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Extraction and partial characterization of pupal-cuticle-melanizing hormone (PCMH) in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera, Papilionidae)

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ABSTRACT—The pupae of *Papilio xuthus* show green, brown, and orange color polymorphism. The color of the pupal body is determined by a hormone that produces brown coloration, a hormone called pupal-cuticle-melanizing hormone (PCMH) which is secreted from brain-suboesophageal ganglion and prothoracic ganglion (Br-SG-PG) complexes during the pharate pupal stage. PCMH was extracted with 2% NaCl from Br-SG-PG complexes of *P. xuthus* green pupae. When pharate pupae producing brown pupae (brown-pupa-producers) were ligatured between the thorax and abdomen with cotton thread, the head-thorax complex developed into the brown type and the abdomens into the green type. Ligatured abdomens treated with crude PCMH produced intermediates of green- and brown-pupae (melanization degree of grades 0–3), which was used to assay PCMH-activity. Extracts of Br-SG complexes from *Bombyx mori* adults also shifted the color of the pupal cuticle toward the brown type in ligatured *P. xuthus* abdomens of brown-pupa-producers. The molecular weight of the *B. mori* factor showing PCMH activity (PCMH-active factor) was estimated to be 3,000–4,000 Da by gel filtration. The PCMH-active factor is a hydrophobic peptide(s) that binds to a cation exchange resin at pH 6.9.

INTRODUCTION

Many insects show color polymorphism as larvae, pupae or adults. However, to date, the neuroendocrine mechanisms underlying the control of body colors have been studied little due to the difficulty of establishing a suitable bioassay system.

In the Asian comma butterfly, *Polygonia c-aureum* L., the wing and body colors of adult, seasonal morphs is determined by a summer-morph-producing hormone (SMPH) secreted from the brain-reterocerebral neuroendocrine system early in the pupal stage (Fukuda and Endo, 1966; Endo, 1972, 1984; Endo *et al.*, 1988), while larval body color of the common armyworm, *Leucania separata*, is regulated by a melanization and reddish coloration hormone (MRCH), which had been purified from the subesophageal ganglia of *L. separata* (Matsumoto *et al.*, 1981). In contrast, a pupal melanization reducing factor (PMRF) is present in the nymphalid butterfly, *Inachis io*. This factor is released from the head-prothorax during the pharate pupal stage (Bückmann and Maisch, 1987), and could be extracted from different butterfly species (Koch *et al.*, 1990).

In a number of papilionids, pupal body colors are determined by environmental factors present around the site of pupation, such as scent, curvature, texture, background color, and photoperiod (Hidaka, 1961; Ishizaki and Kato, 1956; Hazel and West, 1996; Shimada, 1983). A physiological mechanism underlying the control of pupal body color involves a neuroendocrine factor (browning hormone) in *P. xuthus* (Hidaka, 1961). The browning hormone is thought to be produced in the brain and secreted from the prothoracic ganglion in the middle of the pharate pupal stage (Awiti and Hidaka, 1982). However, there are only a few studies about the pupal-cuticle-melanizing hormone (PCMH) in *P. xuthus*.

In this study, we extract PCMH from Br-SG-PG complexes of *P. xuthus* pupae and develop a novel bioassay method using ligatured abdomens of *P. xuthus* pharate pupae. In addition, a PCMH-active factor present in Br-SG complexes of *Bombyx mori* adults was partially characterized.

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MATERIALS AND METHODS

Insects

P. xuthus collected in the towns of Yamaguchi and Hofu were used. Eggs and larvae were kept in containers of transparent plastic ($19 \times 13 \times 5$ cm^3) and exposed to long day length, 16 hr light and 8 hr dark, at 23°C. Larvae were fed on leaves of *Fagara ailanthoides*, which were exchanged daily during the photophase. Larvae of *B. mori*, a commercial race (Kinsyu × Showa), were fed on leaves of *Morus tiliaefolia*, and the pupae were allowed to develop into adults at room temperature.

