Identification of a Novel Hemolymph Peptide That Modulates Silkworm Feeding Motivation

Shinji Nagata‡†, Nobukatsu Morooka‡, Kiyoshi Asaoka§, and Hiromichi Nagasawa‡

From the ‡Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan and the §National Institute of Agribiological Sciences (NIAS), Ohwashi 1-2, Tsukuba, Ibaraki 305-8634, Japan

Phytophagous insects do not constantly chew their diets; most of their time is spent in a non-feeding quiescent state even though they live on or around their diets. Following starvation, phytophagous insect larvae exhibit enhanced foraging behaviors such as nibbling and walking similar to the sequential behavior that occurs prior to each meal. Although extensive physiological studies have revealed regularly occurring feeding behaviors in phytophagous insects, little has been elucidated regarding the mechanism at the molecular level. Here, we report identification and characterization of a novel 62-amino acid peptide, designated as hemolymph major anionic peptide (HemaP), from the hemolymph of Bombyx mori larvae that induces foraging behaviors. The endogenous HemaP levels are significantly increased by diet deprivation, whereas re-feeding after starvation returns them to basal levels. In larvae fed ad libitum, hemolymph HemaP levels fluctuate according to the feeding cycle, indicating that locomotor-associated feeding behaviors of B. mori larvae are initiated when HemaP levels exceed an unidentified threshold. Furthermore, administration of exogenous HemaP mimics the starvation-experienced state by affecting dopamine levels in the suboesophageal ganglion, which coordinates neck and mandible movements. These data strongly suggest that fluctuation of hemolymph HemaP levels modulates the regularly occurring feeding-motivated behavior in B. mori by triggering feeding initiation.

Feeding cycle of about 2 h. Similar cyclic feeding rhythms are also observed in other phytophagous insects such as locusts (2, 3) and caterpillars (4–6). This suggests that, cyclic feeding behavior might be a conserved phenomenon among phytophagous insects. It also implies that some endogenous system strictly regulates the initiation and termination of feeding in phytophagous insects (7, 8).

In the case of B. mori larvae, the probability of feeding initiation (hereafter referred to as feeding motivation) drastically increases about 1 h post feeding from the previous meal (1). In addition, B. mori larvae exhibit repetitive feeding behavioral cycles independent of circadian rhythms (1, 9), unlike other phytophagous lepidopteran species, which have feeding cycles that are influenced by other general factors, such as circadian rhythms and visual light stimuli (10). Because B. mori larvae have regularly occurring feeding cycles that are independent of circadian rhythms, it is unlikely that these factors are involved in regulating B. mori feeding cycles. This suggests that B. mori larvae are a good experimental animal for investigating the endogenous regulatory mechanisms underlying regularly occurring feeding behaviors in phytophagous insects.

From observations of B. mori larvae, we found that feeding motivated B. mori larvae exhibit increased foraging activities, including head-swaying or swinging, nibbling, and walking, whereas B. mori larvae rarely move during the periods between meals. Such foraging behaviors are also generally observed immediately before each meal (Fig. 1A). In particular, small head-swaying behavior always occurs immediately before each meal, which sometimes triggers exaggerated behaviors related to feeding initiation. These excited feeding related behaviors associated with locomotor activation of legs, mouth parts, and mandibles following starvation, or prior to feeding, have also been observed in other phytophagous lepidopteran species such as the tobacco hornworm, Manduca sexta (11, 12). It appears that activation of foraging behaviors and the subsequent initiation of consumption are necessary for a feeding motivated condition. It also appears that some endogenous factor(s) drives the initiation of feeding and the accompanying activation of locomotor activities in phytophagous insects.

Several physiological studies have proposed that some unknown endogenous factor(s) regulate these behaviors in phytophagous insects, such as locusts (3, 13) and caterpillars (5, 8). The factor may detect the imbalance of some nutrients or metabolites in hemolymph. In addition, physiological studies...
have demonstrated that the hemolymph is the site where nutritional condition of the insect is perceived. However, because of the scarcity of accepted bioassay systems for evaluating feeding motivation in phytophagous insects, these factors have not been characterized.

To address the molecular basis of feeding motivation in *B. mori* larvae, we established a bioassay to characterize the factor involved in feeding motivation by observing larval foraging behaviors. Using this bioassay, we purified and identified a novel peptide from *B. mori* larval hemolymph that drove stereotypical foraging behavior following injection into *B. mori* larvae. We also demonstrated that hemolymph levels of the peptide fluctuated according to feeding cycles.

**EXPERIMENTAL PROCEDURES**

*Animals*—Silkworm eggs from the hybrid *B. mori* strain (Kinshu × Showa) were purchased from UEDA SANSHU Ltd. (Ueda, Japan). Larvae were reared in plastic containers at 26 ± 1 °C with 70 ± 10% relative humidity under long day lighting conditions (16 light/8 dark), using the SILKMATE 2S artificial diet purchased from NIPPON NOSAN Co. Ltd. (Yokohama, Japan).

