Purification and Characterization of Arylphorin of the Chinese Oak Silkmoth, *Antheraea pernyi*

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The arylphorin was purified from the pupal haemolymph of the Chinese oak silkmoth, *Antheraea pernyi,* and characterized physiologically and biochemically. The protein was purified by a simple preparative polyacrylamide gel electrophoresis (PAGE) and subsequent diffusive elution. The preparation was shown to be homogeneous by 7.5% native-PAGE. The native molecular weight of arylphorin was 450 kDa with a 80 kDa single subunit, suggesting hexamer. The protein contained high amounts (18.3%) of aromatic amino acids, phenylalanine (9.7%) and tyrosine (8.6%). Therefore, the protein was identified as a kind of a storage protein referred to as an arylphorin. The protein was stained by Schiff's reagent, suggesting a glycoprotein. The protein contained 4.9% (w/w) sugar and mannose and N-acetylglucosamine were major components. Also, degradation of the protein was begun by heat treatment at 90 for 20 minutes. These results showed that the *A. pernyi* arylphorin in the study is hexamer associated with the six subunits consisting of a 80kDa single subunit, and is different from that of Kajiura *et al.* (1998) in the subunit composition.

Introduction

Since the first report of calliphorin as the major haemolymph protein in the pupae of the blowfly, *Calliphora erythrocephala*, by Munn *et al.* (1967), the insect storage proteins have been extensively investigated in hemimetabola, holometabola and non-insect arthropoda (Ghiretti-Magaldi *et al.*, 1966; Carpenter and VanHolde, 1973; Kinnear and Thomson, 1975; Markl *et al.*, 1976; Tojo *et al.*, 1980; deBianchi *et al.*, 1983; Duhamel and Kunkel, 1983; Ryan *et al.*, 1984, 1985a, 1985b, 1986b; Rimoldi *et al.*, 1989; Ancsin and Wyatt 1996; Seo *et al.*, 1998; Cheon *et al.*, 1998; Cheon *et al.*, 2001).

These insect storage proteins can be classified into three categories: arylphorin containing a high content of aromatic amino acids (18 – 26%), methionine-rich storage protein distinguishing it from the arylphorin by a relatively high methionine content (4 – 8%), and other storage proteins (Kanost *et al.*, 1990). Of these classess arylphorin is best-studied storage proteins in many holometabolous insects such as Diptera, Hymenoptera, Lepidoptera (Kanost *et al.*, 1990). Arylphorins are usually associated with hexamers with the molecular weight of around 80 kDa that differ greatly in the subunit composition. Kanost *et al.* (1990) reviewed that lepidopteran insects

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possess two types of arylphorin, e.g. arylphorins with a single subunit and/or with different subunits. Whereas lepidopteran insects such as *Calpodes ethlius* (Palli and Locke, 1987), *Hyalophora cecropia* (Telfer *et al.*, 1983), *Heliothis zea* (Haunerland and Bowers, 1986a, 1986b), *Papilio polyxenes* (Ryan *et al.*, 1986b) have arylphorins with a single subunit type, other lepidopteran insects including *Bombyx mori* (Tojo *et al.*, 1980) and *Manduca sexta* (Ryan *et al.*, 1985a, 1985b) possess arylphorins with two types of subunits that can be separated and identified by SDS-PAGE. Kajirura *et al.* (1998) firstly reported that the arylphorin of the lepidopteran wild silkmoth, *Antheraea pernyi*, was consisted of two types of subunits each weighing 83,000 and 82,000. In the present study, however, we purified and characterized a different *A. pernyi* arylphorin distinguishing it in molecular properties from that of Kajiura *et al.* (1998).

Materials and Methods

Insects

The Chinese wild oak silkmoth, *A. pernyi*, was reared indoors with the fresh leaves of Japanese oak, *Quercus acutissima*, at room temperature under the laboratory condition of a natural photoperiod. *A. pernyi* is a holometabolous lepidopteran wild silkmoth as depicted in Fig. 1. This wild silkmoth changes its external morphology several times during the developmental process from egg to adult. In this process, the names e.g., eggs, larvae, pupae and adults are derived from their morphological characteristics.

Haemolymph collection

The abdominal legs of the above larvae or abdominal parts of the pupae and the adults were injured with sharpened scissors or needles, and the haemolymph bled from the wound was directly collected into the precooled test tube with a few crystals of 1-phenyl-2-thiourea to inhibit phenol oxidase. The haemolymph was centrifuged at $5,000 \times$ g for 15 minutes to remove haemocytes and other debris. The haemolymph prepared was kept at $-70^{\circ}C$ until protein analysis. In some cases, females and males were separately used for determination of sex specificity of the haemolymph protein during developmental and metamorphic stages.

Native- or SDS (Sodium dodecyl sulphate)-PAGE (polyacrylamide gel electrophoresis)

Native-PAGE was carried out in 7.5% acrylamide slab gels with a buffer system containing 0.5 mM Tris-3.8 mM glycine, pH 8.3. Gels were run at 40 mA constant current for 2 hrs. After electrophoresis, proteins in the slab gels were stained overnight with 0.5% Coomassie Brilliant Blue R-250 (Sigma Chemical Co.) in a solution containing methanol, water and acetic acid in a ratio of 5:4:1. SDS-PAGE was performed according to Laemmli (1970) and Weber and Osborn (1969) on 10% polyacrylamide gels containing 0.1% SDS. The proteins in the gel were also stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co.). Molecular weights were estimated with SDS-PAGE size marker (Pharmacia). The proteins were stained using Schiff's reagent for sugar (Zacharius *et al.*, 1969) and Sudan Black B for lipid (Swahn, 1953).

Purification of target protein

The pupal haemolymph proteins of the Chinese wild oak silkmoth were separated on 7.5% non-denaturing polyacrylamide slab gels. The target protein bands on the slab gels recognized by naked eyes using light refraction were cut out without any staining for visualization. The protein bands collected after cutting out the target protein bands were chopped into pieces in 1.5 ml Eppendorf tubes. The preparation was equilibrated overnight at 4°C against 0.5 mM Tris-3.8 mM glycine buffer with pH 8.3. The preparation was centrifuged at $5,000 \times g$ for 15 minutes, and the supernatant was collected. After filtration of the supernatant using Millipore filter with 0.45 pore size, the purity was tested on a 7.5% native polyacrylamide gel. In some cases, the above process was repeated for acquisition of better purity of the protein.

Characterization of physicochemical properties

10 mg of the protein was hydrolyzed with constant boiling of 6 N HCl (Sigma) at 110°C for 24 hrs. The released amino acids were derivatized with phenylisothiocyanate, and derivatized amino acids were separated on PicoTag column (4.6 × 250 mm) according to the instruction from the manufacturer (Waters). Native molecular weight of the protein was determined by gel filtration with HPLC. 50 mM sodium phosphate, pH 6.5 containing 0.1 M ammonium sulfate was used to separate proteins on Shodex KW-804. Flow rate was 0.5 ml/min. Proteins were detected with absorbency at 280 nm. Native standards, obtained from Pharmacia, had the following molecular weights: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa).

