Purification, developmental profile and biosynthesis of arylphorin in the wild silkmoth, *Antheraea pernyi*

Zenta Kajiura, Marcia Noriko Yokoyama, Masao Nakagaki and Ryuzo Takei*

Laboratory of Silkworm Genetics and Pathology, Faculty of Textile Science and Technology, Shinshu University, Ueda Nagano, 386-8567, Japan

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Abstract

We purified *Antheraea pernyi* arylphorin (ApA) from the larval hemolymph by gel permeation chromatography, DE-52 cellulose column chromatography, and hydroxypatite column chromatography. Our results show that ApA is a hexameric protein with a native molecular weight of 450,000, and consists of three molecules of two subunits each weighing 83,000 and 82,000. ApA is rich in tyrosine (8.2%) and phenylalanine (8.4%) and poor in methionine (1.4%). The amounts of ApA in the hemolymph increased during the feeding stage and decreased during the molting stage. ApA was partially recaptured by the fat bodies during the larval-pupal metamorphosis. ApA remained in the hemolymph and also in the fat bodies at a constant level throughout the diapause pupal stage but was then reduced to traces just before adult emergence. *In vitro* translation of RNA from the fat bodies and immunoprecipitation using anti-ApA serum showed that ApA synthesis occurs in the fat bodies through the feeding stage but is reduced remarkably after spinning. The translatable ApAmRNA was not detected at pupation at all.

Key words: *Antheraea pernyi, Antheraea yamamai*, arylphorin, female methionine-rich storage protein

INTRODUCTION

An arylphorin is the most abundant storage protein found in insect hemolymph. This protein is synthesized by the fat body during the larval stage and is primarily used mainly during adult development. Synthesis is most pronounced during the last larval instar, when arylphorin generally becomes the principal hemolymph protein.

Arylphorins are characterized by a hexameric structure and an unusually high content of aromatic amino acids (Riddiford and Law, 1983). The proteins have been found in Diptera (Munn et al., 1971; Thomson et al., 1976; Wolfe et al., 1977) and Lepidoptera (Kramer et al., 1980, Telfer et al., 1983, Tojo et al., 1980).

Amino acids of arylphorin are primarily incorporated into new tissues and proteins during adult development (Levenbook and Bauer, 1984), but they may also be incorporated into a cuticle as an intact protein (Riddiford and Hice, 1985; Scheller et al., 1980). Some storage proteins have ligand binding and transport capabilities (Hauenerland and Bowers, 1986b; Telfer and Masey, 1987). Storage protein 2 (SP2) serves as a xanthomatin-carrier in the hemolymph of the red blood (rb) mutant strain of the silkworm, *Bombyx mori* (Maki et al., 1995).

We have investigated hemolymph proteins and fat body proteins of the wild silkworms, *Antheraea yamamai, Antheraea pernyi*, and their hybrids (Yokoyama et al., 1993). Immunoblot analysis using anti-SP2 serum showed that an 83 kDa peptide was an arylphorin of *A. pernyi* and of *A. yamamai*. On the other hand, Immunoblot analysis using anti-SP1 serum, a female-specific methionine-rich storage protein of *B. mori*, showed that a 75 kDa peptide was a female specific methionine-rich storage protein of *A. pernyi* and of *A. yamamai* (Yokoyama et al., 1993).

This paper details isolation procedures, analysis, physical properties and the course of appearance and disappearance of *A. pernyi* aryl-

*To whom correspondence should be addressed at: Fax: 0268-21-5331, E-mail: zkajiur@iptc.shinshu-u.ac.jp*
phorin (ApA) in the hemolymph and the fat bodies during adult developmental stage. We show both similarities to, as well as some significant differences from the known arylphorins.

MATERIALS AND METHODS

Animals. Antheraea pernyi and Antheraea yamamai were reared as previously described (Kobayashi et al., 1992). Larvae were reared on an artificial diet (Nihon-nosankogyo Co., Yokohama, Japan) until the third ecdisis. After the third ecdisis they were reared on the leaves of the Japanese oak, Quercus acutissima, in a field. Diapausing pupae of A. pernyi were stored at 5°C for five months. After acclimation at 15°C for 5 days and then at 20°C for 7 days, they were incubated at 25°C for 15 days until adult emergence. A. yamamai diapauses in the pharate first instar stage before hatching. Diapausing A. yamamai eggs were stocked at 5°C for five months. After acclimation at 15–20°C for 1 day, the eggs were incubated at 25°C until the first instar larvae hatched.

Preparation of tissues. Each sample of the collected hemolymph was mixed with nine volumes of phosphate-buffered saline (10 mM phosphate buffer pH 7.0, 0.15 mM NaCl containing a few crystals of phenylthiourea) and centrifuged at 10,000 \( \times \) g for 10 min to remove hemocytes. The supernatants were stored at −20°C. The fat bodies dissected from animals were homogenized with 10 volumes of phosphate-buffered saline after weighing. The homogenates were centrifuged at 10,000 \( \times \) g for 10 min. The supernatants were stored at −20°C until the experiments were conducted (Kajiura and Yamashita, 1989).

