The Proteinic Brain Hormone in an Insect, *Bombyx mori* L. (Lepidoptera: Bombycidae)

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This paper deals with both the procedure of extraction and the chemical characteristics of the proteinic brain hormone from pupal brains of the silkworm. Acetone dried brains were extracted with phosphate buffer solution and the extract was lyophilized. The 60 μ g crude extract yielded positive result on *Bombyx*-bioassay. The chemical properties of the active extract were examined with heat treatment at 60 or 100°C, buffer treatment at pH 9.9 or 1.4, digestive procedure with some proteolytic enzymes, and fractional precipitation by ammonium sulfate, all results showing that the extract belonged to proteinic substance. Then, the active fraction was separated from the crude extract by means of chromatographic studies with Sephadex and cellulose ion exchanger. When 1 μ g of the fraction-V obtained by CM-cellulose column was injected into each of test-objects, the latter became an imago in 3 weeks following the injection. Moreover, the highly purified, active ingredient was separated from the fraction-V on CM-cellulose by means of the zone electrophoresis on acrylamide gel, showing that the ingredient migrated to the anode. Thus two different constituents in the brain extract, sterol and protein showed brain hormone activity.

INTRODUCTION

In ordinary pupae of the silkworm, *Bombyx mori* L., the moths emerged in 11–14 days after pupation, in contrast to the pupae which were extirpated of thier brains immediately after pupation, in which no imaginal differentiation was observed for more than 40 days after pupation, although the operated pupae survived for 300 days. Such a pupa was called “Dauer-pupa” (KOBAYASHI, 1955, 1957).

When a fresh brain which was obtained either from larva, pupa or moth was implanted into the head of “Dauer-pupa”, it became an imago in 16–25 days following implantation (KOBAYASHI, 1957). Therefore, the “Dauer-pupa” was used as test-object for the bioassay of brain hormone activity.

The crude hormone extract from insect brains was first reported by KOBAYASHI and KIRIMURA (1958). Since 1957, about 1,000,000 brains of pupae, dissected and set-free from corpora cardiaca and corpora allata, have been used as material in the purification of the hormone. In 1962, KOBAYASHI *et al.* carried out isolation and crystallization of one of active ingredients of the brain hormone through several treatment from a fraction which was separated by the counter-current distribution method from crude but active extract of brain. It was determined that the substance finally obtained from the brain was identical with cholesterol according to the chemical characteristics and was so active that 0.02 μ g of it

caused the imaginal development of the "Dauer-pupa" (KIRIMURA *et al.*, 1962; KOBAYASHI *et al.*, 1962 a, b).

Furthermore, as shown in the previous paper (KOBAYASHI, 1963; SAITO *et al.*, 1963), the chemical properties of the active fraction other than cholesterol, obtained by the counter-current distribution method showed proteinic nature.

In 1961, ICHIKAWA and ISHIZAKI reported that an active ingredient of the brain hormone was contained in water soluble fraction of the brain extract. Thereafter, they claimed that an active ingredient of the brain hormone belonged to proteinic substance (ICHIKAWA and ISHIZAKI, 1963).

Considering the importance of ICHIKAWA and ISHIZAKI'S reports and our experiments on the proteinic brain hormone, we have done fractionation and purification of the proteinic hormone without inclusion of sterol using the methods as described below.

EXTRACTION OF THE CRUDE EXTRACT

Acetone dried brains of *Bombyx*-pupae were extracted with 0.1 M phosphate buffer solution and the extract was lyophilized immediately following the extraction. The 60 μ g extract as protein content showed positive result on the *Bombyx*-bioassay.

This crude but active extract was used as the starting material for the separation of active ingredient of the hormone.

CHEMICAL PROPERTIES OF THE CRUDE EXTRACT

Initially, inactivation of the crude but active extract by heat treatment was carried out as follows: When 200 μ g of the extract dissolved in 0.01 ml of distilled water was injected into each of "Dauer-pupae", after the fraction dissolved in distilled water was heated at 100°C for 20 minutes, its activity was not affected in this experiment as presented in Table 1. Also when 200 μ g of the extract was treated in 0.01 ml of each buffer solution at pH 9.9 (M/10 sodium veronal) or 1.4 (N/10 HCl) for 1 hour at 20°C, no change in hormonal activity was observed (Table 1).