Monosaccharide composition analysis

Each 10 mg of the protein was subjected to three conditions of acid hydrolysis. For analysis of amino acids and neutral sugars, the protein was hydrolyzed in 2 M TFA or 6 N HCl at 100°C for 2 – 6 hrs. The hydrolysate was evaporated to dryness using a Speed Vac (Sarvant Instruments
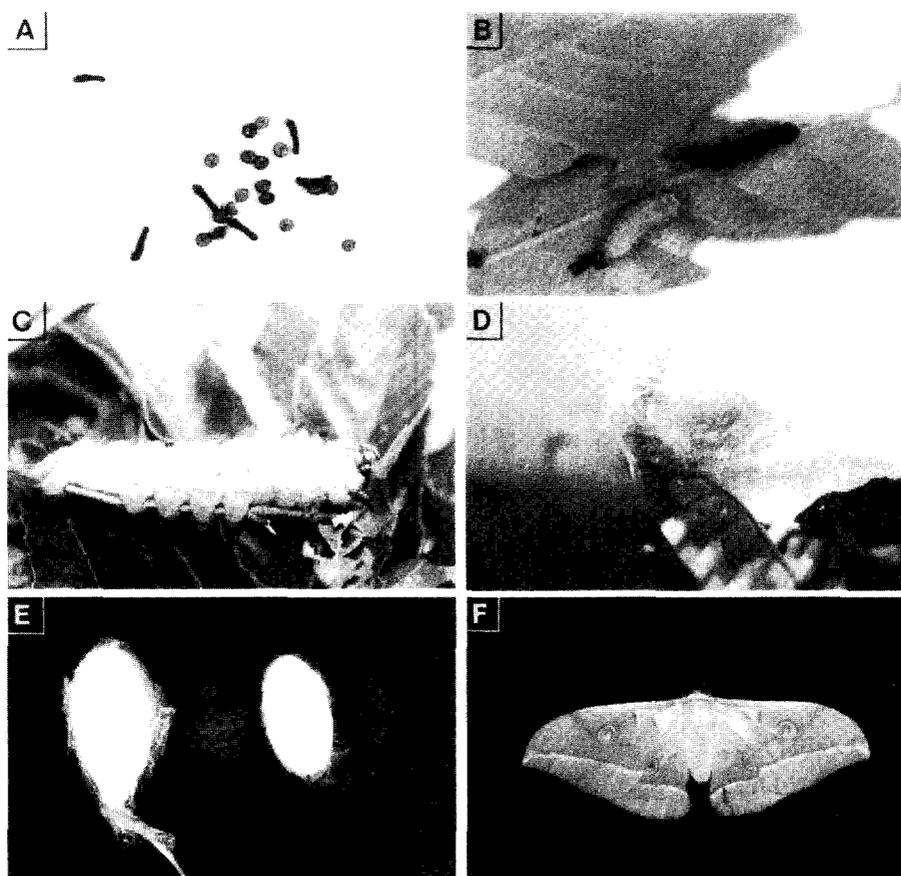
Fig. 1. Life cycle of the Chinese wild oak silkmoth, *A. pernyi*. A, The eggs and newly hatched larvae; B, The 1st instar larvae (black) and newly ecdysed 2nd instar larvae; C, The 5th instar larvae; D, A matured larvae is spining the cocoon; E, Pupae and cocoon; F, Moth.

Inc., Holbrook, NY), resuspended in dH_2O and injected to a Bio-LC DX-300 (Dionex, Sunnyvale, CA) high-pH anion exchange chromatograph (HPAEC) with pulsed amperometric detection. 16 mM NaOH at a flow rate of 1 ml/min was used to separate monosaccharides on a CarboPac PA-1 column (Dionex, 4×250 mm) and a guard column (4×50 mm). Sialic acids were released in a separate, milder hydrolysis condition of 0.1 M HCl for 1 h at 80°C and analyzed with 0.1 M NaOH containing 0.15 M sodium acetate on the same column.

Heat treatment

To test the heat resistance of the pupal major haemolymph protein, the whole haemolymph (typically diluted 5 folds (V/V) in PBS, pH 7.0 at room temperature) of middle-age pupae of the *A. pernyi* was incubated for 20, 40, 60 minutes at every 70, 80, 90, 100°C in a constant temperatured water incubator, respectively. After the treatments of heat resistance, the sample haemolymph was centrifuged at 10,000 rpm for 15 minutes. The supernatant was taken for further successive experiments.

Results

Changes of haemolymph proteins during the development and metamorphosis

The Chinese wild oak silkmoth, *A. pernyi*, is a holometabolous bivoltine insect which undergoes complete metamorphosis (egg → larva → pupa → moth), and pass the winter in the pupal stage (Fig. 1). It takes about 50 to 70 days in outdoor rearing from newly hatched larvae to molt into adults. During this development and metamorphosis, the haemolymph proteins of the Chinese wild oak silkworm were analysed by native-PAGE and SDS-PAGE (Fig. 2 and Fig. 3).

The electrophoretic bands patterns of the haemolymph can be grouped into three distinct patterns: first, young larval haemolymph protein pattern from the 1st instar to the 4th instar (P-1, Pattern 1), second, grown larval haemolymph protein pattern from the 5th larval instar to pupal stage (P-2, Pattern 2), and third, adult haemolymph protein pattern (P-3, Pattern 3).

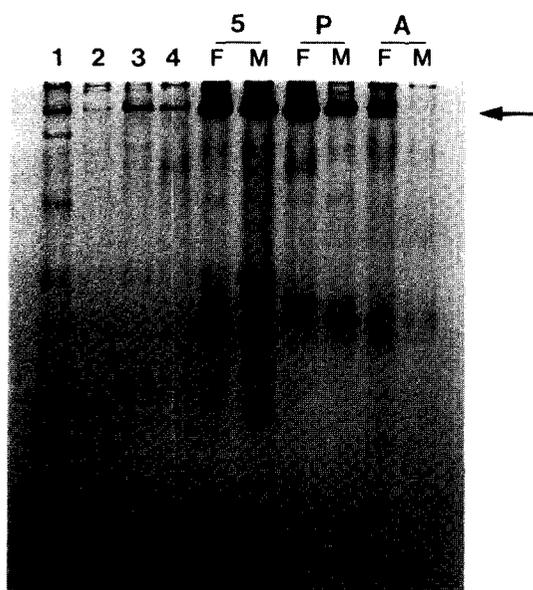Many protein bands were detected from the larval,

Fig. 2. Developmental visualization of haemolymph proteins in the Chinese wild oak silkmoth, *A. pernyi*, by 7.5% native-PAGE stained with Coomasie Brilliant Blue R-250. The arrow shows the developmental occurrence of *A. pernyi* arylphorin during the life cycle of the wild silkmoth. Lane 1, 1st instar larvae; Lane 2, 2nd instar larvae; Lane 3, 3rd instar larvae; Lane 4, 4th instar larvae; Lane 5, 5th instar larvae; P, Pupae; A, Adult; F, Female; M, Male.

