Pure & Appl. Chem., Vol. 61, No. 3, pp. 349–352, 1989.
Printed in Great Britain.

Isolation and structures of peptide hormones from the silkworm *Bombyx mori*: bombyxins – peptides structurally related to insulin

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Abstract - Bombyxins have been isolated from the heads silkworm, Bombyx mori, and stimulate the adult formation of the debrained pupae of Samia cynthia ricini. The amino acid sequence of bombyxins has suggested that bombyxins are the first insulin-related peptides isolated from the invertebrate animal.

INTRODUCTION

In 1917, Kopec first described the endocrinological function of insect brain in the metamorphosis. Until early-1950's, the role of a brain hormone, prothoracicotropic hormone (PTTH), in the insect metamorphosis had been established. In 1958, Kobayashi and Kirimura reported that the adult formation of brainless pupae of Bombyx mori was occured by injecting the brain extract of Bombyx pupae. Soon after that, Ichikawa and Ishizaki showed that the extract of Bombyx pupal brains provoked the adult formation of the brainless dormant pupae of Samia cynthia ricini and that the active principle should be proteinic. In early-1970's, we have started colaborating to isolate PTTH active to the brainless Samia pupae (ref. 1).
Starting with large amounts of the silkworm heads, we have succeeded in isolating PTTH active to Samia pupae in 1982 (ref. 2). In the course of purification of PTTH, we have confirmed that the extract of Bombyx heads contains two species-specific PTTHs, one (temporarily termed 22k-PTTH) is active to the brainless Bombyx pupae and other (recently termed bombyxin) active to the debrained Samia pupae (ref. 3). Recently, we have isolated three molecular species of bombyxins, bombyxin-I, -II and -III, and determined the whole amino acid sequence of bombyxin-II, which is suggested to be the first insulin-related peptide isolated from the invertebrate animal (ref. 4 and 5).

ISOLATION AND CHARACTERIZATION

For purification of bombyxins, the bioassay was made by using the brainless dormant pupae of S. cynthia ricini and the titers of bombyxins were quantified by Samia unit; one Samia unit was defined as the minimal dose of bombyxins necessary to cause the adult formation in an assay pupa (ref. 1). The starting materials for extracting bombyxins were the adult heads of B. mori, which were easily collected in large amounts by a simple operation cutting the heads with a razor blade.
The purification procedure is summerized in Fig. 1 (ref. 6). The first 8 steps consisted of simple precipitations, by which we could overcome the disadvantage of using the whole heads of Bombyx adults as starting materials in stead of the brains, and from step 9 to 14 the conventional chromatographieere used. The final step was a reversed phase high performance liquid chromatography (HPLC) using Partisil ODS-3 column to afford the isolation of bombyxin-I. Bombyxin-II and -III were also isolated by a reversed phase HPLC using Develosil ODS-5 column. The yields of bombyxin-I, -II and -III were 50, 36 and 63 ug, respectively , from about 650,000 Bombyx adult heads and each bombyxin was active to a brainless Samai pupa at a dose of 0.1 to 0.4 ng (0.1 to 0.4 ng/Samia unit). Then, the overall purification was about 1.7×10^6 fold. When 5 to 500 pg of bombyxin was added to 100 ul of the culture medium of a prothoracic gland taken out freshly ecdysed Samia pupa, the ecdyson release from the prothoracic gland was markedly increased, three times as much as that of control.
Bombyxins were suggested to be heterodimeric peptides connected by disulfide bonds with molecular weight of 4 to 5 kDa. The amino terminal amino acid sequences of bombyxins were determined by a gas phase protein sequencer and cystein residues were confirmed through sequencing the S-carboxamide methyl derivatives, indicating the homology in these bombyxins.

The replacement of amino acid residues in these bombyxins occures in such a way that the hydrophylic and hydrophobic nature of amino acid residues is retained at their respective positions and the biological function of bombyxins can tolerate these amino acid substitution, because three bombyxins show almost same level of biological activity (ref. 4).