*Chemicals and Reagents*—Chemicals and reagents used in the present study were purchased from Nacalai-tesk (Osaka, Japan). Dopamine sodium salt, and serotonin (5-hydroxtryptamine), octopamine, and tyramine were purchased from Sigma.

*Bioassay for Foraging Behavior*—Only populations of larvae growing synchronously were used in assays. Short-term starved larvae were deprived of artificial diet blocks during day 2 of the last instar for 6–8 h. Artificial diet blocks were then placed in front of the larvae. In most cases, larvae fed for 20–30 min. After feeding, the larvae were anesthetized by submerging in ice-cold water for 10 min. After drying, lyophilized samples dissolved in distilled water were injected into the dorso-abdominal portion (at the fifth thoracic segment). For assays, individual larvae were injected with 100–1,000 μl samples. After sample injection, larvae were placed in a plastic container or on a large sheet of wax paper facing an artificial diet block (*supplemental Movie S1*). Larvae were spaced so as not to interrupt the feeding behavior of other animals. Larvae were observed for 1–2 h. Throughout the assay period bench- and wind-vibrations were carefully controlled. The time of initiation and termination of foraging behaviors were individually recorded. The first noticeable head movement, which is observed at the beginning of head-swaying and is usually characterized by a figure eight movement pattern consisting of ~1–2 mm movements with mandible vibrations, which are generally observed at the beginning of nibbling behavior, were counted as active behaviors. Because of difficulty in observation, antennae and maxillary movements were not counted as active behaviors. Biological activity was evaluated by calculating the proportion of time spent in foraging behaviors. Observations were carried out using at most 10 larvae in a single assay. Because video monitoring as described below limited observations of small mouthpart movements, the general HemaP assay was carried out by one of us (S. N.) using a different lot of larvae.

*HemaP Purification*—The 28-amino acid residue peptide initially isolated was purified as follows. Hemolymph was collected from 2000 day-2 last instar *B. mori* larvae starved for 2 days (~500 ml) and maintained on ice. The collected hemolymph was stored at −80 °C until purification. The hemolymph was acidified with trifluoroacetic acid (TFA), the final concentration was 0.2%, and then boiled for 15 min. The denatured pellet was removed by centrifugation at 5,000 × g for 15 min. The supernatant was subjected to Sep-Pak Vac C18. The column was washed with 0.1% TFA aqueous solution and then eluted with 60% acetonitrile, 0.1% TFA. The eluent was subjected to reversed-phase HPLC (RP-HPLC) on a Hitachi L-6250 (pump with controller; L-6050, pump; L-4000, UV detector; Hitachi, Tokyo, Japan) with a Senshu Pak Pegasil-300 ODS column (10 mm inner diameter × 250 mm, Senshu Kagaku; Tokyo, Japan) using a different lot of larvae.

*Analytical Indices of HemaP Activity*—To evaluate the bio- logical effects of HemaP, we used several biological indices related to feeding behavior. The amount of ingested diet was measured by the difference in weights of individual artificial diet blocks before and after sample injection. The approximate digestibility (AD)2 (14) is a conventional index representing the uptake efficiency of ingested diet and is calculated as follows: AD = (weight of ingested diet − (weight of fecal pellets)/weight of ingested diet).

*MALDI-TOF MS Analysis*—Hemolymph and other samples were desalted with Sep-Pak Vac C18 (100 mg) used as described above (eluited with 60% acetonitrile, 0.1% TFA). Mass spectra were measured on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE™ STR, Applied Biosystems) with α-cyano-4-...
hydroxycinnamic acid as matrix in the positive ion mode. Matrix solution was prepared by saturating α-cyano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.1% TFA. The samples were applied after mixing with matrix solution in a 1:1 ratio.

