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cDNA CLONING AND GENE EXPRESSION OF LEBOCIN, A NOVEL MEMBER OF ANTIBACTERIAL PEPTIDES FROM THE SILKWORM, *BOMBYX MORI*

Subrata Chowdhury, Kiyoko Taniai, Seiichi Hara[+], Keiko Kadono-Okuda, Yusuke Kato, Masanori Yamamoto, Jinhua Xu, Su Kyung Choi, Nitish C. Debnath, Hong Kyu Choi, Akihiro Miyanoshita, Masao Sugiyama[#], Ai Asaoka and Minoru Yamakawa[*]

Laboratory of Biological Defense, National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki 305, Japan

[+]Noda Institute for Scientific Research, Noda, Chiba 278, Japan

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A cDNA encoding lebocin, a novel member of insect antibacterial peptides, was isolated from the fat body cDNA library of *Bombyx mori* larvae immunized with *Escherichia coli*. The cDNA was 844 bp long and had an open reading frame (ORF) containing a probable signal peptide (16 amino acids), a putative prosegment (104 amino acids) and a mature peptide (32 amino acids) followed by 27 additional amino acids at its carboxyl-terminus. Northern blot analysis showed that lebocin gene expression was inducible by bacterial injection, occured tissue-specifically in the fat bodies and continued at least for 48 h post-infection. These results suggest that lebocin has a unique precursor structure and shows typical gene expression pattern as insect antibacterial peptide.

The invasion of foreign material in the body cavity of insects provokes cellular and humoral responses which are harmful to a wide variety of microorganisms (1). The efficiency of antimicrobial defense reactions of insects is remarkable for their immediate and transient nature, and the mechanism involved in eliciting the immune reaction, although not well understood, is intriguing. As a humoral reaction to bacterial infection in insects, a battery of bactericidal proteins start to accumulate in the hemolymph. To date, more than 50 antibacterial proteins have been identified and characterized, and they are broadly classified into a few major groups (2). With the exception of lysozyme, these induced proteins were first discovered in insects.

Previously, we have investigated the antibacterial response in the silkworm, *Bombyx mori*, a representative of lepidopteran insects and identified a novel antibacterial family named "lebocin" (lebocin 1, 2 and 3) (3). The peptides are rich in proline residues and unique threonine residue is glycosylated. Natural lebocins showed antibacterial activity against

[#]Present address: Hokko Chemical Industry Central Research Laboratories, Atsugi, Kanagawa 243, Japan.

[*]Corresponding author. FAX:+81-298-6028.

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Acinetobactor sp. and *E. coli* whereas synthetic lebocin without sugar chains were less active against those bacteria indicating the importance of O-glycosylation for expression of the antibacterial activity of the peptides (3).

Since we are interested in the mechanisms involved in the induction and the regulation of immune response, we now have cloned the lebocin cDNA from *B. mori* fat body cDNA library. In this paper, we present data on the nucleotide sequence of lebocin cDNA, expression pattern in different tissues and expression kinetics of lebocin gene.

MATERIALS AND METHODS

Biological materials: Silkworms, *B. mori* (Tokai x Asahi) were reared on an artificial diet (Nihonnosanko) at 25°C. *Escherichia coli* K12 strain HB101 (4,5) were grown in LB medium (6) at 37°C with shaking, and used for injection into silkworms.

