Dissertation

Study on the protective effect of *Phaseolus vulgaris* lectin against virus infection

Laboratory of Host Defense and Responses
Division of Pharmaceutical Sciences
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Submission Date : January 2018

ACKNOWLEDGEMENTS

Alhamdulillahi rabbil ‘aalamiin… All praises for Allah… the Lord of universe… Allah gives a strength on the completion of this thesis. He gives me a beautiful chance to meet so many wonderful people in this world…There is no a single thing occurred coincidently…Thank YOU…

First of all, I would like to express my gratitude to Professor Yoshinobu Nakanishi, my principal research supervisor. From the beginning in December 2015, I joined in his laboratory, he guide me, step by step, reached my hand and lead me until now. He is not only a brilliant scientist to admire on, but also a friendly person in daily life. In his very limited time, he always spare his time for a discussion. I always remember, in our discussion, he just spend 30 minutes, the longest times and he amazed me by all of his ideas and suggestions. He will always be a part of me for the rest of my life.

My grateful thanks to Assoc. Prof. Akiko Shiratsuchi for her kind help for my research. For me, she is a good lecture, like a mother, gives her shoulder and calm me, like a friend, I could talk to. Thank you Sensei. The next person, I would like to offer my sincere thanks is Assist. Prof. Saori Nonaka. She is very kind person, indeed. While I have to do overnight experiment for almost 6 months, she’s the one who always close the Minano hea for me, usually around 1 am and often said in the next day: Heny san, do you perform overnight experiment? Taihen ne…Thank you Nonaka Sensei, one of the beautiful woman in our laboratory.

My special thanks to Ms. Junko Arai and Dr. Teizo Murata of J-Oil Mills, Inc. for a gift of lectin and advice, also to Mr. Takumi Matsuda of Kanazawa University for his help in the analysis of digestive tracts of *Drosophila*.

For my senpai Firzan Nainu, Ph.D of Hasanuddin University: I almost lose my faith, until we met in the Asahimachi bus stop, and you say: My Sensei will help you. Like a water in the

dessert, you make me alive and give me a chance to meet an awesome Sensei. Thank you for every help. You teach me everything, from what is western blot to how inject the flies. For Ayu Sekarani Damana Putri (Puput) of Tadulako University…. Thank you for your precious PHA-P lectin data. Really, I surprise, finally…I am the one who continue your experiment. One day you told me you have to stay overnight and soon, and in the end you said : I've got a good data. Good luck for your activity...Thank you.

Lots of lab mate have provided their help for me. Yumiko Tanaka, a pretty woman, thank you for cell culturing training. For Takumi Matsuda, hey…please be healthy person. I always count on you every time, if there is no you, I have to do all by myself. For Chiharu Komiyama, a beautiful woman, thank you for become a friend until the middle of night… For Naoto Shimamoto…you are good teacher…I really don't know, how could I present my graphs without your help…Thank you. For other lab member : Kuraishi Sensei, Dini Rahmatika, Koichiro Kawamura, Yuto Kido, Kazuki Fukushima, Min Zhang, Nozoe Ami, Yoshitaka Furuta, Chiharu Hoshi, Yuta Amagasa, Mai Sono, Mayo Kawabata, Miho Mimatsu, Natsumi Yamada, Emil Salim, Aki Hori, Kato Horoyuki, thank you for your help during I stay in our lab. My best friends in CRI…Dewi Yuliana…You always there for me, a shoulder to cry on… I love you mba…really I do… d Ketut Gunarta, the one who help me "escape" from a death destiny… Terima kasih ya d…

My grateful to Indonesia government, the ministry of Research, Technology and Higher Education, for financial support via BPLLN DIKTI program.

My last grateful thanks are to my father (the deceased), my mother, my mother in law… I love you… For my husband, Ponco Iswanto, you are my everything. For my precious: Ahmad Farruq, Hasna Khairunnisa, and Muhammad Farras. Your mom get a Ph.D now. Hore.. For my sisters: Harty Khristina, Hesty Diana, Hervina Dewi… I love you all… Thank you for everything…

Then, which of the blessings of your Lord will you deny? (QS. Ar-Rahman)

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SUMMARY

Phytohemagglutinin (PHA) isolated from the family of *Phaseolus vulgaris* beans, which is composed of a tetramer of subunits E and L, is a promising agent against viral infection. However, this has not yet been demonstrated in vivo. I investigated this issue using the fruit fly *Drosophila melanogaster* as a host. Adult flies were pre-fed lectin 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. I 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, there is 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. It is conclude that orally administered *Phaseolus vulgaris* lectin protects *Drosophila* against viral infection by augmenting the level of host immunity.

