Isolation and Identification of Adipokinetic Hormone of the Silkworm, *Bombyx mori*

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An adipokinetic hormone (AKH) was isolated from the adult heads of the silkworm, *Bombyx mori*. Fast atom bombardment mass spectrometry (FAB-MS) of the intact AKH and sequence analysis of the AKH after deblocking with pyrogulamate aminopeptidase revealed that the structure of *Bombyx* AKH is pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH₂, which is identical with those of *Manduca sexta* and *Helothis zea* AKH. *Bombyx* AKH is released just after adult eclosion and elevates the lipid level but not the carbohydrate level in the hemolymph.

In 1976, adipokinetic hormone (AKH) was first isolated from locust corpora cardiaca.\(^1\) It stimulates the mobilization of lipid from the fat body to provide energy during flight in insects. By now, AKHs from many species of Orthoptera, Phasmida, Blattariae, and Lepidoptera, have been isolated and characterized.\(^2,3\) and now constitute a large family of structurally related peptides including crustacean red-pigment concentration hormone (RPCP).\(^4\) In addition to lipid mobilization, AKH has hyperglycemic and myotropic activity in some species.\(^2,3\)

Although the silkworm, *Bombyx mori*, lost its ability to fly through evolution, it needs to use energy stored in the larval stage because it takes no nutrition during the adult stage. In this paper we report the presence of AKH in *B. mori*, and its purification and chemical identification.

Materials and Methods

**Experimental animals.** A racial hybrid, J-106 × Daizo, of *B. mori* was used. Larvae were reared on an artificial diet, "Silkmate" (Kyoto-shiryo, Yokohama), at 25 ± 1°C under a photoperiod of 16-hr light and 8-hr dark. Pupae and adults were kept under the same conditions.

**Measurement of lipid and sugar concentrations in hemolymph.** Adult silkworms were blled by pricking the intersegmental membrane of the abdomen, and 5 μl of hemolymph was collected with a micropipette. Total lipids were measured by the phosphoric acid vanillin method\(^5\) and sugars by the anthrone method.\(^6\)

**Bioassay for adipokinetic activity.** Adipokinetic activity in fractions from each purification step was measured using adult males ligated between head and thorax just after eclosion. Test samples were lyophilized after adding 100 μg of bovine serum albumin (BSA) to minimize loss due to non-specific adsorption to the containers, then dissolved in adequate volume of 0.9% NaCl solution. Five microliters of the sample solution was injected into the abdomen between the segments without anesthesia. One hour after the injection, hemolymph was collected and lipid concentration measured as mentioned above.

**Source of AKH.** The source material, male adult heads of the silkworm, *B. mori*, was the same as used for purification of prothoracotrophic hormone,\(^7\) bombexin\(^8\) and pheromone biosynthesis activating neuropeptide.\(^9\)

**Purification of AKH.** Defatting, extraction, and heat treatment (steps 1—3): Seventy-five thousand adult *Bombyx* heads were homogenized in 4400 ml of cold (20°C) acetone with a Polytron homogenizer and filtered. Residues were extracted three times each with 1100 ml of 80% aqueous ethanol, and the combined filtrates were concentrated using a rotary evaporator. The aqueous extracts were heated in a boiling water bath for 10 min and cooled rapidly in an ice-water bath, then the precipitates were removed by centrifugation (10,000 rpm, 20 min).

Ion-exchange chromatography (steps 4 and 5): The supernatant of the previous step was adjusted to pH 8.5 by addition of aqueous ammonia and applied to a QAE-Sephadex A-25 column (5.5 × 6 cm) equilibrated with 0.02 M ammonium acetate buffer (pH 8.5). The column was eluted with 500 ml of the same buffer. The unadsorbed fraction from the QAE-Sephadex column was adjusted to pH 4.0 with acetic acid and applied to an SP-Sephadex C-25 column (5.0 × 9.0 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 4.0). The column was eluted with 500 ml of the same buffer.

