Research Journal of Biological Sciences 2 (3): 315-318, 2007
© Medwell Journals, 2007

Cloning and Sequence Analysis of Bombyxin G Gene from Silkworm *Bomby mori* Larvae

Zhaohui Gong, Yanping Le, Junming Guo and Qiong Liu
Institute of Biochemistry and Molecular Biology, School of Medicine,
Ningbo University, Ningbo 315211, People Republic of China

Abstract: Bombyxin is an insulin-related peptide synthesized in the brain of the silkworm *Bombyx mori*. Here we cloned a new member of bombyxin gene family (named G gene) from the Chinese-race silkworm larvae. The Open Reading Frame (ORF) of G gene encodes a precursor molecule which consists of four domains in the order of signal peptide, B chain, C-peptide and A chain and shares 99% sequence identity with bombyxin previously characterized. The result of protein prediction demonstrates that there are 45.6% of α-helix, 10.0% of extend strand, 4.4% of β-turn and 40.0% of random coil in the secondary structures. Whereas the natural function in insect biology of this newly cloned bombyxin G protein need to be further investigated.

Key words: Bombyxin, insulin, *Bombyx mori*, gene family

INTRODUCTION

Bombyxin was first identified as a neurosecretory hormone in the silk moth, *Bombyx mori* (Nagasawa *et al*., 1984). It was isolated because of its ability to stimulate ecdysone secretion by the prothoracic glands of the moth *Samia Cynthia* and was therefore believed to be the long-sought prothoracicotropic hormone of insects (Nagasawa *et al*., 1986; Nijhout, 1981). However, bombyxin has no significant stimulating effect on the prothoracic glands of *B. mori* either *in vivo* (Ishizaki *et al*., 1983; Suzuki and Ishizaki, 1986) nor *in vitro* (Kiriishi *et al*., 1992).The bombyxin gene encodes a precursor molecule which consists of 4 domains in the order of signal peptide, B chain, C-peptide and A chain and shares nucleotide sequence similarity with the vertebrate insulin gene (Iwami *et al*., 1989).

The copy number of the bombyxin gene is more than 32 perhaploid Bombyx genome and the 32 gene copies have been classified into 7 families, A, B, C, D, E, F and G, according to their sequence similarity (Kondo *et al*., 1996; Tsuzuki *et al*., 1997; Yoshida *et al*., 1997; Yoshida *et al*., 1998). The Bombyx haploid genome thus includes 10family-A, 12 family-B, 6 family-C, 1 family-D, 1 family-E, 1 family-F and 1 family-G gene. The bombyxin genes except the family-E gene cluster in two DNA segments in unique distribution patterns. Their arrangement is classified into three categories: gene pairs, gene triplets and single genes. All bombyxin genes so far characterized lack introns, indicating that they are presumably the functional processed genes or genes derived from the processed genes (Iwami *et al*., 1989; Iwami *et al*., 1990; Iwami, 1990). Bombyxin genes are expressed predominantly in the brain (Iwami *et al*., 1989; Kawakami *et al*., 1989; Iwami, 1990) and at low levels in a number of other larval tissues (Iwami *et al*., 1996), in contrast to the insulin gene which is expressed in the gastroenteromic organ and is almost silent in the brain.

In the present study, we cloned a new member of bombyxin gene family (named G gene) and analyzed the DNA sequence and secondary structure prediction. The natural function in insect biology of this newly cloned bombyxin G protein need to be further investigated.

MATERIALS AND METHODS

Insects: A racial hybrid of *B. mori*, Jingsong×Haoyue (Showa) was fed fresh mulberry leaves and reared at 25±1°C under a photoperiod of 12-h light and 12-h darkness. Larvae were staged on the day of ecdysis to the fifth-instar used for experiments on the next day (day 1 of the fifth-instar).

Genomic DNA extraction: The genomic DNA was extracted from the second instar larvae by using the universal genomic DNA extraction kit (TaKaRa) according to the manufacturer's protocol. Briefly, fresh larval brain tissue was processed by freezing with liquid nitrogen and grinding into a fine powder using a mortar and pestle. Add 50 mg of this larvae powder to a 1.5 mL microcentrifuge tube. The remainder of the procedure was identical to the manufacturer's protocol.