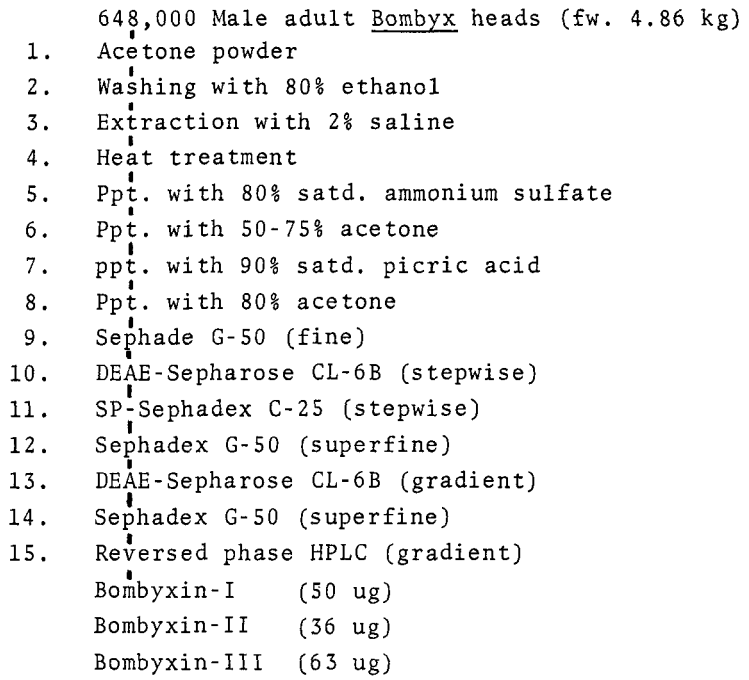

Fig. 1 Purification procedure of bombyxin-I, -II and -III

	1								10										20
Bombyxin-I	G	V	V	D	E	C	C	F	R	P	C	T	L	D	V	L	L	S	Y	C
Bombyxin-II	G	I	V	D	E	C	C	L	R	P	C	S	V	D	V	L	L	S	Y	C
Bombyxin-III	G	V	V	D	E	C	C	L	Q	P	C	T	?	D	V	V	A	T	Y	C

Fig. 2 Amino terminal amino acid sequences of bombyxin-I, -II and -III

AMINO ACID SEQUENCE OF BOMBYXIN-II

Bombyxin-II was reductively alkylated to yield the carboxamide methyl derivative, which was separated into two peptides, A and B chain peptides, by HPLC, indicating that bombyxin-II was a heterodimeric peptide, in which A and B chains were connected with disulfide bonds. The amino acid sequences of A and B chain peptides were determined by the combination of enzymatic digestion with trypsin, α-chymotrypsin and pyroglutaminase, and sequencing the resulted fragmental peptides (ref. 5). The carboxyl terminal amino acid sequences of both peptides were confirmed by the results of carboxypeptidase A digestion.

A chain

	1								10										20	
Human insulin	G	I	V	E	Q	C	C	T	S	I	C	S	L	Y	E	L	E	N	Y	C	N
Bombyxin-II	G	I	V	D	E	C	C	L	R	P	C	S	V	D	V	L	L	S	Y	C	

B chain

	1								10								20						
Human insulin	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	
Bombyxin-II	X	Q	P	Q	A	V	H	T	Y	C	G	R	H	L	A	R	T	L	A	D	L	C	W	E	A

X: pyroglutamic acid

	G	F	F	Y	T	P	K	T
	G	V	D					

Fig. 3 Amino acid sequences of bombyxin-II and human insulin

To determine the mode of disulfide bonds, bombyxin-II was digested with thermolysin to give five fragments, Th1 - 5. Since Th4 and 5 contained only one disulfide bond, the disulfide bond, A_{20}- B_{22}, was unequivocally established. Because Th3 contained two disulfide bonds, there were three possible structures, (a), (b) and (c), for Th3. All of these three peptides were synthesized and compared with Th3 by HPLC, indicating that Th3 was coeluted with peptide (b). Thus the disulfide bonds were estabilished to be A_6 - A_{11} and A_7 - B_{10} and the whole amino acid sequence of bombyxin-II was determined along with the mode of three disulfide bonds, which was exactly the same as that of insulin (ref. 7).

