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Ontogeny and Characterization of Major Haemolymph Protein (MHP) in *Helicoverpa assulta*

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A persistent major haemolymph protein (MHP) was confirmed, and its ontogeny and physicochemical characteristics were investigated in *Helicoverpa assulta*.

The MHP existed continually during larval-pupal-adult development, and its ontogeny was similar to that of total haemolymph protein concentration during development. Its content increased with larval growth, and kept to high level during pupal-adult development except for temporary decrease at the early pupal and adult stages. The MHP was purified by ammonium sulfate precipitation, gel filtration and ion exchange chromatography. The purified MHP was determined to be hexamer glycolipoprotein (pI 5.9, M.W. 414kDa) consisted of single type subunit (69kDa). Amino acid analysis suggested that the MHP contained a relatively high content of aromatic amino acids (18.27 mole % of tryptophan, 7.47 mole % of tyrosine and 6.51 mole % of phenylalanine) compared to storage proteins from other insects. Immunodiffusion test and electrophoretic analysis of the organ proteins (gut, fat body, and Malphigian tubule) suggested that the major haemolymph protein was present in the fat body.

KEY WORDS: Major Haemolymph Protein (MHP), Glycolipoprotein, Hexamer, *Helicoverpa assulta*

In insects, the major haemolymph proteins (also named as storage proteins) accumulate in high amounts in the haemolymph during development. These proteins are synthesised in the fat body of late larval stage and released into the haemolymph (Seong *et al.*, 1985; Kim *et al.*, 1989; Telfer and Kunkel, 1991; Faria *et al.*, 1994). Once the insect ceases feeding and prepares to pupate, these proteins are taken up into the fat body where they are stored in dense protein granules (Tojo *et al.*, 1980; Roberts and Brock, 1981; Wang and Haunerland, 1991).

Most MHPs or SPs have native molecular weights of nearly 400~500kDa, and are always composed of six subunits between 70,000 and 85,000 daltons, but the amino acid composition varies widely among different classes of these proteins. Nevertheless, all holo- metabolous insect species seem to possess at least one major storage protein that is rich in aromatic amino acids(Telfer *et al.*, 1983; Levenbook, 1985; Lenz *et al.*, 1987; Kim *et al.*, 1989; Martinez *et al.*, 1993; Yokoyama *et al.*, 1993).

The physiological roles suggested for these proteins include: amino acid storage for protein synthesis during metamorphosis (Wyatt and Pan, 1978; Levenbook, 1985; Telfer and Kunkel, 1991), participation in the formation of pupal and adult cuticle (Kaliafas *et al.*, 1984; Konig *et al.*, 1986; Palli and Locke, 1987), and serum

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transport of ecdysteroids or insecticide (Haunerland and Bowers, 1986; Chrysanthis *et al.*, 1994).

In the present study, we named the major haemolymph protein (MHP) because of it's abundant distribution in the haemolymph during development, and we describe the developmental profiles and characterization of the major haemolymph protein (MHP) in *Helicoverpa assulta*.

Materials and Methods

Animals

Helicoverpa assulta was fed on an artificial diet (Yoo *et al.*, 1994), and reared at 27°C±1°C, 65±5% r.h., and 16L:18D photoperiod. The sexes were not distinguished because no difference of MHP was observed in preliminary tests.

Preparation of haemolymph and tissue extracts

Haemolymph was bled by cutting foreleg or piercing with a fine needle. The experiments were carried out at the following developmental stages; third, fourth and fifth instar larvae, 1,3,5 and 7-day old pupae, and 1,3 and 5-day old adults. A few crystals of phenylthiourea (PTU) were added to the haemolymph to prevent melanization. The haemolymph was centrifuged at 10,000 rpm for 20min.

Whole body and tissue extracts from 3-day old last instar larvae were obtained by homogenizing in 50 mM Tris-HCl (pH 7.0) containing a few crystal of PTU. The homogenates were centrifuged for 20 min at 10,000 rpm, then the supernatants were stored at 70°C until use.