Probe preparation : cDNA was first synthesized with 8 μg mRNA extracted from immunized fat bodies and ligated into λ-gt10 arms (7). A probe to screen the lebocin cDNA was prepared by polymerase chain reaction (PCR) (8) with 100 ng cDNA, Taq polymerase (Promega) and synthesized primer. Degenerate primers were prepared based on the amino acid sequences of mature lebocin (3). For 5'-primer (20 mer), nucleotide sequences were deduced from Asp(1) to Pro(7) and for 3'-primer from Tyr (25) to Arg (31) of the mature protein. The sequences were (5')GA(T/C)(C/T)T(A/T/G/C)(C/A)G(A/T/G/C)TT(C/T)(C/T)T(A/T/G/C)TA (C/T)CC (3') and (5')C(G/T)(G/A)TT(A/T/G/C)CCCAT(G/A)T(G/A)TC(T/G/A)AT(G/A)TA (3') for 5'- and 3'- oligomer respectively. λgt10 primers were also used in different combination. These primers were (5') GCAAGTTCAGCCTGGTTAAG (3') and (5') TATGAGTATTTCTTC CAGGG (3') for 5'- and 3'- oligomer, respectively. Thirty cycles of PCR were performed at 50°C as annealing temperature for first polymerase chain reactiom (PCR) using temperature control system PC-700 (Astec). An aliquot of sample from the first PCR product was used as a template for a second PCR with 5 cycles at 37°C and 25 cycles at 55°C as annealing temperature. The PCR products were subcloned into T-vector (9) and sequenced by dideoxy nucleotide chain termination method (10) and by dye-terminator method using auto sequencer (ABI).

Plaque hybridization: Plaques ($1x10^5$) were transferred onto a nylon membrane filters (Schleicher and Schuell) and screened with a lebocin probe prepared by PCR (Clontech Inc.). The DNA fragment was labeled with [α-^{32}P]dCTP (ICN) and a DNA labeling kit (Nippon gene). The prehybridization was performed for 3 h at 42°C in a solution containing 5x Denhardt's solution, 5x SSPE, 0.1mg/ml salmon sperm DNA and 50% formamide (6). Hybridization was carried out for 16 h at 42°C with the probe mentioned above. Washing conditions were as described previously (6). The membranes were exposed to X-ray film for 24 h at -80°C. Two positive plaques were confirmed three times by repeating plaque hybridization under the same conditions as described above.

Nucleotide sequencing: The cDNA clone was digested with *Eco*RI and the insert was purified from the low melting agarose gel. The purified insert was ligated into Bluescript SK$^+$ vector and sequenced by labeling with [α-^{32}P]dCTP (ICN) following the dideoxy termination method (6) using reverse, M13 or synthetic primers and Sequenase in the presence of A-, T-,G-,C-specific dideoxynucleotide mixtures (United States Biochemical Corp.). The samples were also sequenced by dye-terminastor/dye-primer method using auto sequencer (ABI).

Northern blot analysis: Four or five day old fifth instar silkworm larvae were kept on ice to paralyze them and injected with 20 μl of *E. coli* strain HB101 (10^8 cells/ml in insect physiological saline containing 150 mM NaCl and 5 mM KCl) through the hind leg. Fat bodies, silk glands, hemocytes, midguts, Malphigian tubules were excised 8 h after the injection. A total RNA was extracted by guanidine thiocyanite-cesium chloride method (11). RNA was electrophoresed in 1.2% agarose gel containing 6.6% formaldehyde (6) and transferred onto a nylon membrane (Schleicher and Schuell) using a Vacugene XL Vacuum transfer apparatus (Pharmacia). Lebocin DNA was labeled with [α-^{32}P]dCTP by random priming (6) and hybridization was carried out as described previously (6). A genomic clone of Bm18S rRNA (12) was used as a probe for internal control. For kinetic experiments, fat bodies were excised at indicated time intervals after injection with *E. coli* as described above.

RESULTS

Sequence analysis

Three lebocins were isolated from the hemolymph of immunized *B. mori* larvae (3). Lebocin 1 and 2 differ from each other only by their sugar chain, for example, lebocin 1 has Galβ3GalNacα whereas bomocin 2 has GalNacα (3). However, bomocin 3 contains the same sugar chain as that of lebocin 2 but differs from both lebocin 1 and 2 by the presence of a leucine residue instead of a proline residue next to the O-glycosylated site, the threonine residue. Degenerate primers were constructed on the basis of lebocin 1 amino acid sequence. These primers were used to screen the cDNA library derived from the fat body poly (A^+) mRNA from *B. mori* larvae by PCR. A 300bp PCR product was generated using λgt10 5'-primer (vector side) and lebocin 5'-primer. The PCR product was subcloned into T-vector (9) and sequenced by chain termination method (10). The sequence analyses of the PCR generated fragment revealed a complete agreement with the amino acid sequence of the mature lebocin 1 and 2. Subsequently, we used this fragment as a probe to screen the full length cDNA from the cDNA library by plaque hybridization and isolated 10 positive clones out of 10^5 plaques suggesting a low prevalence of lebocin mRNA (about 0.01%) in the library. Two positive clones were subcloned into Bluescript SK^+ vector and sequenced by both chain termination method and automated sequencer. The complete nucleotide sequence is shown in Fig. 1.