Fig. 3. Developmental visualization of haemolymph proteins in the Chinese wild oak silkmoth, *A. pernyi*, by 10% SDS-PAGE stained with Coomasie Brilliant Blue R-250. The arrow shows the developmental occurrence of the putative subunits of *A. pernyi* arylphorin during the life cycle of the wild silkmoth. Lane 1, 1st instar larvae; Lane 2, 2nd instar larvae; Lane 3, 3rd instar larvae; Lane 4, 4th instar larvae; Lane 5, 5th instar larvae; P, Pupae; A, Adult; F, Female; M, Male; Ma, Marker proteins denoted in kDa.
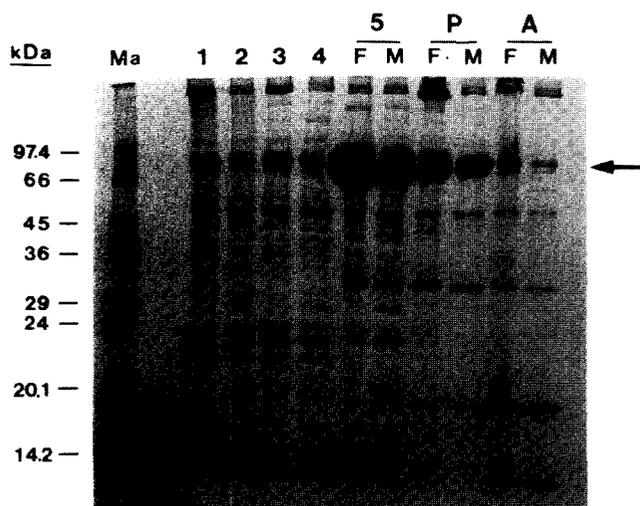
pupal and adult stages in the Chinese wild oak silkmoth. A constantly and densely present protein band (indicated by arrow) with a very slow electrophoretic mobility during all the developmental and metamorphic stages from the 1st instar larvae to adult excluding the male adult was prominantly detected. The concentration of this haemolymph protein is very high throughout the period from the 5th instar larvae to the pupal stage when it was compared with each other by the density and width of the corresponding bands, although not observed in the haemolymph of male adult in the moth stage (Fig. 2). So, the concentration of the protein in the haemolymph remained rather highly constant throughout this developmental period. Accordingly, our increasing interest was concentrated on this haemolymph protein, and the protein was named *A. pernyi* arylphorin by the molecular characterization in the present study.

Also, several protein bands other than the arylphorin (arrow in Fig. 2) were also observed in stage-dependent changing patterns throughout the developmental and metamorphic stages. In addition, relatively fast moving protein bands and medium moving protein bands were very distinctive in stage-dependent manner. In male adult of Fig. 2, however, the arylphorin band was not observed obviously. In SDS-PAGE (Fig. 3), the bands around 80 kDa proteins which was putatively considered as the subunits of the arylphorin by the band density were very prominant during the developmental period from the 5th larval to pupal stages. The electrophoretic bands patterns of the haemolymph in SDS-PAGE can also be grouped in the same manner as described in Fig. 2.

Purification of *A. pernyi* arylphorin

In the study of insect plasma proteins, the importance of protein purification was strongly stressed along with immunological identification, camparing and quantitating haemolymph proteins (Wyatt and Pan, 1978). In recent years, molecular biological studies such as gene cloning, analysis of gene structure and recombinant-DNA methods etc. have been considered essential for deeper research of the target or interesting protein to the researchers. Therefore the purification of the target protein is the first step in the corresponding investigation. There are many methods including gel filtration, ion-exchange chromatography, affinity chromatography, and high performance liquid chromatography, electrophorectic methods by one dimensional gel electrophoresis or 2-D electrophoresis, and isolectric focusing, *etc.* for protein purification. Here, we chose the method of one dimensional gel electrophoresis and its subsequent elution of protein from gel for the purification of the target protein. The arylphorin was purified from the haemolymph in the pupal stage of the Chinese
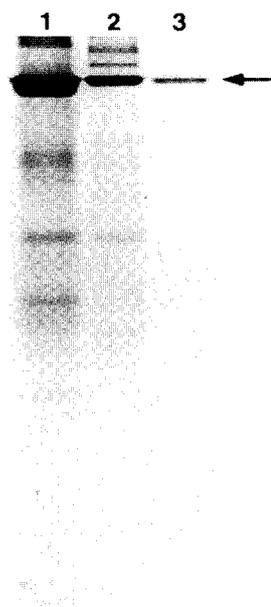
Fig. 4. Purification of *A. pernyi* arylphorin from the whole haemolymph of male pupae collected at the pupal stage of the Chinese wild oak silkmoth *A. pernyi*. Lane 1, Female pupae; Lane 2, Male pupae; Lane 3, The AMHP purified (arrow).
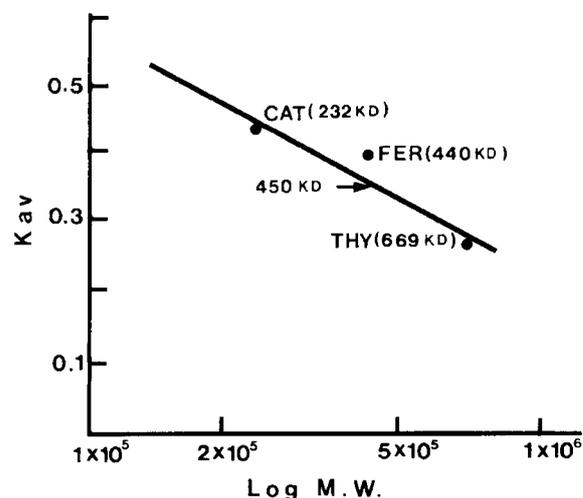

Fig. 5. Molecular weight determination of the native arylphorin by gel filtration with HPLC. 50 mM sodium phosphate, pH 6.5 containing 0.1 M ammonium sulfate was used to separate arylphroin on Shodex kW-804. The protein was eluted at 0.5 ml/min with the above buffer, and detected at 280 nm. Standard protein; CAT, Catalase (232 kDa); FER, Ferritin (440 kDa); THY, Thyroglobulin (669 kDa); 450 KDa, Native molecular weight of the arylphorin.

wild oak silkmoth, *A. pernyi* (Fig. 4). Immediately after electrophoretic separation of the whole pupal haemolymph, the protein bands of the arylphorin of interest were excised without staining the proteins in the gel. This excising was possible by using viewing angle and light refraction by naked eyes. At this time, any kinds of prestained mark proteins on both sides of the sample of interest were notloaded on the gels for the recognition of location of the protein during the electrophoretic separation. The arylphorin band gels excised immediately after the one dimensional 7.5% native-PAGE were chopped in pieces into the 1.5 ml eppendorf tubes by using a small probe. The proteins in the gels chopped were diffusively eluted against phosphate buffered saline (PBS), pH 8.6, or distilled water in the test tubes, overnight. The purity was tested by 7.5% native-PAGE analysis (Fig. 4). The preparation eluted diffusively overnight was shown to be homogenous from the visualization test of the proteins in the gel (Fig. 4). Lane 1 of female pupae and lane 2 of male pupae which are not purified showed several protein bands including the arylphorin, but lane 3 loaded with sample eluted after excising the target protein band from gel showed only one single band, which indicates a nearly perfect purification of the arylphorin.

Physicochemical characteristics of the arylphorin
To elucidate the physico-chemical characteristics of the

arylphorin, the native molecular weight, the subunit molecular weight and its composition, the presence of sugar or lipid on the protein, the heat resistance and the amino acid composition of the protein were determined.

As shown in Fig. 5, the native molecular weight of the arylphorin was estimated to be 450 kDa from a gel filtration chromatographic graph of the logarithm of the molecular weight against Kav (a cloumn-independent measure of the protein behavior) which is the fraction of the stationary gel volumn being accessible to the protein. The gel filtration was performed through a KW-804 column on HPLC, comparing their retention times with those of standard proteins.

The size of subunit molecule was estimated to be 80 kDa by comparing the relative mobilities on SDS-PAGE with those of marker proteins (Fig. 6). These results indicate that the native arylphorin protein molecule is a hexamer consisted of a single subunit type. In many cases insect storage proteins referred to as arylphorin contains covalently bound sugars or lipids. The presence or not of sugars or lipids on the arylphorin was detected by PAS (periodic acid Schiff's reagent) staining or SBB (Sudan black B) staining respectively. As shown in Fig. 7, the arylphorin was stained by PAS, but not stained by SBB. These results indicate that arylphorin is a glycoprotein.