Protein determination

Protein contents were determined by the method of Lowry *et al.* (1951) with slight modifications. Bovine serum albumin (BSA) was employed as a standard protein.

Protein purification

All procedures were carried out at 4°C. Haemolymph from 3-day old last instar larvae was precipitated into five ammonium sulfate ($(NH_4)_2SO_4$) fractions (0~25%, 25~40%, 40~55%, 55~70%, 70~85%), respectively. The first ammonium sulfate fraction (0~25%) containing MHP was applied to a DEAE-cellulose column (2×20 cm) equilibrated with 0.01M sodium phosphate buffer (pH 7.0) and eluted at a flow rate of 0.5 ml/min with a linear gradient of sodium chloride (0~0.5 M). The fractions(fraction No. 37~42) containing MHP was collected and loaded on Ultrogel A6 column (1.6×90 cm) to elute at a flow rate of 0.3 ml/min with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M sodium chloride. The fractions (fraction No. 96~107) containing the MHP were subjected to gel filtration with a Sephadex G-100 column (1.6×70 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). The column was developed with the same buffer at a flow rate of 0.25ml/min, and MHP containing fractions (No. 29~35) were collected. The resulting protein was dialyzed against 50 mM Tris-HCl buffer (pH 8.0), and then freeze dried.

Native-PAGE

Native polyacrylaminde gel electrophoresis was carried out on 7% acrylamide gel at 4°C. Electrophoresis was performed in 1.2 mM Tris-HCl (pH 8.6), for 1hr at 10 mA, then 17hrs at 20 mA. After electrophoresis, the gels were fixed and washed overnight in 12% (W/V) trichloroacetic acid, stained for 4hrs in 0.2% (W/V) Coomassie brilliant blue R-250 in EtOH:acetic acid:D.W. (45:10:45, V/V/V), then destained in EtOH:acetic acid:D.W. (15:7:78, V/V/V).

Molecular weight of the native MHP was determined as described by Hedrick and Smith (1968). Standard proteins [albumin (M.W. 67,000), lactate dehydrogenase (M.W. 140,000), catalase (M.W. 232,000), ferritin (M.W. 440,000), and thyroglobulin (M.W. 669,000)] and MHP were run on 5-9% polyacrylamide gels, and the mobility of each protein relative to the dye front was plotted versus acrylamide gel concentrations. The molecular weights of the standard proteins and the slope of the lines are noted on the above plot.

SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) with slight modifications. The protein samples were denatured for 4 min in 0.5 mM Tris-HCl buffer (pH 6.8) containing 1.25% β-mercaptoethanol, 1% SDS at boiling water bath. Gel staining and destaining were performed by the same manner as in native-PAGE.

Molecular weight of MHP subunits were measured by Weber and Osborn (1969) with some modifications. Standard proteins were phosphorylase (M.W. 94,000), albumin (M.W. 67,000), ovalbumin (M.W. 43,000), carbonic anhydrase (M.W. 30,000), and trypsin inhibitor (M.W. 20,000).

Detection of conjugated proteins

Glycoproteins were identified by the method of Caldwell and Pigman (1965). After electrophoresis, the gels were incubated for 1hr at room temperature in 7.5% acetic acid, then for 1hr in 0.2% periodic acid at 4°C. They were subsequently transferred to Schiff's reagent after being rinsed in 15% acetic acid.

For the detection of lipoproteins, the method of Chippendale and Beck (1966) was used. Excess stain was removed after 10hrs in 70% ethanol.

