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cDNA Cloning and Developmental Expression of Hemolin in *Bombyx mandarina*

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In this study, we describe the *Bombyx mandarina* hemolin cDNA. A sequence analysis of cDNA revealed a single open reading frame (ORF) of 1233 nucleotides. The deduced 410 amino acid sequence of *B. mandarina* hemolin contains 4 imunoglobulin (Ig) *C-2* type domains. *B. mandarina* hemolin cDNA showed the highest sequence homology to known those of *B. mori*. The developmental profile in terms of expression level of hemolin mRNA was determined in the absence of a bacterial challenge. Hemolin mRNA was detected only in mid-gut, but not in hemocytes, fat body, testis, and silkglands. Hemolin mRNA in mid-gut was not detected until the spinning stage of the last instar larva, however, it dramatically increased at the beginning of spinning and gradually decreased until pupal stage.

Key words: *Bombyx mandarina,* Hemolin, Imunoglobulin, Developmental profile, Spinning stage

Introduction

Immune responses in insects include phagocytosis, nodule formation, encapsulation, proteolytic activation of henoloxidase, and induced synthesis of antimicrobial proteins and peptides (Gillespie *et al.*, 1997; Bulet *et al.*, 1999).

Bacterial infections in insects stimulate the synthesis of several proteins that are secreted into the hemolymph (Gillespie *et al.*, 1997; Hetru *et al.*, 1998). Many antibacterial proteins were previously reported: lysozymes (Powning and Davidson, 1973; Hultmark *et al.*, 1980), cecropins (Hultmark *et al.*, 1980), attacins or attacin-like proteins (Hultmark *et al.*, 1983), defensins (Matsuyama and Natori, 1988), proline-rich proteins (Casteels *et al.*, 1989), apidaecins (Casteels *et al.*, 1989), diptericins (Dimarcq *et al.*, 1988), hyphancins (Park *et al.*, 1994), lectins (Jomori and Natori, 1992) and hemolins (Sun *et al.*, 1990). These proteins do not have direct antibacterial activity, but it can bind to bacterial lipopolysaccharide (Daffre and Faye, 1997) and may function as an opsonin to increase the efficiency of phagocytosis (Kanost and Zhao, 1996).

Hemolin, the major antibacterial protein, was known as a member of the immunoglobulin superfamily (IgSF) (Ladendorff and Kanost, 1991). It has been isolated only in lepidopteran insects of *Hyalophora cecropia* (Rasmuson and Boman, 1979), *Manduca sexta* (Ladendorff and Kanost, 1990), *Hyphantria cunea* (Shin *et al.*, 1998) and *Lymantria dispar* (Lee *et al.*, 2002). Recently, in the silkworm, *Bombyx mori*, the hemolin gene was cloned and sequenced (Kim *et al.*, 2004). The current study describes a hemolin gene of the wild silkworm, *B. mandarina.* This species resembles *B. mori* in morphology and developmental characteristics. In addition, these two species can be crossed with each other and the hybrid progeny is fertile. For these reasons, *B. mandarina* is believed to be the closest ancestor of *B. mori.* In this paper, we describe the cloning and expression of *B. mandarina* hemolin for the purpose of finding differences and similarities with *B. mori.*

Materials and Methods

Insects

The *B. mandarina* was collected in the Chilgok area in

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Korea. We collected eggs, larva and pupa. They were reared according to Nho and Kim (1992).

Preparation of RNA

Total RNA were prepared with a TRIZOL reagent (Gibco-BRL, USA) according to the manufacturer's instructions. The amount and quality of the total RNA was checked by spectrophotometry (OD = 260/280) and by formaldehyde-containing 1.0% agarose gel electrophoresis.

RACE

The following gene-specific primers were designed based on the sequence of the fbpv0322 clone in the silkbase (www.ab.a.u-tokyo.ac.jp/silkbase). The sequences for the primers were as follows: 3-RACE primer (5-AAAGC-GACTTTGGGGTCGCCAGCAC-3) and 5-RACE primer (5-CGGGCACTGGACAGCGGAGTTCAAA-3). The RACE reactions were performed according to the instructions of the Marathon cDNA amplification kit (Clontech, USA).