Preparation of Recombinant HemaP (rHemaP)—To construct GST-fused HemaP, RT-PCR was carried out to produce two cDNA fragments, one encoding GST with a C-terminal factor Xa recognition site and the other encoding HemaP with a factor Xa recognition site (primer sets used are described below). The full-length GST-factor Xa-HemaP (GST-X-Hmp) was generated via overlap extension PCR. We used the Ndel and Xhol sites of GST-X-Hmp to subclone into the Ndel and Xhol sites of pET28a. The resulting plasmid (pGST-X-Hmp) was transferred into BL21(DE3) bacterial cells to produce recombinant GST-X-Hmp. Production of GST-X-Hmp was initiated following addition of isopropyl 1-thio-galactopyranoside (0.5 mM final concentration) at a bacterial density of 0.5–0.7 at 600 nm. After a 5-h incubation, cells were collected by centrifugation and sonicated in ice-cold PBS containing 5 mM EDTA and 0.1 mM PMSF. Sonicated cells were re-centrifuged. GST-X-Hmp was predominantly located in the insoluble/inclusion body fraction. The precipitate was solubilized by addition of a small volume of PBS, 1% SDS. The solubilized protein was passed through Sephacryl G-15 using 50 mM Tris-Cl (pH 8.0) as a mobile phase. Fractions containing proteinaceous substances were collected and digested with Factor Xa (Merck, Novagen, Darmstadt, Germany) at room temperature for 16 h after addition of 100 mM NaCl, 5 mM CaCl2. The reaction mixture was passed over a glutathione column (GE Healthcare) to remove the digested GST portion. The flow through was then subjected to RP-HPLC using the same linear gradient program as described for HemaP isolation. The resulting recombinant HemaP (rHemaP) was confirmed by MALDI-TOF MS analyses and N-terminal sequencing. The yield of rHemaP was ~150 µg from a 1-liter culture. First RT-PCR was performed by following primers using cDNA derived from B. mori larval fat body as a template DNA: Fw-1 primer: 5′-TCTGTACGAGGGTGATGCTCT- CTAAGAAG-3′, Rv-1 primer with a Xhol restriction site: 5′-CCGGCTCGAGTTATTCAACTTTCTCCCT-3′. Secondary PCR for GST amplification was performed by the following primers using pGEX-5X3 as a template DNA: Fw-2 primer with a Ndel restriction site: 5′-CAGCCATATGGCTCCCTAT- ACTAGATTGG-3′, Rv-2 primer: 5′-CTTCCTAGGA- GCATACGACCTCGATCAGT-3′. A third PCR was performed with primers Fw-2 and Rv-1 using a mixture of the first and second PCR products as DNA template.

Antiserum against HemaP—Rabbit anti-HemaP antiserum was generated using a synthetic peptide corresponding to the N-terminal portion of HemaP from Asp1 to Phe40.

HemaP Quantification by ELISA—Hemolymph was collected by piercing the ventral thoracic portion of the fifth segment with a 31-gauge needle every 20 min for 3 h. Hemolymph (1 µl) was collected with a micropipette and diluted immediately with 50 µl of ice-cold distilled water. The diluted hemolymph was centrifuged at 12,000 × g at 4 °C to remove hemocytes, and then the supernatant was boiled for 5 min to inactivate contaminating proteases, and then centrifuged at 12,000 × g again to remove denatured proteins. The supernatant using 0.05 µl of hemolymph equivalents was quantified by ELISA in triplicate.

Iodination of HemaP—rHemaP was iodinated using radio-active iodine and the chloramine T method described previously (15). rHemaP (10 µg) in PBS was mixed with 2 µl of NaI (1 mg/ml), 2 µl of Na2125I (GE Healthcare), and 15 µl of chloramine T (2 mg/ml). The mixture was incubated on ice for 90 s. The reaction was stopped by addition of 50 µl of sodium metabisulfite (5 mg/ml). The resulting iodinated rHemaP was purified using reversed-phase HPLC (Jasco SC-802, PU-980, UV-970) and eluted using the same linear gradient program as used in the final HemaP purification step. Iodinated rHemaP (125I-rHemaP) was isolated based on retention time and UV absorbance at 225 nm. The retention time and iodination of 125I-rHemaP were confirmed by comparing with MALDI-TOF MS analyses of unlabeled rHemaP subjected to the chloramine T method in parallel. Based on data from cold iodinated HemaP, the major chloramine T reaction product was confirmed to be di-iodinated HemaP by MALDI-TOF MS analysis. The biological activity was confirmed using cold di-iodinated rHemaP and the radioactivity of 125I-rHemaP was approximated at 3–15 × 106 cpm/µg. 125I-rHemaP was used within 1 week of radiolabeled iodination. The stability of 125I-rHemaP stock maintained at ~80 °C was confirmed with no substantial degradation by RP-HPLC.

125I-rHemaP Tracing—125I-rHemaP was injected into 12-h starved larvae following a 10-min ice-cold water anesthetization. Tissues from 125I-rHemaP-injected larvae were dissected 30 min after injection when larvae had terminated feeding, and 125I-rHemaP distribution in refed larvae was analyzed by...
A Novel Feeding Motivation-related Peptide in the Silkworm

Comparing with that of continuously starved larvae. The tissue distribution was evaluated by dividing the weight of measured tissues. Radioactivity was measured using a γ-counter (ALOKA, Tokyo, Japan).