Amino acid analysis can be an important key in the characterization of the isolated arylphorin, because the
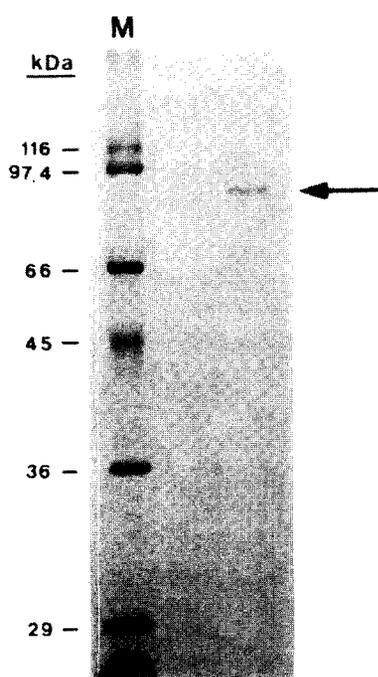
Fig. 6. Determination of the subunit composition and molecular weight of the arylphroin (arrow) by 10% SDS-PAGE. Size markers denoted in kDa are shown on the lane M. The arrow is indicating the location of a single subunit of the arylphroin with the molecular weight of 80 kDa.

Table 1. Amino acid composition of the arylphorina

Residue	Mol%
Asparagine/aspartic acid	11.2
Threonine	6.6
Serine	3.8
Glutamine/glutamic acid	7.9
Proline	5.6
Glycine	5.2
Alanine	3.7
Valine	6.7
Methionine	1.4
Isoleucine	5.0
Leucine	7.9
Tyrosine	8.6
Phenylalanine	9.7
Histidine	3.2
Lysine	9.9
Arginine	3.5

Cysteine and tryptophan were not determined.

content of aromatic amino acids or methionine is a criterion for the classification of storage proteins studied in many insects. To see a category of the arylphorin, compositional analysis of amino acids was performed. The arylphorin had 18.3 mol% and 1.4 mol% for aromatic

amino acids (Phenylalanine and tyrosine) and methionine, respectively (Table 1). In addition, heat resistance of the arylphorin purified was tested (Fig. 8). At the temperature of 70 to 80°C, heat treatments for 20 to 60 minutes were not effective for degradation of arylphorin, but the heat treatment for 20 minutes at over 90°C degradated the arylphorin.

Monosaccharide analysis of the arylphorin

The arylphorin in pupal stage was stained with PAS for sugars, indicating that it is a glycoprotein (Fig. 7). The
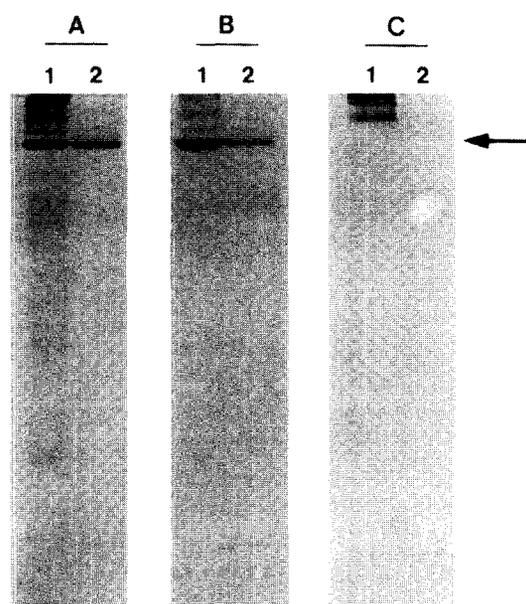
Fig. 7. Detection of sugar and lipid on the purified arylphorin. The proteins were stained with Coomasie Brilliant Blue R-250 (A), with periodic acid Shiff's (PAS) reagent for sugar (B), and with Sudan black B for lipid (C), respectively. Lane 1, Whole haemolymph of pupae; Lane 2, Arylphroin purified. Arrow is indicating the presence or absence of sugar or lipid on the arylphorin.

Table 2. Physicochemical properties of the arylphorin

Physicochemical properties	Size/content
Molecular weight	
Native molecules	450 kDa
Subunit	80 kDa
Aromatic amino acid content (%)[a]	18.3
Methionine content (%)[a]	1.4
Carbohydrate content (%, w/w)	4.9
GlcNAc (mol/mol protein)	4.2
Man (mol/mol protein)	16.3
Lipid[b]	Non-detected

[a]Molar percentages in determined amino acids.
[b]Detected by Sudan black B.

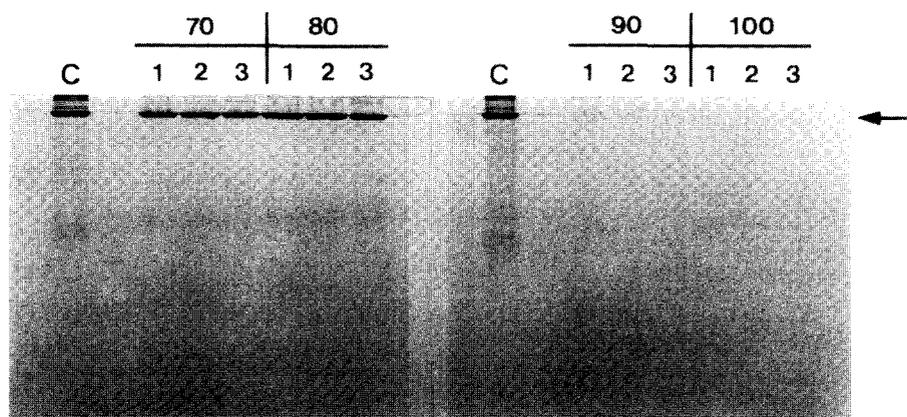**Fig. 8.** Heat resistance of the arylphorin. The numerals, 70, 80, 90 and 100 mean the corresponding temperatures denoted in centigrade scale (°C), respectively. Lane 1, 2 and 3 indicate heat treatments for 20, 40 and 60 min. in the each temperature tested. C, Control haemolymph.

monosaccharide composition of the arylphorin oligosaccharides, following acid hydrolysis, was determined by HPAEC (Table 2). The arylphorin had a fairly medium amount of sugar (4.9%,w/w) based on composition analysis. GlcNAc and Mannose (Man) were main sugar components of the arylphorin. 4.0 mole of GlcNAc per protein suggested that it had two N-glycosylation sites. The high portion of Man compared to GlcNAc suggested that the major glycans of the arylphorin are only neutral glycans (Bigge *et al.*, 1995; Butters and Hughes, 1978, 1981; Davidson *et al.*, 1991; Davidson and Castellino, 1991; Guile *et al.*, 1996). The analysis provided no evidence of existence of Fucose (Fuc) which was commoly found in N-glycosylation of insect or plant. It was unlikely that the protein had mucin type O-linked oligosacharides since there was no detected galactose and N-acetylgalactosamin. However it could not exclude possibilities of another type of O-glycosylations like O-GlcNAc in lectin or on a membrane glycoprotein for modulation (Kelly and Hart, 1989).