Expression of pupal cuticular color

P. xuthus larvae entered the wandering stage after gut-purge. Larvae in the wandering stage were selected from a stock culture and placed in either smooth plastic boxes containing pieces of *F. ailanthoides* leaves or in cardboard boxes with a rough interior surface. A large proportion of larvae kept in plastic boxes with *F. ailanthoides* leaves developed into green types, while those kept in cardboard boxes mostly developed into brown types. The degree of melanization of the pupal cuticle was classified into one of five grades, 0–4. Grade 4 was the typical brown type whereas grade 0 was the typical green type. Intermediates were classified grade 1–3 depending on the degree of cuticular melanization (Fig. 1A). Similarly, mela-

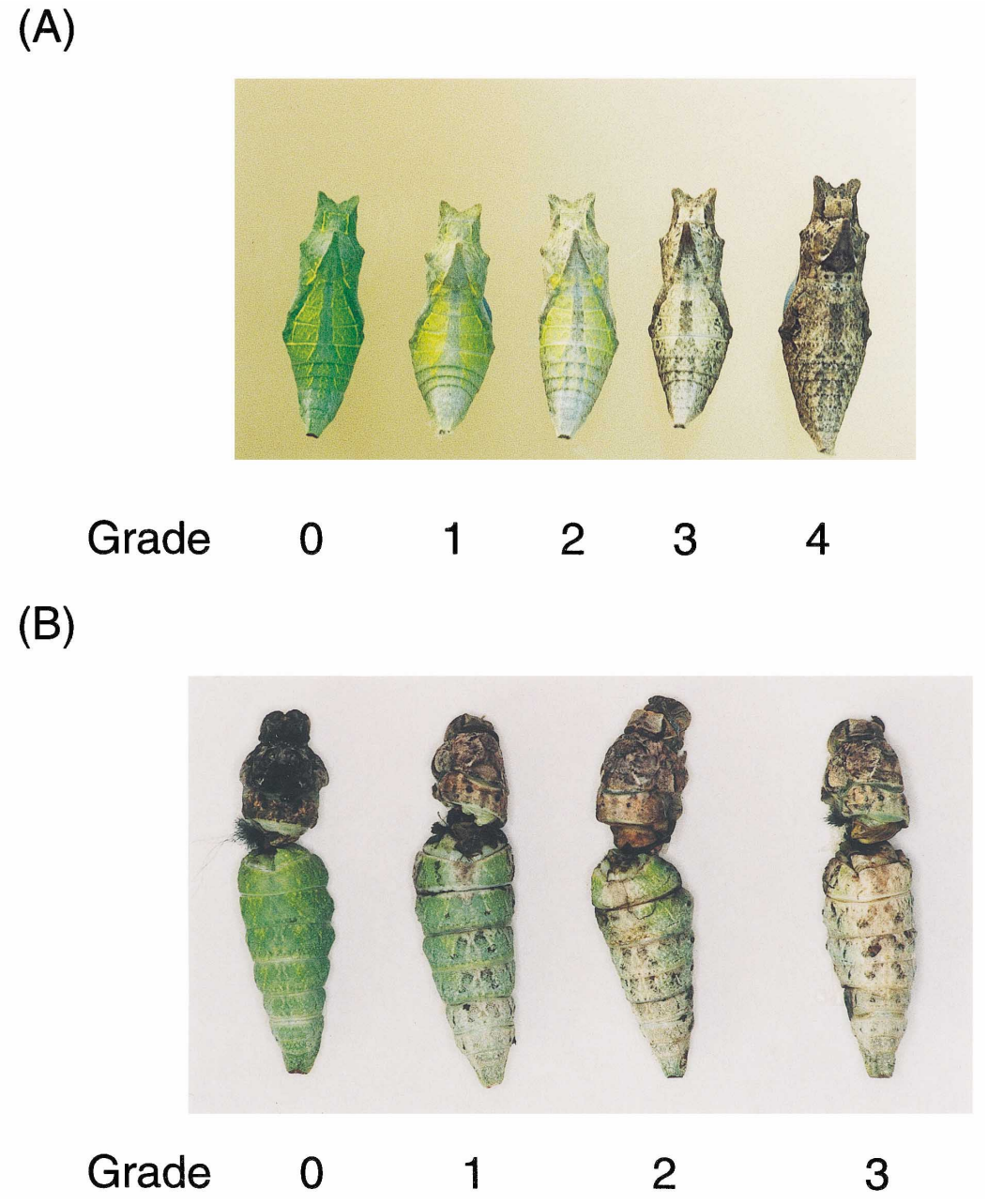

Fig. 1. (**A**) Classification of pupal cuticular melanization of *P. xuthus*. Grade 4 showed the typical brown type form whereas grade 0 showed the typical green form. Intermediates were classified grades 1–3 depending on their degree of cuticular melanization. (**B**) Classification of ligated pupal cuticular melanization of *P. xuthus*. Grade 3 showed the brown type whereas grade 0 showed the green. Intermediates were classified grade 1 and 2 depending on their degree of cuticular melanization. Grades 3, 2, and 1 were made by injection of 50, 20, and 5 Br-SG equivalents of 2 % NaCl fraction from *B. mori*. Grade 0 was made by injection of H_2O.

nization of the cuticle of ligatured pupal abdomens was classified into one of four grades, 0–3. Grade 3 was the brown type, whereas grade 0 was the typical green type. Intermediates were classified as grade 1 or 2, depending on the degree of cuticular melanization (Fig. 1B).

Staging of pharate pupae

The stages of pharate pupae (stage P1–6) were identified by observing the development of the antennal buds and eye pigment visible through the head capsule, as described by Hidaka (1961).

Extraction of PCMH and the PCMH-active factor

Brain-suboesophageal ganglion and prothoracic ganglion (Br-SG-PG) complexes were obtained from 0-day-old *P. xuthus* green pupae by dissection in 0.9% NaCl. Brain-suboesopha-geal ganglion (Br-SG) complexes were obtained from the silkmoth, *B. mori*. One hundred Br-SG-PG complexes from *P. xuthus* and 100 Br-SG complexes from *B. mori* were grouped and stored at –85°C. The PCMH (or PCMH-active factor of *B. mori*) was extracted by the method of Endo *et al*. (1988), with modifications as follows. A batch of 100 