Isoelectric focusing (IEF)

Isoelectric focusing was performed as described by O'Farrell (1975) with some modifications. Glass tubes (120 mm long, i.d. 3 mm) were filled to 90 mm with a mixture containing polyacrylamide (5.5%) and ampholyte (2%). After electrofocusing, gels were equilibrated for 30 min in 2.5 ml, 0.1 M Tris-HCl (pH 6.8) containing 20% glycerine, then applied to the second dimension for electrophoresis, immediately or after storage at −20°C. The pH gradient of IEF gels was determined by slicing one gel into 5 mm segments and incubating with 1 ml of distilled water for 24hrs at room temperature.

Amino acid analysis

MHP was hydrolyzed in 1 ml, 6N HCl for 16hrs at 110°C. The hydrolysate was analyzed on HPLC system (TOSOH 8010 series).

Preparation of the antiserum

Purified MHP (50 μg/ml) was mixed with an equal volume of Freund's complement adjuvant (0.5 ml), and injected subcutaneously into the rabbit. Three injections at 1-week intervals were made and a booster injection was given 1 week after the final injection. Blood was collected 1 week later and serum was stored at −70°C until use.

Immunodiffusion

Immunodiffusion test was conducted on 1% agarose gel in 10 mM veronal buffer (pH 8.6) containing 0.1% sodium azide for 2 days at room terperature as described by Ouchterlony (1949).

Results

Quantitative and qualitative changes of haemolymph proteins were analyzed by means of

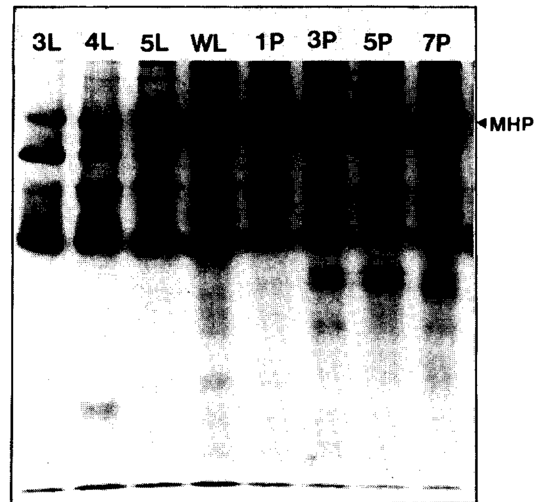

Fig. 1. Native-PAGE (7% gel) of haemolymph protein during larval- pupal development of *Helicoverpa assulta*. 15 μl of haemolymph was applied to each lane. 3L, 3-day old third instar larvae; 4L, 3-day old fourth instar larvae; 5L, 3-day old fifth instar larvae; WL, wandering larvae; 1P, 1-day old pupae; 3P, 3-day old pupae; 5P, 5-day old pupae; 7P, 7-day old pupae; MHP, major haemolymph protein.

Lowry method and native-PAGE to show changes in MHP during larval-pupal-adults development (Table 1, Fig. 1, 2).

These results clearly demonstrated the presence of persistent major haemolymph protein (MHP) in *H. assulta* which occurs as a major protein through 3-day old third instar larvae until 5-day old adults, and decrease when approaching to larval-pupal and pupal-adult metamorphosis periods (Fig. 1, 2).

MHP was obtained in the 0~40% $(NH_4)_2SO_4$ fraction in relatively high purity (Fig. 3), providing an alternative simple way for isolating this protein. The 0~25% $(NH_4)_2SO_4$ fraction was further purified by ion exchange chromatography (DEAE cellulose column) and gel fitration (Ultorgel A6 and Sephadex G-100 column). The purify of the MHP fractions after each purification step was assayed by native-polyacrylamide gel electrophoresis (not shown).