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 as either 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.

Legume lectins are classified into two category: (i) mannose/glucose-specific group, such as concanavalin A and pea lectin; and (ii) galactose/*N*-acetylgalactosamine-specific group, such as peanut lectin and soybean agglutinin. An exception is a group of lectins called phytohemagglutinin (PHA) isolated from the seeds of red kidney bean *Phaseolus vulgaris*. PHA is classified as a "complex type-specific" group, since they show preference for complex type *N*-glycan, not for monosaccharide (1).

PHA is a tetramer of two subunits called E and L, and its tertiary structure is known as a "jelly roll" fold (Figure 1). In contrast with other legume lectins, short α-helices are usually found in the structure of PHA (1). Both E and L subunits contain one carbohydrate-binding site and two metal ion-binding sites for calcium and manganese ions, and the carbohydrate-binding sites of PHA are located mostly in the β folds of a 7-chain sheet (3). There are five different tetramer structures, i.e., E4 (PHA-E4), E3L1, E2L2, E1L3 and L4 (PHA-L4) (4). All these *P. vulgaris* isolectins 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 (5). There are additional isolectins of PHA, namely PHA-P and PHA-M,

which are intermediate forms composed of both L and E subunits. PHA-E4 binds di-galactosylated and bisected *N*-glican. The binding specificity of PHA-L4 is different from that of PHA-E4 although PHA-L4 possesses 70% identity in amino-acid sequences with PHA-E4. The minimal structural unit for high-affinity binding of PHA-L4 is the penta-saccharide unit Gal(β1-4)GlcNac(β1-2)[Gal(β1-4)GlcNAc(β1-6)Man, which is found in tetra- and tri-antennary complex-oligosaccharide of mammalian origin. For some of the legume lectin family, 11 members including PHA-L4 (6) and PHA-E4 (1) crystallographic co-ordinates have been deposited at the protein data bank.

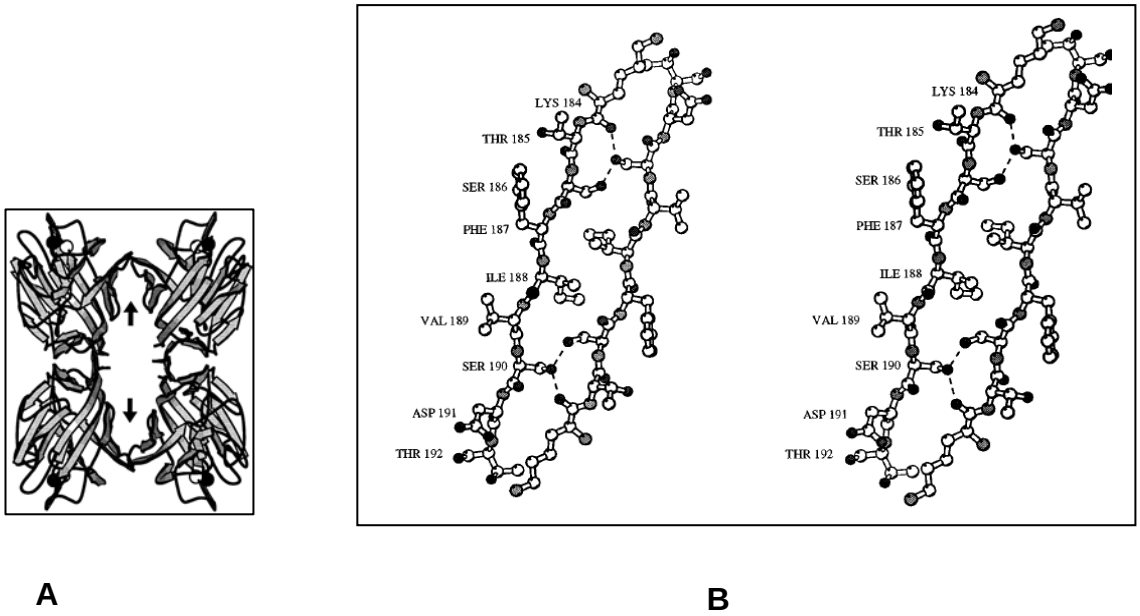


Figure 1. Structure of PHA-L4 (**A**) Two canonical dimers of PHA-L4 are shown. The arrows denote the same orientation of dimer. (**B**) A stereo-view of the two strands that form a two-fold symmetrical interface between the two canonical dimers in PHA-L4 is shown (7).