RT-PCR—Total RNA was extracted from the fat body, silk glands, foregut, midgut, hindgut, Malpighian tubules, hemocytes, central nervous ganglia, brain, ovary, and testis using Trizol reagent according to the manufacturer’s protocol. After treatment with DNase I (TakaRaBio, Shiga, Japan), cDNA was synthesized using a reverse transcriptase, Superscript III (Invitrogen) by priming with an oligo(dT)$_{30}$ primer. cDNA was used for a template DNAs in RT-PCR analyses. Utilized primers were HemaP-Fw (5′-GATGCTCTCA-TAAGAGACAT-3′) and HemaP-Rv (5′-TCAGGCTTTCG-GTGCTTCCG-3′). Ribosomal protein 49 (rp49) was used for an experimental control. Utilized primers were Rp-Fw (5′-CTATAAGACCTGTTCAGGGCAGAATCG-3′) and Rp-Rv (5′-TTATATTTATCTTTCTGAGGGAGGG-3′). Amplified cDNAs were electrophoresed in a 1.2% agarose gel. The resulting PCR products were detected by ethidium bromide staining.

**Immunohistochemistry of HemaP**—Fat body of *B. mori* larvae on day 2 of last instar was dissected and fixed in 4% paraformaldehyde for 16 h at 4 °C, and then washed with PBS three times for 10 min each. The cells were permeabilized with PBS containing 0.1% collagenase type I (Sigma) and 0.2% Triton X-100 for 1 h at room temperature. After blocking with 2% BSA for 30 min at room temperature, tissues were incubated with an antiserum against HemaP (1:100 dilution) for 16 h at 4 °C. Goat anti-rabbit HRP (diluted to 1:500) was used as a secondary antibody and incubated for 16 h at 4 °C. The positive signal was detected by staining with 0.1 mg/ml of 3,3′-diaminobenzidine (DAB) for a 15-min incubation at room temperature. Negative control was incubated with pre-immune serum instead of the antiserum against HemaP.

**Quantitative PCR (qPCR)**—Extraction of total RNA and RT reaction were performed as described above. The qPCR primers for HemaP were: forward 5′-CTTGGCCCGGAGACTGT-3′; reverse 5′-CCCGGGGCTTCCAGATTT-3′. As a control, the following primers for ribosomal protein L3 (RpL3) were used: forward 5′-TGGGAGTTCCCTCCCAT-3′; reverse 5′-CCATGCAGCACAICTTTGAT-3′. PCR was performed using a 7300 Real Time PCR system (Applied Biosystems). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min and then 40 cycles of 95 °C, 15 s; 60 °C, 1 min. The single targeted qPCR product was confirmed by constructing a melt curve to all reactions. The reaction was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) and 50 nM of each primer. Data were analyzed using the 7300 Real Time PCR system software version 1.3 (Applied Biosystems). Results are represented as a ratio of HemaP and RpL3 by C$_r$.

**Circular Dichroism Analyses**—HemaP and rHemaP were lyophilized and dissolved in PBS at a final concentration of 0.4 mg/ml. Circular dichroism (CD) spectra of HemaP were recorded from 200 to 260 nm using a spectropolarimeter (J-720, Jasco) at room temperature with a 1-mm path length cell.

**Quantification of Biogenic Amines (BAs)**—The conditions for HPLC and pulse amperometric detection were modified from previous reports (16, 17). HPLC was carried out using a Shimadzu HPLC (Class VP) system (controller, SIL10AD; Pump, LC-10AT; Degasser, DGU-20A; Shimadzu, Kyoto, Japan). Samples were injected using an autoinjector (SIL-10AD; Shimadzu) through an injection valve with a 100-μl sample loop. Samples were extracted from lyophilized brain or subesophageal ganglion (SOG) using an ultrasonicator (Ultrasonic processor UP200S, Ultrasound Technology, Heilsher, Germany) in 50 μl of 0.1 M perchloric acid containing 0.15 mM EDTA. Samples were injected onto an HPLC system using a column oven (CTO-6A, Shimadzu) at 40 °C and a Capcell-pak C$_{18}$ (250 × 4.6 mm inner diameter, 3-μm average particle size, Shisei-do, Tokyo, Japan) fitted with a pre-column (35 × 4.6 mm inner diameter, packed with the same resin of Capcell-Pak C$_{18}$). BAs were detected with a Coulochem II amperometric detector (ESA, Inc., MA) using a high sensitivity analytical cell containing an enhanced response amperometric electrode coupled to a graphite coulometric electrode as a reference electrode. The detector potential was maintained at 900 mV for octopamine and tyramine, and 400 mV for dopamine (DA). The mobile phase contained 0.1 M citric acid, 0.1 M sodium acetate (pH 4.5), EDTA-2Na (6 mg/liter), sodium 1-octanesulfonate (250 mg/liter), and 7.5% acetonitrile. The flow rate was 1.0 ml/min. Standards were prepared using commercial BAs diluted into extraction buffer and ranged from 2 to 100 pg/30 μl. Amounts of BAs were calculated by referencing peak areas from the standard curve. Under these conditions, we confirmed that there were no other contaminating peaks derived from brain and SOG extracts, and all detected BA peaks were resolved as single peaks with no overlap on the chromatogram.