The MHP was found to have a native molecular weight of 414 kDa by electrophoretic analysis (Fig. 7, 8), and hexamers composed of single type subunit with a molecular weight of 69,000 daltons when determined with SDS-PAGE (Fig. 9, 10). Purified MHP was isoelectric focused on 5.5% polyacrylamide gel, and isoelectric points of the

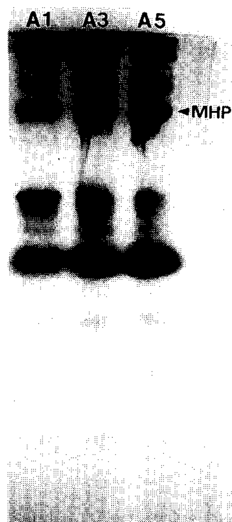

Fig. 2. Native-PAGE (7% gel) of haemolymph protein of adult *Helicoverpa assulta*. 15 μl of haemolymph was applied to each lane. A1, 1-day old adults; A3, 3-day old adults; A5, 5-day old adults; MHP, major haemolymph protein.

Table 1. Concentration of haemolymph protein during development of *Helicoverpa assulta*

	Developmental stages	Haemolymph protein (mg/ml ± SD)*
Larvae	3-day old third instar	35.2±1.1
	3-day old fourth instar	46.3±1.7
	3-day old fifth (last) instar	85.7±1.4
	6-day old fifth (wandering) instar	97.6±2.3
Pupae	1-day old	84.4±1.9
	3-day old	90.2±2.5
	5-day old	100.7±1.6
	7-day old	101.3±3.2
Adults	1-day old	88.0±3.6
	3-day old	94.4±2.1
	5-day old	82.5±3.0

* Each value represents the mean ± SD obtained from three replicate experiments.

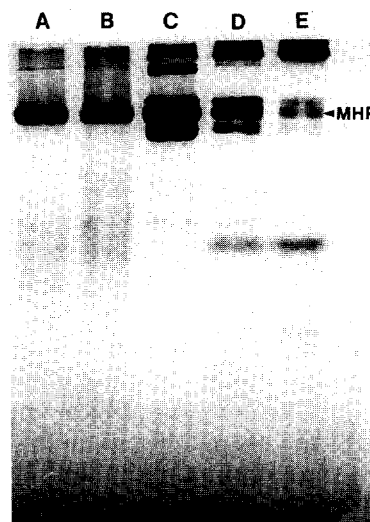

Fig. 3. Native-PAGE of haemolymph protein at 3-day old last instar larvae in *Helicoverpa assulta*. The haemolymph protein was precipitated into five ammonium sulfate $[(NH_4)_2SO_4]$ fractions. A, 0~25% $(NH_4)_2SO_4$; B, 25~40% $(NH_4)_2SO_4$; C, 40~55% $(NH_4)_2SO_4$; D, 55~70% $(NH_4)_2SO_4$; E, 70~85% $(NH_4)_2SO_4$.

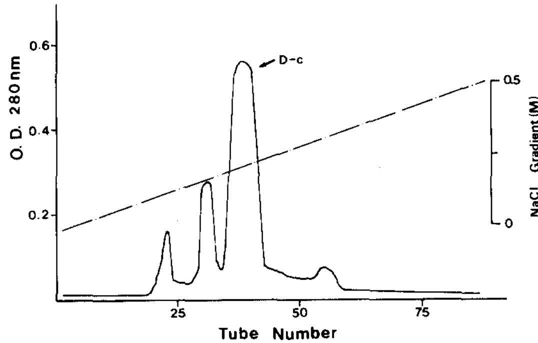

Fig. 4. Gel filtration of fraction No. 96-107 (secondary peak) collected by gel filtration (Ultrogel A6) chromatography. The fraction (No. 96-107) containing MHP was applied to a Sephadex G-100 column (1.6 × 70 cm), and then eluted at a flow rate 0.25 ml/min with 0.01M sodium phosphate buffer (pH 7.0). The bar indicates the area (fraction No. 29~35) in which a single band of MHP was detected by native-PAGE (Fig. 7).

