Original Article

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Protective effects of *Phaseolus vulgaris* lectin against viral infection in *Drosophila*

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Summary **Phytohemagglutinin (PHA) isolated from the family of *Phaseolus vulgaris* beans is a promising agent against viral infection; however, it has not yet been demonstrated *in vivo*. We herein investigated this issue using *Drosophila* as a host. Adult flies were fed lectin approximately 12 h before they were subjected to a systemic viral infection. After a fatal infection with *Drosophila* C virus, death was delayed and survival was longer in flies fed PHA-P, a mixture of L4, L3E1, and L2E2, than in control unfed flies. We then examined PHA-L4, anticipating subunit L as the active form, and confirmed the protective effects of this lectin at markedly lower concentrations than PHA-P. In both experiments, lectin feeding reduced the viral load prior to the onset of fly death. Furthermore, we found a dramatic increase in the levels of the mRNAs of phagocytosis receptors in flies after feeding with PHA-L4 while a change in the levels of the mRNAs of antimicrobial peptides was marginal. We concluded that *P. vulgaris* PHA protects *Drosophila* against viral infection by augmenting the level of host immunity.**

Keywords: Antiviral effect, innate immunity, plant lectin

1. Introduction

Lectins are carbohydrate-binding proteins that have been found in a number of organisms, including microorganisms, plants, and animals (*1*). Lectins exist in intracellular and extracellular compartments and bind mono- and oligosaccharides with specificity in certain sugar residues. When lectins act intracellularly, lectin-tagged cellular components may change their functions and/or localization. In contrast, plasma membrane-bound or extracellular lectins serve either as ligands or receptors as well as bridging molecules to link cells (*1*). When extracellular lectins function as ligands, they stimulate receptors at the surface of target cells in order to activate signal transduction pathways for the induction of a number of biological phenomena.

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Phytohemagglutinin (PHA) in the seeds of the red kidney bean *Phaseolus vulgaris* consists of a tetramer of subunits E and L, and five different compositions of PHA have been identified, i.e., E4, E3L1, E2L2, E1L3, and L4 (*2*). All these *P. vulgaris* PHAs possess an agglutination activity against red blood cells as well as a mitogen activity against lymphocytes, and subunits E and L appear to be responsible for the former and latter activities, respectively (*3*). *P. vulgaris* PHA has the potential as an agent against viral infection. Lectins isolated from the family of *P. vulgaris* beans, such as extra-long autumn purple beans, French beans, and Anasazi beans, exert inhibitory effects on the reverse transcriptase of human immunodeficiency virus *in vitro* (*2,4*). However, the antiviral activity of *P. vulgaris* PHA has not yet been demonstrated *in vivo*.

Insects have provided researchers with animal models suitable for the study of human diseases (*5*). Evidence for the application of insect models for the development of medicines to treat human diseases has been accumulated (*6-8*). The fruit fly *Drosophila melanogaster* has been widely used in research to elucidate the underlying

mechanisms of self-defense systems (*9,10*). In addition to the availability of robust genetic approaches, the small size of *Drosophila* allows us to utilize a large number of flies in each experiment, which in many cases results in accurate and reliable data. Despite size differences, the organization of the genome is very similar between humans and *Drosophila*, and a number of *Drosophila* models of human diseases exist including infectious diseases, diabetes mellitus, and cancer (*11-14*). Furthermore, similarities have been identified in antiviral mechanisms between humans and *Drosophila* (*15-18*). Therefore, we herein used *Drosophila* as a host for viral infection in order to elucidate whether *P. vulgaris* PHA exhibits antiviral activity *in vivo*.

2. Materials and Methods

2.1. *Materials*

The fly line w^{1118} obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, Indiana, U.S.A.) was maintained with standard cornmeal/agar medium at 25°C and used throughout this study. *P. vulgaris* lectin was extracted from red kidney beans, purified to homogeneity by affinity chromatography, and sub-types were separated by ion-exchange column chromatography, according to established procedures (*3*) with slight modifications. A mixture of L4, L3E1, and L2E2, which we operationally named PHA-P (J-Oil Mills code number J113), and purified L4 (PHA-L4) (J-Oil Mills code number J112) were individually tested for their antiviral activities. The amount of proteins was measured by the Bradford method using the Bio-Rad Protein Assay with bovine serum albumin as a standard protein. PHA-L4 and purified E4 (PHA-E4) labeled with fluorescence isothiocyanate (FITC), J-Oil Mills code numbers J512 and J511, respectively, were used to locate the lectin in the digestive tracts of flies.