Gel electrophoresis. Native-polyacrylamide gel electrophoresis (Native-PAGE) was carried out using a slab gel (Davis, 1964). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Molecular weights were estimated using high and low molecular weight calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden).

Amino acid analysis. The purified protein was exhaustively dialyzed against water, and then hydrolyzed in 6 N HCl at 110°C in vacuo for 24, 48 and 72 h. Amino acids in the hydrolysate were analyzed by an OPA system (Jasco Co., Japan).

Immunoblot analysis. Immunoblot analysis was carried out according to the method of Burnette (1981). An antiserum of purified ApA was prepared as described by Kajiura and Yamashita (1989). The peptides separated by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane sheet in the blotting buffer [100 mM Tris, 192 mM glycine and 5% (v/v) methanol] for 60 min at 2 mA/cm² using Horizblot (Atto Co., Tokyo, Japan). The electroblotted nitrocellulose sheet was incubated for 20 min in PBS [10 mM Na-phosphate buffer, pH 7.0, containing 0.8% NaCl and 10 mM phenylmethylsulfonylfluoride (PMSF)] and then incubated for 60 min with antiserum diluted with 1 : 5,000 in 2% skim milk-PBS. After being washed with PBS/skim milk three times, the nitrocellulose sheet was incubated for 60 min with a 1 : 2,000 dilution of peroxidase-conjugated goat anti rabbit IgG in PBS/skim milk. The bound antibodies were visualized with a POD immunostain set (Wako Pure Chemicals, Osaka, Japan).

Fat body mRNA isolation and in vitro translation. Fat bodies were dissected and rinsed with ice-cold PBS and then quickly frozen by liquid nitrogen. The frozen tissues were stored at −80°C until use. Total RNA was extracted by guanidinium thiocyanate and SDS-phenol according to the methods of Maniatis et al. (1989). Polyadenylated RNA was isolated using oligo (dT) cellulose (Pharmacia, Type 7) as described by Aviv and Leder (1972). In vitro translation of the total RNA and polyadenylated RNA was performed in a rabbit reticulocyte lysate cell free translation system (Amersham, England) in the presence of [35S]methionine (37 Tbd/mol, ICN) under the conditions suggested by the supplier. The translation products were analyzed by fluorography.

Immunoprecipitation of translation products. Poly(A)+ RNA was prepared from total RNA of fat bodies of day 12 of the fifth instar larva. Fifty microliters of in vitro translation products were mixed with 10 \( \mu l \) 10% SDS to dissociate the translation products, followed by the addition of 10 \( \mu l \) of 20% Triton X-100. The
mixture was incubated at 30°C for 1 h with 50 μl of anti-ApA serum. The immunoprecipitates were recovered from the mixture using Staphylococcus protein A cellulofine (Seikagaku Kogyo Co., Osaka, Japan) was suspended in a 0.05 M phosphate buffer (pH 7.0) (Watanabe and Price, 1982) and analyzed by SDS-PAGE and fluorography.

**Fluorography.** SDS-polyacrylamide gels were stained with Coomassie Brilliant Blue R250 and processed for fluorography according to the method of Skinner and Griswold (1983). A dried gel was exposed to a preflashed Fuji X-ray film at -80°C.

**RESULTS**

**Purification of Antheraea pernyi arylphorin (ApA)**

ApA was purified from 15 ml of the hemolymph of male fifth instar larvae (day 12) by column chromatography (Yokoyama et al., 1994): gel-filtration with cellulofine GCL-1000 and cellulofine GCL-300 (Seikagaku Co., Tokyo, Japan), anion-exchange chromatography with DE-52 (Whatman International Ltd., Madison, England), and adsorptive chromatography with hydroxyapatite (Seikagaku Co., Tokyo, Japan). The purity of ApA was examined by SDS-PAGE after each purification step.

In the following procedures, the presence of ApA in the fractions (5 ml each) obtained at each step was checked by SDS-PAGE.

To separate the various hemolymph proteins, we used gel permeation chromatography on GCL-1000 cellulofine (2.0 cm × 90 cm) equilibrated with a phosphate-buffered saline PBS [50 mM Na-phosphate, 0.10 M NaCl, and 0.02% (w/v) NaN₃, pH 7.0]. The absorbance of proteins in each fraction was read at 280 nm by a JASCO UVIDEC-610 (Japan Spectroscopic Co., Ltd.). Fractions containing ApA (fraction numbers 12–24) (Fig. 1A) were lyophilized and applied to the second gel permeation chromatography on GCL-300 cellulofine according to the same method as GCL-1000 cellulofine (Fig. 1B).