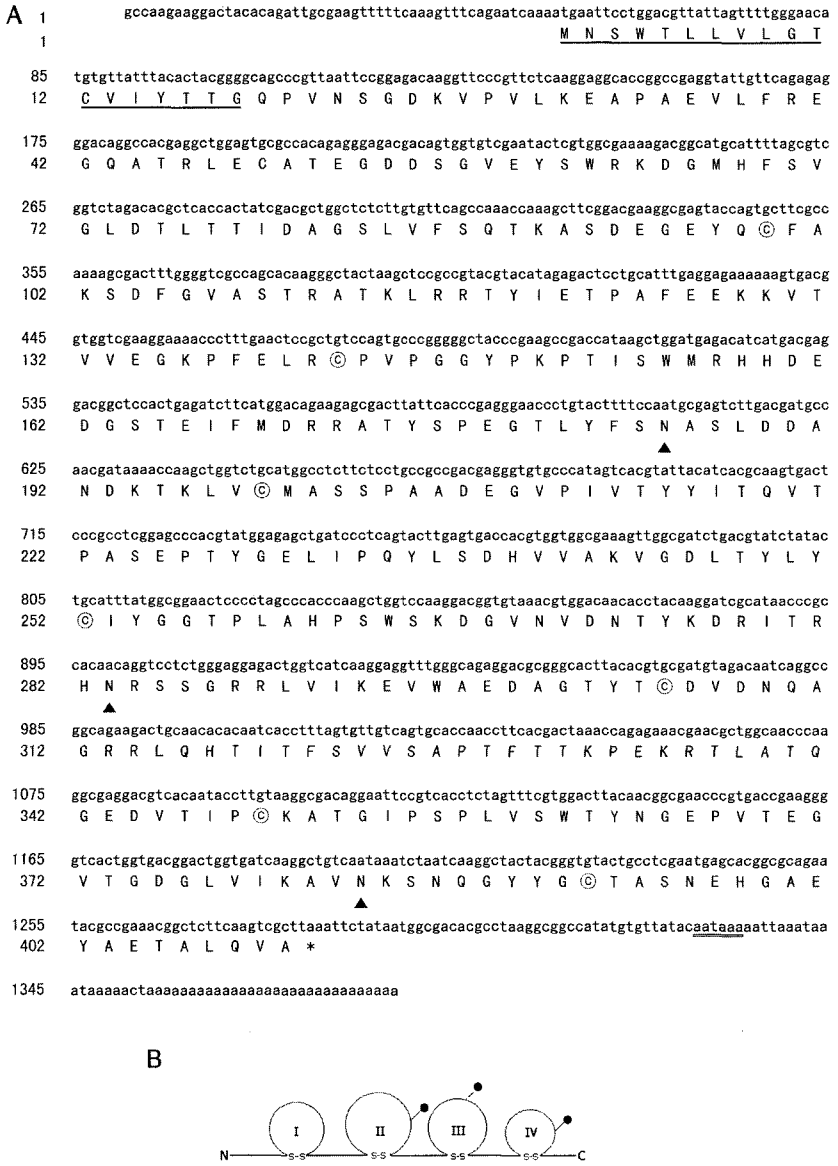

Fig. 1. Nucleotide and deduced amino acid sequences of *B. mandarina* hemolin cDNA. (A) The deduced amino acid sequence is shown below the nucleotide sequence and is numbered from the initiating methionine. The termination codon is shown by asterisk. The putative signal peptide is underlined in bold and the polyadenylation site is double underlined. The cysteine residues which define the four Ig domains are circled. Three potential sites for *N*-glycosylation (Asn-X-Asn/ser) are marked by dark triangles. (B) Schematic representation of *B. mandarina* hemolin. Four Ig domains (I-IV) are symbolized as loops closed by intra-domain disulfide (S-S) bond. The GenBank accession number is AY515323.

Double-stranded cDNA was synthesized from the mid-gut poly(A^+) RNA, ligated to the Marathon adapter, and used as a template for the 5- and 3- RACE reactions with the Adapter primer 1 and the above gene-specific primers. The resulting RACE products were separated on a 1% low melting point (LMP) agarose gel. A 3'-RACE product (1.0 kb) and a 5'-RACE product (0.5 kb) were excised from the gel and subcloned into pCRII-TOPO (Invitrogen, USA).

RT-PCR

Using total RNA and Oligo(dT)$_{18}$ primer (Ambion, USA) a cDNA synthesis was performed at 42°C for 60 min and 94°C for 5 min. Subsequently, PCR was performed as follows: the first denaturation for 2 min at 94°C, 3 steps of 30 cycles and the last extention for 2 min at 72°C. The 3 step cycles is consisted of 30 sec at 94°C, 30 sec at 44°C and 1 min at 72°C. Reactions were performed in a GeneAmp PCR System 2400 (Perkin Elmer, USA). The primers were designed based on full-length cDNA. The primer sites are 1 – 19 nt and 1267 – 1287 nt, respectively.