2.2. *Lectin feeding of flies*

Males of *Drosophila* adults, 3-7 days after eclosion, were collected in vials (30-35 flies per vial) in which filter paper (Whatman 3MM Chr) was placed at the bottom and kept for 3 h with no food or water for starvation. A total of 0.3 mL of water containing or not containing *P. vulgaris* PHA at various concentrations was added to the vials, and flies were given free access to water absorbed by filter paper for 19 h. Flies were then maintained with regular food for 12 h for recovery from starvation before the abdominal infection with *Drosophila* C virus (DCV).

2.3. *Preparation of DCV, infection, and viral titer assays*

DCV, a natural pathogen of *Drosophila*, is a non-enveloped positive-strand RNA virus assigned to the Dicistroviridae genus *Criparivus*. In order to prepare DCV stocks, cultures of S2 cells, a *Drosophila* cell line established from embryonic hemocytes, were incubated with DCV, and culture media were collected, subjected to the assessment of viral titers with S2 cells as a host, and stored at –80°C until used, as described previously (*19*). Adult flies, with and without lectin feeding, were infected with DCV (2,750 or 27,500 50% tissue culture-infective dose ($TCID_{50}$)) by the abdominal injection of the viral suspension using a nitrogen gas-aided microinjector and maintained in vials containing regular fly food, as described previously (*19*). An assay for fly survivorship and the assessment of the viral load in flies were conducted as described previously (*19*).

2.4. *Messenger RNA analysis of antimicrobial peptides*

Males of *Drosophila* adults (approximately 15 flies), with and without lectin feeding, were suspended in TRIzol reagent (Invitrogen) in a plastic microtube and homogenized using a plastic pestle on ice. After centrifugation, RNA in the supernatants was precipitated with isopropanol and dissolved with water. Extracted total RNA was subjected to reverse transcription using a random hexamer as a primer to generate cDNA. Sequences corresponding to those of antimicrobial peptide mRNAs were amplified from the cDNA by PCR using primers specific to attacin-A, defensin, diptericin, drosomycin, and metchnikowin. The levels of the mRNAs of Draper and integrin αPS3-βv, engulfment receptors responsible for the phagocytic removal of apoptotic cells in *Drosophila* (*18*), were similarly determined. A sequence corresponding to the mRNA of the ribosomal protein rp49 was also amplified as an unchanged internal control. We first determined the ratio of two cDNA preparations, obtained from RNA of flies fed lectin or water, that gives a similar level of the signal derived from rp49 mRNA. Then, PCR amplification of the sequences corresponding to the mRNAs of antimicrobial peptides and engulfment receptors was carried out using two cDNA preparations at the determined ratio. The concentrations of all cDNAs used in PCR were within a quantifiable range. The PCR products were separated by polyacrylamide gel electrophoresis followed by staining with ethidium bromide. The intensity of each PCR product was digitized and shown relative to that obtained with RNA of water-fed flies. The nucleotide sequences of the PCR primers were: 5'-CCCGGAGTGAAGGATG-3' (forward) and 5'-GTTGCTGTGCGTCAAG-3' (reverse) for the mRNA of attacin-A (*20*); 5'-GTTCTTCGTTCTCGTGG-3' (forward) and 5'-CTTTGAACCCCTTGGC-3' (reverse) for the mRNA of defensin (*20*); 5'-GCTGCGCAATCGCTTCTACT-3' (forward) and 5'-TGGTGGAGTGGGCTTCATG-3' (reverse) for the mRNA of diptericin (*20*);