The nucleotide sequence of lebocin cDNA (total 844 bp) contained a single open reading frame spanning 537 nucleotides. Within the reading frame, a predicted signal peptide (16 amino acids) and a long putative prosegment (104 amino acids) were followed by a mature peptide sequence (32 amino acids). The cleavage site between the signal peptide and the pro-segment was predicted according to the "(-3,-1)-rule" (13). Based on this rule, the residue in position -1 must be small, i.e. either alanine, serine, glycine, cysteine, threonine or glutamine; the residue in -3 must not be aromatic (phenylalanine, histidine, tyrosine, tryptophan), charged (aspartic acid, glutamic acid, lysine, arginine), or large and polar (aspartic acid, glutamine). Further, it is suggested that proline must be absent through position -3 to -1. The best probable cleavage site for lebocin has taken into consideration. Although there was complete agreement between the amino acid sequence of mature lebocin 1 and 2 and deduced amino acid residues from 121 to 152 of the precursor, there were 27 additional amino acids at the carboxyl-terminus of the precursor before the stop codon (Fig. 1). Since these amino acids were missing from the mature peptide isolated from hemolymph, they might have been removed by proteolysis during maturation. The deduced amino acid sequence of lebocin revealed that there was no cysteine residue in the entire region of the lebocin precursor, suggesting that the overall secondary structure of lebocin is rather simple. An analysis of the secondary structure of the deduced amino acids of the mature peptide showed that the peptide is mostly hydrophilic except a little hydrophobicity at the amino-terminus (data not shown). A recognition sequence for addition of poly(A^+) tail (14) was not found but a related sequence (AATTAAA) was present at base position 823. The 3'-untranslated region of lebocin was AT rich (73.2%). It has been suggested from the studies on the effect of introducing AT rich sequence of granulocyte macrophage colony stimulating factor (GM-CSF) cDNA into rabbit β-globin gene (15) that AU rich sequences in the 3'-untranslated region of GM-CSF mRNA are the recognition signal for the specific degradation of this mRNA. In addition, ATTA sequence was present in the 3'-untranslated region. This sequence was found to be conserved in the 3'-untranslated region of many cDNAs encoding mediator protein associated with the inflammatory response in mammals (15). Therefore, it can be speculated that the presence of ATTA sequence in the 3'-untranslated region of lebocin mRNA could regulate the stability of the mRNA once the purpose of its induction is accomplished.

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                                                    GCGGCCGCTCAAC    -1

ATG.TAC.AAG.TTT.TTA.GTA.TTC.AGT.TCA.GTT.CTG.GTG.CTG.TTC.TTT.    45
Met-Tyr-Lys-Phe-Leu-Val-Phe-Ser-Ser-Val-Leu-Val-Leu-Phe-Phe     15

GCT.CAG.GCT.TCG.TGC.CAG.AGG.TTC.ATC.CAG.CCG.ACC.TTC.AGG.CCA.    90
Ala-Gln-Ala-Ser-Cys-Gln-Arg-Phe-Ile-Gln-Pro-Thr-Phe-Arg-Pro     30
    △

CCG.CCA.ACA.CAG.CGT.CCG.ATA.ATA.CGT.ACA.GCG.CGA.CAA.GCT.GGC.   135
Pro-Pro-Thr-Gln-Arg-Pro-Ile-Ile-Arg-Thr-Ala-Arg-Gln-Ala-Gly     45