Sequence anlysis and BLAST search

The sequence of the full-length cDNA and RT-PCR products, cloned in pCRII-TOPO (Invitrogen, USA), were sequenced with a BigDye termination cycle sequencing ready reaction kit (PE Applied Biosystems, USA). Elctrophoresis was performed by an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, USA). Sequence homology was determined with BLAST (National Center for Biotechnology Information, USA) (Altschul *et al.*, 1990).

Results and Discussion

Structure and characteristic of *Bombyx mandarina* hemolin cDNA

The *B. mandarina* hemolin full-length cDNA revealed a single open reading frame (ORF) of 1383 nucleotides, beginning with a methionine at residue 52 (Fig. 1A) (GenBank accession number AY515323). The ORF codes for a protein of 410 amino acids. The deduced amino acids have four immunoglobulin domains (Fig. 1B). The four Ig-like domains between the conserved cystein residues have sizes different from the 58 amino acids (domain I) to 44 amino acids (domain IV). The signal peptide is the amino-end 18th Glycine. Asparagine, which had the possibility of a *N*-linked glycosylation site (Asparagine-X-Threonine/Serine) is localized in 185 (domain II), 283 (III) and 383 (IV), respectively (Fig. 1A).

B. mandarina hemolin cDNA has great similarity with *B. mori* (Fig. 2). Only six bases were substituted within the ORF. A similarity search using the BLASTP algorism

Fig. 2. Nucleotide sequences of hemolin cDNA in *Bombyx*. Alignments of the sequence in *Bombyx mandarina* (*B. man*) and *Bombyx mori* (*B. mor*). The dots indicate the identity and the hyphens indicate the gaps assumed.

(Altschul *et al.*, 1990) revealed that it shared homology with *Manduca sexta* (Wang *et al.*, 1995; accession number, U11879) and *Hyalophora cecropia* (Sun *et al.*, 1990; S65948) at 47% and *Lymantria dispar* (Lee *et al.*, 2002; AF453868) and *Hyphantria cunea* (Shin *et al.*, 1998; AF023276) at 42% (Fig. 3). The great similarity with *B. mori* (Kim *et al.*, 2003; AY515320), the same genus of *B. mandarina*, is 99.6%. These results coincided with the

