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Identification and Characterization of Male Specific Protein in the Hemolymph of *Galleria mellonella* L.

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Male specific protein (MSP) was identified and purified from the hemolymph of *Galleria mellonella* L. by electrophoresis and anion exchange chromatography. MSP has a native molecular weight of 55 kDa as determined by gel filtration chromatography and consists of a single unit with the apparent molecular weight of 27 kDa and has the pI of approximately 5.8. MSP is present in the hemolymph from day 8 pupae throughout the male adult. MSP was also found in pupal fat body, adult fat body, and adult testis.

Insect hemolymph proteins have been intensively studied so far. Especially, female specific proteins (FSP), such as vitellogenin, vitellin and yolk protein, have been widely studied along with storage protein and lipophorin (Engelmann, 1979; Hagedorn and Kunkel, 1979; Kunkel and Nordin, 1985; Lee et al., 1995; Yun and Kim, 1996). FSPs were synthesized in part by the ovary but most of them synthesized by the fat body. FSPs were released into the hemolymph as a form of vitellogenin and taken up into the maturing ovary and accumulated there as vitellin (Engelmann, 1979; Hagedorn and Kunkel, 1979; Kim and Lee, 1994). FSP appears only in female hemolymph during oocyte maturation but not in male hemolymph.

On the while, male specific proteins (MSP) were known to be synthesized mostly by the accessory gland. These proteins were reported to repress female receptability after copulation or to control the change of copulation behavior (Chen and Balmer, 1989; Ohashi et al., 1991). But MSP present only in male hemolymph was little studied since it has been first reported in mediterranian fruit fly, *Ceratitis capitata*, as male specific serum proteins (MSSP) (Katsoris et al., 1990). MSSP of *C. capitata* was known to be of five kinds in the molecular weight range of 13.5 to 14.5 kDa and synthesized by the fat body and released into the hemolymph during early adult developmental stage. The synthesis of two major proteins of them were regulated at the transcriptional and the translational level (Sotiria et al., 1995).

Present work is to identify and purify MSP from the hemolymph of *Galleria mellonella* L. Presence of MSP in various organs was also examined, using antibody against it.

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Materials and Methods

Insect

Larvae of *Galleria mellonella* L. were reared on artificial diet at 27±1℃ and 75±5% relative humidity in dark condition.

Collection of hemolymph and extraction of proteins from various organs

Hemolymph was collected from the last instar larvae and pupae by puncturing larval abdomen and pupal head with a fine needle. Also, hemolymph was collected from the adult by cutting the head and gently squeezing the thorax and abdomen. A few crystals of phenylthiourea (PTU) were added to prevent melanization. The hemolymph was centrifuged at 10,000 g for 10 min at 4℃ to remove hemocytes and cell debris and the supernatant was stored at -70℃ until use. Fat body, ovary, and testis were dissected in cold Ringer's solution (128 mM NaCl, 1.8 mM $CaCl_2$ 1.3 mM KCl, 0.05 M Tris; larvae pH 6.2, pupae and adult pH 7.4) and washed two or three times in the same Ringer's solution. Each tissue was completely homogenized in Ringer's solution and centrifuged at 10,000 g for 10 min at 4℃ and after removing overlaid lipid layer, the supernatant was stored at -70℃ until use.

Electrophoresis

Non-SDS-PAGE (polyacrylamide gel electrophoresis) was conducted on a 6% separating gel as described by Davis (1964). SDS-PAGE was carried out on a 15% separating gel at 15 mA according to the procedure of Laemmli (1970). After electrophoresis, gels

were stained in Coomassie brilliant blue R250.