Br-SG-PG (or Br-SG) complexes was homogenized in 1.5 ml ice-cold acetone with a Teflon homogenizer and centrifuged for 15 min at 12,100*g* at 4°C. The resulting supernatant (acetone fraction) was lyophilized and stored at –85°C. The pellet was extracted twice with 1.0 ml of 80% ethanol and centrifuged under the same conditions. The supernatant (80% ethanol fraction) was lyophilized and stored at –85°C. The pellet was extracted with 1.0 ml of 2% NaCl in a boiling water bath for 4 min, cooled rapidly on ice and centrifuged for 30 min at 12,100*g* at 4°C. The resulting supernatant (2% NaCl fraction) was applied to a PD-10 column for desalting, lyophilized and stored at –85°C until use. The pellet was extracted with 1.0 ml of distilled H_2O and centrifuged. The supernatant (water fraction) was lyophilized and stored at –85°C. Finally, the pellet was extracted with 1.0 ml of acidic solution, 20 mM HCl in acetic acid, and centrifuged. The resulting supernatant (acidic fraction) was lyophilized and stored at –85°C.

Ion-exchange column chromatography

A 2% NaCl fraction prepared from 500 Br-SG complexes of *B. mori* was applied to a PD-10 column equilibrated with 10 mM ammonium acetate (pH 6.9) and eluted with the same solution according to the supplier's instructions. The eluate was lyophilized and the pellet suspended with 1.0 ml of 10 mM phosphate buffer (pH 6.9). The solution was applied to linked Sep-Pak Accell QMA and CM cartridge columns equilibrated with the same buffer. The linked columns were washed with the same buffer containing 20% acetonitrile. Each column was separated and eluted with the same buffer containing 20% acetonitrile and 0.6 M NaCl, respectively. The eluate of each QMA and CM cartridge (QMA or CM fraction) column was applied to a PD-10 column for desalting, lyophilized and stored at –85°C.

Gel filtration

A 2% NaCl fraction of *B. mori* (550 Br-SG equivalents) was supplemented with solid ammonium sulfate, up to 50% saturation. After standing overnight, the precipitate (50% ammonium sulfate fraction) was collected by centrifugation at 12,100*g* for 30 min at 4°C and dissolved in 1.0 ml 0.1 M ammonium acetate. The dissolved solution was applied to a Sephadex G-50 column (1.5×50 cm) pre-equilibrated with the same buffer and eluted with the buffer at a flow rate of 10 ml/hr. Fractions of 3.1 ml each were collected, lyophilized and then stored at –85°C until used for the bioassay. Molecular weight standards were cytochrome c (12,384 Da) and vitamin B_{12} (1,355 Da).