5'-CGTGAGAACCTTTTCCAATATGATG-3' (forward) and 5'-TCCCAGGACCACCAGCAT-3' (reverse) for the mRNA of drosomycin (*20*); 5'-AACTTAATCTTGGAGCGA-3' (forward) and 5'-CGGTCTTGGTTGGTTAG-3' (reverse) for the mRNA of metchnikowin (*20*); 5'-CGGAATTCTCTGCCGCAC GGGTTACATAG-3' (forward) and 5'-CCGCTCGAGC CGGCTCGAATTTTCGCTT-3' (reverse) for the mRNA of Draper (*21*); 5'-AGATACCTACTCCTGGGCTT-3' (forward) and 5'-TCCGCATTTGGAGCTCCATT-3' (reverse) for the mRNA of integrin αPS3 (*21*); 5'-AAGCCAACTCTACCCATGATT-3' (forward) and 5'-GTGGGACAGTTGCAATAGGT-3' (reverse) for the mRNA of integrin βv (*21*); and 5'-GACGCTTCAAGGGACAGTATCTG-3' (forward) and 5'-AAACGCGGTTCTGCATGAG-3' (reverse) for the mRNA of rp49 (*20*).

2.5. *Statistical analysis*

All data were collected from at least three independent experiments except for those presented as Figures 3B and 4B. The numerical data were statistically analyzed by the Log-rank test (the Kaplan-Meier method) for a survivorship assay or the two-tailed Student's *t*-test for other experiments. A p-value < 0.05 between two sets of experimental data was considered to be significant.

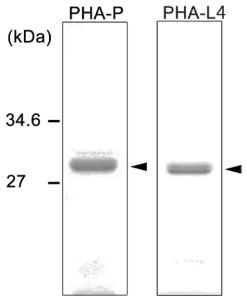

Figure 1. Purity of *P. vulgaris* PHA used in this study. Chromatographically prepared PHA-P (0.5 μg) and PHA-L4 (0.5 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis, and visualized by staining with Coomassie Brilliant Blue. The arrowheads point to the positions of lectin, and the positions and molecular masses of standard proteins are shown on the left. Subunits E and L possess a similar molecular mass of about 28 kDa.

3. Results

3.1. *Prolonged survival of DCV-infected flies after feeding with PHA-P*

Throughout this study, chromatographically purified *P. vulgaris* lectins were used (Figure 1). We first examined whether the procedures for feeding flies with lectin adopted in the present study allowed flies to take lectin into the digestive tract. Male adult flies were starved as described in the Materials and Methods and then fed FITC-labeled PHA-E4 or PHA-L4, which had been dissolved with water and adsorbed by filter paper placed in a vial. The digestive tracts of these flies were dissected out and examined under a fluorescence stereomicroscope. Fluorescence signals derived from FITC were clearly detected in a part of the midgut (*22*) of flies fed either isolectin (Figure 2), indicating the success of the oral administration of lectin in these procedures.

We then investigated the effects of PHA-P on the survival of flies after the abdominal infection with DCV. Adult flies were fed PHA-P, subjected to a fatal infection with DCV, and analyzed for survivorship. We found that flies pre-fed this lectin lived longer after the infection with DCV than unfed control flies (Figure 3A). PHA-P gave effective concentrations of 60-120 μg/mL during feeding (Figure 3B).

3.2. *Delay in the onset of fly death and prolongation in the survival time of DCV-infected flies after feeding with PHA-L4*

We performed similar experiments using PHA-L4, which consists of a tetramer of subunit L (see Figure 1). The administration of PHA-L4 extended the survival time of DCV-infected flies (Figure 4A), as did PHA-P, with effective concentrations of 1.2-6.0 μg/mL (Figure 4B), which were 20-50-fold lower than those of PHA-P. Furthermore, the pre-feeding of flies with PHA-L4 delayed death in flies (Figure 4A). The results obtained from repeated experiments revealed that PHA-L4-

