Molecular Size of the Diapause Hormone of the Silkworm *Bombyx mori*

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The diapause hormone (DH) responsible for arrested development of the silkworm *Bombix mori* consists of two active principles of peptide (A and B). Since both hormones form aggregates in aqueous phases, the molecular weights were determined by gel permeation chromatography of Merckogel OR 6000 using methanol-dichloromethane mixture as the developing agent. Gramicidins and modified peptides soluble in the organic solvents were used as the standard markers for the molecular weight measurements of the hormones. The molecular weights of DH-A and -B are estimated to be 3300 ± 400 and 2000 ± 200, respectively.

Introduction

In 1951 it was demonstrated that the suboesophageal ganglion is involved in embryonic diapause of the silkworm *Bombyx mori* [1]. Then the neurosecretion from the ganglion was extracted and termed as the diapause hormone [2]. The hormone has attracted attention from physiological points of view (see references cited in 3 and 7). On the other hand, the purification of the diapause hormone (DH) was advanced [3] and it was revealed that the hormone consists of two active principles (A and B) [3]. One of these (DH-A) was characterized as a peptide consisting of 14 kinds of amino acids and two kinds of amino sugars [4]. Another active principle (DH-B) has recently been isolated [5] and found to have similar characters to DH-A [6]. Hormonal activity of DH-B was reported to be more than three fold that of DH-A [7].

As already reported, DH-A forms aggregates comprising large molecules in aqueous solutions [3]. The same is true in the case of DH-B [5, 6]. Thus, routine aqueous gel filtration technics for biochemical analyses of peptides cannot be applied in the present case. Accordingly we adopted gel permeation chromatography (GPC) using non-aqueous solvents. A number of modified peptides with certain molecular weights were used as standard markers for the molecular weight measurements of DH-A and -B. Based on the calibration curve obtained with these peptides on the GPC of Merckogel OR 6000 using a mixture of methanol and dichloromethane (1:1, v/v), we were able to determine the molecular weights of these two hormones.

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Abbreviations: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Cl_2Bzl, 2,6-dichlorobenzyl; ClZ, *o*-chlorobenzyloxycarbonyl; Dipmoc, diisopropylmethyloxycarbonyl; OBzl, benzyloxy; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl; Z(OMe), *p*-methoxybenzyloxycarbonyl.

Materials

DH-A was prepared by the methods reported previously [3], and DH-B by the same methods with a slight modification [5].

As the standard markers for molecular weight determination, organic solvent-soluble peptides with molecular weights ranging from 1140 to ca. 6000 were used; gramicidin A and S were purchased from Sigma Chemical Co., U.S.A., and synthetic and modified peptides were kindly furnished by the Protein Research Foundation, and the Shionogi Research Laboratory, Shionogi Co., Ltd., Osaka, Japan. Additionally we used a peptide fraction obtained during DH purification [3]. This fraction was a mixture of polypeptides with high molecular weight, probably exceeding 6000. These markers are listed in the legend to Fig. 1. In addition, commercially available standard polyethylene glycols with molecular weights of 1000, 2000, 3000 and 7500 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were also used for GPC as references.

Methods and Results

Gel permeation chromatography (GPC) was conducted under the same condition as was described already for the purification of DH[3]; 45 g of Merckogel OR 6000 (E. Merck, Darmstadt, Germany) was packed in a glass column (0.8 × 200 cm), and a few milligrams of each sample dissolved in 0.2 ml of methanol-dichloromethane (1:1, v/v) was placed onto the column. The column was developed with the above solvent system at a flow rate of 0.6 – 0.8 ml per hour. Each 0.8 ml (DH-A and -B) or 1.7 ml (peptides and polyethylene glycols) of eluents was collected in a tube. The chromatogram was monitored by weighing the residue of the eluents after evaporation. The effluent volumes of the markers were plotted against the logarithm of their molecular weights (Fig. 1). As indicated in this figure, an almost linear relationship (solid line) between effluent volumes and molecular weights of peptide markers as well as polyethylene glycols (broken line) except the one having the molecular weight of 7500 was observed. In several runs of DH-A and -B, they appeared in the effluent volumes of 23 to 24 ml and 32 to 33 ml, respectively, which are indicated in Fig. 1. Thus, the molecular weights of DH-A and -B were calculated to be 3300 ± 400 and 2000 ± 200, respectively.