Discussion

The electrophoresis has been a rapid and essential analysis technique for the separation of plasma protein in insect science since Davis (1964) applied the native-PAGE technique to human serum research, and Laemmli (1970) used the SDS-PAGE for determination of molecular weight of subunit protein. For the initial analytical separation of members of the insect storage protein including the arylphorin of which characterization was performed physiologically and biochemically in the present study, the electrophoresis of the native proteins is more effective than SDS-PAGE, in which the similarly sized subunits

migrate closely together. We analysed the profile of haemolymph proteins by native-PAGE during the developmental and metamorphic period from the 1st instar to the adult in the Chinese wild oak silkmoth, *A. pernyi*. The species is a holometabolous lepidopteran silkmoth as depicted in Fig. 1. This wild silkmoth changes its external morphology several times during the developmental process from egg to adult. In this process, eggs, larvae, pupae and adults are characterized by their morphological characteristics, albeit we believe that the developmental process can be on the basis of the electrophoretic patterns of haemolymph proteins, because the haemolymph is a central place for storage of proteins, hormones and other metabolites, exchange of biological information, for supply of environment of physiological and biochemicalactivities. From this view the developmental process of the wild silkmoth can be into 3, P-1, P-2 and P-3, during the developmental process from the first instar immature stage to the adult (Fig. 1). Especially in P-2 including 5th larval and pupal haemolymph, external morphological characteristics are surprisingly distinct between the larvae and the pupae, but the electrophoretic protein patterns are very similar each other. Also, this grouping of developmental process based on the electrophoretic protein patterns is possible in the another lepidopteran silkmoths, the Japanese wild oak silkmoth *A. yamamai* and the domesticated silkworm *B. mori* (Lee, 1994; Kim *et al.*, 1989).

We purified the arylphorin from the pupal haemolymph of the Chinese wild oak silkmoth, *A. pernyi*, by electrophoresis and a subsequent simple diffusive elution (Fig. 4). In general, it is very difficult to recognize the protein bands in the gel during or after the electrophoresis by naked eyes without the help of prestaining or illumination of ultraviolet light. In our case, however, we could recognized the target protein band which showed a very slow

mobility and a dense band scar by eyes, because the light refraction allowed the reccognition of the location of the target protein in the separation gels during the electrophoresis and/or after electrophoresis. After electrophoresis, the target gel bands were excised and chopped in very small pieces, and eluted diffusively against distilled water or Tris-glycine buffer. The preparation was shown to be homogeneous by 7.5% native-PAGE and 10% SDS-PAGE (Fig. 4, 6). The protein purification methods employed in the study was very effective because it took a short time to get the homogeneous form of the protein and was simple. Therefore the present purification methods of the target protein performed by light refraction, excising the gel and diffusive elution will be very useful for other protein researches. The protein purified was finally confirmed as an arylphorin from its molecular properties characterized in the present study. Many arylphorins are purified and characterized physiologically, biochemically and molecular biologically in many insect species such as *C. ethlius* (Palli and Locke, 1987), *H. zea* (Haunerland and Bowers, 1986a), *H. cecropia* (Telfer *et al.*, 1983), *P. polyxens* (Ryan *et al.*, 1986b), *S. cynthia ricini* (Shimada *et al.*, 1987), *A. mellifera* (Ryan *et al.*, 1984), *B. mori* (Tojo *et al.*, 1980), *B. orientalis* (Duhamel and Kunkel, 1983), *D. melanoganter* (Roberts and Brock, 1981; Roberts, 1983; Brock and Roberts, 1983), *M. domestica* (Marinotti and deBianchi, 1986), *C. vicina* (Burmester and Scheller, 1992, 1995a, 1995b, 1995c). The storage proteins including arylphorin can be divided into 3 categories as described in introduction. The term arylphorin requires the following molecular properties (Kanost *et al.*, 1990): first, exceptionally high content of aromatic amino acids (18 – 26%), second, hexamers of 72 – 83 kDa subunits, third, covalently bound oligosaccharide moieties, fourth, in some cases, possession of non-covalently associated lipids (Karmer *et al.*, 1980b; Ryan *et al.*, 1984; Levenbook, 1985; Palli and Locke, 1987). The arylphorin studied in the present report showed that the native molecular weight was 450 kDa with a 80 kDa single subunit, it contained an exceptionally high content (18.3%) of aromatic amino acids and had covalently bound oligosaccharide moieties (Fig. 5, 6, 7 and Table 1, 2). However, the arylphorin did not contain lipid. From these results, the protein purified in the study was identified and confirmed as an arylphorin (glycoprotein) containing only sugar such as mannose and N-acetylglucosamin without lipids. The native molecular weight of the arylphorin was similar with those of arylphorins of other moths, 450 kDa in *Manduca sexta* (Kramer *et al.*, 1980a, 1980b), 450 kDa in *A. pernyi* (Kajiura, 1998), 450 kDa in *H. zea* (Haunerland and Bowers, 1986a), 485 kDa in *B. mori* (Tojo *et al.*, 1980), 470 kDa in *S. cynthia ricini* (Shimada *et al.*, 1987). Also, the 80 kDa

subunit molecular weight of the arylphorin is existed within the range of 72 – 83 kDa which are usual in arylphorins characterized from several moths. But it showed a considerable difference from the arylphorins which were consisted of two types of subunits (Tojo *et al.*, 1980; Kramer *et al.*, 1980b; Ryan *et al.*, 1985a; Duhamel and Kunkel, 1983; Kajiura *et al.*, 1998), because the arylphorin in the present study was consisted of a single subunit with the molecular weight of 80kDa. Therefore, the hexamer arylphorin which had a single subunit type confirmed by SDS-PAGE is very similar in the subunit composition with the arylphorins of several other lepidopteran insect species, *C. ethlius* (Palli and Locke, 1987), *H. zea* (Haunerland and Bowers, 1986a), *H. cecropia* (Telfer *et al.*, 1983), *A. mellifera* (Ryan *et al.*, 1984), but is very different from the *A. pernyi* arylphorin which was consisted of two subunits each weighing 83,000 and 82,000 (Kajiura *et al.*, 1989), even though belonged to same lepidopteran species.

The storage hexamers are a family of insect proteins with native molecular weight around 500,000 with six hemologous subunits weighing generally between 70,000 and 85,000 daltons (Telfer and Kunkel, 1991), also are belonged to insect storage proteins. The arylphorin investigated in this study is belonged to the categories of storage proteins. By partially considering the review entitled "The function and evolution of insect storage hexamers" reported by Telfer and Kunkel (1991), we are going to classify in a special and different way the arylphorin by focusing it on amino acid composition and the results of its principal component analysis. Many amino acid compositions reported for 39 storage hexamers plus the arylphorin in 24 species from 3 orders of insects are listed in Table 3 which is partial citation from the original reviewed data in Telfer and Kunkel (1991). For most proteins, a simple percentage composition of amino acids is no longer an interesting topic because the most proteins conform closely to the average composition described by King and Jukes (1969). Amino acid composition is still a vital issue for storage hexamers, however, because it is one of the key selective features that have governed their evolution (Telfer and Kunkel, 1991). Arylphorin is designation for proteins with tyrosine and phenylalanine contents totalling more than 15% (Telfer *et al.*, 1983), and methionine-rich describes those proteins in which this amino acid exceeds 4%, in both cases the values are more than twice the average determined by King and Jukes (1969). Thus, the most important criterion for the identification of storage haemolymph proteins referred to as an arylphorin is an exceptionally higher content of aromatic amino acids in the protein. In Table 3, the total content of the aromatic amino acids tyrosine and

Table 3. Comparison of amino acid composition of hexamerins including arylphorin in different orders of the insects