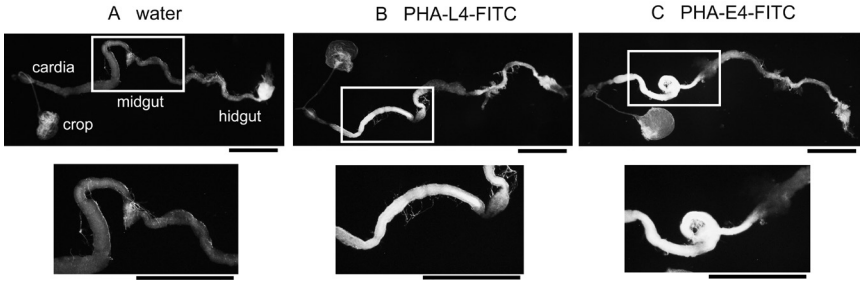

Figure 2. Existence of *P. vulgaris* PHA in the midgut of flies after feeding. Adult flies were fed FITC-labeled PHA-E4 (0.1 μg/mL), FITC-labeled PHA-L4 (0.1 μg/mL), or water alone as a control for 2 h. Digestive tracts were dissected out and examined by fluorescence microscopy. The bottom panels in each set of the data are magnified views of the squared area in the upper panels. Fluorescence signals derived from FITC are shown in white. The data represent one experiment of 4 replicates that showed a similar result. Scale bars = 200 μm.

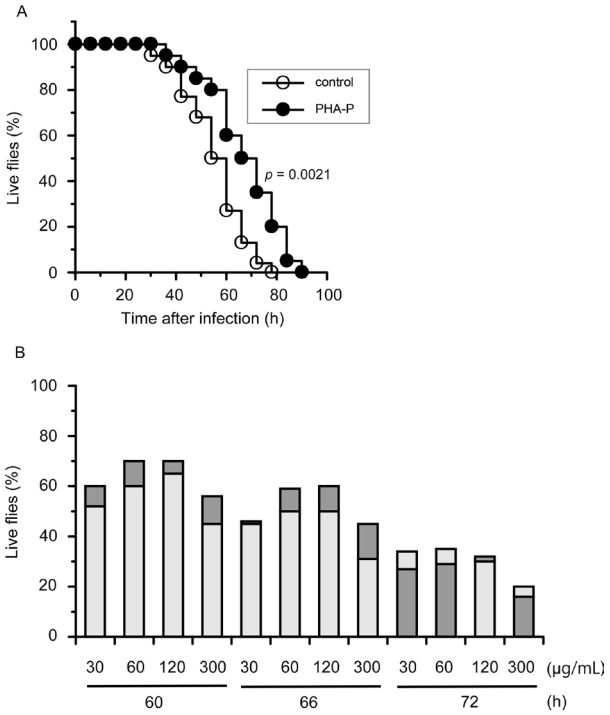

Figure 3. Effects of PHA-P on the survival of flies after DCV infection. (A) Adult flies were fed PHA-P (60 μg/mL) or water alone as a negative control for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$, and subjected to an assay for survivorship. Data obtained from one out of 3 independent experiments with similar results are presented. **(B)** Adult flies were fed PHA-P at various concentrations for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$, and examined for the ratio of live flies at the indicated time points. Data from two independent experiments are shown as white and gray bars.