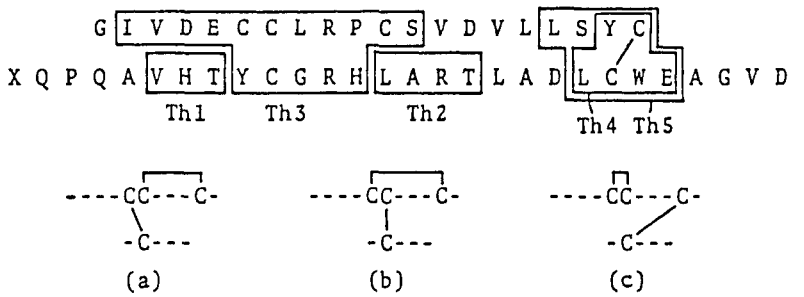

Fig. 4 *Thermolysin digestion of bombyxin-II and possible structures for the fragmental peptide, Th3*

Fig. 5 The whole amino acid sequence of bombyxin-II

The synthesis of bombyxin-II was attempted to confirm its amino acid sequence. The protected A and B chain peptides were synthesized by a peptide synthesizer of Applied Biosystems. After deblocking, the free A and B chain peptides were combined, reduced with dithiothreitol and reoxidazed with air to afford bombyxin-II in a yield of 4%. The peptide maps of the thermolysin digests of the natural and synthetic bombyxin-II were completely identical. Further the synthetic bombyxin-II showed almost the same activity to the assay pupae as the natural one. Thus the amino acid sequence of bombyxin-II was synthetically confirmed (ref. 7).

BOMBYXINS BELONG TO INSULIN FAMILY PEPTIDES

The most striking feature of bombyxins is the homology with insulin; the homology in A chain is approximately 50% and that of B chain about 40%. In addition, the mode of three disulfide bonds are completely identical with each other and the hydrophobic core residues in bombyxin-II, A_2 (Ile), A_{16} (Leu), B_{14} (Leu) and B_{18} (Leu), are also identical with those of insulin. The glycine residues, which may contribute to the fold of peptide chains, at A_1, B_{11} and B_{26} in bombyxin-II is homologous with those of insulin. Based upon these facts, a three dimensional model of bombyxin-II has been constructed using computer graphics, showing that bombyxin-II can assume an insulin-like tertiary structure (ref. 8).
Recently the gene coding a bombyxin has been cloned using a Bombyx gene library and synthetic DNA probe and the DNA sequence of the bombyxin gene indicates that bombyxin are biosynthesized in the form of prepropeptide followed by post-translational processing (ref. 9). Thus bombyxins can be considered to be a member of the insulin family peptides. In spite of considerable homology, insulin was inactive to the adult formation of brainless Samia pupa even at a dose of 1 ug, 10^4 times as much as the minimal active dose of bombyxins, and bombyxins failed to react with guinea pig antibody of porcine insulin.
Bombyxins and insulin family peptides might have arisen from a common ancestral gene, which must be considerably conserved during the evolution to insects and mammals with different functions. From this view point, it is worthy of note that the presence of the gene coding an insulin-related peptide in Mollascan, termed mollascan insulin-related peptide (MIP) has recently been reported irrespective of the biological functions (ref. 10).

Acknowledgements

Auhtors wish to thank Emeritus Professor Saburo TAMURA, The University of Tokyo, for his unfailing encouragement. This work was partly supported by grants from the Ministry of Education, Science and Culture of Japan.

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