Reversed-phase high-performance liquid chromatography (RP-HPLC)

The 50% ammonium sulfate fraction from 1000 Br-SG complexes of *B. mori* was dissolved in 0.1% trifluoroacetic acid (TFA) and subjected to RP-HPLC using a VP-304 column (4.6×250 mm, Senshy Co., Tokyo). The column was eluted with a linear gradient of 20–50% acetonitrile on 0.1% TFA over 60 min at a flow rate of 1.0 ml/min. The elution profile was monitored by UV absorbance at 280 nm, and sample fractions were collected at 4 min intervals, lyophilized and stored at –85°C until use.

Bioassay of PCMH-activity

Larvae in the wandering stage were placed in cardboard boxes with a rough inner surface and ligatured between the thorax and abdomen at stage P2 as described by Hidaka (1961).

Ten μl of sample containing the extract of the desired Br-SG-PG (or Br-SG) equivalents was injected into each ligatured abdomen when the ligatured anterior reached stage P4. The injection was made into the dorso-lateral intersegmental region between the 6th and 7th abdominal segments, and the wound was sealed with paraffin wax. After pupation, the larval cuticle was removed in a 0.9 % saline solution using a fine forceps, and the pupae were classified into one of four grades. An average melanization degree (AMD) was obtained from the response of ligatured pupal abdomens using a classification based on a color gradient of the ligatured pupal cuticle.

Chemicals

Sephadex G-50 and PD-10 were purchased from Pharmacia (Uppsala, Sweden). Sep-Pak Accell QMA and CM cartridge columns were obtained from Waters (Massachusetts, USA). The solvents for chromatography were of HPLC grade. All other chemicals were of analytical grade.

RESULTS

Expression of green or brown type pupae

At the onset of the wandering stage, larvae were placed in either smooth-surfaced plastic boxes with pieces of green plants or in rough-surfaced cardboard boxes. Larvae placed in the plastic boxes mostly developed into green-type pupae; grades 0–1, 2 and 3–4 were 91.2%, 5.3% and 3.5%, respectively. The majority of larvae kept in cardboard boxes developed into brown-type pupae; grades 0–1, 2, and 3–4 were 1%, 0%, and 99%, respectively.

Stage-dependent changes on the effect of pharate pupal ligation on pupal cuticular color

Pharate pupae destined to develop into green- and brown-type pupae (G- and B-pharate pupae) were ligatured between the thorax and abdomen at six different stages (P1–6). All of the head-thorax complexes of B-pharate pupae developed into brown types. However, the proportion of ligatured abdomens which developed into the brown types varied with the stage of ligation. When ligatured at stages earlier than P4, the abdomens of B-pharate pupae developed into the green type. A large proportion of the abdomens of B-pharate pupae ligated at P6, and a few ligated at P5 developed into brown type. In contrast, the head-thorax complexes and abdomens of G-pharate pupae all developed into the green type, regardless of the stage of ligation (data not shown).

The results indicate that Br-SG-PG complexes of B-pharate pupae release the PCMH at stages P4 and P5. It may be the case that PCMH is not released in G-pharate pupae.

Extraction of the PCMH from *P. xuthus* and *B. mori*

Acetone, 80% ethanol, 2% NaCl, water, and acidic fraction were prepared from 400 Br-SG-PG complexes of *P. xuthus*, in addition to those from Br-SG complexes of *B. mori*. As shown in Table 1, PCMH-activity determined by an assay using ligatured abdomens of B-pharate pupae was mainly detected in the 2% NaCl fraction (AMD 2.4 and 2.2, respectively) of both insects. The PCMH-activities recovered in the 80% ethanol fraction (AMD 0.2) and water fraction (AMD 0.4) were lower than in the 2% NaCl fraction in both insects. Furthermore, PCMH-activities were not detected in the acetone or acidic fractions. In addition, all ligatured pharate pupal abdomens which had been treated with distilled water developed into the green types. G-pharate pupae were also ligatured between the thorax and abdomen, and the abdomens were injected with *P. xuthus* (or *B. mori*) factors of 30 Br-SG-PG (or Br-SG) equivalents. However, they did not show a response to all fractions (data not shown).