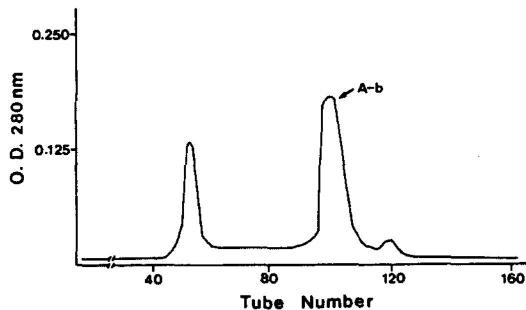

Fig. 5. Native-PAGE (7% gel) of purified major haemolymph protein. HA, haemolymph protein of last instar larvae; MHP, major haemolymph protein.

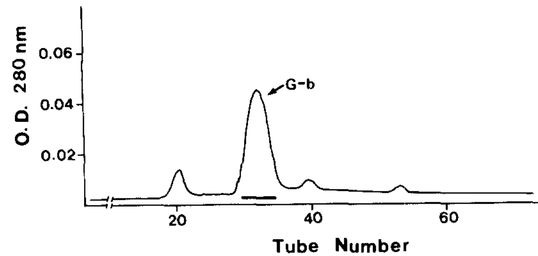

Fig. 6. Determination of molecular weight for native MHP according to the method of Hedrick and Smith (1968). Standard proteins used are as follows: A, lactate dehydrogenase (M.W. 140,000); B, catalase (M.W. 232,000); C, ferritin (M.W. 440,000); D, thyroglobulin (M.W. 669,000).

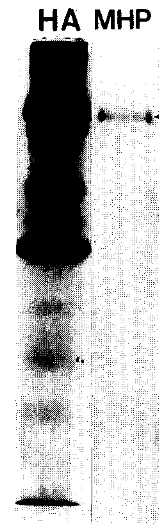

Fig. 7. Native-PAGE (7% gel) of purified major haemolymph protein. HA, haemolymph protein of last instar larvae; MHP, major haemolymph protein.

MHP was determined to be pH 5.9 (Fig. 9). The MHP of *H. assulta* was found to be glycolipoprotein (Fig. 12).

The amino acid composition of MHP is shown in Table 2. As can be seen from this table, the relatively high content of tryptophan, tyrosine and phenylalanine but low content of methionine and cysteine was observed.

As shown in Fig. 13, the MHP was detected in the fat body while not detected in the gut and Malphigian tubule by electrophoretic analysis. In Ouchterlony's immunodiffusion test, anti-MHP showed positive reaction with fat body, but it did not react with gut and Malphigian tubule from the 3-day old last instar larvae (Fig. 14).

Discussion

The MHP existed continually during larval-pupal-adult life cycles, and its ontogeny was similar to that of total haemolymph protein concentration during development (Table 1, Fig. 1, 2). Its content increased with the larval growth, and kept

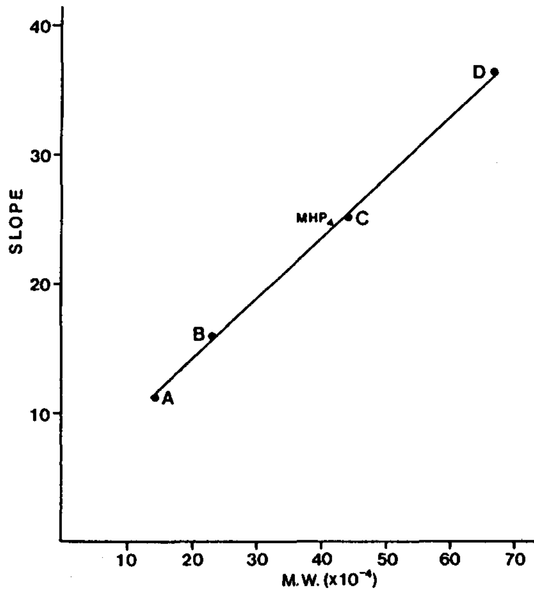

Fig. 8. Determination of molecular weight for native MHP according to the method of Hedrick and Smith (1968). Standard proteins used are as follows: A, lactate dehydrogenase (M.W. 140,000); B, catalase (M.W. 232,000); C, ferritin (M.W. 440,000); D, thyroglobulin (M.W. 669,000).