P. vulgaris PHA has 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 (4,8). However, the antiviral activity of *P. vulgaris* PHA has not yet been demonstrated in vivo. Investigation using animal model will contribute to further understanding the role of PHA in host defense against viral infection. Ms. Ayu Sekarani Damana Putri, who then stayed at the Laboratory of Host Defense and Responses under the program of Kanazawa University Student

Exchange for Science and Technology, found that the oral administration with PHA-P extended the survival period of *Drosophila melanogaster*, adult flies, after the abdominal infection with DCV (Figure 2).

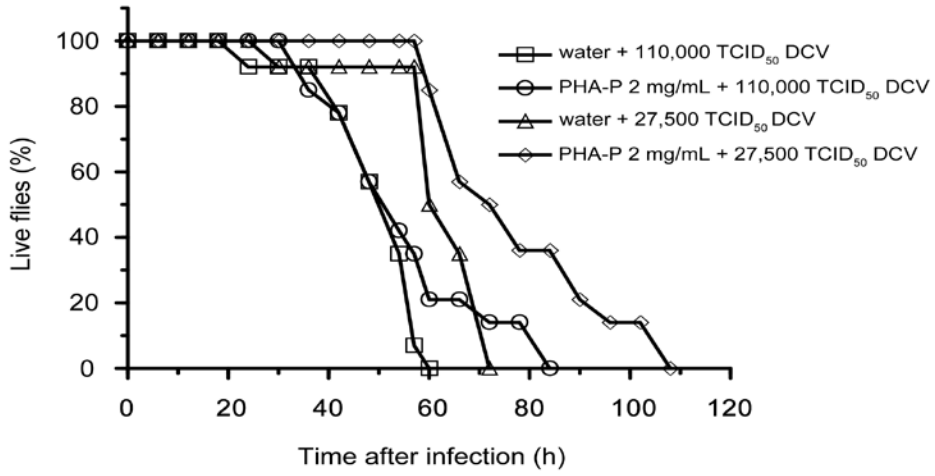


Figure 2. Antiviral effect of PHA-P on the survival of flies infected with DCV at two different doses. An assay for survivorship was conducted with flies infected with DCV at 27,500 and 110,000 $TCID_{50}$ after administration with 2 mg/mL PHA-P lectin or water alone (unpublished data of Damana Putri, 2015).

The fruit fly *Drosophila melanogaster* has been widely used in research to elucidate the underlying mechanisms of self-defense systems (9,10,11) as well as the causes of diseases (12). 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 a 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 (13,14). Furthermore, *Drosophila* has been used in the screening of candidate drugs against intractable human diseases (15), and similarities have been identified in antiviral mechanisms between humans and *Drosophila* (16-19).

I decided to take over Ms. Ayu Sekarani Damana Putri project to elucidate whether *P. vulgaris* lectin exhibits antiviral activity in vivo. I will use *Drosophila melanogaster* as a host for viral infection.

MATERIALS AND METHODS

Fly stocks, cell culture, and lectin

Fly line w^{1118}, a common white-eye laboratory stock of *Drosophila* provided by Bloomington Drosophila Stock Center (Indiana University, Bloomington, Indiana, USA), was raised with a standard laboratory cornmeal-agar medium at 25°C. Schneider's *Drosophila* line 2 (S2) cell, a *Drosophila* embryonic cell-derived cell line, was used as host cells for the determination of virus titer. S2 cells were cultured at 25°C with Schneider's *Drosophila* medium (Life Technologies Japan, Tokyo) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

All lectins used in this study were provided by J-Oil Mills, Inc. (Yokohama, Japan). *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 (5) with slight modifications. A mixture of L4, L3E1, and L2E2, which we operationally named PHA-P (J-Oil Mills, Inc. code number J113), and purified L4 (PHA-L4) (J-Oil Mills, Inc. code number J112) were individually tested for their antiviral activities. The amount of proteins was measured by the Bradford method using 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, Inc. code numbers J512 and J511, respectively, were used to locate lectin in the digestive tracts of flies.