Dose-dependent response to crude PCMH-active extracts from *P. xuthus* and *B. mori*

A crude (2% NaCl) extract prepared from 200 Br-SG-PG complexes of *P. xuthus* and was injected into each ligatured abdomen of B-pharate pupae. The doses of extract injected into each abdomen were 5, 10, and 20 Br-SG-PG equivalents, respectively. In addition, a crude preparation eluted from

Table 1. Activity of the different extracts of PCMH from *P. xuthus* and PCMH-active factor from *B. mori*.

Source of extract and eluate fractions	No.	Degrees of ligatured pupal cuticular melanization				AMD
		0	1	2	3	
Br-SG complexes						
Papilio xuthus						
Acetone	10	10	0	0	0	0.0
80% EtOH	10	10	0	0	0	0.0
2% NaCl	10	0	0	6	4	2.4
Water	10	8	0	2	0	0.4
Acidic	10	10	0	0	0	0.0
Br-SG-PG complexes						
Bombyx mori						
Acetone	10	10	0	0	0	0.0
80% EtOH	10	8	2	0	0	0.2
2% NaCl	10	0	2	4	4	2.2
Water	10	10	0	0	0	0.0
Acidic						
Control						
Water	6	6	0	0	0	0.0
Untreated	9	9	0	0	0	0.0

Ten μl of sample containing the extract of 30 Br-SG equivalents was injected into ligatured abdomen when the ligatured anterior reached stage P4. An average melanization degree (AMD) for cuticular melanization was obtained from the bioassay response of 6–10 pupae.

Table 2. Effect of dose-response to crude PCMH-active extracts from *P. xuthus* and *B. mori* on the development of cuticular melanization.

Dose of Br-SG-(PG) complexes equivalents	No.	Degrees of ligatured pupal cuticular melanization				AMD
		0	1	2	3	
2% NaCl fraction from						
Papilio xuthus						
0 (H_2O)	4	4	0	0	0	0.00
5	4	1	3	0	0	0.75
10	4	0	2	2	0	1.50
20	4	0	0	4	0	2.00
CM fraction from						
Bombyx mori						
0 (H_2O)	4	4	0	0	0	0.00
5	4	0	4	0	0	1.00
10	4	0	3	1	0	1.25
40	4	0	0	1	3	2.75

Ten μl of sample containing an extract of the desired Br-SG-PG (or Br-SG) equivalents was injected into ligatured abdomen when the ligatured anterior reached stage P4.

a CM cartridge column with 0.6 M NaCl was also prepared from 500 Br-SG complexes of *B. mori* and injected into each ligatured abdomen of B-pharate pupae. The doses injected into each recipient were 5, 10 or 40 Br-SG equivalents, respectively.

The recipient abdomens of B-pharate pupae showed dose-dependent responses to a crude Br-SG-PG extract of *P. xuthus* and a crude Br-SG preparation of *B. mori* and developed into intermediates of green- and brown-types. The degree of pupal cuticle melanization increased with the dosage of the extracts (Table 2). When recipient abdomens of B-pharate pupae were injected with a crude *B. mori* preparation of 40 Br-SG equivalents they developed brown type cuticle (AMD 2.75).