SUBCLASS: Order/protein group	Genus species	Asx 1	Thr 2	Ser 3	Glx 4	Pro 5	Gly 6	Ala 7	Val 8	Met 9	Ile 10	Leu 11	Tyr 12	Phe 13	Lys 14	His 15	Arg 16	mol % (aromatic amino acids)
HOLOMETABOLA: Diptera/calliphorin																		
	Calliphora erythrocephalla	116	48	43	100	31	54	33	59	45	40	58	114	111	82	33	33	22.5
	Calliphora stygia	120	47	42	101	41	54	30	53	38	41	68	119	109	79	31	28	22.8
	Ceratinus capitata	139	46	53	110	39	56	47	52	36	45	88	96	71	60	26	36	16.7
	C. capitaia	143	42	47	115	37	53	44	48	42	42	86	103	73	62	26	37	17.6
	C. capitata	142	40	455	112	36	53	46	55	39	43	71	119	75	57	26	41	19.4
	Drosophila melanogaster	128	52	36	103	32	70	41	47	54	37	69	93	94	69	30	42	16.7
	Lucilia cuprina	119	43	37	106	30	51	33	63	35	38	69	118	111	82	29	34	22.9
	Musca domestica	131	41	37	101	39	53	31	53	35	29	52	151	120	94	16	29	27.1
Diptera/second hexamerin																		
	Calliphora stygia	140	34	62	108	71	58	46	52	20	39	87	94	62	72	50	6	15.6
	Ceratinus capitata	134	41	42	131	50	50	54	69	19	53	66	87	72	60	36	39	15.9
	Drosophila crucigera	155	36	77	111	50	83	57	63	20	28	48	74	61	50	56	32	13.5
	Drosophila melanogaster	127	39	47	115	54	62	46	83	18	28	59	83	82	61	70	26	16.5
	Drosophila mimica	140	49	58	134	36	69	59	68	21	29	63	71	65	62	38	41	13.6
	Drosophila mullen	150	37	74	113	47	76	52	65	12	31	62	77	71	56	44	32	14.8
Hymenoptera/arylphorin	*Apis melliferu*	134	42	78	93	47	58	44	32	57	51	78	96	69	54	12	53	17.5
Lepidoptera/arylphorin	*Antheraea pernyi arylphorin*	112	66	38	79	56	52	37	67	14	50	79	86	97	99	32	35	18.3
	Bombyx mori	121	45	40	125	61	40	54	61	27	38	69	89	98	85	13	32	18.7
	Galleria mellonella	125	41	37	120	47	48	51	65	18	57	87	100	65	67	13	56	16.5
	Samia synthia ricini	121	48	48	113	33	46	50	67	14	45	75	94	97	82	31	39	19.1
	Spodoptera litura	121	20	57	122	50	33	48	67	20	48	74	111	85	82	28	35	19.6
	Heliothis zea	107	34	55	108	41	50	56	64	21	46	78	104	90	78	30	37	19.4
	Hyalophora cecropia	111	50	47	111	73	49	44	67	15	44	69	92	87	65	36	39	17.9
	Manduca sexta	128	29	52	107	58	49	53	76	20	32	63	103	88	77	29	35	19.1
	Papilio polyxenes	99	64	48	113	60	48	42	67	9	45	72	113	86	73	22	37	19.9
	Calpodes ethlius	100	49	49	72	58	73	39	77	15	32	70	127	84	98	25	32	21.1
	Lymantria dispar	105	46	65	100	51	95	58	64	14	43	74	83	74	72	27	29	15.7
Lepidoptera/high methione	*Spodoptera litura SL1*	139	61	31	50	35	85	31	83	76	52	89	47	48	82	22	71	9.6
	Spodoptera litura SL2	141	55	23	51	31	140	22	75	35	54	97	65	53	82	18	60	11.8
	Samia synthia pryeri	100	51	43	88	88	48	41	60	50	41	63	68	90	97	35	34	15.8
	Bombyx mori	122	61	52	86	41	48	35	86	108	46	75	55	51	70	11	55	10.6
	Galleria mellonella	130	53	53	70	39	49	42	65	84	49	86	54	38	88	32	68	9.2
	Hyalophora cecropia	133	59	49	95	23	54	46	70	70	45	86	51	66	85	14	56	11.7
	H. cecropia	136	55	49	96	19	39	36	75	49	52	106	68	62	100	9	51	13.0
	Manduca sexta	130	74	29	35	23	50	29	89	65	65	112	59	57	80	29	74	11.6
	Papilio polyxenes	158	68	20	61	32	42	21	86	39	66	116	62	54	81	22	72	11.6
Lepidoptera/others	*Hyalophora cecropia*	109	46	70	108	35	48	45	71	24	68	88	78	48	56	62	43	12.6
	Plodia interpunctella	160	67	34	48	61	47	37	80	48	69	117	36	41	68	19	68	7.7
	Calpodes ethlius SP1	145	67	63	87	30	47	42	59	50	57	101	53	49	63	25	61	10.2
	C. ethlius SP2	134	61	58	30	44	44	38	79	31	54	100	93	68	78	39	50	16.1

*The above amino acid data was partly cited from the review by Telfer and Kunkel (1991), and the mol % of aromatic amino acids was calculated by the author.

phenylalanine in lepidopteran insects/arylphorin is 18.7% in *B. mori* (Tojo *et al.*, 1980), 16.5% in *G. mellonella* (Silhacek and Bean, 1988), 19.1% in *S. cynthia ricini* (Shimada *et al.*, 1987), 19.6% in *S. litura* (Tojo and Yoshiga, 1993), 19.4% in *H. zea* (Haunerland and Browers, 1986a, 1986b), 17.9% in *H. cecropia* (Telfer *et al.*, 1983), 19.1% in *M. sexta* (Kramer *et al.*, 1980a), 19.9% in *P. polyxenes* (Ryan *et al.*, 1986b), 21.1% in *C. ethlius* (Palli and Locke, 1987), 15.7% in *L. dispar* (Karpells *et al.*, 1990). Because the total amino acid content 18.3% of aromatic amino acids, tyrosine (8.6%) and phenylalanine (9.7%) in the arylphorin of this study is well corresponded to around the amino acid compositions mentioned above, the arylphorin can be classified into an arylphorin which is a member of storage proteins in insects as mentioned above (Table 3).

In addition, even though they are belonged to a same arylphorin family, the average aromatic amino acid contents are different each other in different orders. For example, the total aromatic amino acid contents are on average 20.7% in Diptera and 18.7% in Lepidoptera (not shown in Table 3, calculated by the author). Dipteran arylphorins can be also characterized as high in both methionine and aromatic amino acid content, on the contrary the lepidipteran ones as low in methionine contents (Table 3). Therefore, the statistical classification of the storage proteins based on the clustering such as a principal component analysis was performed by Telfer and Kunkel (1991). According to the principal component analysis, the storage hexamers can be grouped into four categories. The first group is the only one whose aromatic and methionine contents are both high, includes dipteran calliphorins (Brock and Roberts, 1983; deBianchi *et al.*, 1983; Katsoris and Marmaras, 1979; Kinnear and Thomson, 1975; Munn and Greville, 1969; Wolfe *et al.*, 1977). The second group is a second hexamerin in Diptera which has much lower methionine and aromatic amino acid contents than calliphorin (Beverley and Wilson, 1982; Roberts and Brock, 1981; Shirras and Bownes, 1989). The third group is a lepidopteran glycoprotein with high aromatic amino acid and low methionine contents (Haunerland and Bowers, 1986a, 1986b; Karpells *et al.*, 1990; Kramer *et al.*, 1980a, 1980b; Kunkel *et al.*, 1990; Palli and Locke, 1987; Ryan *et al.*, 1986a, 1986b; Telfer *et al.*, 1983; Tojo *et al.*, 1980). The fourth group is also lepidopteran storage proteins with high methionine and low aromatic amino acid contents, lacks carbohydrate, and also differs from the other three groups in developmental profile (Bean and Silhacek, 1989). From the above principal component analysis of insect hexamers, it can be concluded that the arylphorin in the study is belonged to the third group which are characterized as lepidopteran arylphorin with high aromatic amino acid and low methionine contents.