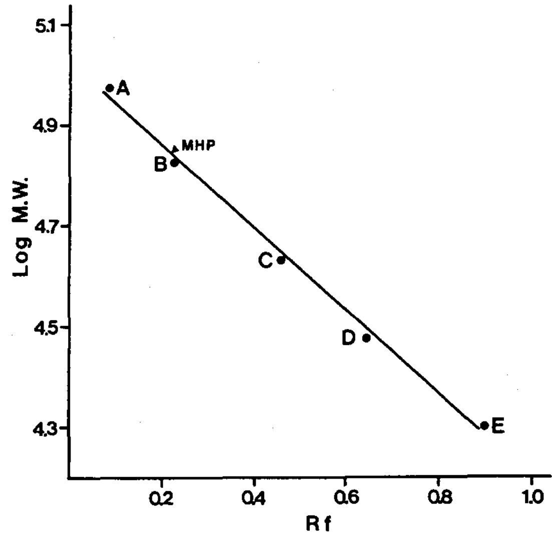

Fig. 10. Determination of molecular weight for subunit MHP by SDS-PAGE according to the method of Weber and Osborn (1969). Standard proteins used are as follows: A, phosphorylase (M.W. 94,000); B, albumin (M.W. 67,000); C, ovalbumin (M.W. 43,000); D, carbonic anhydrase (M.W. 30,000); E, trypsin inhibitor (M.W. 20,000).

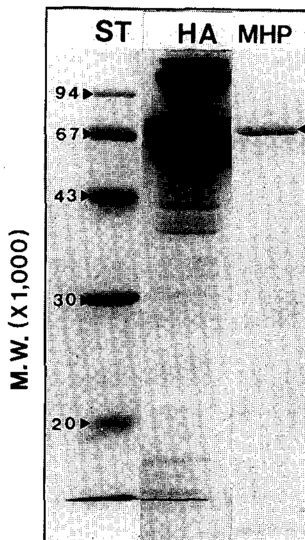

Fig. 9. SDS-PAGE (13% gel) of purified major haemolymph protein. HA, haemolymph protein of last instar larvae; MHP, major haemolymph protein; ST, standard proteins.

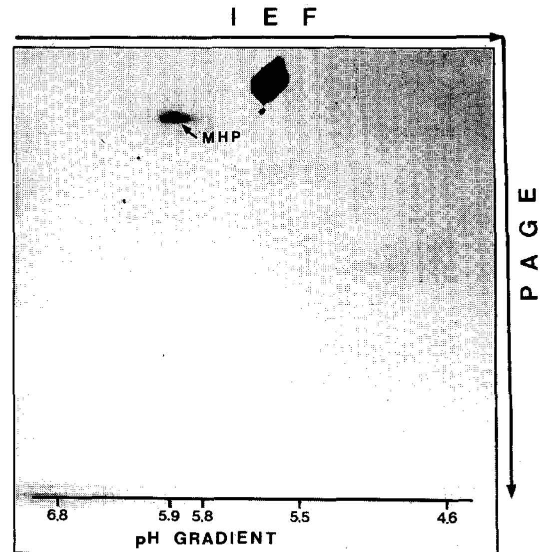

Fig. 11. Two-dimensional native-PAGE (7% gel) of the purified MHP.