Preparation and titration of *Drosophila* C virus

Drosophila C virus (DCV), a natural pathogen for *Drosophila*, was used to infect culture cell lines and adult flies. To prepare a stock of DCV, semi-confluent S2 cells were incubated with an aliquot of original DCV stock at 25°C, and the culture media were collected at day 7. A 50%

tissue culture-infective dose ($TCID_{50}$) of the harvested culture media was determined by the end-point dilution assay. In brief, S2 cells (2.5×10^4) were seeded in wells of 96-well culture dishes with 150 μl medium and incubated overnight. On the next day, they were inoculated with the harvested DCV-containing culture medium at 10-fold serial dilutions (50 μl/well), 8 wells for each dilution, and further incubated at 25°C for 7 days. All wells were assessed for the presence of cell debris and loss of cell confluency, indicative of viral infection, and a virus titer was determined according to an established procedure (20). The culture medium gave a DCV concentration of 5×10^{10} $TCID_{50}$/ml and was kept frozen at –80°C as a stock until use.

Lectin feeding, virus infection in flies, and determination of virus titer

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 water containing or not containing *P. vulgaris* lectin 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 12-24 h for recovery from starvation before the abdominal infection with DCV.

Infection of adult flies with DCV was carried out according to an established procedure (21) with slight modifications. Briefly, male flies, with and without lectin feeding, were anesthetized with CO_2 and abdominally injected with ~50 nl of virus suspension (27,500 or 2,750 $TCID_{50}$) using a glass needle with the aid of a nitrogen gas-operated micro-injector. These flies were maintained at 25°C until examination. We considered fly death within the first 2 h of injection to be due to injury with a needle and excluded those flies from the analyses.

To determine the growth of DCV in flies, lysates of whole flies were subjected to an assay for virus titer. For preparing whole fly lysates, five live flies were randomly chosen and homogenized with 100 μl of *Drosophila* lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 150

mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, and 0.1% (v/v) NP-40) using Treff tubes and plastic pestles. The homogenates were centrifuged at 14,000 rpm for 10 min, and supernatants were collected and diluted by 10–10^{13} for the determination of a virus titer.

Messenger RNA analysis of antimicrobial peptides and engulfment receptors

Males of *Drosophila* adults (approximately 15 flies), with and without lectin feeding, were suspended in TRIzol reagent (Invitrogen) in a Treff tube 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 by PCR using primers specific to attacin-A, defensin, diptericin, drosomycin, and metchnikowin. The levels of the mRNAs of Draper and integrin αPS3-βν, engulfment receptors responsible for the phagocytic removal of apoptotic cells in *Drosophila* (22), were similarly determined. A sequence corresponding to the mRNA of the ribosomal protein rp49 was also amplified as an unchanged internal control. I first conducted PCR using varying doses of cDNA and determined a quantifiable range of cDNA amounts for each primer. At the same time, the ratio of two cDNA preparations obtained from RNA of flies fed lectin or water that give a similar level of the signal derived from rp49 mRNA was determined. Finally, PCR amplification for antimicrobial peptides and engulfment receptors was carried out using two cDNA preparations at that ratio. Amplified DNA was 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 PCR primers were: 5'–CCCGGAGTGAAGGATG–3' (forward) and 5'–GTTGCTGTGCGTCAAG–3' (reverse) for the mRNA of attacin-A (23); 5'–GTTCTTCGTTCTCGTGG–3' (forward) and 5'–CTTTGAACCCCTTGGC–3' (reverse) for the mRNA of defensin (23); 5'–