CAG.GAA.CCG.CTA.TGG.CTG.TAT.CAA.GGT.GAC.AAT.GTT.CCT.CGT.GCG.   180
Gln-Glu-Pro-Leu-Trp-Leu-Tyr-Gln-Gly-Asp-Asn-Val-Pro-Arg-Ala     60

CCA.AGT.ACT.GCA.GAC.CAT.CCG.ATT.CTT.CCT.TCG.AAA.ATC.GAC.GAC.   225
Pro-Ser-Thr-Ala-Asp-His-Pro-Ile-Leu-Pro-Ser-Lys-Ile-Asp-Asp     75

GTG.CAG.CTC.GAT.CCA.AAC.CGA.AGG.TAT.GTT.CGC.AGT.GTC.ACT.AAT.   270
Val-Gln-Leu-Asp-Pro-Asn-Arg-Arg-Tyr-Val-Arg-Ser-Val-Thr-Asn     90

CCA.GAA.AAT.AAC.GAG.GCG.TCC.ATT.GAA.CAT.TCA.CAT.CAT.ACA.GTT.   315
Pro-Glu-Asn-Asn-Glu-Ala-Ser-Ile-Glu-His-Ser-His-His-Thr-Val    105

GAT.ACT.GGA.CTT.GAC.CAG.CCG.ATC.GAG.AGC.CAC.CGT.AAC.ACA.AGG.   360
Asp-Thr-Gly-Leu-Asp-Gln-Pro-Ile-Glu-Ser-His-Arg-Asn-Thr-Arg    120

GAC.CTG.AGG.TTT.TTG.TAC.CCT.CGA.GGG.AAA.CTG.CCT.GTT.CCA.ACG.   405
Asp-Leu-Arg-Phe-Leu-Tyr-Pro-Arg-Gly-Lys-Leu-Pro-Val-Pro-Thr    135
    ▲

CCT.CCT.CCG.TTT.AAC.CCC.AAG.CCA.ATA.TAT.ATT.GAT.ATG.GGA.AAC.   450
Pro-Pro-Pro-Phe-Asn-Pro-Lys-Pro-Ile-Tyr-Ile-Asp-Met-Gly-Asn    150

CGT.TAC.CGA.CGA.CAT.GCG.TCG.GAT.GAT.CAA.GAA.GAA.TTG.CGG.CAA.   495
Arg-Tyr-Arg-Arg-His-Ala-Ser-Asp-Asp-Gln-Glu-Glu-Leu-Arg-Gln    165

TAT.AAT.GAG.CAC.TTT.CTG.ATT.CCG.AGG.GAT.ATT.TTC.CAA.GAA.TAG.   540
Tyr-Asn-Glu-His-Phe-Leu-Ile-Pro-Arg-Asp-Ile-Phe-Gln-Glu-***    179

GGAAAGTTCCAGAAACAAAAGATTTCGGAGTGCACTCCTATATTCATTGAATCGTAATT    599

ACTTAAGTTTAAGTGATATTTTTAATAGTTTTGCATAAAAATACAAAGTATTTTAAAGT    658

GGCCGTTTCAATTCAAATAATGTAAGTACTCGTACGTATTGTTTATCAAATTAAATAAA    717

TCTCAACCAATATTGAACTTATGTTTTGTTAATTTGCTTTGCAACAAATTTCTCTCACC    776

TGTTGTAAAAAATATTTTATAATTGTGCTGTGTATTTAAAGTGTGTAATTAAAAG        831
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Fig. 1. Nucleotide sequence of lebocin cDNA. The nucleotide sequence of lebocin cDNA along with the deduced amino acid sequence is shown. The number of nucleotides and amino acids is indicated. The predicted signal peptide and the prosegment are shown by single and dotted underlines, respectively. The predicted cleavage sites between signal peptide and prosegment and between prosegment and mature peptide are indicated by open and closed triangles, respectively. The translation stop codon, TAG, is shown as three stars. The polyadenylation signal-like sequence (AATTAAA) and ATTTA sequence are indicated by dotted and thick underlines, respectively.