**RESULTS**

**Isolation and Identification of a Novel Peptide Driving Foraging Behaviors from B. mori Larvae**—To address the molecular basis of feeding motivation in *B. mori* larvae, we first established a bioassay to characterize the factor involved in feeding motivation. Foraging behaviors including head-swaying, walking, nibbling, and ingesting were observed in feeding-motivated larvae, such as starved larvae and in the larvae prior to each meal (Fig. 1A). Based on these observations, we then evaluated feeding motivation by measuring the amount of time spent in foraging behaviors (Fig. 1B). By screening extracts of several body parts from *B. mori* larvae, we found that a fraction of hemolymph from starved larvae stimulated foraging behavior upon injection into satiated *B. mori* larvae (supplemental Movie S1). This result indicated the presence of a factor that drives foraging behavior. A single homogenous fraction corresponding to a peptide composed of 28 amino acid residues (Fig. 1, C and D) was obtained following four reversed-phase high performance liquid chromatography (RP-HPLC) purification steps. A search of the *B. mori* EST database, Silkbase (morus.ab.a-u-tokyo.ac.jp/), revealed that the foraging behavior-driving factor was the N-terminal portion of an anionic peptide composed of 62 amino acid residues (calculated pl 4.7) (Fig. 1D, a cDNA sequence, and
supplemental Fig. S1). The truncated biologically active peptide was likely cleaved in the acidic boiling purification process from the native peptide by acid-catalyzed hydrolysis of the peptide bond between Glu<sup>28</sup> and Ser<sup>29</sup>.

Identification of the Undamaged Peptide from Hemolymph—

To confirm the presence of the undamaged peptide deduced from the cDNA sequence in the hemolymph of <i>B. mori</i> larvae, we analyzed peptide fractions of larval hemolymph. RP-HPLC and MALDI-TOF MS analyses of <i>B. mori</i> larval hemolymph demonstrated that the full-length native peptide precursor of the biologically active peptide described above was predominantly present in the hemolymph (Fig. 2, A and B). The N-terminal amino acid sequence of the isolated native peptide was DAPKEDNSINTLAESK, which was consistent with that of the initially isolated 28-amino acid peptide fragment. MALDI-TOF MS analysis also demonstrated that the observed molecular weight coincided with the calculated average mass of the peptide. We consequently designated the undamaged peptide as hemolymph major anionic peptide, HemaP. Consistent with the transcript abundance profile in the EST data base, which contained multiple fat body-derived HemaP encoding cDNAs, RT-PCR analysis of <i>B. mori</i> larval hemolymph showed that the fat body was the main HemaP producing tissue in the larval body (Fig. 2C).

Preparation of Recombinant HemaP—To investigate whether HemaP influences foraging behaviors, we prepared recombinant HemaP (rHemaP) using an <i>Escherichia coli</i> expression system. Analysis by SDS-PAGE and MALDI-TOF MS revealed that the prepared peptide was consistent with those of native HemaP (Fig. 3, A and B) and was thus suitable for further experiments. In addition, we confirmed that rHemaP and the HemaP-containing hemolymph fraction had identical retention times by RP-HPLC (Fig. 3C), and that the native HemaP-including fraction induced foraging behavior at the 10-μl hemolymph equivalent (Fig. 3D).
Biological Activity of HemaP—To test whether HemaP can induce feeding-related activities in B. mori larvae, we observed HemaP-injected B. mori larvae. Injection of both native HemaP and rHemaP into satiated B. mori larvae induced foraging behavior (Fig. 4A), whereas vehicle- and BSA (as a control nonspecific protein)-injected larvae showed little mobility despite the presence of artificial diet blocks. rHemaP injection influenced foraging behavior in a dose-dependent manner; a higher dose of rHemaP had no effect on foraging behavior (Fig. 4A).

We next analyzed several indices related to feeding behavior by rHemaP injection (Fig. 4, B–D). Foraging behaviors in rHemaP-injected larvae were increased (Fig. 4B and supplemental Movie S2) and they ate a larger amount of diet compared with vehicle-injected larvae (Fig. 4C). In addition, rHemaP-injected larvae had a higher efficiency of ingestion (14) (AD) after 12 h (Fig. 4D). Because the increased AD was comparable with that of the starvation-experienced larvae, we compared indices with starvation-experienced larvae. As shown in Fig. 4, C and D, the effects of rHemaP injection are similar to those of starved larvae, indicating that exogenous rHemaP administration can mimic the state of the starvation-experienced larvae.

Quantification Analysis of Hemolymph HemaP Level—Next, we sought to determine whether HemaP levels fluctuate in relation to the feeding state by measuring endogenous HemaP in the hemolymph by ELISA using an anti-HemaP antiserum (supplemental Fig. S3, A and B). HemaP levels in starved larvae were significantly higher than those in larvae fed ad libitum (Fig. 5A). In periods of prolonged starvation, increased HemaP levels were maintained, whereas no significant change in HemaP levels was observed in larvae fed ad libitum at any developmental time (Fig. 5B). These data also indicate that starvation-induced HemaP levels plateaued. Furthermore, HemaP levels in starved larvae returned to basal levels following resumption of feeding (Fig. 5C), indicating that increased HemaP levels are re-established in response to dietary cues. These changes of HemaP
according to feeding states were also observed in larvae fed on the mulberry leaves, an original host plant of *B. mori* (supplemental Fig. S4).