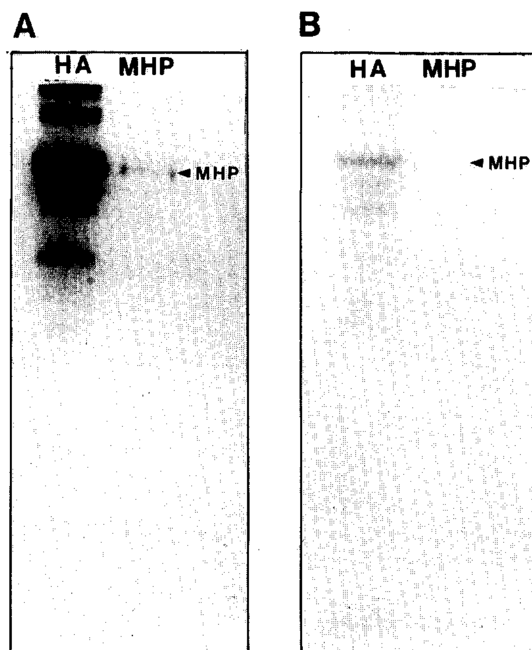

Fig. 12. Native-PAGE (7% gel) of total haemolymph protein and purified MHP. The gels were stained with periodic acid Schiff's reagent (A) and Sudan black B (B). HA, haemolymph protein of last instar larvae; MHP, major haemolymph protein.

to high level during pupal-adult development except temporary decrease at the early pupal and adult stages. Similar development profiles have been observed in the haemolymph levels of the MHPs of other insects (Levenbook, 1985; Lenz *et al.*, 1987).

Most recently, MHPs and SPs have been found to have molecular weights of 400 to 500 kDa and hexamers composed of subunit with molecular weights of 70,000~80,000 daltons(Roberts and Brock, 1981; Lenz *et al.*, 1987; Yun and Kim, 1990; Faria *et al.*, 1994; Tojo and Yoshiga, 1994). These proteins are known to be consisted of either single type of subunit (Tojo *et al.*, 1978; Robert and Brock, 1981; Telfer and Kunkel, 1991) or two types of subunits (Kramer *et al.*, 1980; Levenbook, 1985). In present work with *H. assulta*, molecular weight of MHP was determined to be 414 kDa by electrophoretic analysis (Fig. 7, 8), and composed of single type subunit with a molecular weight of 69 kDa (Fig. 9, 10). This

Table 2. Amino acid composition of major haemolymph protein (MHP) in *Helivoverpa assulta*

Amino acids	Mole %
Aspartic acid	8.14
Threonine	2.82
Serine + Asparagine	3.40
Glutamic acid + Glutamine	10.66
Proline	5.15
Glycine	4.16
Alanine	7.10
Cysteine	1.84
Valine	3.60
Methionine	1.46
Isoleucine	1.95
Leucine	6.29
Tyrosine	7.47
Phenylalanine	6.51
Histidine	3.10
Lysine	4.70
Tryptophan	18.27
Arginine	3.38
Total	100.00

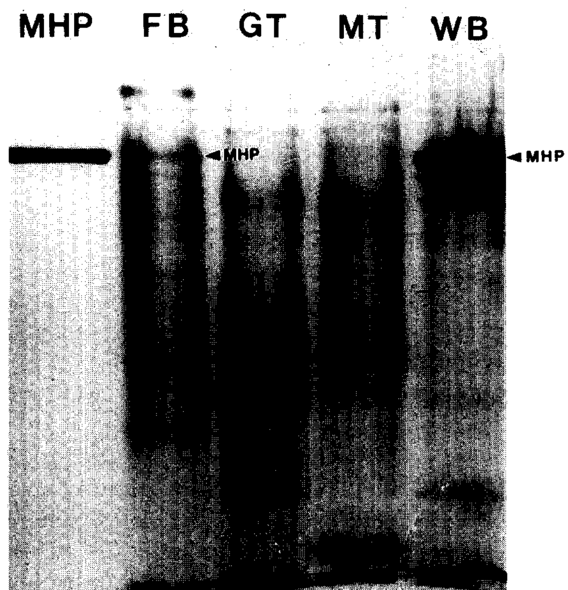

Fig. 13. Native-PAGE (7% gel) of proteins extracted from some organs of the last instar larvae. MHP, major haemolymph protein; FB, fat body; GT, gut; MT, Malphigian tubule; WB, whole body.