Fractions (numbers 10–20) containing ApA were pooled, lyophilized overnight, and dissolved in 3 ml of 25 mM Tris-HCl buffer, pH 7.5. The dissolution was dialyzed against water for 1 h and then overnight against 25 mM Tris-HCl buffer, pH 7.5, and then subjected to

![Graphs showing purification steps and chromatograms](image)

**Fig. 1.** Purification steps of storage proteins from the hemolymph of Antheraea pernyi. Elution profiles of the storage proteins are shown, A, gel-filtration chromatography with cellulofine GCL-1000, B, gel-filtration chromatography with cellulofine GCL-300, C, anion-exchange chromatography with DEAE-cellulose (DE-52) and D, adsorptive chromatography with hydroxyapatite.

![Purified ApA protein](image)

**Fig. 2.** Purified ApA protein. Native-PAGE on a 6% slab gel (A) and SDS-PAGE on a 10% slab gel (B) were carried out by the method described in Materials and Methods. Lane 1, hemolymph; Lane 2, purified protein.
anion-exchange column chromatography (2.0 cm × 20 cm DE-52, Whatman). After washing to remove nonadsorbed proteins, ApA was eluted by a linear NaCl gradient from 0.0 to 0.5 M in 25 mM Tris-HCl (pH 7.5). ApA was detected in the highest peak (Fig. 1C).

Approximately 60 ml of DE52 cellulose anion-exchange elute containing ApA (fractions number 37–48) were concentrated and dialyzed against 10 mM Na-phosphate pH 6.8, and applied to adsorption chromatography on hydroxyapatite column (2.0 cm × 20 cm, Seikagaku). ApA was eluted by a linear phosphate gradient from 10 to 400 mM (fractions number 59–91) (Fig. 1D).

The adsorption column chromatography was effective in separating ApA from other hemolymph proteins. The native molecular weight of ApA is approximately 450,000 (Fig. 2A). SDS-PAGE shows that ApA is composed of two subunits weighing 83,000 and 82,000 (Fig. 2B). These results indicate that ApA is a hexamic protein composed of three molecules of two subunits each weighing 83,000 and 82,000.

**Amino acid composition**

The most prominent feature of arylphorin is its high aromatic amino acid content. Table 1 shows the amino acid composition of purified ApA along with that of *Samia, Hyalophora*, and *Bombyx* arylphorin for comparison (Shimada et al., 1987; Tojo et al., 1978). ApA is rich in tyrosine (8.2%) and phenylalanine (8.4%) and poor in methionine (1.4%). The composition of ApA was quite similar to those of other lepidopteran arylphorins. Based on these results, we decided that the purified protein was arylphorin.

**Developmental changes**

Levels of ApA and *A. yamamai* arylphorin (AyA) in the hemolymph increased remarkably during the last instar stage and decreased steeply after spinning, while these proteins were

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![Fig. 3](image)

Fig. 3. Developmental profiles of *Antheraea* arylphorin in the hemolymph of *A. pernyi* (A) and *A. yamamai* (B) shown by SDS-polyacrylamide gel electrophoresis. Hemolymph was collected every day from second instar day 4 (second apolysis) to fourth instar day 7 (fourth apolysis). Each 40 μg of proteins was resolved on SDS-PAGE. II, III and IV show the second, third, and fourth instar larvae, respectively. Arabic numerals show age in days.
accumulated in the fat bodies after that point (Yokoyama et al., 1993). ApA was found from second instar day 4 until fourth instar day 7 (pharate fifth instar) in the hemolymph of *A. pernyi* (Fig. 3A). AyA was also found during the same stage as in ApA (Fig. 3B). Both arylphorins increased slowly through intermolt period, and remained a constant level in the early molting stage, and then decreased slightly just after ecdysis (Fig. 3).

We analyzed ApA in the hemolymph and the fat bodies by immunoblot analysis during pupal-adult development (Fig. 4). A considerable amount of ApA accumulated until day 12 in both male and female hemolymph and fat bodies. On day 15, ApA declined to an undetectable level in the male hemolymph but remained in the fat bodies (Fig. 4A). In contrast, ApA decreased to a low level in the female hemolymph and was slightly detectable in the fat bodies on day 15 (Fig. 4B).