Characterization of the PCMH-active factor from Br-SG complexes of *B. mori*

A crude PCMH-active preparation (50% ammonium sul-

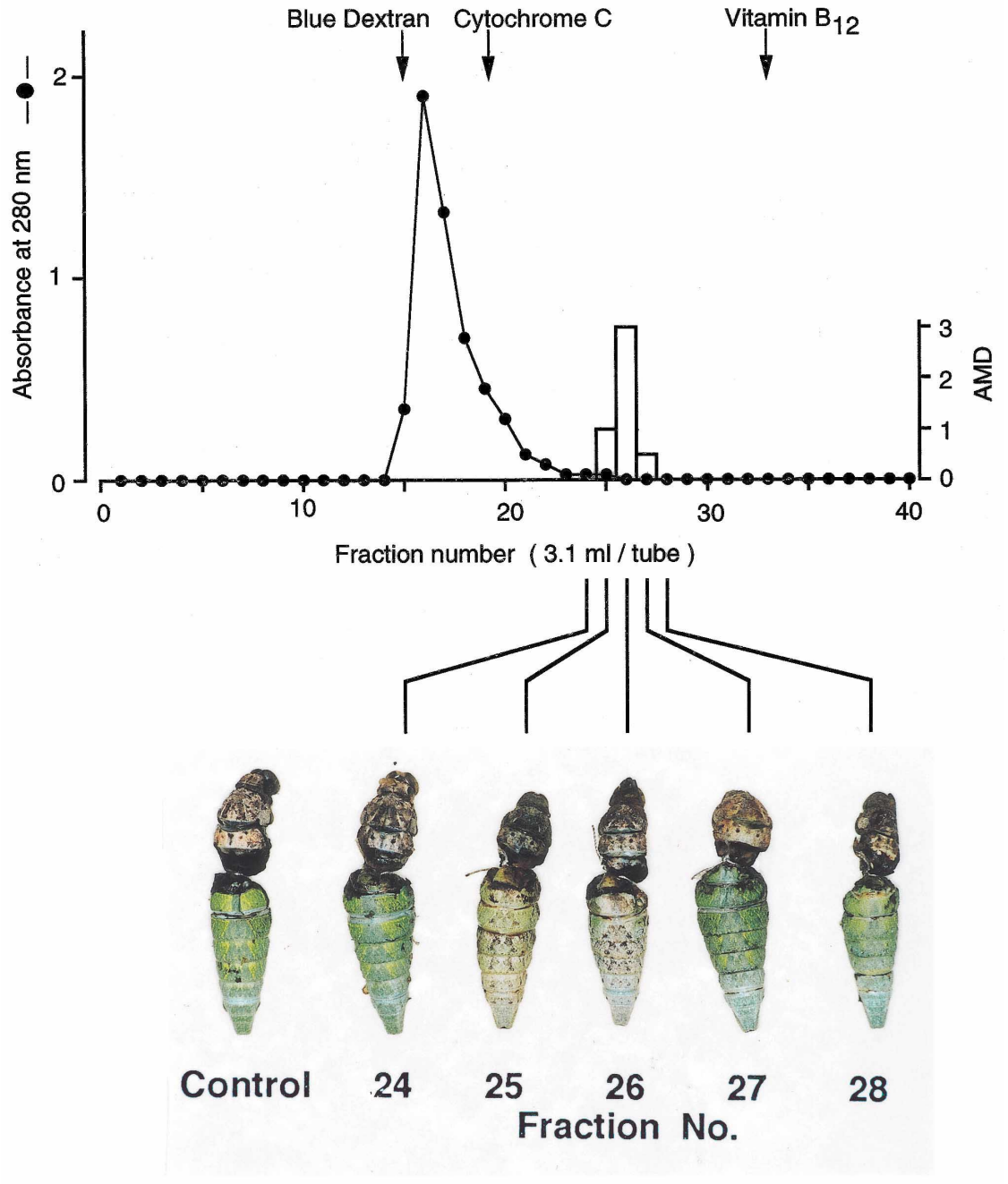

Fig. 2. Gel filtration profile of crude PCMH-active preparation (50% ammonium sulfate fraction) from Br-SG complexes of *B. mori* on a Sephadex G-50 column. Histograms show the AMD of the PCMH-activity detected from the fractions. Photograph shows result for the bioassay of each fraction on Sephadex G-50 column chromatography. Ten µl of sample containing the extract of 50 Br-SG equivalents was injected into the ligatured abdomen when the ligatured anterior reached stage P4. Four pupae were used for bioassays of PCMH-activity in each fraction.

fate fraction) prepared from 550 Br-SG complexes of *B. mori*, dissolved in 1.0 ml of 0.1 M ammonium acetate buffer (pH 6.9) , was applied to a Sephadex G-50 column. PCMH-activity was recovered in three fractions, from which the molecular mass of the factor was estimated to be 3,000–4,000 Da (Fig. 2).

A crude PCMH-active (2% NaCl) preparation was prepared from 1000 Br-SG complexes and applied to a Vydac C_4 cartridge column (Senshy, Co., Tokyo) which had been pretreated with bovine serum albumin. The column was washed with 0.1% TFA and eluted stepwise with 10%, 20%, 30%, 40%, and 50% acetonitrile. Each eluate fraction was lyophilized and bioassayed. PCMH-activity was detected in two fractions, those of 30% and 40% acetonitrile (data not shown). The 30% and 40% acetonitrile fractions were dissolved in distilled water and subjected to a second RP-HPLC with a VP-304 column.

