

dsRNA Binding Activity of Silworm Larval Hemolymph is Mediated by Lipophorin Complex

Sakashita, Kosuke

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Tatsuke, Tsuneyuki

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Masaki, Yuki

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Lee, Jae Man

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University

他

<https://doi.org/10.5109/16122>

出版情報：九州大学大学院農学研究院紀要. 54 (2), pp.401-406, 2009-10-29. Faculty of Agriculture, Kyushu University

バージョン：

権利関係：



dsRNA Binding Activity of Silkworm Larval Hemolymph is Mediated by Lipophorin Complex

Kosuke SAKASHITA¹, Tsuneyuki TATSUKE¹, Yuki MASAKI¹, Jae Man LEE,
Yutaka KAWAGUCH and Takahiro KUSAKABE*

Laboratory of Silkworm Science, Division of Genetics and Plant Breeding, Department of Applied Genetic and Pest Management, Faculty of Agriculture, Kyushu University, Hakozaki 6–10–1,
Fukuoka 812–8581, Japan

(Received June 30, 2009 and accepted July 13, 2009)

RNAi induction by the injection of long dsRNA into hemocoel has been tried to archive efficient gene knockdown in silkworm larvae or pupae, and severas reports successfully applying RNAi to silkworm have been brought out. However, effect of RNAi in silkworm is often insufficient and improvement of efficiency is desirable. On the other hand, the metabolic turnover or organ uptake of dsRNA injected into insects has not been sufficiently clarified so far. In this study, we report that silkworm larval hemolymph has dsRNA binding activity. We also show that the molecular entity of dsRNA binding activity of hemolymph is lipophorin, a major lipoprotein complex in insects. dsRNA binding activity of silkworm larval hemolymph was observed by electrophoretic mobility shift assay. dsRNA binding activity-associated protein was purified by affinity chromatography with immobilized dsRNA as a ligand. Amino acid sequence of the purified protein was determined by Edman degradation and the protein was revealed to be a silkworm homolog of Apolipophorin I, an unexchangeable apolipoprotein subunit of lipophorin. Lipophorin fraction prepared from hemolymph by density gradient ultracentrifugation also exhibited the similar dsRNA binding activity. We determined full-length sequence of Apolipophorin II/I precursor homolog of silkworm, whose structure was highly conserved among insects. Although physiological role of dsRNA binding activity is still unknown, the present study indicates potential effect by injection of dsRNA into silkworm individuals.

INTRODUCTION

In an analysis of gene function, investigation of the effect of loss-of-function is essentially important. As for major eukaryotic model organisms, such as mouse (Kile and Hilton, 2005; Hacking, 2008), fruit fly (St Johnston, 2002; Bischof *et al.*, 2007), nematode (Fire, 1986; Jantsch *et al.*, 2004) or yeast (Fincham, 1989; Wendland, 2003), tools for producing gene knockout mutants have been established and commonly utilized for wide range of studies. In most non-model organisms including silkworms, production of knockout mutants has not been available so far, limiting detailed elucidation of the gene function.

Development of RNAi technology, which principally only requires sequence information of the target gene, enabled conduct of loss-of-function analysis in these organisms by knockdown of the target gene. Especially usefulness of RNAi technology is significant in organisms such as the red flour beetle, *Tribolium castaneum*, in which effective suppress of target-gene expression is easily achieved by RNAi (Brown *et al.*, 1999; Lorenzen *et al.*, 2002; Tomoyasu and Denell, 2004).

One of common approaches to induce gene knockdown by RNAi in invertebrate individual organisms is direct injection of dsRNA solution into hemocoel (Bettencourt *et al.*, 2002; Tomoyasu and Denell, 2004; Hunter *et al.*, 2006; Garver and Dimopoulos, 2007). This

approach is successfully applied to silkworm larva and pupa to investigate functional analysis of several genes (Gui *et al.*, 2006; Ohnishi *et al.*, 2006; Huang *et al.*, 2007; Cheng *et al.*, 2008). On the other hand, it usually requires a large amount of dsRNA to achieve sufficient knockdown of targeted gene in silkworm individuals. Though it is generally believed that unlike mammals, sequence-non-specific effect of long dsRNA is not problematic in invertebrates, influence of injection of dsRNA on host physiology such as innate immunity has little been mentioned or clarified.