Purification of MSP

Approximately 1 ml of adult male hemolymph was mixed with an equal volume of Tris-HCl buffer solution (0.05 M Tris, 1 mM EDTA, pH 8.0) and dialyzed twice within closed cellulose bag (MW<12,000 Da) against the same buffer solution containing small amounts of PTU for 8 h. After dialysis, samples were subjected to anion exchange chromatography. Samples were eluted from DEAE-cellulose (DE-52, Whatman) at a flow rate of 25 ml/h with 2.5 ml per fraction. Unbound proteins were eluted with equilibration buffer, Tris-HCl (pH 8.0) whereas bound proteins eluted with 200 ml of KCl linear gradient (0 to 0.4 M KCl in elution buffer). Absorbance of each fraction was measured at 280 nm and the purity of MSP was confirmed by SDS PAGE and Non-SDS-PAGE.

Determination of molecular weight and isoelectric point of MSP

Molecular weight of MSP subunit was determined on a 12% SDS gel as described by Lambin et al. (1976). Standard molecular weight marker proteins used were phosphorylase b (97.4 kDa), bovine serum albumin (BSA; 66.2 kDa), hen white egg ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa) from Bio-Rad. Native molecular weight of MSP was determined using Sephadex G-200 column chromatography according to the method described by Andrews (1964). Standard molecular weight markers were β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) from Sigma.

Isoelectric focusing of MSP was performed on 5% polyacrylamide gel, according to the procedure described by Righetti and Drysdale (1976), using ampholites in the pH range of 3-10 from Sigma.

Preparation of antibody and western blotting

Five hundred μl of purified MSP (1000 μg/ml) was mixed with an equal volume of Freund's complete adjuvant and injected into a rabbit subcutaneously. Injections were given every other day for the first week, and a fourth injection was made 2 weeks later. Freund's incomplete adjuvant (0.5 ml) and the purified MSP (0.5 ml) were thoroughly mixed and used for a booster injection 2 weeks after the fourth injection. Blood was collected 1 week after the last injection and centrifuged at 10,000 g for 10 min. The supernatant antiserum was stored at -70℃ until used.

Western blotting was used to detect MSP on SDS polyacrylamide gels. Samples were electrophoresed on a 15% SDS gel. After SDS-PAGE, samples were transfered to a nitrocellulose sheet in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 100 V for 2 h (Towbin et al., 1979). After transferring, the nitrocellulose sheet was equilibrated in TBS (Tris buffered saline; 20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min and incubated in blocking solution (3% gelatin in TBS) for 1 h. The sheet was then washed twice for 10 min with TTBS (0.05% Tween 20 in TBS) and incubated for 1 h in a solution containing 500-fold diluted anti-MSP serum. The sheet was again washed twice with TTBS and then incubated for 1 h in a solution containing 5000-fold diluted secondary antibody (GAR-HRP conjugated IgG). After incubation and washing twice with TTBS and once with TBS, the sheet was submerged in HRP color development solution (60 mg color development reagent, 4-chloro-1-naphthol in 20 mM ice-cold methanol, 0.015% H_2O_2 in 100 ml TBS) for development of the purple color.

Results

Identification and purification of MSP

Hemolymph from larvae, adult male and female was electrophoresed on a 15% SDS gel. One band is absent in larval and adult female hemolymph but present in adult male hemolymph (Fig. 1). This protein is designated as male specific protein (MSP) because the protein exists in large amounts only in