Northern blot analysis

A number of tissues from *B. mori* were examined to find out the tissue specificity of lebocin gene expression. Total RNA was isolated from Malphigian tubes, midguts, hemocytes and fat bodies. Lebocin cDNA was used as a probe for Northern blot analysis. The result shown in Fig. 2 indicates that lebocin is an induced peptide and strongly expressed in fat bodies, whereas in hemocytes and midguts, it has given little positive signal suggesting that lebocin is mainly expressed in the fat bodies. In the case of *M. sexta* cecropin B, the main site of expression was fat body (16) which is similar to that of lebocin. Similar expression pattern has been found in some dipteran cecropins (17). On the other hand, in the case of *B. mori*

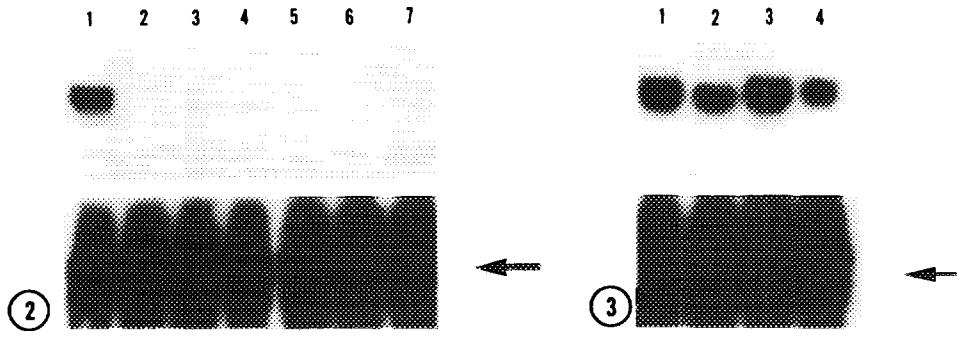

Fig. 2. Tissue-specific expression of lebocin gene. The sites of lebocin gene expression were analyzed by Northern blot analysis using lebocin cDNA as a probe. Total RNA was extracted from fat body (lane 1 and 5), hemocytes (lane 2 and 7), midgut (lane 3 and 6) and Malphigian tubules (lane 4). The tissues were excised from immunized *B. mori* larvae (lane 1-4) 12h post-immunization and from non-immunized larvae (lane 5-7). As an internal marker, *B. mori* 18S rRNA genomic DNA (pBm R161) was used as a probe.

Fig. 3. Time-course of lebocin gene expression. Fat body was extracted from *B. mori* larvae at 4 h (lane 1), 8 h (lane 2), 24 h (lane 3) and 48 h (lane 4) post-immunization. Northern blot analysis was performed using lebocin cDNA as a probe. *B. mori* 18S rRNA genomic DNA was used as a probe for internal marker (an arrow).

cecropin B, strong expression was found both in the fat body and hemocytes (18). Thus, the diversity of expression is dependent on the induced peptide but not on the host, suggesting the functional distribution of the induction of these peptides.

In order to determine the temporal pattern of lebocin gene expression, the time-course of accumulation of lebocin mRNA was also investigated with the RNA extracted from the immunized larvae. Fat bodies were excised at various time intervals after immunization and total RNA was extracted and analyzed by Northern blotting (Fig. 3). The transcripts were detectable with same intensity from 4 h post-infection until the end of time tested (48 h). This is in contrast with some peptides where transcripts reached a maximum level between 8-12 h post-immunization and then gradually decreased. For example, cecropin B from *B. mori* reached a maximum level of expression at 8 h post-infection and then gradually decreased (18). Similar observations have been reported with cecropins of *M. sextra* (16) and *D. melanogaster* (17), and sarcotoxin IA of *S. peregrina* (19). However, the temporal expression pattern of lebocin gene has similarities with that of sarcotoxin IIA (6 to 24 h) (20), attacin from *B. mori* (4 to 48 h)(21), abaecin, hymenoptaecin and bee defensin from the honeybee, *Apis mellifera* (36 h) (22).