Recently, it has been discovered that silkworm larval hemolymph has dsRNA binding activity, though its physiological role is still unclear. In this study, we identified the dsRNA binding activity of silkworm hemolymph is attached to lipid-protein complex, lipophorin.

MATERIALS AND METHODS

Collection of silkworm larval hemolymph

Hemolymph was collected from day three of the fifth instar larvae of silkworm p50 strain nurtured on mulberry leaves. Before collection, larvae were anesthetized at low temperature on ice. Then, the forelegs of larvae were punctuated with a needle and hemolymph was collected into microcentrifuge tubes kept on ice. A few crystals of 1-phenyl-2-thiourea were added to the tubes to prevent from melanization. Hemolymph was centrifuged two minutes at 10000×g for 1 min to remove hemocytes and other cell debris, and the supernatant was stored at –80°C until use.

¹ Laboratory of Silkworm Science, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

* Corresponding author (E-mail: kusakabe@agr.kyushu-u.ac.jp)

dsRNA binding assay

Substrate dsRNA for binding assay was prepared by *in vitro* transcription according to standard methods (Zamore *et al.*, 2000; Tomoyasu and Denell, 2004) with minor modifications. DNA fragment corresponding to firefly luciferase coding sequence was amplified by PCR with primers GCTCACTGAGACTACATCAGCTATTC and ATGAGGATCTCTCTGATTTTCTTG, using pENTR11-luciferase as a template. Obtained fragment was subcloned EcoRV site of pZErO2 vector (Invitrogen) and resulting plasmid was named pZErO2-luc-6. To prepare template for *in vitro* transcription, T7 promoter sequence was attached to the both end of the luciferase fragments by two round of PCR. Primers CAGTGAATTGTAATACGACTCACTATAGGG and TAATACGACTCACTATAGGG CATCAAGCTTGGTACCGAGCTCC were used in the first round PCR, and primer CAGTGAATTGTAATACGACTCACTATAGGG was used in the second round. Obtained PCR product was phenol-extracted, precipitated and used as a template of transcription reaction by T7 RNA polymerase. Both strands were transcribed and dsRNA of about 500 bp in length was obtained. Hemolymph and dsRNA were mixed, incubated for 5 min at 20 °C and analysed by electrophoresis in 1% agarose gel.

Fractionation of dsRNA binding activity of hemolymph using affinity chromatography

In order to obtain dsRNA binding fraction from silkworm hemolymph, affinity chromatography using a column with immobilized dsRNA as a ligand molecule was performed. dsRNA immobilized column was prepared as follows. 50 mg of dsRNA prepared by *in vitro* transcription with T7 RNA polymerase as described above was mixed with 5 ml of 100 mM immobilization primer (TTGTAATACGACTCACTATA, corresponding to T7 promoter sequence, 5'-biotinylated), and made the primer anneal to 3' protruding single-stranded portion of both end of dsRNA, which contained reverse-complement sequence of T7 promoter. Resultant biotinylated oligonucleotide-annealed dsRNA was suspended with 10 ml of TE buffer, added to HiTrap Streptavidin HP 1 ml Column (GE Healthcare Life Sciences) wetted with 10 ml of buffer containing 20 mM Tris-HCl buffer, pH8 and was immobilized. The column was then equilibrated with 10 ml of buffer containing 20 mM phosphate buffer, pH6.6, 100 mM NaCl, 5 mM EDTA, 10% glycerol. 30 ml of 20% Hemolymph containing 20 mM phosphate buffer, pH6.6, 100 mM NaCl, 5 mM EDTA, 10% glycerol was added to the column using a peristaltic pump. The pump was also used in the following procedures. Flow-through was collected as 500 ml aliquots. Then the column was washed with 30 ml of same buffer, and 10 ml of elution buffer containing same as above, except 300 mM NaCl, was added. Eluate was collected 500 ml as fractions. Both fractions of flow-through and eluate were analysed by SDS-PAGE. Flow-through fractions were also subjected to dsRNA binding assay.