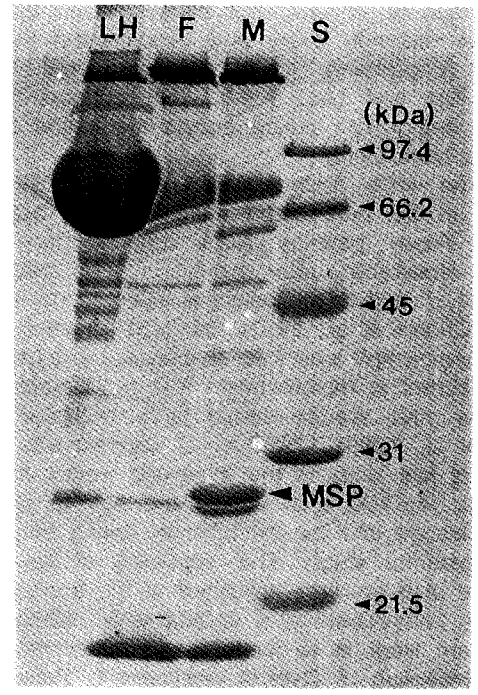

Fig. 1. Identification of male specific protein (MSP) from hemolymph of *Galleria mellonella* L. by electrophoresis. A. SDS-PAGE, S, molecular weight standard markers [phosphorylase b (97.4 kDa); BSA (66.2 kDa); ovalbumin (45 kDa); carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa)]; LH, last instar larvae; F, adult female; M, adult male.

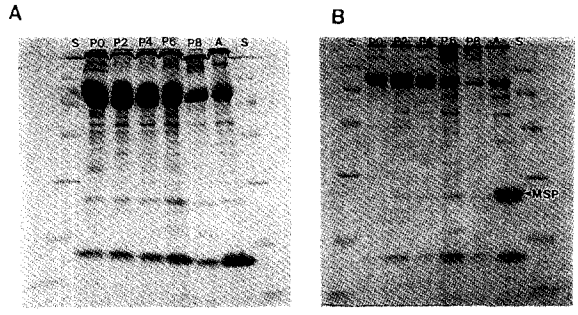

Fig. 2. Stages and sex specificity of MSP by SDS-PAGE electrophoresis. (A) Female hemolymph. (B) Male hemolymph. S, molecular weight standard markers, P0, P2, P4, P6, P8, 0, 2, 4, 6, 8-day-old pupae; A, adult.

adult male hemolymph. Also, male and female hemolymph was electrophoresed during pupal development (Fig. 2). The MSP is present only in adult male hemolymph.

To purify the MSP, dialyzed adult male hemolymph was subjected to anion exchange chromatography using DEAE-cellulose (DE-52) (Fig. 3A). Fractions in bound and unbound protein peaks were separately collected and applied to SDS PAGE. The fraction numbers 12 to 18 of unbound protein peak contain only MSP (Fig. 3B). Purified MSP was submitted to SDS and Non-SDS PAGE, showing that MSP is completely purified (Fig. 4A and C). Also, western blot analysis with antibody against MSP showed a single band (Fig. 4B).