**Vydac C₄ cartridge** (step 6): One-tenth of the unadsorbed fraction from the SP-Sephadex column was directly applied to 10 g of Vydac C₄ packing material (20—30 μm, contained in a 75-ml polypropylene syringe barrel) equilibrated with 0.1% trifluoroacetic acid (TFA). The cartridge was eluted with 40 ml each of 15%, 20%, 25%, 30%, 35%, and 50% acetonitrile in 0.1% TFA. This process was repeated nine times for the rest of the sample. Reversed phase HPLC (RP-HPLC) (steps 7—9): One-fourth of the active fraction from step 6 was diluted twice with water and pumped onto a SensusPak VP-304 semi-preparative column (10 × 250 mm, Sensus-Kagaku) previously equilibrated with 10% acetonitrile in 0.1% TFA. The materials were eluted with a 60-min linear gradient of 10—40% acetonitrile containing 0.1% TFA at a flow rate of 5.0 ml/min. Ten-milliliter fractions were collected. The same procedure was then repeated three times for the rest of sample. The active fractions were combined and diluted twice with water and applied to a SensusPak VP-318 column (4.6 × 250 mm, Sensus-Kagaku) equilibrated with 15% acetonitrile in 0.1% heptfluorobutyric acid (HFBA). The column was eluted with a 40-min linear gradient of 15—35% acetonitrile in 0.1% HFBA at a flow rate of 1.0 ml/min. The active fractions were pooled and diluted twice with water, and applied to the same VP-318 column equilibrated with 15% acetonitrile in 0.1% TFA. The column was eluted with a 40-min linear gradient of 15—35% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.

**Pyrogulamate aminopeptidase digestion.** Pyrogulamate aminopeptidase (Boehringer Mannheim) was dissolved in 90 mM potassium phosphate buffer (pH 7.5) containing 10 mM dithiothreitol at a concentration of 4 munit/10 μl. The purified *Bombyx* AKH (1.6 μg) was lyophilized and resuspended in 90 μl of the same buffer, to which 10 μl of the enzyme solution was added. After incubation at 30°C for 1 hr, 1.0 ml of 0.1% TFA was added and the mixture was directly applied to the VP-318 column equilibrated with 17.5% acetonitrile in 0.1% TFA. The column was eluted with a 40-min linear gradient of 17.5—27.5% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.

Amino acid sequence analysis. The materials from the peak of pyrogulamate aminopeptidase digestion were sequenced using a gas-phase sequencer (Shimadzu PQS-1) equipped with a phenylthiohydantoin (PTH) amino acid analyzer (Shimadzu LC-6A).

Fast atom bombardment mass spectrometry (FAB-MS). One microliter of 0.1% TFA solution containing approximately 10 μg of the purified AKH was added to a matrix of thioglycerol on a stainless steel probe tip, which was introduced into the ion source of the mass spectrometer. Analysis was performed with a JEOL JMS DX303 mass spectrometer using Xe as
the fast atom.

Results

**Lipid level in hemolymph and bioassay for AKH**

The changes in total lipid concentration in hemolymph after adult eclosion is shown in Fig. 1. The concentration of lipid in newly emerged adult was 3.5 μg/μl, and began to increase dramatically 2 hr after eclosion and reached 20 μg/μl at 26 hr. Ligation between head and thorax just after eclosion inhibited such an increase of lipid level and that was maintained at 5 μg/μl until 26 hr after eclosion. This result suggested that a factor, which could be adipokinetic hormone, was secreted from the head just after eclosion to elevate hemolymph lipids.

To confirm the presence of adipokinetic hormone in the heads, *Bombyx* adult heads were extracted with 80% EtOH and the extracts were injected into animals ligated just after eclosion. The concentration of lipid was measured at 1, 2, 4, and 6 hours after injection. The lipid level in the animal injected with 1 head equivalent of 80% EtOH extracts increased up to 20 μg/μl at 1 hr, then decreased to 15 μg/μl at 2 hr and was maintained until 6 hr as shown Fig. 2. These results indicate an adipokinetic hormone is present in the head of *Bombyx* and extracted with 80% EtOH.

When 80% EtOH extracts were injected into the ligated animals at several doses and the lipid level was measured 1 hr after injection, a dose-response relationship was observed, as shown in Fig. 3. The threshold dose for a response is 0.03 head eq. and maximal response is induced by 3 head eq., giving a linear curve between the two doses. Half-maximal activation was effected with a dose of 0.2 head eq. and defined as a *Bombyx* AKH unit.