In general, storage hexamers are known to be glycosylated except methionine-rich storage protein (Haunerland, 1996). Arylphorins have 2 − 10% (w/w) carbohydrate content consisting of GlcNAc and Man in a 1:4 − 5 molar ratio (Telfer *et al.*, 1983; Tojo and Yoshiga, 1993; Ancsin and Wyatt, 1996) The arylphorin confirmed as arylphorin has 4.9% (w/w) of carbohydrate (Table 2). In many cases arylphorin contain covalently bound oligosaccharide moieties, the contents of carbohydrate are 2.1% in *M. sexta*, 4% in *H. cecropia* and 2.5% in *H. zea* (Telfer *et al.*, 1983 ; Ryan *et al.*, 1985a; Haunerland and Bowers, 1986a). Also, the contents of N-acetylglucosamin and mannose were 4.2% and 16.3% (mol/mol protein), respectively, and this result shows that the arylphorin is a high mannose type arylphorin. The carbohydrate content 4.9% of the arylphorin is relatively high compared with those of other insect species as describe above. The exact function of the sugar bound to the arylphorin is uncertain, but we suggested that the sugar may play a role in the folding of arylphorin or in the assembly of the hexamer (Kim *et al.*, 2003).

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References

Ancsin, J. B. and G. B. Wyatt (1996) Purification and characterization of two storage proteins from *Locusta migratoria* showing distinct developmental and hormonal regulation. *Insect Biochem. Mol. Biol.* **26**, 501-510.

Bean, D. W. and D. Silhacek (1989) Changes in the titer of the female-predominant storage protein (81k) during larval and pupal development of the wax moth, *Galleria mellonella. Arch. Insect Biochem. Physiol.* **10**, 333-348.

Beverley, S. and A. Wilson (1982) Molecular evolution in *Drosophila* and higher Diptera. I. Micro-complement hexation studies of a larval hemolymph protein. *J. Mol. Evol.* **18**, 251-264.

Bigge, J. C., T. P. Patel, J. A. Bruce, P. N. Goulding, S. M. Charles and R. B. Parekh (1995) Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Anal. Biochem.* **230**, 229-238.

Brock, H. and D. Roberts (1983) An immunological and electrophoretic study of the larval serum proteins of *Drosophila* species. *Insect Biochem.* **13**, 57-63.

Burmester T. and K. Scheller (1992) Identification of binding

proteins involved in the stage-specific uptake of arylphorin by the fat body cells of *Calliphora vicina. Insect Biochem. Mol. Biol.* **22**, 211-220.

Burmester T. and K. Scheller (1995a) Ecdysteroid-mediated uptake of arylphorin by larval fat bodies of *Calliphora vicina* : involvement and developmental regulation of arylphorin binding proteins. *Insect Biochem. Mol. Biol.* **25**, 799-806.

Burmester T. and K. Scheller (1995b) Complete cDNA sequence of the receptor responsible for arylphorin uptake by larval fat body of the blowfly *Calliphora vicina. Insect Biochem. Mol. Biol.* **25**, 981-989.

Burmester T., U. Matzner and K. Scheller (1995) Effect of 20-hydroxyecdysone on synthesis and uptake of arylphorin by the larval fat body of *Calliphora vicina* (Diptera; Calliphoridae). *Eur. J. Entomol.* **92**, 217-227.

Butters, T. D. and R. C. Hughes (1978) Lectin binding to mosquito *Aedes aegyptii* and human KKB cells: structural comparisons of membrane oligosaccharides. *Carbohyd. Res.* **61**, 159-168.

Butters, T. D. and R. C. Hughes (1981) Isolation and characterization of mosquito cell membrane glycoproteins. *Biochem. Biophs. Acta.* **640**, 655-671.

Carpenter, D. and K. VanHolde (1973) Amino acid composition, amino terminal analysis, and subunit structure of *Caneer magister* hemocyanin. *Biochemistry* **12**, 2231-2238.

Cheon, H. M., I. H. Hwang, D. H. Chung and S. J. Seo (1998) Sequence analysis and expression of met-rich storage protein SP-1 of *Hyphantria cunea. Mol. Cells* **8**, 219-225.

Cheon, H. M., H. J. Kim, D. H. Chung, M. O. Kim, J. S. Park, C. Y. Yun and S. J. Seo (2001) Local expression and distribution of a storage protein in the ovary of *Hyphantria cunea. Archives of Insect Biochemistry and Physiology* **48**, 111-120.

Davidison, D. J., R. K. Bretthauer and F. J. Castellino (1991) a-Mannosidase-catalyzed trimming of high-mannose glycans in noninfected and baculovirus-infected *Spodoptera frugiperda* cells (IPLB-SF-21AE). A possible contributing regulatory mechanism for assembly of complex-type oligosaccharides in infected cells. *Biochemistry* **30**, 9811-9815.

Davidson, D. J. and F. J. Castellino (1991) Structures of the asparagine-289-linked oligosaccharides assembled on recombinant human plasminogen expressed in a *Mamestra brassicae* cell line (IZD-MBO503). *Biochemistry* **30**, 6689-6696.

Davis, B. J. (1964) Disc electrophoresis-: method and application to human serum proteins. *Ann. New York Acad. Sci.* **121**, 404-427.

DeBianchi, A. G., O. Marinotti, F. P. Espinoza-Firentes and S. O. Pereira (1983) Purification and characterization of *Musca domestica* storage protein and its developmental profile. *Comp. Biochem. Physiol.* **76B**, 861-867.

Duhamel, R. C. and J. G. Kunkel (1983) Cockroach larval-specific protein, a tyrosine-rich serum protein. *J. Biol. Chem.* **258**, 14461-14465.

Ghiretti-Magaldi, A., C. Nuzzolo, F. Ghiretti (1966) Chemical studies on hemocyanins. I. Amino-acid composition. *Biochemistry* **5**, 1943-1951.

Guile, G. R., P. M. Rudd, D. R. Wing and R. A. Wing (1996) A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analyzing oligosaccharide profiles. *Anal. Biochem.* **240**, 210-226.

Haunerland, N. H. (1996) Insect storage proteins: gene families and receptors. *Insect Biochem. Mol. Biol.* **26**, 755-765.

Haunerland, N. H. and W. S. Bowers (1986a) Arylphorin from the corn earworm, *Heliothis zea. Insect Biochem.* **16**, 617-625.

Haunerland, N. H. and W. S. Bowers (1986b) A larval specific lipoprotein; purification, and characterization of a blue chromoprotein from *Heliothis zea. Biophys. Biochem. Res. Commun.* **134**, 580-586.

Kajiura, Z., M. N. Yokoyama, M. Nagasaki and R. Takei (1998) Purification, developmental profile and biosynthesis of arylphorin in the wild silkmoth, *Antheraea peryni. Appl. Entomol. Zool.* **33**, 305313.

Kanost, M. R., J. K. Kawooya, J. H. Law, R. O. Ryan, M. C. Van Heusden and R. Ziegler (1990) Insect haemolymph proteins. *Adv. Insect Physiol.* **22**, 299-396.

Karpells, S., D. Leonard and J. Kunkel (1990) Cyclic fluctuations in arylphorin, the principal serum storage protein of *Lymantria dispar*, indicate multiple roles in development. *Insect Biochem.* **20**, 73-82.

Katsoris, P. and V. Marmaras (1979) Characterization of the major haemolymph proteins in *Ceratitus capitata. Insect Biochem.* **9**, 503-507.