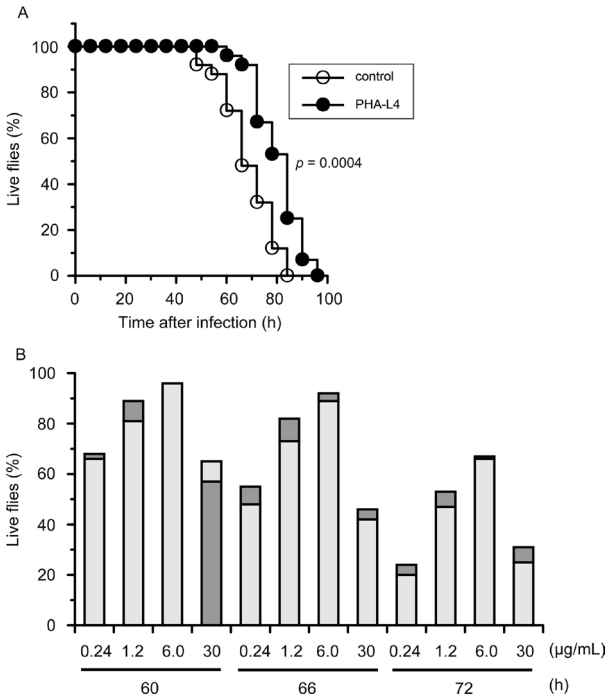

Figure 4. Effects of PHA-L4 on the survival of flies after DCV infection. (A) Adult flies were fed PHA-L4 (6 μg/mL) or water alone as a negative control for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$, and subjected to an assay for survivorship. Data obtained from one out of 5 independent experiments with similar results are presented. **(B)** Adult flies were fed PHA-L4 at various concentrations for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$, and examined for the ratio of live flies at the indicated time points. Data from two independent experiments are shown as white and gray bars.

Table 1. Timings of fly death

Items	2,750 $TCID_{50}$		27,500 $TCID_{50}$	
	Onset	Extinct	Onset	Extinct
Water-fed flies	42 - 48	90	42 - 54	78 - 90
PHA-L4-fed flies	48 - 66	96	54 - 60	84 - 102

Hours post-infection when flies start to die (onset) and all flies are dead (extinct) are shown as a summary of the data from three independent experiments with two different viral doses.

treated flies started to die approximately 10 h later and lived approximately 10 h longer than control flies (Table 1). In order to confirm these effects of PHA-L4, we carried out an infection experiment at a 10-fold lower dose of the viral burden. We found a similar protective effect against DCV infection in flies administered PHA-L4 as that observed in the experiment with a higher viral dose (Table 1).

3.3. *Inhibitory effects of PHA-L4 on the viral load in Drosophila*

We next investigated the level of the viral load in flies at various time points after the abdominal infection with DCV at two different doses. We found that the level markedly increased during the first 12 h, and then more gradually increased in control unfed flies at either viral dose (Figures 5A and 5B). The pre-administration of PHA-L4 to flies inhibited the growth of the virus, apparently in a different manner at the two viral doses used. In flies infected with DCV at a higher dose, lectin reduced the level of the viral load during 30-42 h post-infection, a period when the viral level reached its maximum (Figure 5A). In contrast, an increase in the level of the viral load was inhibited at early time points, 12-24 h post-infection, in flies administered a lower dose of the virus (Figure 5B). The inhibition of DCV growth by PHA-L4 appeared to occur well before the onset of fly death, irrespective of the initial level of the viral load (Table 1).

3.4. *Increases in the level of the mRNAs of immunity-related proteins in flies after feeding with PHA-L4*

Feeding with lectin might alter the antiviral status in flies. Both humoral (*23*) and cellular (*19*) immunity play roles in the protection of flies against viral infection, including that with DCV. Therefore, we compared the amounts of the mRNAs of antimicrobial peptides and

phagocytosis receptors in adult flies before and after lectin feeding by reverse transcription-mediated PCR. The mRNA levels of various antimicrobial peptides, the Toll pathway-induced attacin and diptericin as well as the Imd pathway-induced defensin, drosomycin, and metchnikowin (*10*), were determined, and we found that the mRNA levels of some antimicrobial peptides, such as diptericin and defensin, marginally increased in flies after feeding with PHA-L4 (Figure 6A). We then similarly analyzed the mRNAs of Draper and integrin αPS3-βν, engulfment receptors that activate two partly overlapping signaling pathways for the induction of phagocytosis of apoptotic cells by *Drosophila* hemocytes (*21*). The data indicated a dramatic increase in the levels of the mRNAs of both Draper and integrin βν in flies after lectin feeding (Figure 6B): signals derived from the mRNA of integrin αPS3 were not detected under the conditions adopted in this study (data not shown). These results suggest that *P. vulgaris* PHA enhances Toll and Imd pathways as well as the phagocytic activities of hemocytes in adult flies.