**In vitro translation of fat body mRNA**

Total RNA extracted from the fat bodies was used for *in vitro* translation in the presence of $[^{35}S]$-methionine. The translated products were analyzed by SDS-PAGE followed by fluorography. Figure 5 shows that many pro-
Discussion

Arylphorins differ considerably with respect to subunit composition. On the one hand, some lepidopteran species, *Calpodes ethlius* (Palli and Locke, 1987), *Heliothis zea* (Haunerland and Bowers, 1986a), *Hyalophora cecropia* (Tel-fer et al., 1983), *Papilio polyxenes* (Ryan et al., 1986), and the hymenopteran *Apis mellifera* (Ryan et al., 1984), contain arylphorin with a single subunit type. On the other hand, other lepidopteran species, *Bombyx mori* (Tojo et al., 1980), *Galleria mellonella* (Ray et al., 1987), *Manduca sexta* (Ryan et al., 1985), and the dictyopteran *Blatta orientalis* arylphorins (Duhamel and Kunkel, 1983) posses two types of subunits separated by SDS-PAGE. These sub-units are generally present in a 1:1 ratio (Kanost et al., 1990). The present studies show that ApA consists of two subunits with apparent molecular weights of 83,000 and 82,000 (Fig. 2). It remains to be determined whether these arylphorin subunits posses different amino acid sequences or whether there is some modification at the molecular level which occurs in one of the subunits.

An 83 kDa peptide of *Antheraea* was clas-
A. pernyi  A. yamamai
T I T I

Fig. 6. Immunoprecipitation of peptides translated in vitro through RNA from the fat bodies using anti-ApA serum. Total RNA of fat bodies were extracted from the fifth instar larvae (day 12) and was used as the template for in vitro translation in a rabbit reticulocyte lysate. Total translation products and immunoprecipitates were analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by fluorography. The letters “T” and “I” represent “total translation products” and “immunoprecipitates.”

sified in aryrophorin from its molecular weight, developmental profile and immunoblot analysis (Yokoyama et al., 1993). The amino acid composition of our purified protein supports that the 83 kDa peptide is an aryrophorin subunit (Table 1). A female-specific 75 kDa peptide of Antheraea, another storage protein, was classified in a female-specific methionine-rich storage protein from its molecular weight, developmental profile and immunoblot analysis (Yokoyama et al., 1993). However, it remains unknown whether or not the A. pernyi female-specific storage protein is methionine-rich.

Aryrophorin exists as a predominant component in the hemolymph of A. pernyi and A. yamamai from second instar day 4 to fourth instar day 7 (Fig. 3). This suggests that aryrophorin synthesis occurs in early larval instars such as Manduca sexta, Bombyx mori, and Galleria mellonella (Fujii et al., 1989; Ray et al., 1987; Riddiford and Hice, 1985).

The ratio of aryrophorin to total proteins increased remarkably from the final instar and reached maximal levels at the commencement of spinning (Yokoyama et al., 1993). However, it decreased during the larval-pupal transformation and accumulated concurrently in the fat body (Yokoyama et al., 1993). Even after pupation, a considerable amount of aryrophorin remains in the hemolymph of A. pernyi as it did in Hyalophora cecropia and Manduca sexta (Fig. 3, Riddiford and Hice, 1985; Tojo et al., 1978).

Figure 4 shows that aryrophorins in the hemolymph and the fat bodies decreased gradually to day 12 of adult-developing pupa. This suggests a slow turnover or use by some tissues. However, aryrophorin decreased rapidly for the last three days to adult emergence (Fig. 4), suggesting that turnover or use was accelerated.

Interestingly, ApA disappeared in the male hemolymph earlier than in the female hemolymph (Fig. 4A), while it was exhausted in the female fat bodies earlier than in the male fat bodies (Fig. 4B). Each concentration of Bombyx mori aryrophorin in the hemolymph and in the fat bodies is higher in females than in males (Tojo et al., 1980; Kajiura and Yamashita, 1989), and also each concentration of hemolymph ApA and fat body ApA was higher in females than in males based on some preliminary experiments (data not shown). Therefore, it is reasonable that hemolymph ApA disappeared earlier in males than in females, and we suppose that the rate of degradation of ApA would be more higher in the female fat bodies than in the male fat bodies.

In vitro translation shows that aryrophorin synthesis started again after the last larval ecdysis and continued through the feeding stage, then stopped at pupation by a decrease in aryrophorin mRNA (Fig. 5). This suggests that aryrophorin synthesis closely parallels aryrophorin mRNA titres. The developmental time course of the appearance and disappearance of aryrophorin mRNA may be in agreement with that of other Lepidoptera aryrophorins. Immunoprecipitation revealed that an 83 kDa peptide is a major subunit of Antheraea aryrophorin (Fig. 6), suggest-
Table 1. Amino acid composition of arylphorins in Antheraea, Samia, Hyalophora and Bombyx

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Values are the average of three samples hydrolyzed for 24 h.

$^a$Shimada et al. (1987).

$^b$Telfer et al. (1983).

$^c$Reported as "SP-2" by Tojo et al. (1980).

$^d$Tryptophan was not determined.

ing that some modification at the molecular level may be occurring with one subunit. Further studies are focused on post-translational modification and arylphorin cDNA polymorphism.

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