GCTGCGCAATCGCTTCTACT–3’ (forward) and 5’–TGGTGGAGTGGGCTTCATG–3’ (reverse) for the mRNA of diptericin (23); 5’–CGTGAGAACCTTTTCCAATATGATG–3’ (forward) and 5’–TCCCAGGACCACCAGCAT–3’ (reverse) for the mRNA of drosomycin (23); 5’–AACTTAATCTTGGAGCGA–3’ (forward) and 5’–CGGTCTTGGTTGGTTAG–3’ (reverse) for the mRNA of metchnikowin (23); 5’–CGGAATTCTCTGCCGCACGGGTTACATAG–3’ (forward) and 5’–CCGCTCGAGCCGGCTCGAATTTTCGCTT–3’ (reverse) for the mRNA of Draper (24); 5’–AGATACCTACTCCTGGGCTT–3’ (forward) and 5’–TCCGCATTTGGAGCTCCAAT–3’ (reverse) for the mRNA of integrin αPS3 (24); 5’–AAGCCAACTCTACCCATGATT–3’ (forward) and 5’–GTGGGACAGTTGCAATAGGT–3’ (reverse) for the mRNA of integrin βν (24); and 5’–GACGCTTCAAGGGACAGTATCTG–3’ (forward) and 5’–AAACGCGGTTCTGCATGAG–3’ (reverse) for the mRNA of rp49 (23).

Data processing and statistical analysis

Unless otherwise stated, data obtained from three independent experiments were analyzed and expressed as means ± standard deviations. Number of replication in each experiment is specified in the corresponding figure legends. In an assay for fly survival, a single-vial experiment was first conducted, and the experiments were repeated at least three times to confirm reproducibility. Statistical analyses were performed by log-rank test (Kaplan-Meier method) for the data obtained in an assay for fly survival or two-tailed Student *t*-test for all other data. *p* values less than 0.05 were considered significant and are indicated in figures.

RESULTS

Preceding data showed that the oral administration with PHA-P extended the survival period of adult flies after the abdominal infection with DCV. To determine a maximum period of lectin feeding, adult flies were starved for 3 h, fed water alone, and monitored for their death every 1 h. The death of first fly was observed at 19 h (data not shown) and, thus, decided the period of 19 h as a feeding period. The effect of PHA-P on the survival of flies that were infected with DCV at two different viral loads was examined (Figure 2).

Confirmation of the preceding data on the antiviral actions of PHA-P

I decided to take over this project and began my doctoral study by confirming Damana Purtri's preliminary data. Through this study, chromatographically purified *P. vulgaris* lectins were used (Figure 3).

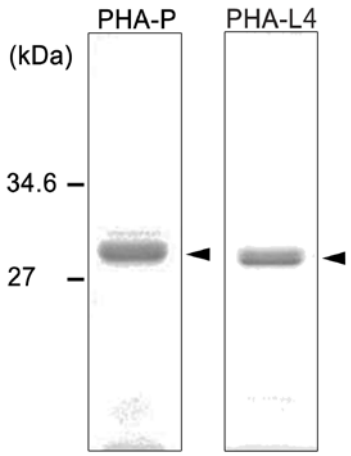


Figure 3. Purity of *P. vulgaris* PHA used in this study. Chromatographically prepared PHA-P (0.5 μg) and PHA-L4 (0.5 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis, and visualized by staining with Coomassie Brilliant Blue. The arrowheads point to the positions of lectin, and the positions and molecular masses of standard proteins are shown on the left. Subunits E and L possess a similar molecular mass of about 28 kDa.

Based on the preceding data, I set up the initial conditions where the antiviral effects of *P. vulgaris* lectins were examined, as the followings. I also determined the criteria that ensure the proper implement of viral infection: flies start to die at 24 h or longer and all die by 60 h after infection.

1. Starvation period : 3 h
2. Lectin concentration : 2 mg/ml
3. Time for lectin feeding : 19 h
4. Time for recovery from starvation : 24 h
5. Dose of DCV for infection : 27, 500 $TCID_{50}$

Under these conditions, I carried out 3 consecutive experiments to determine the ratio of survived flies after viral infection. In the 3rd experiment, flies were injected with phosphate-buffered saline (PBS) alone, a buffer with which DCV was suspended, and no killing effect was observed. The data shown as Table 1 and Figure 4 indicated that viral infection in all 3 experiments met the above-described criteria. The pre-administration with PHA-P reproducibly brought about an extension of fly survival. When the data obtained with flies that had been fed lectin and water alone were statistically analyzed using the log-rank test, there observed a significant difference between the two fly groups in all 3 experiments. Therefore, I successfully demonstrated the longer survival of DCV-infected flies by pre-administration with PHA-P, as did Damana Putri, and concluded that this lectin possesses an activity to protect *Drosophila* against infection with DCV.