Identification of dsRNA binding protein as lipophorin

N-terminal sequencing of the specific band in dsRNA binding fractions was conducted. dsRNA binding fraction obtained by dsRNA affinity column was separated by 10% SDS-PAGE and transferred onto the PVDF membrane. Then the membrane was stained with Amido Black and the corresponding band was excised and used for N-terminal sequencing by Edman degradation, resulting in successful identification of nineteen residues out of twenty readings. Obtained sequence was used for homology-search with public databases at NCBI BLAST server and identified as a silkworm homolog of Apolipophorin I. Full-length open reading frame of silkworm Apolipophorin II/I precursor was determined using public and private EST sequences and sequence analysis was conducted. Signal peptide prediction was conducted using signalP (Bendtsen *et al.*, 2004). Conserved domain search was conducted using Pfam (Finn *et al.*, 2006). Potential glycosylation site prediction was conducted using NetNGlyc (www.cbs.dtu.dk/services/NetNGlyc/). UniProt entry information of Apolipophorin II/I precursor homologs (accession numbers: Q9V496, *Drosophila melanogaster*, Q9U943 *Locusta migratoria*, Q25490, *Manduca sexta*) was used for comparison.

Preparation of Lipophorin by KBr density gradient ultracentrifugation

Lipophorin was isolated from larval hemolymph by single spin density gradient ultracentrifugation as previously reported (Shapiro *et al.*, 1984; Tsuchida *et al.*, 1997). Hemolymph-KBr solution consisting of 20% hemolymph, 20 mM sodium phosphate, pH 6.6, 150 mM NaCl, 1 mM glutathione, 1 mM PMSF, 5 mM EDTA and 0.45 g/ml KBr was prepared. The hemolymph/KBr mixture solution was placed into Beckman thick-walled polycarbonate tubes, 8 ml/tube, and overlaid with 4 ml of 150 mM NaCl. Tubes were placed into a Beckman 70.1Ti rotor and centrifuged at 40,000 rpm for 4 hr at 4 °C in a Beckman model L7-55 ultracentrifuge. After centrifugation, the contents of the tubes were fractionated from the top into 300 µl fractions and the fractions subjected to SDS-PAGE. The fractions containing lipophorin were examined for dsRNA binding activity after dialysis against buffer containing 20 mM sodium phosphate, pH 6.6, 100 mM NaCl, 5 mM EDTA, 1 mM MgCl₂, 10% glycerol.

RESULTS

Identification of dsRNA binding activity-associated protein in silkworm larval hemolymph

dsRNA binding activity of silkworm larval hemolymph was examined using gel mobility shift assay (Fig. 1A). dsRNA mixed with larval hemolymph showed remarkable mobility shift in 1% agarose gel. Mobility shift was inhibited by the addition of EDTA, indicating the binding was dependent on divalent cations (Fig. 1B).

In order to uncover the molecular basis of observed activity, purification of dsRNA binding substance from hemolymph was conducted using affinity chromatogra-

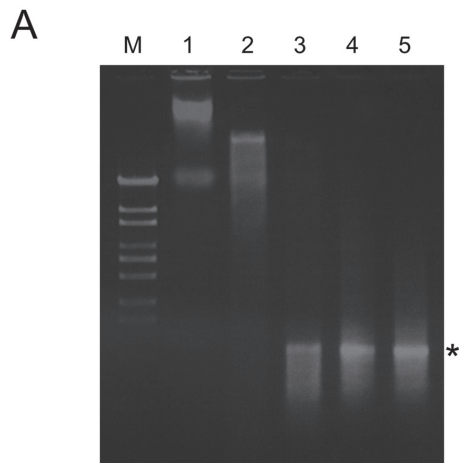


Fig. 1A. Electrophoretic profiles of hemolymph and dsRNA mixture using 1% agarose gel. dsRNA was incubated with silkworm hemolymph of fifth instar larvae and separated by 1% agarose gel electrophoresis. Hemolymph ($2\mu\text{l}$, $0.5\mu\text{l}$, $0.12\mu\text{l}$, $0.03\mu\text{l}$ or $0.008\mu\text{l}$, lanes 1–5) was mixed with 500 ng of dsRNA in volume of $10\mu\text{l}$, and $6\mu\text{l}$ of each sample were subjected to electrophoresis. Asterisk indicates the position of the band corresponding to free dsRNA. M, marker.