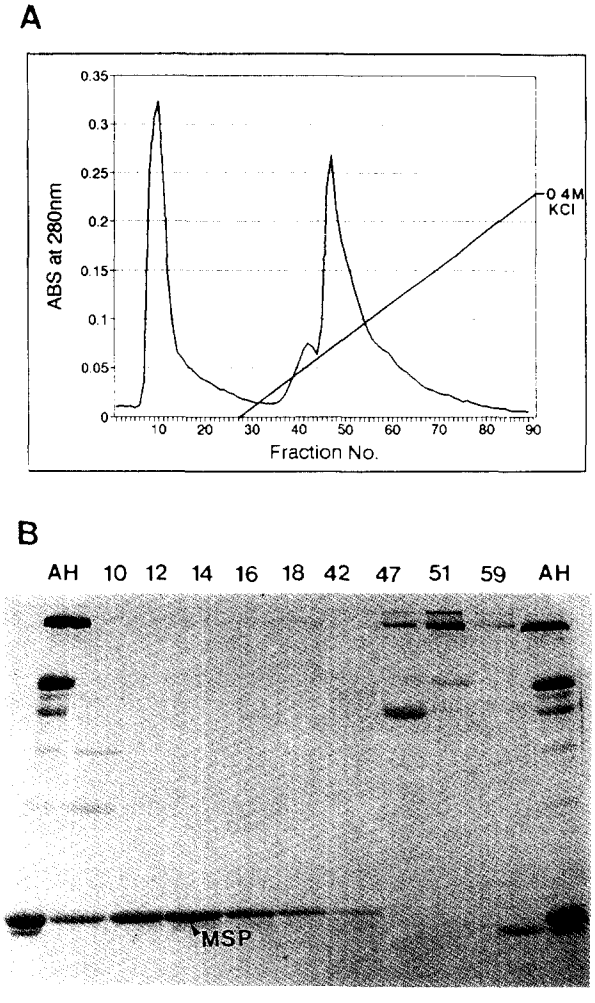

Fig. 3. Purification of MSP by Ion exchange chromatography. (A) Chromatogram obtained by a DE-52 column chromatography of adult male hemolymph. Tris-HCl buffer (0.05 M Tris, 1mM EDTA, pH 7.8) was used as elution buffer. (B) SDS-PAGE of column fractions. AH, adult male hemolymph; Numbers indicate fractions of DE-52 column chromatography.

Characterization of MSP

Molecular weight of MSP was determined to be approximately 55 kDa by gel filtration and the subunit was estimated to be 27 kDa as determined by SDS-PAGE (data not shown). Also, the isoelectric point was estimated to be 5.8 (data not shown).

SDS-PAGE and Western blotting were conducted with hemolymph from white pupae (1 day-old-pupae) to adult to see the appearing stage of MSP in the male insect. MSP is absent in the hemolymph of white

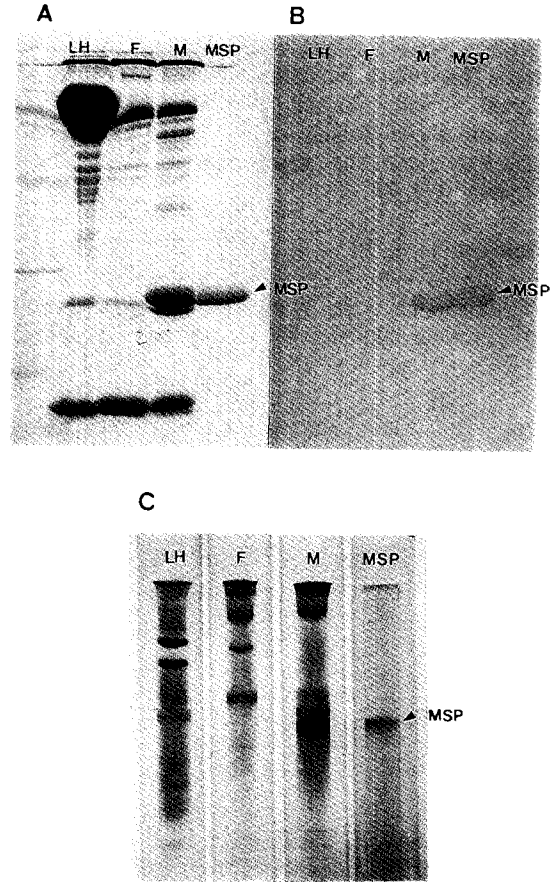

Fig. 4. SDS-PAGE (A), Western blot (B), and native PAGE (C) of purified MSP. LH, last larval hemolymph; F, female adult hemolymph; M, male adult hemolymph; MSP, purified MSP.