PCR amplification: Based on the structure and expression of bombyxin G1 gene (Yoshida *et al*., 1998) two primers were designed, P1: 5'-GCGGATCC

Corresponding Author: Zhaohui Gong, Institute of Biochemistry and Molecular Biology, School of Medicine, Ningbo University, Ningbo 315211, People Republic of China

ATGAAGCTCATCA-3'(*Bam*HI) and P2: 5'-GGGAATTC TTAACAATAAGAAC-3'(*Eco*RI). Two microliters of serially diluted genomic DNA samples were added to 50 μL reaction mixture containing 0.2U Taq DNA polymerase (TaKaRa) for PCR. Then the PCR was performed as follows: (a) Denaturation: 4 min at 94°C; (b) Amplification: 35 cycles for all cytokines, 20 sec at 94°C, 20 sec at 55°C and 30 sec at 72°C and (c) Extension: 10 min at 72°C. Twelve microliters of each sample was run on a 2% agarose gel, stained with ethidium bromide and viewed using ultraviolet light.

DNA sequencing and sequence analysis: The PCR product (obtained in the above section) was purified by a PCR fragment recovery kit (TaKaRa) and then sequenced by a DNA sequencer (ABI). By using different biosofts to analysis the sequence of this cloned gene and predict the secondary structure of its deduced protein.

RESULTS

PCR amplification: The previous study indicated that all bombyxin genes so far characterized lack introns and hold high sequence homology (Yoshida *et al*., 1998). Therefore direct PCR amplification was performed with a pair of synthesized primers. The result of PCR showed the size of specific gene fragment was approximately 270 bp (Fig. 1). This evidence indicated that the new bombyxin G gene was cloned.

DNA sequencing and sequence analysis: The result of DNA sequencing showed that the length of the bombyxin G gene was 270 bp and encoded 90 amino acids (Fig. 2). By comparing to others genes in the bombyxin gene family, the bombyxin G gene encodes a precursor peptide which shows 99% sequence identity with bombyxin G1 and G1' genes previously characterized, except for two positions (+105 and +257).

The result of amino acid sequence alignment showed one-amino acid difference was found among bombyxin G, G1 and G1' (Fig. 3). Furthermore, the open reading frame of the G gene with four domains in the order signal peptide (1-19)/B chain (20-46)/C-peptide (49-67)/A chain (70-90), as in human insulin.

Protein secondary structure prediction: To investigate the secondary structure of bombyxin G protein, the prediction was performed online via web (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = npsa_sopma.html). The result showed that 41 amino acids formed α-helix (45. 6%), 9 amino acids formed extend strand (10.0%), 4 amino acids formed β-turn (4.4%) and 36 amino acids formed random coil (40.0%) in the secondary structures (Fig. 4).

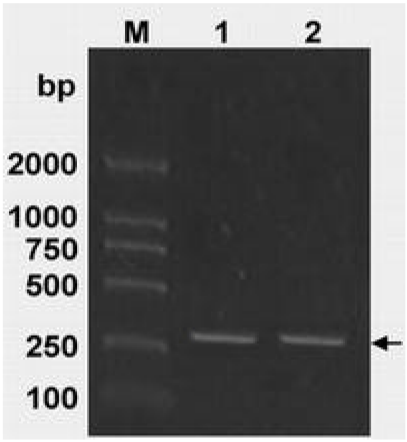

Fig. 1: The fragment of bombyxin G by PCR amplification. M, DNA marker DL 2000 (TaKaRa); lane 1 and 2, PCR products of bombyxin G gene. The arrow indicates the 270 bp target gene

```
atgaagctcatcatatttgttgttttctgcatcacaatctacggttcaacgtcgggacaa
 M  K  L  I  I  F  V  V  F  C  I  T  I  Y  G  S  T  S  G  Q
caagaagtcgctcgcagatattgcggaagacacttagcagttacgatggcagatctgtgc
 Q  E  V  A  R  R  Y  C  G  R  H  L  A  V  T  M  A  D  L  C
ttcggagttcagtttgataaacgcaatacgcagtacgaaggctatcactggccgttactg
 F  G  V  Q  F  D  K  R  N  T  Q  Y  E  G  Y  H  W  P  L  L
gcgtactccgaagagcgtatcaagcgccaaggcatagctgatgagtgctgtctcgttccg
 A  Y  S  E  E  R  I  K  R  Q  G  I  A  D  E  C  C  L  V  P
tgcacgactaatgtttcgagttcttattgttaa
 C  T  T  N  V  S  S  S  Y  C  -
```

Fig. 2: Nucleotide sequence and deduced amino acid sequence of bombyxin G gene. The dash (-) indicates the termination codon

```
                    10                  20                  30
1  MKLIIFVVFCITIYGSTSGQQEVARRYCGR  G
1  MKLIIFVVFCITIYGSTSGQQEVARRYCGR  G1
1  MKLIIFVVFCITIYGSTSGQQEVARRYCGR  G1

                    40                  50                  60
31 HLAVTMADLCFGVQFDKRNTQYEGYHWPLL  G
31 HLAVTMADLCFGVQFDKRNTQYEGYHWPLL  G1
31 HLAVTMADLCFGVQFDKRNTQYEGYHWPLL  G1

                    70                  80                  90
61 AYSEERIKRQGIADECCLVPCTTNVSSSYC  G
61 AYSEERIKRQGIADECCLVPCTTNVLSSYC  G1
61 AYSEERIKRQGIADECCLVPCTTNVLSSYC  G1
   —
   —
91 .                                  G
91 .                                  G1
91 .                                  G1
```