Table 1. SOME TREATMENTS OF ACTIVE EXTRACT OF THE BRAIN HORMONE

Treatments	No. of test-objects	No. of moths emerged (days)	No. of pupal fatalities
Temperature			
60°C	10	8 (17), 2 (20)	0
100°C	10	6 (17), 2 (20)	2
pH			
9.9	10	3 (17), 3 (20)	4
1.4	10	5 (17), 3 (20) 1 (21)	1

The results obtained for 4 weeks following the injection at 25°C.

Secondly, inactivation of the extract by digestion with proteolytic enzymes was tried. In the first experiment, when 0.1 μg of each of the following enzymes, Pronase (the proteinase purified from *Streptomyces griseus*, supplied by the Kaken Co., Ltd.), Nagase (commercial name of crystalline subtilisin, the bacterial proteinase purified from *Bacillus subtilis*, supplied by the Nagase Co., Ltd.), trypsin and chymotrypsin, respectively, was injected into 5 groups of 10 "Dauer-pupae", all pupae did not show any sign of imaginal development for 4 weeks following the injection. In the second experiment, each of 200 μg of the extract was treated with 4 μg of each of the following enzymes, trypsin, chymotrypsin, Nagase, and Pronase at pH 8 for 60 minutes at 37°C. Thereafter, the digested fraction was treated by heating at 90°C for 30 minutes for the inactivation of those enzymes.

Each of fractions after the afore-going treatment was injected into 4 groups of 10 "Dauer-pupae" respectively. The results obtained were presented in Table 2.

Table 2. DIGESTION OF ACTIVE EXTRACT OF THE BRAIN
HORMONE BY PROTEOLYTIC ENZYMES

Digestion by	No. of test-objects	No. of moths emerged (days)	No. of pupal fatalities
Trypsin	10	3 (15), 4 (20)	3
Chymotrypsin	10	3 (15), 5 (20)	2
Nagase	10	0	1
Pronase	10	0	1

The results obtained for 4 weeks following the injection at 25°C.

From the afore-going results, it is concluded that the brain hormone extract is stable on heat treatment and it is inactivated by Nagase and Pronase, though it is scarcely inactivated by trypsin or chymotrypsin.

Thirdly, it was revealed by fractional precipitation of the crude extract by ammonium sulfate that active principle was obtained in a precipitate produced between 40 and 55 per cent saturations.

From the above-mentioned characteristics of the crude extract, it seems that the active ingredient of this hormone belongs to proteinic substance. Therefore, we have performed chemical works on the purification of the proteinic hormone as described below.

GEL-FILTRATION OF THE CRUDE EXTRACT

Each of 100 mg of the crude extract mentioned above was dissolved in 3 ml of 0.05 M phosphate buffer at pH 6.9 and applied to four different Sephadex columns (1.5×90 cm in size) equilibrated previously with the same buffer in cold room at 1–2°C respectively. The flow rate was 20 ml in an hour.

After the fraction was collected by tube, 4 ml each, and lyophilized in the same tube, the brain hormone activity of these fractions was examined with *Bombyx*-test. Positive bioassay was obtained in the fractions of tube number 10–20 on Sephadex G-50, 18–36 on G-75, 20–30 on G-100, and 42–52 on G-200 as shown

Fig. 1. Gel-filtration of the crude extract with Sephadex column. The black band showed a range of brain hormone activity.

with black band in Fig. 1.

CHROMATOGRAPHY ON DEAE-CELLULOSE

The crude but active extract of 150 mg dissolved in 2 ml of 0.01 M phosphate

buffer at pH 7.5 was applied to DEAE-cellulose column (0.9×30 cm) equilibrated previously with the same buffer in cold room at $1-2^{\circ}\text{C}$. The flow rate was 20 ml in an hour.

Fig. 2. Chromatography of the crude extract on DEAE-cellulose column by gradient elution with sodium chloride as shown. The dotted line: The sodium chloride concentration. The black band showed a range of brain hormone activity.

The gradient elution of absorbed protein was developed, after the material which was not absorbed in resin flowed out with the initial buffer. The material was eluted with a linear gradient of 0.01 M phosphate at pH 7.5 to 1 M sodium chloride in 0.01 M phosphate (pH 7.5) and finally with 0.5 N HCl solution. Meanwhile, the elute was collected in tubes, 4 ml each. The most active ingredient was not absorbed on DEAE-cellulose column, though the fraction-II had weak activity on the bioassay (Fig. 2).