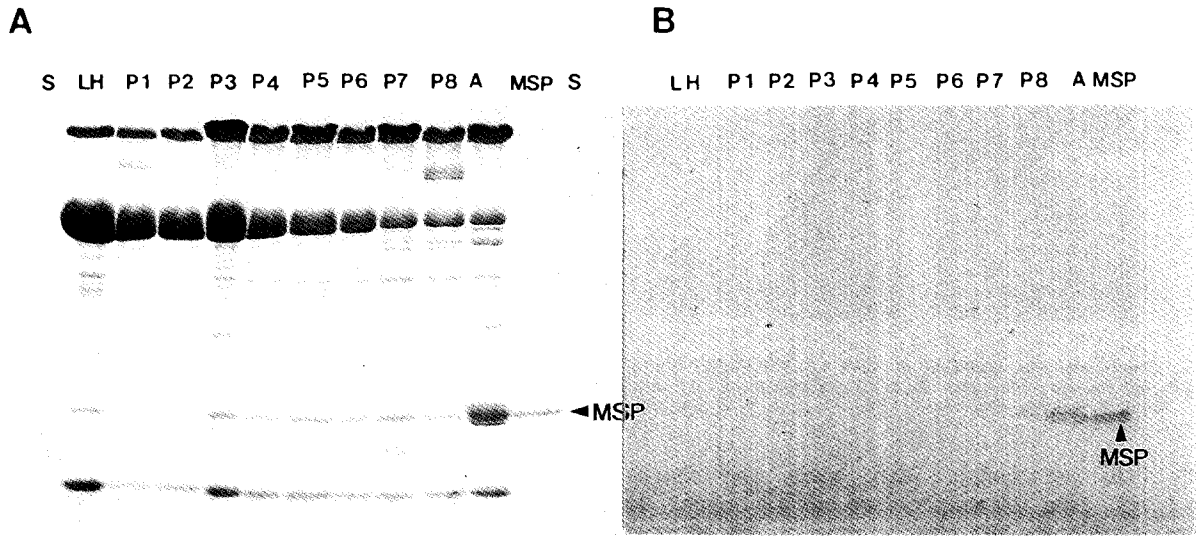

Fig. 5. SDS-PAGE (A) and Western blot (B) of pupal hemolymph proteins of male insects. S, molecular weight standard markers; LH, last instar larvae; P1, P2, P3, P4, P5, P6, P7, P8, 1, 2, 3, 4, 5, 6, 7, 8-day-old pupae; A, adult; MSP, purified MSP.

pupae to day 7 male pupae but appears from day 8 pupae to adult with increasing amounts (Fig. 5B).

To examine the presence of MSP in various organs, various organs of larvae and adult male hemolymph were applied to SDS-PAGE and Western blotting. The result indicates that MSP is absent in all larval organs tested (Fig. 6). MSP was also absent in pupal testis, ovary and laid eggs but present the pupal and adult male fat body and also adult testis (Fig. 7).

Discussion

MSPs in insect were known to be low molecular weight proteins synthesized mostly by the accessory gland and involved in spermatogenesis (Jans et al., 1984; Friedländer, 1989) or to control female receptivity and stimulate ovulation during the reproduction process (Chen and Balmer., 1989; Ohashi et al., 1991). Protein (170 kDa) synthesized by the accessory gland of *Rhodnius prolixus* was found in the hemolymph (Balker and Davey, 1982; Sevala and Davey, 1991). On the while, MSSP (male specific serum proteins) in the hemolymph of *C. capitata* is different from other MSPs in that they are synthesized in fat body (Katsoris et al., 1990). In the present work with *G. mellonella*, hemolymph protein of larvae and adult male and female were compared by electrophoresis, showing the presence of MSP. The MSP of *G. mellonella* was purified and their charac-