**Purification of Bombyx AKH**

The 9-step procedure of the extraction and purification of *Bombyx* AKH from adult heads as well as weight, total activity and specific activity after each step are summarized in Table I.

Step 1—3: After defatting with cold acetone, *Bombyx* AKH was extracted with 80% EtOH. The 80% EtOH extracts from 75,000 adult heads had an activity of 3.8 × 10^5

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**Fig. 1.** Hemolymph Lipid Concentration of Newly Emerged Male *B. mori*.

Open circles indicate the hemolymph lipid concentration of intact animals. Closed circles indicate that of animals ligated between head and thorax just after eclosion. Each value was obtained from different animals and data are presented as the means ± S.D. (n=5).

**Fig. 2.** Changes in Hemolymph Lipid Concentration after Injection of One-head Equivalent of 80% EtOH Extracts.

Each value was obtained from different animals and data are presented as the means ± S.D. (n=5).

**Fig. 3.** Dose–Response Curve for 80% EtOH Extracts.

Each value was obtained from different animals and data are presented as the means ± S.D. (n=5).

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**Table I.** Summary of Purification of *Bombyx* AKH from 75,000 *Bombyx* Adult Heads

<table>
<thead>
<tr>
<th>Step</th>
<th>Weight (μg)</th>
<th>Total activity (units)</th>
<th>Specific activity (ng/unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75,000 Male Adult Bombyx Heads</td>
<td>560,000,000</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.</td>
<td>Acetone Powder</td>
<td>22,000,000</td>
<td>380,000</td>
</tr>
<tr>
<td>2.</td>
<td>Extraction with 80% EtOH</td>
<td>15,000,000</td>
<td>380,000</td>
</tr>
<tr>
<td>3.</td>
<td>Heat Treatment</td>
<td>14,000,000</td>
<td>98,000</td>
</tr>
<tr>
<td>4.</td>
<td>QAE-Sephadex A-25</td>
<td>12,000,000</td>
<td>60,000</td>
</tr>
<tr>
<td>5.</td>
<td>SP-Sephadex C-25</td>
<td>30,000</td>
<td>100,000</td>
</tr>
<tr>
<td>6.</td>
<td>Vydac C4 Cartridge</td>
<td>3,000</td>
<td>64,000</td>
</tr>
<tr>
<td>7.</td>
<td>VP-304 RP-HPLC (Acetonitrile/TFA)</td>
<td>450</td>
<td>58,000</td>
</tr>
<tr>
<td>8.</td>
<td>VP-318 RP-HPLC (Acetonitrile/HFBA)</td>
<td>20</td>
<td>49,000</td>
</tr>
</tbody>
</table>
Bombyx AKH units, i.e., an adult head contains 5 units. Bombyx AKH was stable to heat treatment at 100°C for 10 min.

Steps 4 and 5: The superantant after heat treatment was applied to a QAE-Sephadex column after adjusting to pH 8.5. AKH activity was found in the breakthrough fraction. This active fraction was adjusted to pH 4.0 and applied to an SP-Sephadex column. AKH activity was again recovered from the unadsorbed fraction. These results suggest that Bombyx AKH has blocked amino- and carboxyl-termini, and no charged amino acid, like others of the AKH/RPCH family of peptides.

Steps 6—9: The unadsorbed fraction from SP-Sephadex was applied to a Vydac C₄ cartridge and the materials were eluted with a stepwise gradient of acetonitrile containing 0.1% TFA. AKH activity was recovered in the 25% acetonitrile/0.1% TFA fraction. After dilution with water, this fraction was applied to a VP-304 semipreparative column and eluted with a linear gradient of acetonitrile containing 0.1% TFA. AKH was recovered in the 28—30 min fraction as shown in Fig. 4. The active fraction was further purified by two steps of HPLC using an analytical VP-318 column with different solvent systems as shown in Fig. 5. Finally 20 μg of Bombyx AKH were isolated from 75,000 adult heads with 14% recovery of biological activity.