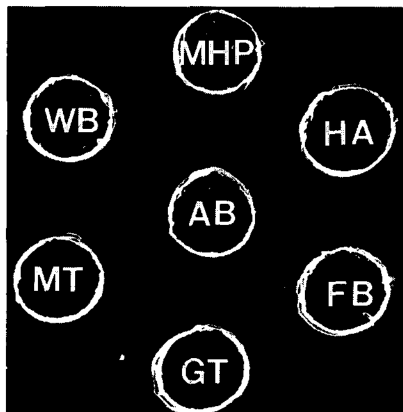

Fig. 14. Immunodiffusion reaction between proteins extracted from some organs extracts and anti-MHP. MHP, major haemolymph protein; FB, fat body; GT, gut; MT, Malphigian tubule; WB, whole body.

value was similar to MHPs of other insects. The pI value of purified MHP was determined to be 5.9 (Fig. 11). Generally, the isoelectric points of these proteins are known to be pH 5.5-5.9 (Lenz *et al.*, 1987; Kim *et al.*, 1989).

The presence of tyrosine and phenylalanine is a common feature of most MHPs and SPs studied today (Mintzas and Rina, 1986; Telfer and Kunkel, 1991). Their amino acids are subsequently used to fuel the synthesis of adult proteins (Levenbook and Bauer, 1984; Levenbook, 1985), although additional roles have been proposed, such as cuticle composition (Konig *et al.*, 1986; Palli and Lock, 1987; Telfer and Kunkel, 1991). In present work *H. assulta*, MHP was contained a relatively high content of aromatic amino acids (tryptophan, tyrosine, phenylalanine) compared to storage proteins from other insects(Mintzas and Rina, 1986; Palli and Lock, 1987).

In the present work, MHP was detected in the fat body by electrophoretic analysis and immunodiffusion test (Fig. 13, 14), and these results suggested that MHP is synthesized by the fat body. In general, SPs and MHPs are synthesized in the fat body during last larval stages, reased into the haemolymph, and then selectively reabsorbed into the fat body after pupation (Izumi *et al.*, 1981; Roberts and Brock, 1981; Palli and Lock, 1987; Martinez and Wheeler, 1993; Faria *et al.*, 1994).

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담배나방(*Helicoverpa assulta*)의 발생중 Major Haemolymph Protein(MHP)의 변화 및 특성

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담배나방(*Helicoverpa assulta*)의 발생기간 중 존재하는 major haemolymph protein(MHP)을 확인하였으며, 그 발생에 따른 변화상 및 물리화학적 특성을 조사하였다. MHP는 유충-용-성충의 발생단계에서 확인되었으며, 그 함량은 유충의 성장에 따라 증가하여 용기와 성충기 동안에도 높게 유지되고 용초기와 성충 초기에서 일시적인 감소를 보여, 총 혈림프 단백질의 농도 변화와 유사한 변화양상을 보였다. Ammonium sulfate precipitation, gel filtration 그리고 ion exchange chromatography를 통해 정제한 MHP의 전기영동 분석 결과, 단일 구성소단위(69 kDa)가 hexamer를 이룬 분자량 414 kDa의 glycolipoprotein(pI 5.9)인 것으로 확인되었다. MHP의 아미노산 조성에 있어서 18.27 mole %의 tryptophan, 7.47 mole %의 tyrosine 그리고 6.51 mole %의 phenylalanine이 검출되어 다른 곤충류의 저장단백질들에 비해 비교적 높은 aromatic amino acid의 함량을 보였다. 지방체, 장, 말피기관 단백질의 전기영동 및 이들 단백질과 anti-MHP간의 immunodiffusion test 결과 MHP는 지방체에 존재하고 있음이 확인되었다.