Using RP-HPLC of the 30% and 40% acetonitrile fractions, PCMH-activity was eluted at 26%–34% and 30%–34% acetonitrile, respectively (Fig. 3). PCMH-activity was approximately 3 times greater in the eluate of the 40% acetonitrile fraction than in 30% fraction.

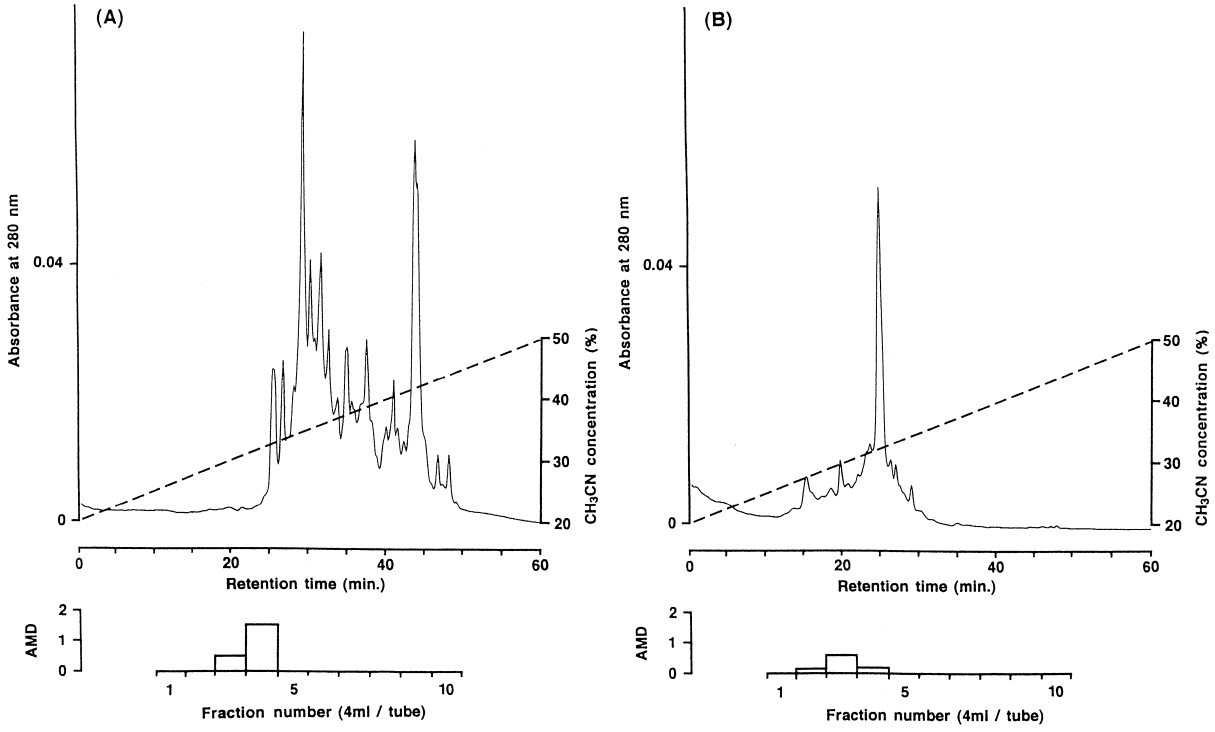

Fig. 3. Reverse phase HPLC of 40% acetonitrile (A) and 30% acetonitrile (B) fraction after C_4 column chromatography. The absorbance of fractions at 280 nm (–) is shown. Histograms show the AMD of PCMH-activity in the fractions. Ten µl of sample containing the extract of 20 Br-SG equivalents was injected into the ligatured abdomen when the ligatured anterior reached stage P4. Seven pupae were used for bioassay of PCMH-activity in each fraction.

Table 3. Affinity of the PCMH-active factor from SG-PG complexes of *B. mori* to anion- and cation-exchange columns.