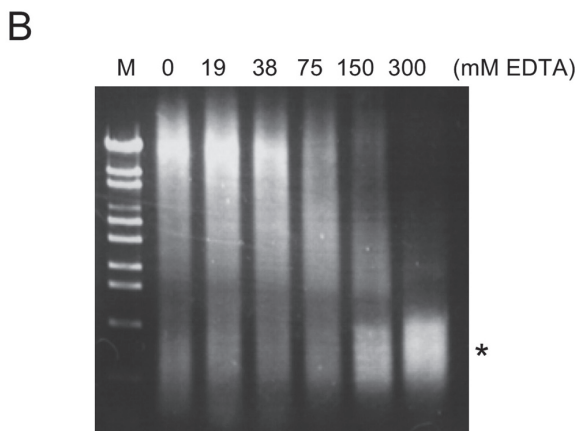


Fig. 1B. Electrophoretic profiles of hemolymph and dsRNA mixture using 1% agarose gel. Hemolymph ($0.5\mu\text{l}$) was mixed with 500 ng of dsRNA and indicated concentration of EDTA in volume of $10\mu\text{l}$, and $6\mu\text{l}$ of each sample were subjected to electrophoresis. Asterisk indicates the position of the band corresponding to free dsRNA. M, marker.

phy. Silkworm larval hemolymph was added to a dsRNA-immobilized column, and proteins bound to dsRNA were eluted by high salt buffer as described in Materials and Methods. A notable observation was that dsRNA binding fraction exhibited pale yellow color suggesting existence of carotenoids (data not shown). In SDS-PAGE analysis, specific band at around molecular weight of 210 kDa was observed in dsRNA binding fractions (Fig. 2). dsRNA binding fraction was desalted by dialysis and examined

for dsRNA binding activity. It showed very low binding activity, which might be due to lack of cofactor, or that the dsRNA binding site is blocked by uncoupled dsRNA removed from the column.

The 210 kDa band was also observed in flow-through and wash fractions (Fig. 2). To verify the involvement of the protein in dsRNA binding, correlation of the existence of the protein with dsRNA binding activity was further examined. The flow-through fractions of dsRNA column affinity chromatography were examined for dsRNA binding activity (Fig. 3A). While the former fractions (fraction numbers 1 to 16) had no dsRNA binding activity, the latter fractions (fraction numbers 19 to 40) exhibited the activity. This result indicates that dsRNA binding activity was depleted in former fractions, and then

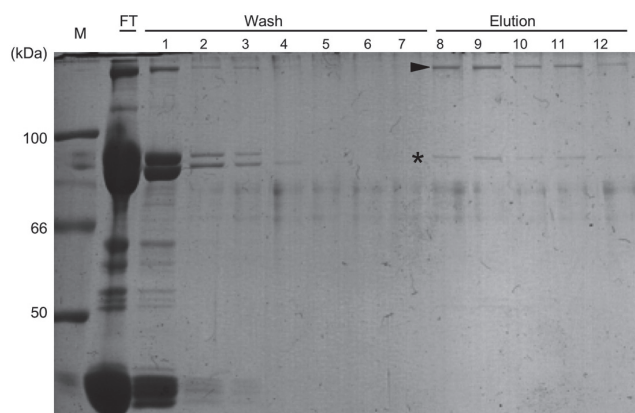


Fig. 2. SDS-PAGE of fractions of dsRNA binding activity by affinity column chromatography. Silkworm larval hemolymph was subjected to dsRNA-immobilized column, washed with mild salt buffer and eluted by high salt buffer. Flow-through, wash and elution fractions are indicated by bars above. Arrowhead indicates elution fraction-specific band. Asterisk indicates the position of a concomitant band unidentified in this study. M, molecular weight marker. FT, flow-through fraction.