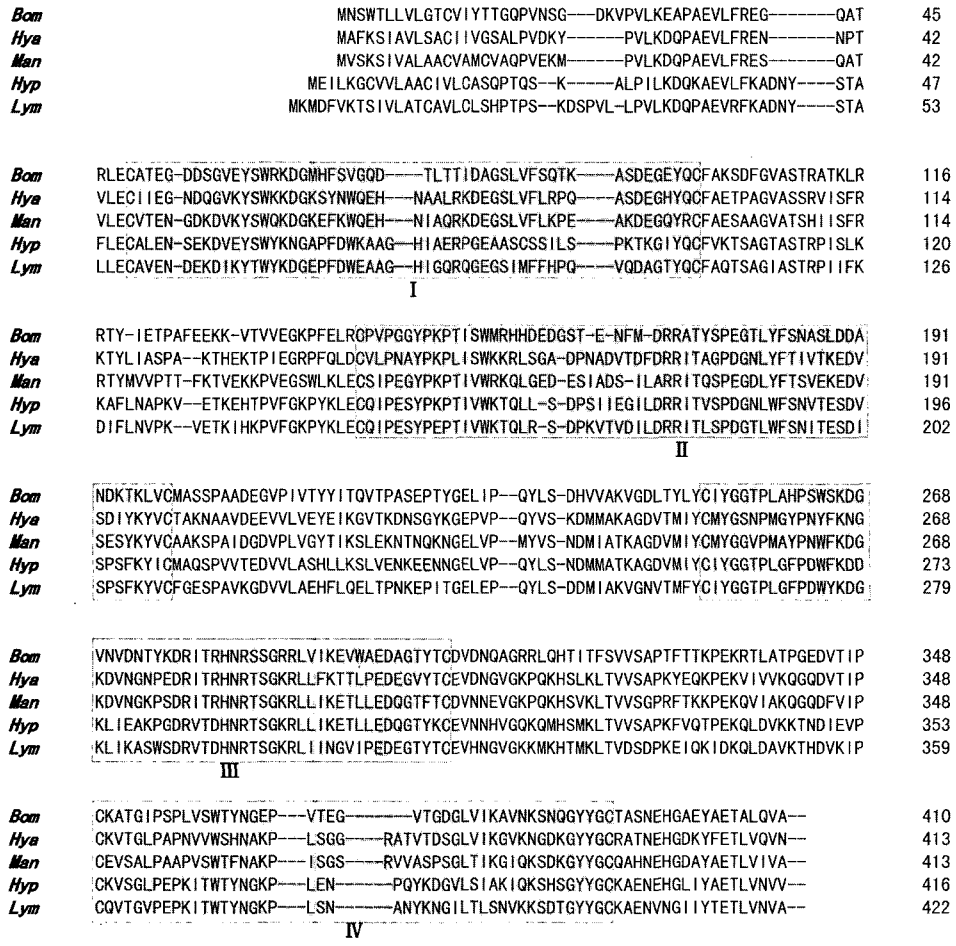

Fig. 3. Alignments between *B. mandarina* hemolin and their related homologs. Alignments of the deduced amino acid sequence in *Bombyx mandarina* (*B. man*) with four other hemolins of, *H. cecropia* (*Hya*), *M. sexta* (*Man*), *H. cunea* (*Hyp*) and *L. dispar* (*Lym*). The four Ig domains (–) are highlighted in gray between the conserved cystein residues.

phylogenetic relationships of the two species.

Developmental expression of hemolin

The developmental expression profile of hemolim mRNA was determined by RT-PCR. Hemolin mRNA was not detected in every tusses as fat body, mid-gut, gonads, silk glands, or hemocytes of *B. mandarina* larva in the absence of bacterial infection (data not shown). At the beginning of the spinning stage, however, a high amount of hemolin was detected in the mid-gut. It was expressed only in the mid-gut at the spinning stage (Fig. 4A). Although hemolin is an immune protein, it is expressed in the mid-gut not in the fat body. So, we investigated the expression pattern of the hemolin mRNA in the fat-body from the final larval stage to the pupa (Fig. 4B). Hemolin mRNA was not detected in the fat body of the feeding stage of the forth instar larva, spinning stage (pre-pupa) and pupa stage. In another lepidopteran insect, *M. sexta*, hemolin mRNA appeared in the fat body at the beginning of the wandering stage and increased in abundance, then gradually declined until the pre-pupal period (Yu and Kanost, 1999). Fig. 4C represents the hemolin developmental expression profile in mid-gut. Hemolin mRNA was expressed at the beginning of the spinning stage. Its level dramatically increased and reached a maximum on day 2 of the spinning stage. The hemolin mRNA level then gradually declined, but it was detected until the pupa stage. Thus, the function of hemolin is supposed to be unique for the physiological process at the post-spinning stage in *B. mandarina.*

In the beginning, hemolin was known as a hemolymph protein whose synthesis is induced after the infection of bacteria only in Lepidoptera (Rasmuson and Boman, 1979; Ladendorff and Kanost, 1990). Until recently, it is suggested that hemolin has many functions in the developmental stages (Yu and Kanost, 1999, 2002; Bettencourt *et al.*, 2000). *B. mandarina* hemolin mRNA was up-reg-

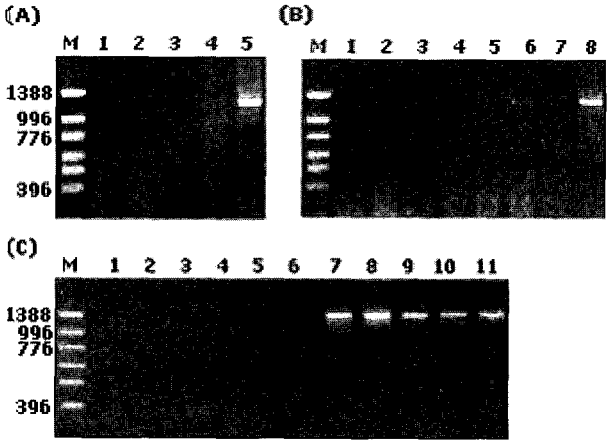

Fig. 4. Expression profile of hemolin mRNA in *B. mandarina*. (A) Tissues at the spinning stage. M, maker; 1, fat body; 2, testis; 3, hemolymph; 4, silkglands; 5, mid-gut. (B) The fat body during developmental stages. M, maker; 1, 4th instar; 2, beginning of the spinning stage; 3, 2 days of spinning; 4, finished spinning (pre-pupa); 5, white pupa; 6, 1 day of pupa; 7, 2 days of pupa; 8, mid-gut (1day of the spinning). (C) The mid-gut during developmental stages. M, maker; 1, 2nd instar; 2, 1 day of the 3rd instar; 3, 3 days of the 3rd instar; 4, 1 day of the final instar; 5, 3 days of the final instar; 6, 5 days of the final instar; 7, 1 day of the spinning; 8, 2 days of the spinning; 9, finished the spinning (pre-pupa); 10, white pupa; 11, 1 day of pupa.

ulated in mid-gut at the spinning stage. Further research is needed to better understand the physiological functions including that of the egg and moth.

Acknowledgements

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