at room temperature.

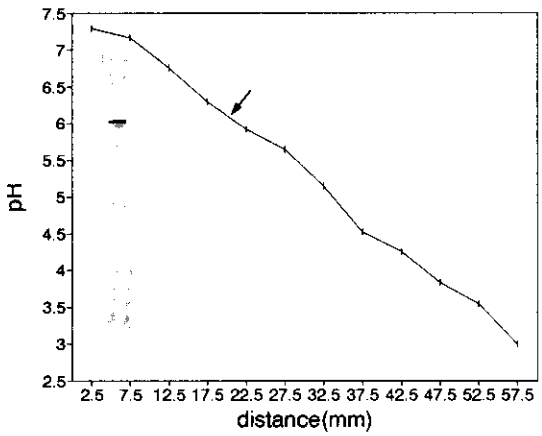

Fig. 9. Estimation of isoelectric point for SP-1 receptor by the method of Giulian *et al.* (1984)

somewhat deviation because of imperfect standard pH value at both limits of the gel. Partially purified SP-1 receptor protein was subjected to SDS-PAGE under reducing and nonreducing conditions and alternative temperatures of sample incubation and during electrophoresis. The results were shown as different band patterns (Fig. 10). These results suggest that the receptor protein molecule can exist separately or form a micelle in solubilized state, depending on the temperature and the state of disulfide bond breakage.

Discussion

While the specific uptake into fat body at the end of the larval stadium is characteristic for all insect storage proteins, very little is known about the exact mechanism that mediate this process. It has been proposed that the proteins are taken up by receptor mediated endocytosis, and evidence for several potential storage proteins receptors has been found in dipteran species. While a single receptor protein for calliphoran has been identified in the fleshfly, *Sarcophaga peregrina* (Ueno and Natori, 1984), three binding proteins for a storage protein have been reported for the blowfly, *Calliphora vicina* (Burmester and Scheller, 1992). But in this case, only one protein was regarded as a real receptor protein. But so far in this field, there is no exception in that if only one protein band is shown by proper ligand blotting method, it is a genuine receptor protein

band (Daniel *et al.*, 1983, Ueno and Natori, 1984, Wang and Haunerland, 1993). However, the vice versa is not true. Wang and Haunerland (1993) reported that a SP receptor of *H. zea* is present only in periviceral fat body and only during the period around pupation when storage proteins are sequestered. Recently, a storage protein receptor was identified and purified to homogeneity from *Helicoverpa zea* (Wang and Haunerland, 1994).

In the present work with *H. cunea*, SP-1 receptor protein on fat body was identified and partially purified, and their property was studied. Although CHAPS and Triton X-100 are considered to be excellent in dissolving fat body cell membrane proteins, Triton X-100 is thought to be more suitable, based on the fact that sample dissolved in Triton X-100 showed denser band. SP-1 receptor protein was partially purified through anion exchange chromatography. Based on the elution profile and pI value, SP-1 receptor protein is thought to be eluted in bound peak because the protein has lower pI value than the pH of elution buffer. M.W. of SP-1 receptor protein was determined on SDS gel under nonreducing condition. It was estimated to be approximately 80 kDa. On the while, molecular weight of the protein was measured to be a little lower value under reducing condition. This is quite contrast to the fact that M.W. of lipophorin receptor is 160 kDa under reducing condition but approximately 130 kDa under nonreducing condition (Daniel *et al.*, 1983). Although at now we could not confirm the precise reason for it, it is considered that besides of the shape of the molecule which vary largely depending on intramolecular disulfide bond, the change of the amounts of SDS which interchelates into the molecule in alternative situation, may work greatly.

Isoelectric focusing was conducted to determine pI value of SP-1 receptor protein as described by Giulian *et al.* (1984). It was estimated to be 6.1 which belongs to acidic receptor. It is thought very important that the receptor protein molecules are influenced by reducing and nonreducing condition and temperature, because these are closely connected with structure and activity of the receptor protein.

While the properties of the storage protein receptor and its temporal and spatial distribution strongly suggest that storage proteins are taken up by the process of receptor mediated endocytosis, conclusive evidence for this mechanism could require ultrastructural analysis. Of particular interest could be to follow the endocytosis process from the recognition of storage proteins at the cell membrane to their final deposition in protein storage granules.

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미국흰불나방 지방체에서 저장단백질-1의 수용체의 특성과 부분정제

채권석 · 여성문[1] · 김학렬(고려대학교 생물학과, [1]단국대학교 생물학과)

미국흰불나방(*Hyphantria cunea*)의 지방체 조직을 [^{35}S] 메타이오닌이 포함된 배지에서 조직배양한 결과, 저장단백질-1(SP-1)이 전용기부터 용 1일 사이에 지방체로 흡수됨을 알았다. CHAPS, Triton X-100 등의 계면활성제를 농도별로 처리하여 막단백질의 용해도를 스크리닝한 뒤, anti-SP-1 polyclonal 항체를 쓴 Western blotting과 ligand blotting, 그리고 *in vitro* reductive methylation으로 ^{14}C을 표지한 저장단백질-1을 사용한 fluorography 등으로 1개의 수용체 밴드를 확인하였다. 1% Triton X-100으로 용해시킨 지방체 세포막단백질 용액을 시료로 이온교환크로마토그래피를 시행하여 수용체를 부분정제하였고, SDS-PAGE에 의해서 분자량을 측정한 결과 약 80 kDa로 나타났고 isoelectric focusing 시행 결과 등전점은 약 6.1로 계산되었다. 수용체 분자는 환원조건과 비환원조건의 차이와 전기영동 중의 온도에 따라서 SDS-PAGE상의 뚜렷한 밴드 양상의 차이를 나타내었다.