In contrast, we measured HemaP levels in a long developmental period from the last instar larvae to adults. After the feeding period, the HemaP level was significantly decreased, whereas HemaP levels did not change during the feeding period (Fig. 5D). After pupation, HemaP levels increased again, indicating some function of HemaP different from the feeding regulation. In addition, a similar tendency of temporal change was observed in the transcriptional level of *hemap* in the fat body (supplemental Fig. S5).

**DISCUSSION**

In this study, we identified a novel peptide that modulates *B. mori* feeding behavior. Data base searches showed that HemaP-like peptides are conserved across lepidopteran species (supplemental Fig. S6A), whereas HemaP-like peptides were...
not observed in other insect orders. Among the HemaP-like peptides, a peptide from the honey moth, Galleria mellonella, has been identified as anionic peptide 2 by peptidomics searching for antimicrobial activity (26). They demonstrated that antimicrobial activity of anionic peptide 2 was very weak, consistent with our preliminary data that HemaP has little antimicrobial activities (data not shown).

Although identities of HemaP-like peptides are less than 30%, their structures appear to have characteristics of \( \alpha \)-helix-rich proteins (supported by CD spectral analysis, supplemental Fig. S6B). In addition, HemaP is abundant in the hemolymph (\( \sim 20 \mu M \)). Although the complete amino acid sequence of HemaP shows no significant similarity with other proteins, similar \( \alpha \)-helical characteristics and partial sequence similarities have been observed in other hemolymph proteins, such as lipophorins (insect lipoproteins) (27) and odorant-binding proteins (28). Therefore, HemaP might associate with some compounds possibly related to nutrients or metabolites. As demonstrated in the present study, in which HemaP hemolymph levels oscillate in response to feeding with HemaP transfer from the hemolymph to the fat body (Fig. 6, C and D), HemaP might shuttle between the hemolymph and fat body similar to that observed for lipophorins. We, however, have not identified those HemaP-associated components in

\[
\begin{align*}
\text{FIGURE 5. Changes in hemolymph HemaP levels and HemaP dynamics in relationship to different feeding states.} & \\
A, \text{ hemolymph HemaP levels in } & \\
\text{B. mori} \text{ larvae (last instar, day 2) fed } ad \text{ libitum and starved for 4 days. Bars indicate the average of measured HemaP levels. Open and closed circles} \text{ indicate HemaP levels from individual starved and fed larvae (} n = 20). \text{ } p \text{ value was calculated by unpaired } t \text{ test.} & \\
B, \text{ time course of HemaP hemolymph levels in last instar larvae. Open circles} \text{ indicate the HemaP levels of starved larvae. Closed circles} \text{ indicate the HemaP levels of larvae fed } ad \text{ libitum.} \text{ } * , p < 0.05; ** , p < 0.01; \text{ unpaired } t \text{ test (compared with larvae fed } ad \text{ libitum on each day) (mean } \pm \text{ S.D. } n = 10). \text{ C, effects of refeeding on HemaP levels in starved larvae. Larvae starved for 2 days were fed artificial diet blocks for 12 h. } * , p < 0.05; ** , p < 0.01; \text{ one-way analysis of variance, then a post hoc Tukey HSD test (mean } \pm \text{ S.D. } n = 10). \text{ D, change in HemaP levels from the last instar larval stage to adults. } ** , p < 0.01; *** , p < 0.001; \text{ one-way analysis of variance, then a post hoc Dunnett test (compared with first day of the last instar larvae) (mean } \pm \text{ S.D. } n = 10). \text{ E, change in HemaP levels in two representative larvae fed } ad \text{ libitum. Gray boxes with a letter } f \text{ indicate feeding period. Data represent two of } 10 \text{ larvae examined.} & \\
\end{align*}
\]
Feeding/swaying

JOURNAL OF BIOLOGICAL CHEMISTRY

MARCH 4, 2011 • VOLUME 286 • NUMBER 9

FIGURE 6. Association of hemolymph HemaP with the fat body after feeding diet. A, preparation of iodinated HemaP; RP-HPLC profile of rHemaP (upper panel) and iodinated HemaP (lower panel). The identity of iodinated rHemaP (indicated by the arrow), which eluted faster than rHemaP, was confirmed by MALDI-TOF MS analyses (data not shown). The main product was confirmed to be di-iodinated rHemaP (I-rHemaP). *, a peak corresponding to unreacted rHemaP. X, a peak derived from excess reagents. Bioassay of I-rHemaP induced foraging activity at levels similar to rHemaP (5 μg) compared with vehicle injection. **, p < 0.01; one-way analysis of variance, then a post hoc Dunnett test (compared with vehicle-injected larvae) (mean ± S.D. n = 10). C, decrease of 125I-rHemaP in the hemolymph following a single meal. 125I-rHemaP was injected into short-term starved B. mori larvae. Data were compared with those of larvae that were not fed after 125I-rHemaP injection. p = 0.023 (unpaired t test) (mean ± S.D. n = 10). D, tissue distribution of 125I-rHemaP after feeding. Gray and white bars represent radioactivity in tissues from refed and continuously starved larvae, respectively. **, p < 0.01; unpaired t test (refed versus continuously starved larvae) (mean ± S.D. n = 10).