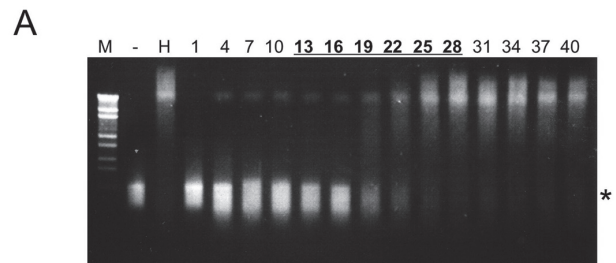


Fig. 3A. Coexistence of 210 kDa protein with dsRNA binding activity of silkworm larvae hemolymph.

dsRNA binding activity of flow-through fractions of dsRNA column-passed silkworm larval hemolymph. dsRNA binding activity was depleted in fractions 1 to 16, while appeared in fractions 19 to 40. Fraction numbers are indicated above. Underlined numbers indicates fractions examined in Fig. 17B. M, marker. -, dsRNA without hemolymph. H, untreated hemolymph. Asterisk indicates the position of the band corresponding to free dsRNA.

B

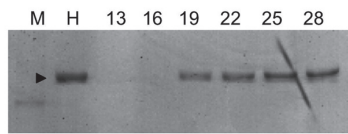


Fig. 3B. Coexistence of 210 kDa protein with dsRNA binding activity of silkworm larvae hemolymph. Flow-through fractions shown in Fig. 17A were separated by SDS-PAGE. Fraction numbers corresponding to these in Fig. 17A are indicated above. The existence of the 210 kDa band (arrowhead) was correlated with dsRNA binding activity shown in Fig. 17A. M, marker. H, hemolymph.

recovered in latter fractions due to the saturation of the dsRNA-immobilized column. Next, the fractions without dsRNA binding activity (fractions 13 and 16) and the fractions exhibiting the activity (fractions 19, 22, 25 and 28) were examined for existence of the 210 kDa protein by SDS-PAGE (Fig. 3B). While the band of 210 kDa protein was observed in fractions harboring dsRNA binding activity (fractions 19 to 28), disappeared in fractions without binding activity (fractions 13 and 16), suggesting this protein was involved in dsRNA binding activity.

Apolipophorin I associates with dsRNA binding activity

N-terminal sequence of the 210 kDa protein with the dsRNA binding activity was determined by Edman degradation as described in Materials and Methods. The N-terminal amino acid sequence was successfully obtained except for one undetermined residue (Fig. 4A). A database search revealed the sequence shared high degrees of sequence homology with Apolipophorin I of *Manduca sexta* (tobacco hornworm), whose molecular weight was around 210 kDa, consistent with my observation (Fig. 4B). An EST entry corresponding to the cDNA that shows high homology with *Manduca* Apolipophorin precursor was found in a silkworm cDNA library, and its full-length sequence was compiled by further analysis (Fig. 5). Apolipophorin I was known to be processed from precursor protein by a subtilisin-like prohormone convertase, furin. The nucleotides sequence determined was corresponding to the deduced sequence for processed N-terminal, indicating the silkworm Apolipophorin I obtained was similarly processed. These results suggested that the band was a functional homolog to silkworm Apolipophorin I, and lipophorin, a lipid-protein complex, was bearing dsRNA binding activity. Lipophorin plays a role in transfer of carotenoids from midgut epithelium to the middle parts of the silkgrand, consistent with the observed color of the fraction.

A

¹SVKTEIDSF^XDKNXKAEAPY₂₀

Fig. 4A. Identification of 210 kDa protein as silkworm homolog of Apolipophorin I. N-terminal sequence of the 210 kDa protein corresponding to the band shown in Fig. 17 was determined by Edman degradation. X indicates an unidentified residue.

B

Bm SVKTEIDSF⁺DKNLKAEAPY
S+K+EID FDKN KAE+APY
Ms ...KVEDSLSRGRRS⁺IKSEIDVFDKNLKAESAPYNNELDLDIY...

Fig. 4B. Identification of 210 kDa protein as silkworm homolog of Apolipophorin I.