Table 1. Determination of the ratio of survived flies after infection with DCV or water

Hour elapsed	1st Experiment				2nd Experiment				3rd Experiment					
	PHA-P + DCV (p = 0.040)		Water + DCV		PHA-P + DCV (p = 0.029)		Water + DCV		PHA-P + DCV (p = 0.0080)		Water + DCV		Water + PBS	
	∑	%	∑	%	∑	%			∑	%	∑	%	∑	%
0	17/17	100	18/18	100	21/21	100	21/21	100	26/26	100	24/24	100	23/23	100
6	17/17	100	18/18	100	21/21	100	21/21	100	26/26	100	24/24	100	23/23	100
12	17/17	100	18/18	100	21/21	100	21/21	100	26/26	100	24/24	100	23/23	100
18	17/17	100	18/18	100	21/21	100	21/21	100	26/26	100	24/24	100	23/23	100
24	17/17	100	18/18	100	20/21	95	19/21	90	26/26	100	24/24	100	23/23	100
30	17/17	100	18/18	100	20/21	95	17/21	80	26/26	100	24/24	100	23/23	100
36	16/17	94	17/18	94	19/21	90	16/21	76	26/26	100	24/24	100	23/23	100
42	16/17	94	17/18	94	18/21	85	13/21	61	26/26	100	24/24	100	23/23	100
48	15/17	88	16/18	88	15/21	71	11/21	52	26/26	100	23/24	95	23/23	100
54	12/17	70	7/18	38	10/21	47	5/21	23	26/26	100	18/24	75	23/23	100
60	8/17	47	4/18	22	3/21	14	0/21	0	20/26	76	12/24	50	23/23	100
66	5/17	29	2/18	11	1/21	4			15/26	57	7/24	29	23/23	100
72	3/17	11	0/18	0	0/21	0			8/26	30	3/24	12	23/23	100
78	1/17	5							4/26	15	0/24	0	23/23	100
84	0/17	0							0/26	0			23/23	100