FIGURE 7. Effects of HemaP on biogenic amines in the brain and suboesophageal ganglia of B. mori larvae. Effects of HemaP injection, starvation for 2 days, and refed after starvation on DA levels in larval brain (A) and SOG (B). HemaP was injected into larvae after feeding. *, p < 0.05; **, p < 0.01; one-way analysis of variance, then a post hoc Dunnett test (compared with larvae fed ad libitum) (mean ± S.D. n = 10).

FIGURE 8. Schematic model of endogenous feeding regulation via HemaP (HemaP turnover). In brief, once fat body-derived hemolymph HemaP levels reach a certain threshold, DA in the SOG is consumed for foraging conditioning. After feeding, the dietary cue causes association of HemaP partially with the fat body, and hemolymph HemaP returns to basal levels.

The hemolymph. It is intriguing that similar proteins (i.e. lipophorin, odorant-binding protein, and HemaP) are produced and secreted by the fat body, indicating that the fat body might contribute to the responses of some hemolymph nutritional changes such as metabolites and nutrients.

In developmental analyses of HemaP, HemaP levels were decreased at wandering and spinning periods, and increased after pupation (Fig. 5D). In addition, the changed HemaP levels were similar to those of hemap transcriptional levels (supplemental Fig. S5). These data indicate that hemolymph HemaP levels might be regulated by secretion from the fat body and by association again with this organ in short feeding cyclic periods. In contrast, HemaP levels might be transcriptionally regulated in long developmental periods. Interestingly, HemaP levels were increased after pupation, indicating the different function of HemaP from feeding regulation in vivo.

As observed in Fig. 5A, HemaP levels increased in starved larvae. In contrast, 10 μg of rHemaP injection did not result in any foraging behavior (Fig. 4A). These results indicate that different responsiveness to the increased HemaP level might be affected by different physiological feeding states between satiated and starved larvae.

Our results clearly demonstrate that when HemaP levels exceed a yet undefined threshold level, B. mori larvae initiate locomotor activation and exhibit the characteristic behaviors of motivated feeding. The idea of a threshold HemaP level for triggering locomotor activation corresponds well with the simulation model in locust (3, 13). These models illustrate a threshold level that separates the initiation level of feeding or locomotor activation. As demonstrated in the present study (Fig. 5E), the hemolymph HemaP level fluctuated as depicted in the locust feeding threshold model. Therefore, HemaP is the first identified molecule to date that has been shown at the molecular level to regulate feeding motivation cycles.

To date, it has been reported that a number of physiological events, including conditioning for pheromone sensing, are involved in the regulation of BAs (29–32). More specifically, in Drosophila melanogaster, feeding motivation is conditioned by dopaminergic neurons of the mushroom body in the brain (31), and pheromone sensing is conditioned by SOG DA levels (32). Similarly, HemaP levels contribute to the motivated foraging condition via SOG DA as a model of HemaP turnover depicted...
A Novel Feeding Motivation-related Peptide in the Silkworm

in our present study (Fig. 8). HemaP is secreted from the fat body and accumulates in the hemolymph. Once hemolymph HemaP levels reach a certain threshold, DA in the SOG is consumed for foraging behavior. After feeding, the dietary cue introduces excess hemolymph HemaP to the fat body with hemolymph HemaP returning to basal levels. In fact, previous reports demonstrated that DAergic neurons in the SOG of B. mori larvae are specifically located on the anterior portion of SOG (23), which are similar to neuronal sites implicated in nerve cord innervations of the mandible locomotor (25). Although it remains to be elucidated whether HemaP can directly affect SOG DA levels, DAergic neurons appear to impact mandible locomotor neurons.

More detailed investigations of the regulatory mechanisms in insect feeding behavior, such as DA tracing following feeding or starvation, and analyses of hormonal influences on HemaP levels, as well as identification of the unknown factors that drive HemaP secretion from the fat body are issues awaiting elucidation. However, our discovery of a specific, abundant hemolymph peptide, HemaP, provides a new framework for understanding the endogenous regulation of feeding motivation. Also, the present work confirms earlier reports at the molecular level that the hemolymph plays a crucial role in insect perception of nutritional status (33–35).