Comparison of deduced amino acids sequence of an EST entry (Genbank accession number) corresponding to the obtained sequence with *Manduca sexta* homolog. Residues 701–740 of *M. sexta* apolipophorin II/I precursor (Genbank accession number AAB53254) are presented. Identical residues are shown. + indicates similar residues. Underlined letters and arrowhead indicate the furin recognition sequence and the processing site, respectively, for cleavage of Apolipophorin precursor into two mature subunits.

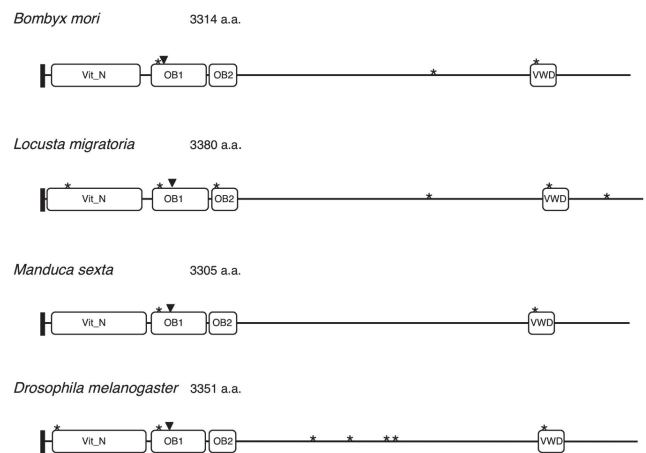


Fig. 5. Schematic diagrams of some selected insect Apolipophorin II/I precursors.

Conserved domains of insect apolipophorins are presented to scale. Detailed procedures of sequence analysis is described in Materials and Methods. Solid boxes at N-terminal represent signal peptides. Triangles and Asterisks indicate furin cleavage site and potential glycosylation sites, respectively. Vit_N, Lipoprotein amino terminal region. OB1, domain of unknown function consisting of several large open beta-sheets (Pfam DUF1943). OB2, domain of unknown function represents a conserved open beta-sheet domain, found in proteins including vitellogenin and apolipophorin (Pfam DUF1081). VWD, von Willebrand factor type D domain.

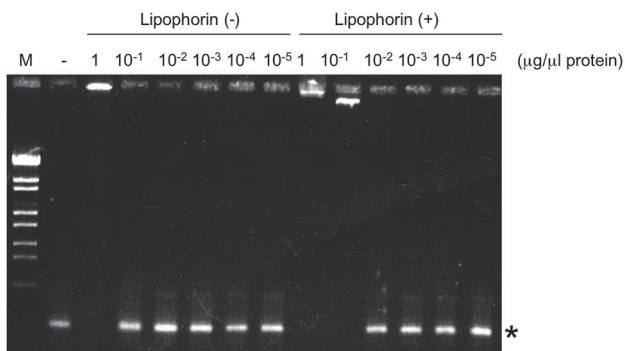


Fig. 6. dsRNA binding activity of lipophorin prepared from larval hemolymph.

Lipophorin little-containing (–) and containing (+) fractions were prepared by KBr density gradient ultracentrifugation and dsRNA binding activity was examined. Protein concentration of reaction solution is indicated. M, marker. –, dsRNA used as substrate. Asterisk indicates the position of the band corresponding to free dsRNA

Lipophorin confers dsRNA binding activity of larval hemolymph

To confirm Lipophorin has dsRNA binding activity, silkworm larval hemolymph lipophorin was purified by ultracentrifugation as previously reported (Shapiro *et al.*, 1984; Tsuchida *et al.*, 1997) and dsRNA binding activity was examined (Fig. 6). Purified lipophorin showed dsRNA binding activity, verifying that lipophorin was responsible for dsRNA binding activity of silkworm larval hemolymph.

DISCUSSION

In this study, we examined molecular basis of dsRNA binding activity of silkworm larval hemolymph. Affinity chromatography using dsRNA as a ligand was conducted and dsRNA binding fraction-specific protein was identified. Determined amino acid sequence of the protein showed high homology with apolipophorin I, a subunit of lipid-protein complex, Lipophorin purified from hemolymph by KBr density gradient ultracentrifugation exhibited dsRNA binding activity. These results suggest that dsRNA binding activity is exerted by lipophorin.