Kelly, W. G. and G. W. Hart (1989) Glycosylation of chromosomal proteins: localization of O-linked N-acetylglucosamine in *Drosophila* chromatin. *Cell* **57**, 243-251.

Kim, K. M., J. Y. Moon, S. M. Lee and H. J. Yoon (1989) Studies on the purification and biochemical properties of vitellin in the *Antheraea yamamai* 1. Isolation and purification of vitellin and its changes to embryonic development. *Korean J. Seric. Sci.* **31**, 72-81.

Kim, S. H., S. K. Hwang, R. A. Dwek, P. M. Rudd, Y. H. Ahn, E. H. Kim, C. J. Cheong, S. I. Kim, N. S. Park and S. M. Lee (2003) Structural determination of the N-glycans of a lepidopteran arylphorin reveals the presence of a monoglucosylated oligosaccharide in the storage protein. *Glycobiology* In press.

King, T. and J. Jukes (1969) Nondarwinian evolution. *Science* **164**, 788-789.

Kinnear, J. and J. Thomson (1975) Nature, origin and fate of major haemolymph proteins in *Calliphora. Insect Biochem.* **5**, 531-552.

Kramer, S. J., E. C. Mundall and J. H. Law (1980a) Purification and properties of manducin, an amino acid storage protein of the haemolymph of larval and pupal *Manduca sexta. Insect Biochem.* **10**, 279-288.

Kramer, S. J., E. C. Mundall and J. H. Law (1980b) Purification and properties of manducin, an amino acid storage protein of the haemolymph of larval and pupal *Manduca sexta. Insect Biochem.* **10**, 279-288.

Laemmli, U. K. (1970) Cleavage of structural proteins during

the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, S. M. (1994) Physiological and biochemical characteristics of the adult major haemolymph protein of the silkworm, *Bombyx mori*. Thesis of Ph. D. Thesis, Seoul National University. Seoul, Korea.

Levenbook, L. (1985) Insect storage proteins; in *Comprehensive Insect Physiology, Biochemistry and Phamacology* (Vol. **10**). Gillbert L. I. and G. A. Kerkut (eds.), pp. 307-346, Pergamon Press, Oxford.

Marinotti, O. and A. G. deBianchi (1986) Uptake of storage protein by *Musca domestica* fat body. *J. Insect Physiol.* **32**, 819-825.

Markl, J., R. Schmid, S. Czichos-Tiedt and B. Linzen (1976) Hemocyanins in spiders, Chemical and physical properties of the proteins in *Dugesiella and Cupiennius* blood. *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1713-1725.

Munn, E. A., A. Feinstein and G. D. Greville (1967) A major protein constituent of pupae of the blowfly *Calliphora erythrocephala* (Diptera). *Biochem. J.* **102**, 5.

Munn, E. A. and G. D. Greville (1969) The soluble proteins of developing *Calliphora erythrocephala*, particularly calliphorin, and similar proteins in other insects. *J. Insect Physiol.* **15**, 1935-1950.

Palli, S. R. and M. Locke (1987) Purification and characterization of three major haemolymph proteins of an insect *Calpodes ethlius* (Lepidoptera, Hesperiidae). *Arch. Insect Biochem. Physiol.* **5**, 233-244.

Rimoldi, O., J. Soulages, S. Gouzalez, F. Peluffo and R. Bremer (1989) Purification and properties of the very high density lipoprotein from the haemolymph of adult *Triatoma infestans*. *J. Lipid Res.* **30**, 857-864.

Roberts, D. B. and H. W. Brock (1981) The major serum proteins of dipteran larvae. *Experientia* **37**, 103-110.

Roberts, D. B. (1983) The evolution of larval serum protein genes in *Drosophila*; in *The larval Serum Proteins of Insects*. Scheller, K. (ed.), pp. 86-101, Thieme, Stuttgart.

Ryan, R. O., D. R. Anderson, W. J. Grimes and J. H. Law (1985a) Arylphorin from *Manduca sexta*: carbohydrate structure and immunological studies. *Arch. Biochem. Biophys.* **243**, 115-124.

Ryan, R. O., J. O. Schmidt and J. H. Law (1984) Arylphorin from the haemolymph of the larval honeybee, *Apis mellifera*. *Insect Biochem.* **14**, 515-520.

Ryan, R. O., P. S. Keim, M. A. Wells and J. H. Law (1985b) Purification and properties of a predominantly female specific protein from the haemolymph of the larva of the tobacco hornworm, *Manduca sexta. J. Biol. Chem.* **260**, 782-

786.

Ryan, R. O., S. V. Prasad, E. J. Henriksen, M. A. Wells and J. H. Law (1986a) Lipoprotein interconversions in an insect, *Manduca sexta*. Evidence for a lipid transfer factor in the hemolymph. *J. Biol. Chem,* **261**, 563-568.

Ryan, R. O., X. Y. Wang, E. Willot and J. H. Law (1986b) Majorhaemolymph proteins from larvae of the black swallowtail butterfly, *Papilio polyxenes. Arch. Insect Bichem. Physiol.* **3**, 539-550.

Seo, S. J., Y. J. Kang, H. M. Cheon, H. R. Kim (1998) Distribution and accumulation of storage protein-1 in ovary of *Hyphantria cunea drury. Arch. Insect Biochem. Physiol.* **37**, 115-128.

Shimada, T., M. Nagata and N. Yoshitake (1987) Charcterization of arylphorin of the Eri-silkmoth, *Samia cynthia ricini* (Donovan) (Lepidoptera: Saturniidae) *Appl. Ent. Zool.* **22**, 543-552.

Shirras, A. D. and M. Bownes (1989) Cricklet: A locus regulating a number of adult functions of *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA* **86**, 4559-4563.

Silhacek, D. and D. Bean (1988) Storage protein physiology in *Galleria mellonella*. Cymborowski, B., I. Sehnal and A. Zabza (eds.), pp. 1007-1011, Wroclaw Unl. Press Wroclaw, Poland.

Swahn, B. (1953) Studies on blood lipids. *Scan. J. Clin. & Lab. Invest.* **5**, *Suppl.* 9.

Tefler, W. H. and J. G. Kunkel (1991) The function and evolution of insect storage hexamers. *Annu. Rev. Ent.* **36**, 205-228.

Telfer, W. H., P. S. Keim and J. H. Law (1983) Arylphorin, a new protein from *Hyalophora cecropia*: comparisons with Calliphorin and Manducin. *Insect Biochem.* **13**, 601-613.

Tojo, S., M. Nagata and M. Kobayashi (1980) Storage proteins in the silkworm, *Bombyx mori. Insect Biochem.* **10**, 289-303.

Tojo, S. and T. Yoshiga (1993) Purification and characterization of three storage proteins in the common cutworm, *Spodoptera litura. Insect Biochem. Mol. Biol.* **23**, 729-738.

Wyatt, G. R. and M. L. Pan (1978) Insect plasma proteins. *Ann. Rev. Biochem.* **47**, 779-817.

Weber, K. and M. Osborn (1969) The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406-4412.

Wolfe, J., M. E. Akam and D. B. Roberts (1977) Biochemical and immunological studies on larval serum protein I, the major haemolymph protein of *Drosophila melanogaster* third-instar larvae. *Eur. J. Biochem.* **79**, 47-53.

Zacharius, R. M., T. E. Zell, J. H. Morrison and J. J. Woodlock (1969) Glycoprotein staining following electrophoresis on acrylamide gels. *Analyt. Biochem.* **30**, 148-152.