Fig. 3: Amino acid sequence comparison among bombyxin G, G1 and G1' genes. The dot (.) represents the termination of protein translation

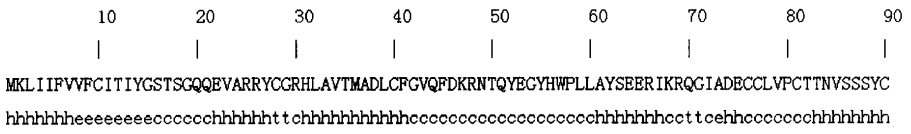

Fig. 4: Secondary structure prediction of bombyxin G protein by SOPMA software. The lowercase h indicates α-helix, e indicates extend strand, c indicates random coil and t indicates β-turn

DISCUSSION

Like insulin, bombyxin is a heterodimer, with A and B chains connected by disulfide bonds. Bombyxin genes have also been identified in *Samia cynthia* (Saturniidae), which has six genes and *Agrius singulatus* (Sphingidae), which appears to have three genes. In both the latter species the bombyxin genes belong to the same two families, related to the A and B families of *B. mori*. The insulin-like receptor was suggested to act in the hypodermal and intestinal target tissues, in which a change in metabolism is triggered by the dauer regulatory cascade and to regulate the metabolism and remodelling of tissues indirectly by controlling the production of other hormones (Kimura *et al*., 1997). The insulin-like receptor cascade also controls the reproductive maturation of the germ line as well as morphogenetic aspects of the pharynx and hypodermis. Bombyxin was reported to induce meiosis in *Bombyx* ovarian cells *in vitro* (Orikasa *et al*., 1993) and morphological changes in BM-N4 cells, a cell line derived from a *Bombyx* ovarian tissue (Tanaka *et al*., 1995). In addition, the hemolymph titer of bombyxin attains a peak value during pupal-adult development in which tissue remodel- ling and reproductive maturation are in maximum progress (Saegusa *et al*., 1992). In the development of silkworm, the hemolymph bombyxin titer may fluctuate dynamically to a level, which is close to the actually determined bombyxin titer at middle stages of pupal-adult development (Suenobu *et al*., 2004). It is therefore highly probable that bombyxin plays an important role in metamorphosis and reproduction through its ability to metabolise energy and that bombyxin G1 shares this important role in view of the conserved amino acid similarity in the receptor binding site with other bombyxin members.

The role of bombyxin in *Bombyx* larvae seems to differ from that of insulin in mammals, although both hormones share the same function in terms of being in control of carbohydrate metabolism. Ancient insulin may have evolved to acquire divergent metabolic actions in different animal groups in accordance with their mode of life while preserving its response to the glucose signal.

ACKNOWLEDGMENT

This resaerch was supported by the National Science Fundation of China (30670447) and K.C.Wong Magna Fund in Ningbo University. We would thank Dr. Yaozhou Zhang and Dr. Yongfeng Jin for their helpful discussions.

REFERENCES

Ishizaki, H., A. Suzuki, I. Moriya, A. Mizoguchi, M. Fujishita, H. O'oka, H. Kataoka, A. Isogai, H. Nagasawa and A. Suzuki, 1983. Prothoracicotropic hormone bioassay: Pupal-adult *Bombyx* assay, Development, Growth and Differentiation, 25: 585-592.

Iwami, M., 1990. The Genes Encoding Bombyxin, A Brain Secretory Peptide of *Bombyx mori*: Structure and expression. In: Ohnishi E, Ishizaki H (Eds.), Molting and metamorphosis (Tokyo/Springer, Berlin Heidelberg New York, Japan Science Society Press).

Iwami, M., T. Adachi, H. Kondo, A. Kawakami, Y. Suzuki, H. Nagasawa, A. Suzuki and H. Ishizaki, 1990. A novel family C of the genes that encode bombyxin, an insulin-related brain secretory pep-tide of the silkmoth *Bombyx mori*: isolation and characterization of gene C-1, Insect Biochem., 20: 295-303.