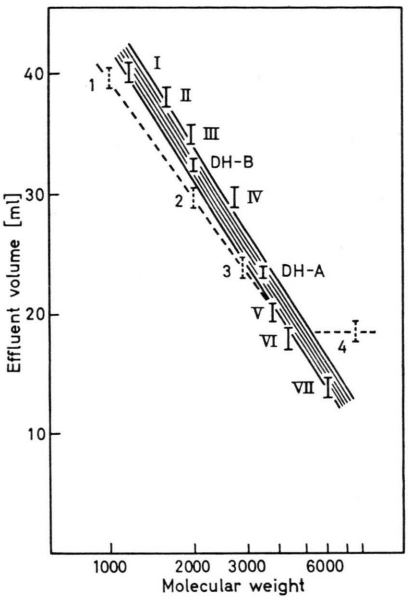

Fig. 1. Relationship between molecular weights and effluent volumes of peptides (solid line) and polyethylene glycols (broken line). The effluent volumes of samples are protted against the logarithms of molecular weights. Roman numerals denote the following peptides;
I, gramicidin S (molecular weight, 1140);
II, protected bradykinin (1493) [Z(OMe)-Arg(NO_2)-Pro-Pro-Gly-Phe-Ser(Bzl)-Pro-Phe-Arg(NO_2)-OBzl];
III, gramicidin A (1880);
IV, protected neutrotensin fragment (2609) [Z-Leu-Tyr(Cl_2Bzl)-Glu(OBzl)-Asn-Lys(ClZ)-Pro-Arg(Tos)-Arg(Tos)-Pro-Thr(Cl_2)-Ile-Leu-OBzl];
V, protected calcitonin fragment (3604) [Boc-Lys(Dipmoc)-Leu-Ser(Bzl)-Gln-Glu(OBzl)-Leu-His-Lys(Dipmoc)-Leu-**Gln-Thr(Bzl)-Tyr(Bzl)-Pro-Arg(Tos)**-Thr(Bzl)-**Asp(Obzl)-Val-Gly-Ala-Gly-Thr(Bzl)-Pro-NH_2**];
VI, protected ACTH fragment (4183) [a nonacosapeptide fragment of ACTH which has new protective groups [9]];
VII, peptide fraction (>6000) [obtained by Sephadex LH-20 column chromatography during DH purification (see text)].
Arabic numerals denote polyethylene glycols with molecular weights, 1000, 2000, 3000 and 7500. The effluent volumes of the hormones are indicated in 'DH–A' and 'DH–B'. **Fraction size in the gel permeation chromatography is 0.8 ml** in the case of DH and 1.7 ml, peptides and polyethylene glycols.

Discussion

Previously, by gel permeation chromatography (GPC) with Sephadex LH-20 and Merckogel OR 6000 using solvent system of methanol-dichloromethane, the molecular weight of DH-A was roughly estimated to be between 2000 and 4000, and that of DH-B was suggested to be slightly smaller[3]. In the series of works to elucidate the chemical structures of these hormones, it is necessary to determine their molecular weights more accurately. For this purpose, relationship between the molecular weights and effluent volumes in GPC should be compared among the similar kinds of compounds. Polyethylene glycols, simply constructed polymers, exhibited a simple relationship between those two factors (broken line in Fig. 1). However, such a relationship is not always observable among fairly complex molecules even in the same category of compounds. For example, the molecular weights of complex lipids prepared from bovine brain did not run parallel to their effluent volumes on methylated Sephadex G-25 column developing with methanol-chloroform mixture; some lipid of a higher molecular weight was eluted after the one with the smaller[8]. In the present case, fortunately, among various kinds * of peptides and their derivatives, an almost

* Gramicidin S is a cyclic decapeptide, and gramicidin A a linear peptide modified in the form of formamide and β-hydroxyethylamide at the N- and C-terminal, respectively. Protected calcitonin fragment has an amide group at the carboxyl end, and protected ACTH fragment has a free N-terminal but an esterified C-terminal[9]. Functional groups of other peptides are fully blocked by various kinds of protective groups.

linear relationship between those effluent volumes and molecular weights was observed with the Merckogel GPC system as shown in Fig. 1 (solid line). Incidentally, considering these points, the Merckogel GPC system we adopted here would be useful not only for the molecular weight determination but also separation for the intermediates in peptide syntheses since they have many protective groups and have little or no solubility to water. Anyhow, the molecular weights of DH-A and -B estimated from this GPC system (3300 ± 400 and 2000 ± 200, respectively) appear to be reliable.

The above estimation of the molecular weights of two diapause hormones was further substantiated as follows; amino acid analysis suggests that DH-A contains 23 to 24 molecules of amino acids and two molecules of amino sugars (see Table 3 in Isobe *et al.*[4]). The molecular weight, therefore, becomes 2800 or 2900 which is obtained by a simple summation of molecular weight of each amino acid residue. DH-B consists of about 20 amino acids and no amino sugar[6]; the calculated molecular weight being ca. 2200. The analysis of amino acid sequence as well as other chemical studies on the two hormones are in progress.

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