Lipophorin is a major lipoprotein in insect hemolymph playing a role in lipid transportation. Three species of Apoprotein, named apolipophorins, are contained in lipophorin. Apolipophorin-I (ApoLp-I) and apolipophorin-II (ApoLp-II) are non-exchangeable structural components of lipophorin, whereas apolipophorin-III (ApoLp-III) is known to associate with lipophorin during lipid loading to precursor. ApoLp-I and ApoLp-II was processed from the same precursor by furin (Smolenaars *et al.*, 2005). Full sequence of silkworm homolog of apolipophorin I/II precursor was determined by cloning and database search. While cloning of silkworm ApoLp-III was reported (Yamauchi *et al.*, 2000), sequence of silkworm homologs of ApoLp-I and ApoLp-II has determined

in this study for the first time. Determined sequence of dsRNA binding fraction-specific band was corresponding to the processed N-terminal sequence of ApoLp-I, and processing site of furin reported in other insects was conserved.

Lipophorin is suggested to play a role in innate immunity in insects. In wax moth *Galleria mellonella*, Lipophorin was shown to recognize LPS in hemolymph and form detergent-insoluble aggregates and inactivate LPS (Ma *et al.*, 2006). dsRNA might be regarded as an unusual and viral infection-related substance in hemolymph. Thus, dsRNA binding activity of lipophorin might be involved in innate immune system. In this aspect, potential dsRNA-induced response in hemolymph, which might affect the effect of larval RNAi, remains to be clarified.

ACKNOWLEDGEMENTS

This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), by a grant from the Ministry of 248 Agriculture, Forestry and Fisheries of Japan (Integrated research project for plant, insect and animal using genome technology INSECT-1201), and by a grant-in-aid, no. 17380037, from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- Bendtsen, J. D., H. Nielsen, G. von Heijne and S. Brunak 2004 Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.*, **340**: 783–795
- Bettencourt, R., O. Terenius and I. Faye 2002 Hemolin gene silencing by ds-RNA injected into *Cecropia* pupae is lethal to next generation embryos. *Insect Mol. Biol.*, **11**: 267–271
- Bischof, J., R. K. Maeda, M. Hediger, F. Karch and K. Basler 2007 An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proc. Natl. Acad. Sci. U. S. A.*, **104**: 3312–3317
- Brown, S. J., J. P. Mahaffey, M. D. Lorenzen, R. E. Denell and J. W. Mahaffey 1999 Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. *Evol. Dev.*, **1**: 11–15
- Cheng, D., Q. Xia, J. Duan, L. Wei, C. Huang, Z. Li, G. Wang and Z. Xiang 2008 Nuclear receptors in *Bombyx mori*: Insights into genomic structure and developmental expression. *Insect Biochem. Mol. Biol.*, **38**: 1130–1137
- Fincham, J. R. 1989 Transformation in fungi. *Microbiol. Rev.*, **53**: 148–170
- Finn, R. D., J. Mistry, B. Schuster-Bockler, S. Griffiths-Jones, V. Hollich, T. Lassmann, S. Moxon, M. Marshall, A. Khanna, R. Durbin, S. R. Eddy, E. L. Sonnhammer and A. Bateman 2006 Pfam: clans, web tools and services. *Nucleic Acids Res.*, **34**: D247–251
- Fire, A. 1986 Integrative transformation of *Caenorhabditis elegans*. *EMBO J.*, **5**: 2673–2680
- Garver, L. and G. Dimopoulos 2007 Protocol for RNAi assays in adult mosquitoes (*A. gambiae*). *J. Vis. Exp.*: 230
- Gui, Z. Z., K. S. Lee, B. Y. Kim, Y. S. Choi, Y. D. Wei, Y. M. Choo, P. D. Kang, H. J. Yoon, I. Kim, Y. H. Je, S. J. Seo, S. M. Lee, X. Guo, H. D. Sohn and B. R. Jin 2006 Functional role of aspartic proteinase cathepsin D in insect metamorphosis. *BMC Dev. Biol.*, **6**: 49
- Hacking, D. F. 2008 ‘Knock, and it shall be opened’: knocking out and knocking in to reveal mechanisms of disease and novel