Iwami, M., A. Kawakami, H. Ishizaki, S.Y. Takahashi, T. Adachi, Y. Suzuki, H. Nagasawa and A. Suzuki, 1989. Cloning of a gene encoding bombyxin, an insulin-like brain secretory peptide of the silkmoth *Bombyx mori* with prothoracicotropic activity, Development, Growth and Differentiation, 31: 31-37.

Iwami, M., A. Tanaka, N. Hano and S. Sakurai, 1996. Bombyxin gene expression in tissues other than brain detected by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and *in situ* hybridization, Experientia, 52: 882-887.

Kawakami, A., M. Iwami, H. Nagasawa, A. Suzuki and H. Ishizaki, 1989. Structure and organization of four clustered genes that encode bombyxin, an insulin-related brain secretory peptide of the silkmoth *Bombyx mori*, Proceed. National Academy of Sciences of the United States of America, 86: 6843-6847.

Kimura, K.D., H.A. Tissenbaum, Y. Liu and G. Ruvkun, 1997. daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*, Sci., 277: 942-946.

Kiriishi, S., H. Nagasawa, H. Kataoka, A. Suzuki and S. Sakurai, 1992. Comparison of the *in vivo* and *in vitro* effects of bombyxin and prothoracicotropic hormone on prothoracic glands of the silkworm, *Bombyx mori*, Zoological Sci., 9: 149-155.

Kondo, H., M. Ino, A. Suzuki, H. Ishizaki and M. Iwami, 1996. Multiple gene copies for bombyxin, an insulin-related peptide of the silkmoth *Bombyx mori*: structural signs for gene rearrangement and duplication responsible for generation of multiple molecular forms of bombyxin, J. Molecular Biol., 259: 926-937.

Nagasawa, H., H. Kataoka, A. Isogai, S. Tamura, A. Suzuki, H. Ishizaki, A. Mizoguchi, Y. Fujiwara and A. Suzuki, 1984. Amino- terminal amino acid sequence of the silkworm prothoracicotropic hormone: Homology with insulin, Sci., 226: 1344-1345.

Nagasawa, H., H. Kataoka, A. Isogai, S. Tamura, A. Suzuki, A. Mizoguchi, Y. Fujiwara, A. Suzuki, S.Y. Takahashi and H. Ishizaki, 1986. Amino acid sequence of a prothoracicotropic hormone of the silkworm *Bombyx mori*, Proc. Nat. Acad. Sci. USA., 83: 5840-5843.

Nijhout, H.F., 1981. Physiological control of molting in insects, Am. Zoologist, 21: 631-640.

Orikasa, C., H. Yamauchi, H. Nagasawa, A. Suzuki and M. Nagata, 1993. Induction of oocyte-nurse cell differentiation in the ovary by the brain during the initial stage of oogenesis in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae), Applied Entomol. Zool., 28: 303-311.

Saegusa, H., A. Mizoguchi, H. Kitahora, H. Nagasawa, A. Suzuki and H. Ishizaki, 1992. Changes in the titer of bombyxin-immunoreactive material in hemolymph during the postembryonic development of the silkmoth *Bombyx mori*, Development, Growth and Differentiation, 34: 595-605.

Suenobu, A., A. Mizoguchi and T. Ichikawa, 2004. Relationship between firing activity of bombyxin-producing neurosecretory cells and hemolymph bombyxin titer in the silkworm *Bombyx mori*, General and Comparative Endocrinol., 137: 219-226.

Suzuki, C. and H. Ishizaki, 1986. Prothoracicotropic hormone bioassay: *Bombyx* larva assay, Invertebrate Reprod. Dev., 10: 259-274.

Tanaka, M., H. Kataoka, K. Nagata, H. Nagasawa and A. Suzuki, 1995. Morphological changes of BM-N4 cells induced by bombyxin, an insulin-related peptide of *Bombyx mori*, Regulatory Peptides 57: 311-318.

Tsuzuki, S., T. Masuta, M. Furuno, S. Sakurai and M. Iwami, 1997. Structure and expression of bombyxin E1 gene, a novel family gene that encodes bombyxin-IV, an insect insulin-related neurosecretory peptide, Comparative Biochem. Physiol., B 117: 409-416.

Yoshida, I., K. Moto, S. Sakurai and M. Iwami, 1998. A new member of the bombyxin gene family: Structure and expression of bombyxin G1 gene, an insulin-related peptide gene of the silkmoth *Bombyx mori*, Dev. Genes and Evol., 208: 407-410.

Yoshida, I., S. Tsuzuki, S.E. Abdel Salam, M. Ino, A.M. Korayem, S. Sakurai and M. Iwami, 1997. Bombyxin F1 gene: structure and expression of a new bombyxin family gene that forms a pair with bombyxin B10 gene, Zoological Sci., 14: 615-622.