**Chemical identification of Bombyx AKH**

Before sequence analysis, Bombyx AKH was treated with pyroglutamate aminopeptidase because other AKHs isolated from many species have pyroglutamate at the N-terminus. In fact, Bombyx AKH treated with pyroglutamate aminopeptidase was eluted 8 min earlier than intact AKH as shown in Fig. 6, suggesting that Bombyx AKH has a pyroglutamate at the N-terminus. Sequence analysis of Bombyx AKH after removal of pyroglutamate...
Adipokinetic Hormone of *Bombyx mori*

Fig. 7. FAB-MS of Bombyx AKH.

$m/z$ 1,008 and 1,030 correspond to $(M + H)^+$ and $(M + Na)^+$, respectively.

gave a sequence of Leu–Thr–Phe–Thr–Ser–Ser–Trp–Gly.

To identify the N-terminal pyroglutamate and the C-terminal structure, the molecular weight of intact AKH was measured by FAB-MS. As shown in Fig. 7, two quasi-molecular ion peaks were observed at $m/z$ 1008 and 1030 which corresponded to $(M + H)^+$ and $(M + Na)^+$, respectively. Therefore, the molecular weight of Bombyx AKH is 1007, and the N-terminal of Bombyx AKH was pyroglutamate and the C-terminus was glycine with an amide form. This structure, pGlu–Leu–Thr–Phe–Thr–Ser–Ser–Trp–Gly–NH$_2$, was identical with those of *Manduca sexta* and *Heliothis zea* AKH.

To confirm this structure, natural AKH was compared with synthetic *Manduca* AKH, purchased from Peninsula Laboratories, INC. USA, by HPLC. As shown in Fig. 8, the natural *Bombyx* AKH was completely coeluted with the synthetic peptide. Also, the biological activity of the synthetic peptide could not be distinguished from that of natural *Bombyx* AKH. Thus, the complete structure of Bombyx AKH is pGlu–Leu–Thr–Phe–Thr–Ser–Ser–Trp–Gly–NH$_2$.

Since there are some indications that AKH can control carbohydrate levels in some species of insect, we tested the hypotrehalosemic activity of Bombyx AKH in the adult insect. When the changes in carbohydrate concentration in hemolymph after eclosion was measured, no change was observed during 6 hr after eclosion in both intact and ligated insects. Furthermore, 10 units (4 ng) of Bombyx AKH was injected and the hemolymph carbohydrate concentration was measured, but it remained constant even though the lipid level was elevated dramatically (data not shown).

**Discussion**

The silkworm, *B. mori*, takes no nutrition during the adult stage. It is reported that fat body glycogen decreases drastically at the spinning and pupal stage in *Bombyx*. It suggests that adult Bombyx mainly uses lipid, stored in the larval stage and mobilized by AKH, as an energy source for reproductive behavior including mating and egg laying. Though only adult males were used for measuring adipokinetic activity, adult females also had the same response to AKH and the concentration of hemolymph lipid was the same as that of males.

Although AKHs of other insect species were purified from corpora cardiaca by a few steps of HPLC, Bombyx AKH was isolated from the adult heads by the 9-step purification procedure because the corpora cardiaca of Bombyx is too small to take out. In this procedure, total activity increased after step 6 as shown in Table I. It is perhaps due to the underestimation of AKH activity of the samples after steps 4 and 5. Since the samples were not desalted before bioassay, the high concentration of salt might interfere with the AKH activity. Finally, 20 μg of Bombyx AKH were obtained from 75,000 heads with a recovery of 14%. The amount of AKH in an adult head would be about 2 pmol, lower than other insect species. For example, *Locusta migratoria* contains 200 pmol of AKH in a pair of adult corpora cardiaca.

The structure of Bombyx AKH was elucidated by sequence analysis of the peptide deblocked with pyroglutamate aminopeptidase and FAB-MS measurement of the intact peptide. Bombyx AKH has no basic or acidic amino acids, and has pyroglutamate at the N-terminus and an amidated form at the C-terminus. This structure coincides with the behavior on ion exchange chromatography.

The structure of Bombyx AKH is identical to those of *Manduca sexta* and *Heliothis zea* AKH. All AKHs of Lepidoptera already reported have the same sequence, suggesting that the same molecule is distributed widely in Lepidoptera as AKH.

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