CHROMATOGRAPHY ON CM-CELLULOSE

The crude but active extract of 150 mg dissolved in 2 ml of 0.01 M phosphate buffer at pH 6.0 was applied to CM-cellulose column (0.9×30 cm) equilibrated with the same buffer in cold room at $1-2^{\circ}\text{C}$. The material was gradually eluted by a linear gradient with 0.01 M phosphate at pH 6.0 to 7.5, 0.01 M phosphate at pH 7.5, a gradient with 0.01 M phosphate added with 1 M sodium chloride at pH 7.5 and finally 0.5 N NaOH solution. The flow rate was 20 ml in an hour. Positive bioassay was obtained in the fraction-V in this column (Fig. 3).

Fig. 3. Chromatography of the crude extract on CM-cellulose column by gradient elution with sodium chloride as shown. The dotted line: The sodium chloride concentration. The black band showed a range of brain hormone activity.

ACRYLAMIDE GEL ELECTROPHORESIS OF FRACTION-V ON CM-CELLULOSE

The fraction-V on CM-cellulose was used as material of acrylamide gel electrophoresis.

Zone electrophoresis was carried out on acrylamide gel using micro-free boundary electrophoretic apparatus, following MORIYA's method (MORIYA, 1964), with some modifications (NAKASONE and KOBAYASHI, 1965).

The electrophoresis on acrylamide gel was carried out with the afore-mentioned material with a current at 400 V for 1 hour at 5°C in refrigerator using discontinuous buffer system following TACHIBANA and YAMAURA (1965): 0.019 M tris-(hydroxymethyl)-aminomethane-0.001 M citric acid buffer of pH 8.8 was used for gel and 0.3 M boric acid-0.05 M sodium hydroxide of pH 8.1 for electrode compartments. The dye employed in the present work was 1 per cent solution of Amido Black 10 B.

For convenience the 5 major constituents were named A,B,C,D, and E, starting with the designation of A for the most rapidly migrating component and components, D and E appeared on the cathode side (Fig. 4). After the electrophoresis, components of both sides, the anode and the cathode, were eluted with deionized water and lyophilized respectively. The components of the anode side showed strongly positive bioassay, while those of the cathode side were negative, though the most active component of anode side was not determined. Therefore, it seems that component or components in the anode side is highly purified and active ingredient of the proteinic brain hormone.

Fig. 4. Electrophoretic patterns on acrylamide gel of the fraction-V on CM-cellulose column.

BIOASSAY OF THE PROTEINIC BRAIN HORMONE

"Dauer-pupae" of *Bombyx mori* were used as test-objects for the assay of the brain hormone activity. They were observed for about 5 weeks at 25°C prior to utilization. Each test animal was injected with 0.01 ml of various fractions of proteinic substance dissolved in distilled water. As shown in Table 3, the organisms emerged as moths in about 3 weeks following the injection at 25°C. However, no moth emerged from 40 control organisms, that were not injected or a mock injection of 0.01 ml of distilled water without proteinic substance was performed.

Table 3. THE BRAIN HORMONE ACTIVITY OF PROTEINIC SUBSTANCE ISOLATED FROM BRAIN EXTRACT

Kind of fractions	Dose (μ g)	No. of test-objects	No. of moths emerged	Weeks from injection till emergence	No. of pupal fatalities
G-50-I	26	22	20 ^a	3	2
G-75-II	18	22	20 ^a	3	2
G-100-III	32	16	16 ^a	3	0
G-200-III	33	12	11 ^a	3	1
DEAE-I	45	16	14 ^a	3	2
DEAE-II	100	20	11 ^a	3	3
DEAE-II	20	20	3	3	2
CM-V	3	20	17 ^a	3	3
CM-V	1	10	9 ^a	3	1
CM-V	0.5	10	5	3	2
CM-V	0.02	10	4	3	1
Control	0	40	0	—	0

^a Positive: Tests were considered positive when 55 per cent or more of the specimens pupated.

The results obtained for 4 weeks following the injection at 25°C.

Dose of each fraction from G-50-I to DEAE-II was determined by LOWRY-FOLIN method.

From the afore-going results of chromatographies on Sephadex and cellulose ion exchanger, the filtration with Sephadex was not good for the isolation of proteinic hormone. Gel-filtration was suitable for the preliminary purification.

CM-cellulose was suitable for the isolation of the brain hormone as presented in Table 3. Consequently, it has been cleared that isolation of proteinic brain hormone can be carried out by the suitable combination of both chromatographies on Sephadex and cellulose ion exchanger.

Therefore, considering the importance of steroid as one of active ingredients of the brain hormone (KIRIMURA *et al.*, 1962; KOBAYASHI *et al.*, 1962 a, b), it seems that the brain hormone in insects consists of steroid and proteinic substances.

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