- therapies. *Early Hum. Dev.*, **84**: 821–827
- Huang, J., Y. Zhang, M. Li, S. Wang, W. Liu, P. Couble, G. Zhao and Y. Huang 2007 RNA interference-mediated silencing of the bursicon gene induces defects in wing expansion of silkworm. *FEBS Lett.*, **581**: 697–701
- Hunter, C. P., W. M. Winston, C. Molodowitch, E. H. Feinberg, J. Shih, M. Sutherlin, A. J. Wright and M. C. Fitzgerald 2006 Systemic RNAi in *Caenorhabditis elegans*. *Cold Spring Harb. Symp. Quant. Biol.*, **71**: 95–100
- Jantsch, V., P. Pasierbek, M. M. Mueller, D. Schweizer, M. Jantsch and J. Loidl 2004 Targeted gene knockout reveals a role in meiotic recombination for ZHP-3, a Zip3-related protein in *Caenorhabditis elegans*. *Mol. Cell. Biol.*, **24**: 7998–8006
- Kile, B. T. and D. J. Hilton 2005 The art and design of genetic screens: mouse. *Nat Rev Genet.*, **6**: 557–567
- Lorenzen, M. D., S. J. Brown, R. E. Denell and R. W. Beeman 2002 Cloning and characterization of the *Tribolium castaneum* eye-color genes encoding tryptophan oxygenase and kynurenine 3-monooxygenase. *Genetics*, **160**: 225–234
- Ma, Y., A. Creanga, L. Lum and P. A. Beachy 2006 Prevalence of off-target effects in *Drosophila* RNA interference screens. *Nature*, **443**: 359–363
- Ohnishi, A., J. J. Hull and S. Matsumoto 2006 Targeted disruption of genes in the *Bombyx mori* sex pheromone biosynthetic pathway. *Proc. Natl. Acad. Sci. U. S. A.*, **103**: 4398–4403
- Shapiro, J. P., P. S. Keim and J. H. Law 1984 Structural studies on lipophorin, an insect lipoprotein. *J. Biol. Chem.*, **259**: 3680–3685
- Smolenaars, M. M., M. A. Kasperaitis, P. E. Richardson, K. W. Rodenburg and D. J. Van der Horst 2005 Biosynthesis and secretion of insect lipoprotein: involvement of furin in cleavage of the apoB homolog, apolipophorin-II/I. *J. Lipid Res.*, **46**: 412–421
- St Johnston, D. 2002 The art and design of genetic screens: *Drosophila melanogaster*. *Nat. Rev. Genet.*, **3**: 176–188
- Tomoyasu, Y. and R. E. Denell 2004 Larval RNAi in *Tribolium* (*Coleoptera*) for analyzing adult development. *Dev. Genes Evol.*, **214**: 575–578
- Tsuchida, K., J. L. Soulages, A. Moribayashi, K. Suzuki, H. Maekawa and M. A. Wells 1997 Purification and properties of a lipid transfer particle from *Bombyx mori*: comparison to the lipid transfer particle from *Manduca sexta*. *Biochim. Biophys. Acta*, **1337**: 57–65
- Wendland, J. 2003 PCR-based methods facilitate targeted gene manipulations and cloning procedures. *Curr. Genet.*, **44**: 115–123
- Yamauchi, Y., C. Hoeffler, A. Yamamoto, H. Takeda, R. Ishihara, H. Maekawa, R. Sato, S. Su-II, M. Sumida, M.A. Wells and K. Tsuchida 2000 cDNA and deduced amino acid sequences of apolipophorin-IIIs from *Bombyx mori* and *Bombyx mandarina*. *Arch. Insect Biochem. Physiol.*, **43**: 16–21
- Zamore, P. D., T. Tuschl, P. A. Sharp and D. P. Bartel 2000 RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, **101**: 25–33