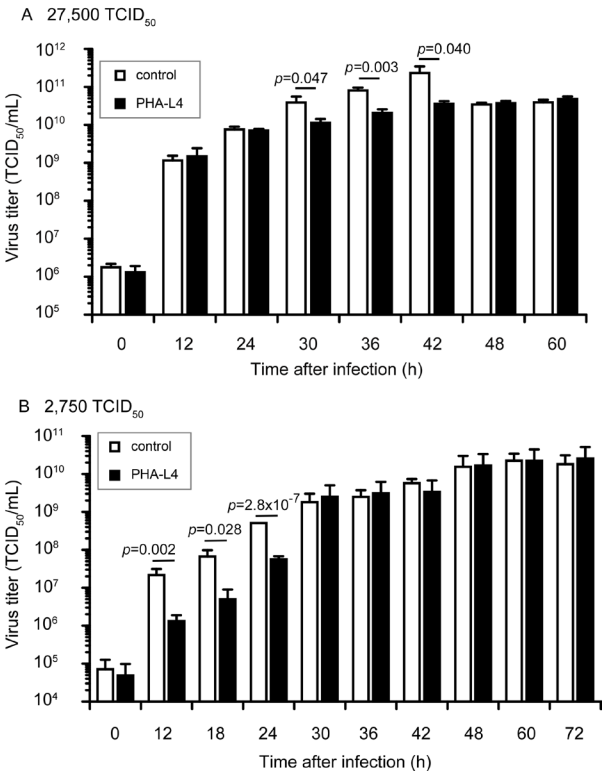

Figure 5. Effects of PHA-L4 on the viral load in adult flies. Adult flies were fed PHA-L4 (6 μg/mL) or left untreated as a negative control for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$ **(A)** or 2,750 $TCID_{50}$ **(B)**, and subjected to an assay for the viral load at the indicated time points. The means ± SD of data obtained from 3 independent experiments are shown. The data from two groups were statistically analyzed, and *p*-values are indicated when they were found to be significantly different.

4. Discussion

Pre-feeding with lectin isolated from *P. vulgaris* beans extended the survival period of *Drosophila* adults after an infection with DCV. This result indicates that *P. vulgaris* lectin exhibits antiviral activity in *Drosophila*. Based on the similarities in antiviral mechanisms between insects and humans, the present results confirmed that *P. vulgaris* PHA protects a host organism against viral infection.

Lectin-fed flies started to die later and survived longer than control unfed flies, and decreased the levels of the viral load. Antiviral actions accompanied by a decrease of the viral load suggest that the lectin treatment enhanced fly resistance, not tolerance against the virus (*24*). An analysis with fluorescence-labeled lectin indicated the presence of lectin in the midgut of the digestive tracts of flies. However, we speculate that lectin did not enter the body across the wall of the tract due to the peritrophic matrix, which serves as a barrier against microbial pathogens (*25*). Therefore, the antiviral effects of *P. vulgaris* PHA do not appear to occur through a direct interaction with the virus, but by indirectly altering host responses against viral infection. The antiviral defenses of *Drosophila* have been shown to rely on RNA interference, the Toll-Dorsal pathway, the JAK-STAT pathway, the phagocytosis of virus particles, and the phagocytosis of virus-infected cells (*16-19,26-28*). Therefore, humoral and cellular innate immune responses appear to participate in the protection of *Drosophila* against viral infection. We showed a dramatic increase in the levels of the mRNAs of phagocytosis receptors, which are responsible for the removal of cells undergoing apoptosis (*18*), whereas an increase of antimicrobial peptide mRNAs was only marginal. Taking our previous finding that the apoptosis-dependent phagocytosis of DCV-infected cells plays an important role in the antiviral actions of *Drosophila* immunity (*19*) into consideration, an increase in the phagocytic activity of immune cells could be a mechanistic interpretation of the antiviral effects of *P. vulgaris* lectin. Subunit L appears to be responsible for the antiviral actions of *P. vulgaris* lectin, as in the induction of lymphocyte growth (*3*). It is, thus, likely that PHA-L4 is a major *P. vulgaris* isolectin that influences the level of immunity in both vertebrate and invertebrate animals. Further investigation is required to clarify the mechanisms of actions of *P. vulgaris* PHA to protect animals from microbial infections.

In conclusion, *P. vulgaris* lectin, particularly PHA-L4, was shown to exhibit protective activity against DCV infection in *Drosophila* by augmenting the resistance of the host.