Acknowledgments—We thank Drs. David A. Schooley (University of Nevada, Reno) and J. Joe Hull (USDA-ARS, Maricopa, AZ) for critical reading and assistance in manuscript preparation. We thank Drs. K. Sasaki and J. Ishibashi for analyses of antimicrobial activity of HemaP against Gram negative and positive bacteria. We also thank Dr. K. Sasaki for critical comments.

REFERENCES
1. Nagata, S., and Nagasawa, H. (2006) J. Insect Physiol. 52, 807–815
2. Simpson, S. I. (1982) Physiol. Entomol. 7, 325–336
3. Simpson, S. I. (1995) in Regulatory Mechanisms of Insect Feeding (Chapman, R. F., and de Boer, J., eds) pp. 137–156, Chapman & Hall, New York
4. Reynolds, S. E., Yeomans, M. R., and Timmins, W. A. (1986) Physiol. Entomol. 11, 39–51
5. Bernays, E. A., and Singer, M. S. (1998) Physiol. Entomol. 23, 295–302
6. Bernays, E. A., and Woods, H. A. (2000) J. Insect Physiol. 46, 825–836
7. Simpson, S. J. (1990) in Biology of Grasshoppers (Chapman, R. F., and Joern, A., eds) pp. 73–103, John Wiley & Sons, New York
8. Timmins, W. A., and Reynolds, S. E. (1992) Physiol. Entomol. 17, 81–89
9. Hiro, T., and Yamaoka, K. (1981) J. Seric. Sci. Jpn 50, 335–342
10. Brady, J. (1974) Adv. Insect Physiol. 10, 1–115
11. Bowdan, E. (1988) J. Insect Behav. 1, 31–50
12. Bowdan, E. (1988) Entomol. Exp. Appl. 47, 127–136
13. Simpson, S. J., and Ludlow, A. R. (1986) Anim. Behav. 34, 480–496
14. Waldbauer, G. P. (1968) Adv. Insect Physiol. 5, 229–288
15. Hunter, W. M., and Greenwood, F. C. (1962) Nature 194, 495–496
16. Hirashima, A., Yamaji, H., Yoshizawa, T., Kuwano, E., and Eto, M. (2007) J. Insect Physiol. 53, 1242–1249
17. Hardie, S. L., and Hirsh, J. (2006) J. Neurosci. Methods 153, 243–249
18. Sasaki, K., and Asaoka, K. (2005) Neurosci. Lett. 374, 166–170
19. Rohrbacher, J. (1994) J. Comp. Physiol. A 175, 619–628
20. Bräunig, P., and Burrows, M. (2004) J. Comp. Neural 478, 164–175
21. Stocker, R. F., and Schorderet, M. (1981) Cell Tissue Res. 216, 513–523
22. Agrawal, N., Padmanabhan, N., and Hasan, G. (2009) PLoS ONE 4, e6652
23. Hwang, J. S., Kang, S. W., Goo, T. W., Yun, E. Y., Lee, J. S., Kwon, O. Y., Chun, T., Suzuki, Y., and Fujiwara, H. (2003) Biotechnol. Lett. 25, 997–1002
24. Miles, C. I., and Booker, R. (1998) J. Exp. Biol. 201, 1785–1798
25. Sasaki, K., and Asaoka, K. (2006) J. Insect Physiol. 52, 528–537
26. Brown, S. E., Howard, A., Kasprzak, A. B., Gordon, K. H., and East, P. D. (2009) Insect Biochem. Mol. Biol. 39, 792–800
27. van der Horst, D. J., van Hoof, D., van Marrewijk, W. J., and Rodenburg, K. W. (2002) Mol. Cell. Biochem. 239, 113–119
28. Graham, L. A., Brewer, D., Lajoie, G., and Davies, P. L. (2003) Mol. Cell. Proteomics 2, 541–549
29. Kaufmann, L., Schürmann, F., Viallouros, M., Harrewijn, P., and Kayser, H. (2004) Comp. Biochem. Physiol. C Toxicol. Pharmacol. 138, 469–483
30. Socha, R., Kodrik, D., and Zemek, R. (2008) Comp. Biochem. Physiol. B Biochem. Mol. Biol. 151, 305–310
31. Krashes, M. J., DasGupta, S., Vrede, A., White, B., Armstrong, J. D., and Waddell, S. (2009) Cell 139, 416–427
32. Selcho, M., Pauls, D., Han, K. A., Stocker, R. F., and Thum, A. S. (2009) PLoS One 4, e5897
33. Simpson, S. J., and Abisgold, J. D. (1985) Physiol. Entomol. 10, 443–452
34. Timmins, W. A., Bellward, K., Stamp, A. J., and Reynolds, S. E. (1988) Physiol. Entomol. 13, 303–314
35. Simpson, S. J., and Reubenheimer, D. (1993) Physiol. Entomol. 18, 395–403