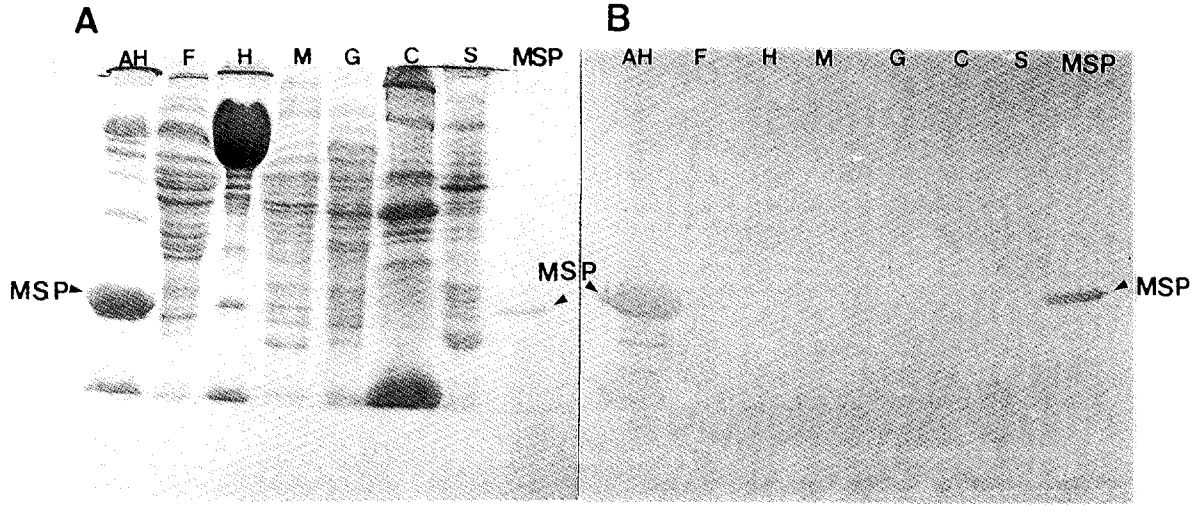

Fig. 6. SDS-PAGE (A) and Western blot (B) of MSP in several organs from last instar larvae. AH, adult male hemolymph; F, fat body; H, hemolymph; M, malphighian tubule; G, gut; C, cuticle; S, silk gland; MSP, purified MSP.

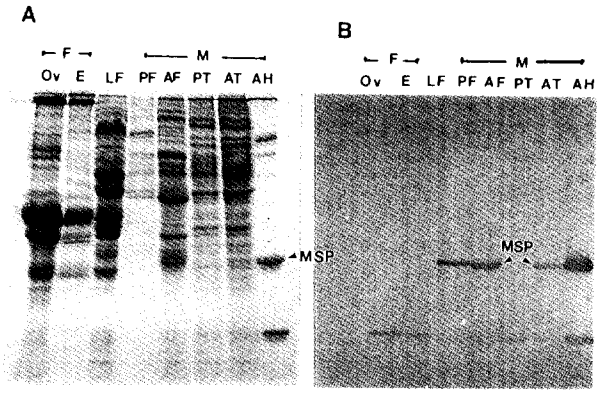

Fig. 7. SDS-PAGE (A) and Western blot (B) of several organs from male and female insects. F, female; M, male; Ov, ovary; E, egg; LF, larval fat body; PF, pupal fat body; AF, adult fat body; PT, pupal testis; AT, adult testis; AH, adult hemolymph.

teristics were examined.

MSP of *G. mellonella* has native molecular weight of 55 kDa and is composed of one subunit which has the molecular weight of 27 kDa. This is a little larger than that of *C. capitata* (Katsoris et al., 1990). Isoelectric point of *G. mellonella* MSP was determined to be approximately 5.8. Also, *G. mellonella* MSP appears as of one kind in the hemolymph during the period of day 8 pupae to adult, whereas MSSP of *C. capitata* is present as five kinds only in the hemolymph of adult male. Especially MSP of *G. mellonella* continues to increase as time goes by after emergence (data not shown).

The antibody against MSP of *G. mellonella* was made and reacted with proteins from other organs. The result is that the MSP of *G. mellonella* is absent in various organs of larvae and adult female but present in fat body of pupae and adult male. MSP of *G. mellonella* is also present in adult testis. When the time of appearance in hemolymph, fat body, and testis were compared, MSP of *G. mellonella* is suggested to be synthesized in pupal fat body and released into the hemolymph and taken up in part into the adult testis.

The peculiar thing is that antibody against MSP of *G. mellonella* reacts with a 17 kDa protein constantly present in the hemolymph of all stages in addition to MSP, indicating that MSP of *G. mellonella* has a partial immunological homology with the 17 kDa hemolymph protein. There was little known about transport process and physiological function of MSP since fat body-origin MSP was first reported in *C. capitata*. Based on the fact that MSP of *G. mellonella* is detected in testis, hemolymph MSP is expected to play some role in spermatogenesis or some other function in testis during adult stage. Detailed role of *G. mellonella* MSP is now being examined in our laboratory.

Acknowledgements

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