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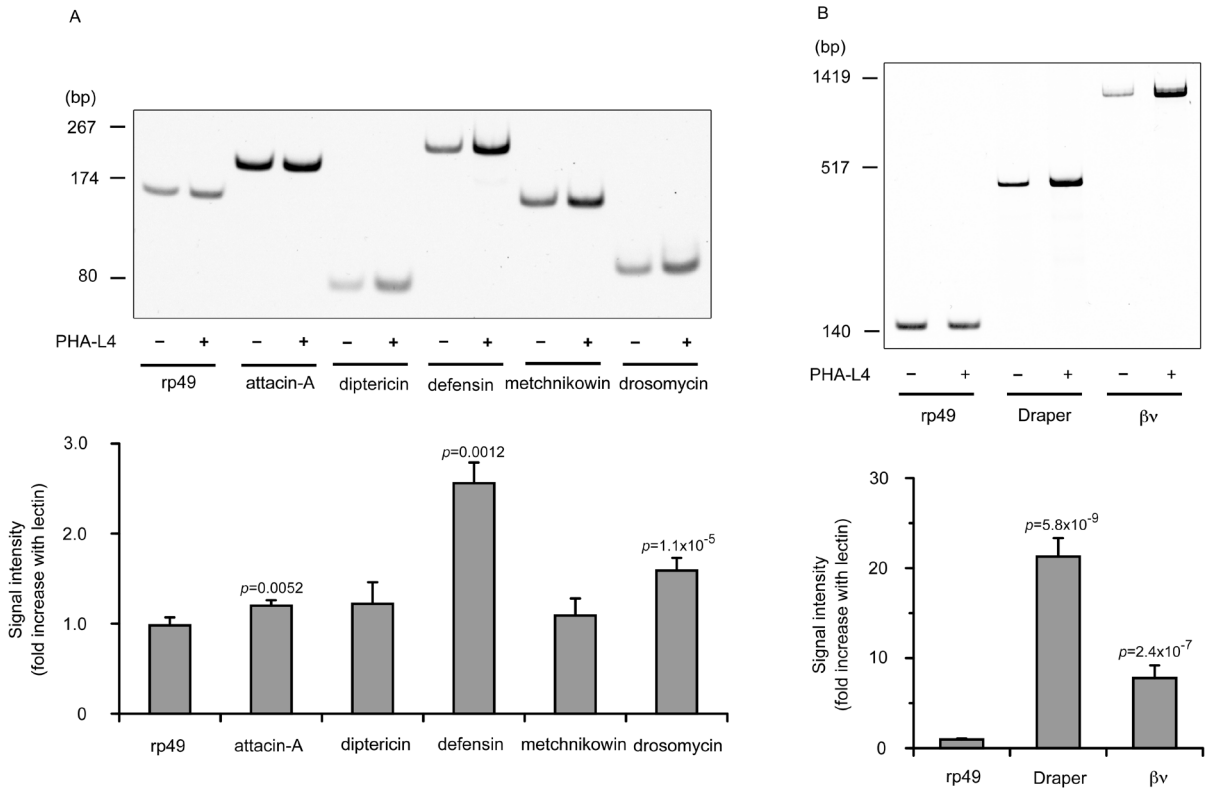

Figure 6. Effects of PHA-L4 on the levels of the mRNAs of antimicrobial peptides and phagocytosis receptors. Adult flies were fed PHA-L4 (6 μg/mL) or water alone for 19 h. Total RNA was extracted and analyzed by reverse transcription-mediated PCR for the mRNAs of the indicated antimicrobial peptides **(A)** and phagocytosis receptors **(B)**, together with that of rp49 as an unchanged internal control. Products in PCR were separated on a 6% polyacrylamide gel and visualized by the staining with ethidium bromide, and the intensities of the signals were digitized. Shown at the top are examples of ethidium bromide-stained gels with the positions and sizes, in base pairs (bp), of standard DNAs on the left. The intensities of the signals in four independent experiments were determined and shown relative to those obtained with RNA of water-fed flies, taken as 1, as means ± SD at the bottom. βν, integrin βν.

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