DISCUSSION

Insect antibacterial proteins are classified into five major groups, namely, insect defensins, cecropins, attacin-like proteins, lysozymes and proline-rich proteins (2). Lebocin belongs to the proline-rich group, containing drosocin from the fruit fly (23), pyrrhocoricin from the sap-sucking bug (24), abaecin (25) and apidaecin (26) from the honeybee. Of these antibacterial peptides drosocin and pyrrhocoricin retain a glycosylated threonine residues. A computer search for the amino acid identity of lebocin with that of other antibacterial proteins indicated a high identity with abaecin (41%) (25) and amino-terminal part of sarcotoxin IIA (37%)

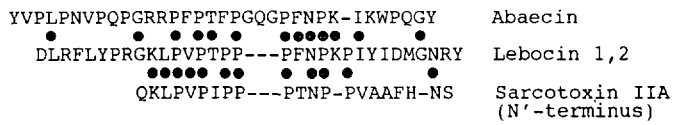

Fig. 4. Sequence identity of lebocin, abaecin and Sarcotoxin IIA. Identical sequences are indicated by a filled circle. In the case of Sarcotoxin IIA, only amino-terminal region is shown.

(19) (Fig. 4). The identical region is proline rich and could indicate some functional similarity among these peptides. The deduced amino acid sequence of lebocin revealed a possible O-glycosylation site (Pro-Thr/Ser-Xaa-Xaa-Pro) (27) at positions 14-18 of the mature peptide and the presence of sugar chain has been proved by sugar analysis (3). It is noteworthy that 8 proline residues are found in the middle part of the peptide and 5 of them formed a cluster (Fig. 4). This suggests that the remarkable arrangement of proline rich region of this peptide provides a rigid bend, having a conformation that may play a significant role in "receptor recognition" by the polypeptide N-acetylgalactosaminyltransferase as suggested in bovine basic myelin protein (A1 protein) (28). In the case of A1 protein, threonine residue in the proline rich region is known to be O-glycosylated (28). In other cases, O-glycosylation of threonine residue in such proline rich region was also observed in IgG (29) and other insect antibacterial peptides such as drosocin (23) and pyrrhocoricin (24). However, although abaecin also contains the proline rich region like lebocin (Fig. 4), its unique threonine residue is not O-glycosylated, since Edman degradation of abaecin resulted in the high yield of phenylthiohydantoin-threonine (25). This discrepancy might depend on the amino acid sequence around the threonine residue. Indeed, the proline rich sequence of abaecin, Pro-Phe-Pro-Thr-Phe-Pro-Gly, is not in accord with the above described O-glycosylation site (27).

A unique characteristic of the deduced amino acid sequence of lebocin precursor is its long putative prosegment (Fig. 1). Although such a long prosegment has not yet been reported in insect antibacterial peptides, a deduced preproregion of a pig bone marrow cDNA encoding antibacterial peptide designated as PMAP-37 revealed that its prosegment consists of 101 amino acid residues (30). The preproregion of this peptide is highly similar to corresponding regions in congeners from pig, cattle and rabbit (31-42). A general aspect of this family of precursors is the function of this region. One possibility which was initially explored was that it might act as a protease inhibitor (43). In contrast to the pig antibacterial peptide, computor search for the amino acid identity of the preproregion of lebocin did not show significant similarity to that of other peptides. Thus, roles of the proregion in processing remain to be defined.

Lebocin precursor also contains a relatively long extra amino acid sequence at the carboxyl-terminus (Fig. 1). We assume that the 27 additional amino acid sequence is processed during maturation of lebocin. Analysis of carboxyl-termini of lebocin 1, 2 and 3 showed that no modification such as amidation occurs on the termini (data not shown). Interestingly, such a long additional amino acid sequence was also observed in drosocin precursor (24 amino acids) (23), suggesting resemblance of the structural framework at carboxyl-termini between O-glycosylated insect antibacterial peptieds. Thus, it is of interest to examine whether the precursor peptide encoding pyrrhocoricin, another O-glycosylated insect antibacterial peptide, also contains such a long additional amino acid sequence.

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