Source of extract and eluate fractions	No.	Degrees of ligatured pupal cuticular melanization				AMD
		0	1	2	3	
Br-SG complexes						
Bombyx mori						
CM-fraction	5	1	2	2	0	1.2
QMA-fraction	3	2	1	0	0	0.3
Pass through	3	3	0	0	0	0.0
Control						
Water	7	7	0	0	0	0.0
Untreated	8	8	0	0	0	0.0

Ten µl of sample containing an extract of 30 Br-SG equivalents was injected into the ligatured abdomen when the ligatured anterior reached stage P4.

A PCMH-active fraction from a 2% NaCl extract of 500 Br-SG complexes that had been passed through a PD-10 column was applied to Accell QMA- and Accell CM-cartridge columns at neutrality (pH 6.9). Peptides bound to Accell QMA- and Accell CM-cartridge columns were eluted with 0.6 M NaCl, lyophilized and used for the bioassay, in addition to a fraction passed through the cartridge columns. PCMH-activity was mainly detected in the CM fraction (AMD 1.2), which was far greater than that from QMA fraction (Table 3).

The results indicate that the factor responsible for PCMH-activity from Br-SG extracts may be a hydrophobic peptide(s) that binds to a cation exchange resin at neutrality.

DISCUSSION

In the swallowtail, *P. xuthus*, a neuroendocrine factor plays a significant role in melanizing the pupal cuticle. The browning hormone, which has not been identified to be the same factor as the pupal-cuticle-melanizing hormone (PCMH), has been demonstrated to be produced in the brain and secreted from the prothoracic ganglion in the late pharate pupal stage (Hidaka, 1961).

PCMH was extracted from Br-SG-PG complexes of 0-day-old green-type pupae and assayed using ligatured abdomens of B-pharate pupae. In addition, a factor showing the same activity as *P. xuthus* PCMH was extracted from Br-SG complexes of the silkmoth, *B. mori*, and was quantified using the same bioassay.

PCMH is thought to be secreted by the Br-SG-PG complexes between developmented stages P4 and P5 in B-pharate pupae. That is, ligatured abdomens of B-pharate pupae developed into the green types when they were ligatured at stages earlier than P4. Abdomens of the brown or intermediate types were obtained when B-pharate pupae were ligatured at stages later than P5.

Ligatured abdomens of B-pharate pupae showed a dose-dependent response to crude PCMH extracts. When treated with different dosages of the crude PCMH extracts, the ligatured abdomens showed variable responses ranging from green to intermediate types (Fig. 2). For development of the brown type, a crude extract of PCMH greater than 40 Br-SG equivalents was required. All ligatured abdomens of G-pharate pupae treated with crude PCMH of 30 Br-SG equivalents developed into green type and did not show response to it.

It may be that the Br-SG-PG complex of B-pharate pupa secrets a relatively large amount of PCMH, causing the cuticle to be grade 4. Alternatively, a factor inhibiting the action of PCMH may be present in the hemolymph of G-pharate pupae or factors which are involved in the melanizing cascade reactions may be in short supply in the ligated abdomen of G-pharate pupae.

A factor with PCMH activity (PCMH) was also extracted from *B. mori* Br-SG complexes using almost the same procedure reported for extracting a SMPH-active factor (Endo *et al.*, 1988). The PCMH-active factor may be a different molecule from the one responsible for SMPH-activity: PCMH (3,000–4,000 Da) is smaller than the SMPH-active peptide (4,500–5,000 Da) (Fig. 2) and binds to CM resin while the SMPH-active factor is not bound at neutrality (Table 3). In addition, the acetonitrile concentration at which the PCMH-active factor was eluted by a RP-HPLC (30–40%) was less than that of the SMPH-active peptide (40–45%) (Tanaka *et al.*, 1997).

Recently, MRCH, diapause hormone (DH), and pheromone biosynthesis activating neuropeptide (PBAN) were purified from the silkworm, *B. mori*, and amino acid sequences were determined (Matsumoto *et al.*, 1986; Imai *et al.*, 1991; Kitamura *et al.*, 1989). These peptides share a common C-terminal amide (FXPRL amide). In contrast, cerebral neuropeptides of butterflies (SMPH and PCMH) were difficult to purify, and have not yet been isolated. We can not provide amino acid sequence data for the C-terminals as has been demonstrated in the *B. mori* peptides having FXPRL amide (Starnecker *et al.*, 1994, 1996). It remains to be investigated whether the neuropeptides of the butterflies have a common C-terminal amide. Attempts to purify PCMH are currently in progress.

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