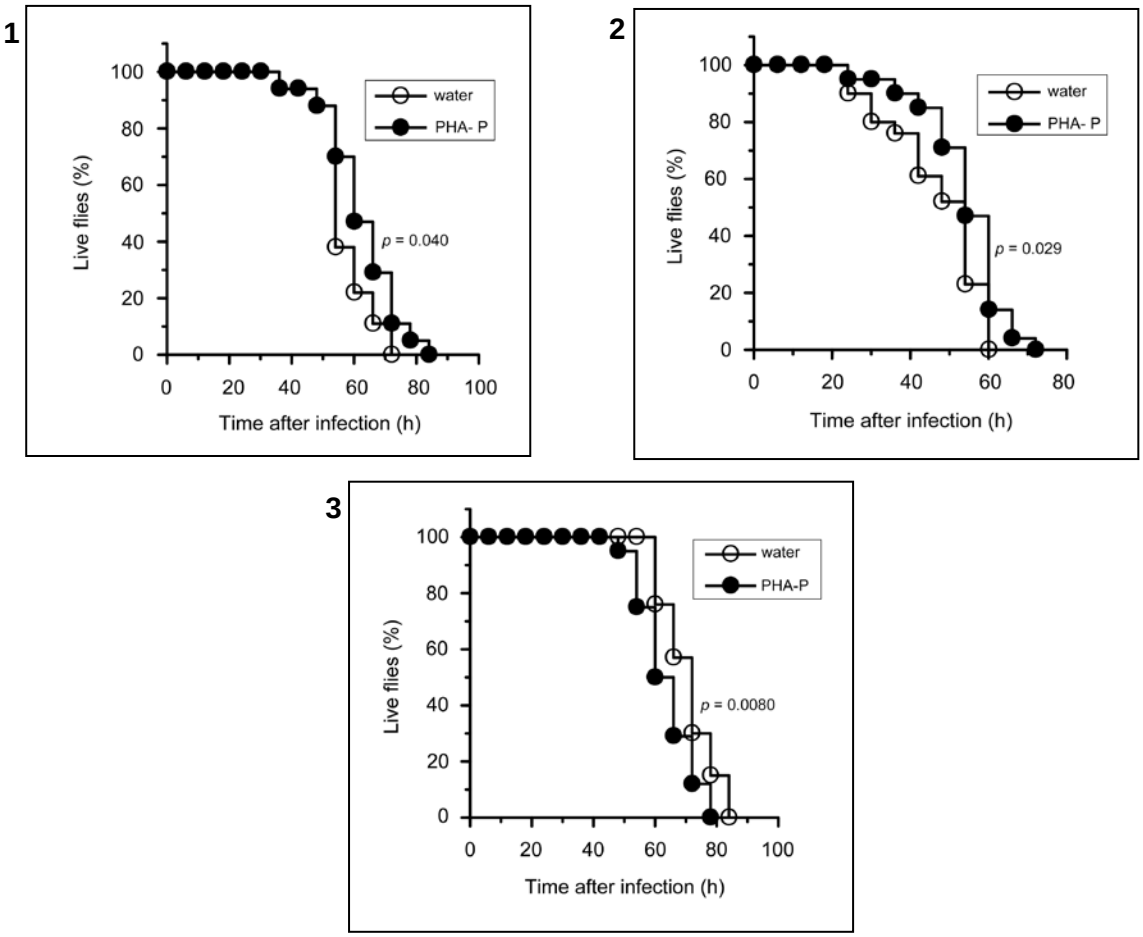


Figure 4. **Antiviral effect of PHA-P.** Flies were administered with PHA-P or water alone as a negative control, followed by the infection with DCV. The ratio of live flies at the indicated time points in 3 independent experiments are shown. The data of lectin- and water-fed flies were statistically analyzed using the log-rank test.

Preparation-to-preparation variations of PHA-P in antiviral activities

After the first preparation of PHA-P was used up, I obtained another preparation from J-Oil Mills, Inc. and tested for its activity. However, this preparation of PHA-P did not significantly increase the survival of DCV-infected flies (Table 2 and Figure 5). To examine a possible impurity of this preparation, I analyzed it by SDS-polyacrylamide gel electrophoresis. However, a dye-stained pattern of PHA-P did not show a difference between previous and this preparations (data not shown).

Table 2. Effect of new preparation of PHA-P on survival of DCV-infected flies

Hour elapsed	PHA-P + DCV (p = 0.46)		Water + DCV		Water + PBS	
	∑	%	∑	%	∑	%
0	23/24	100	24/25	100	23/23	100
6	23/23	100	24/24	100	23/23	100
12	23/23	100	24/24	100	23/23	100
18	23/23	100	24/24	100	23/23	100
24	23/23	100	24/24	100	23/23	100
30	23/23	100	24/24	100	23/23	100
36	23/23	100	24/24	100	23/23	100
42	23/23	100	24/24	100	23/23	100
48	20/23	86	22/24	91	23/23	100
54	19/23	82	20/24	83	23/23	100
60	15/23	65	14/24	58	23/23	100
66	7/23	30	7/24	29	23/23	100
72	4/23	17	2/24	8	23/23	100
78	1/23	4	0/24	0	23/23	100
84	0/23	0			23/23	100

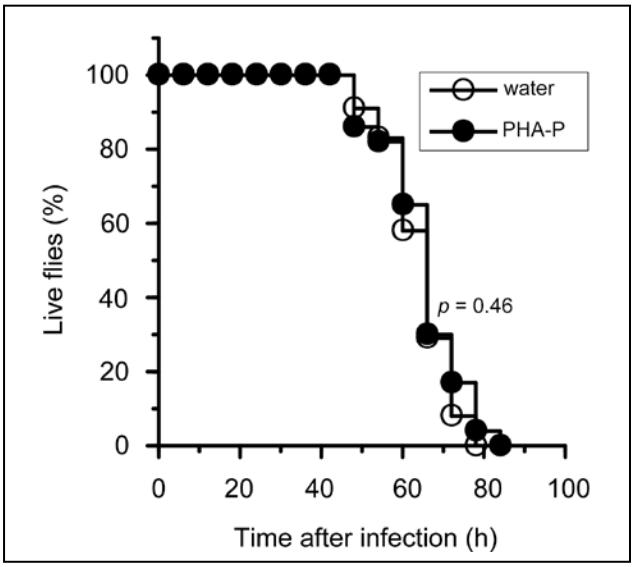


Figure 5. Effect of new preparation of PHA-P on survival of DCV-infected flies. Flies were administered with PHA-P or water alone, followed by the infection with DCV. The ratio of live flies at the indicated time points is shown. The data of lectin- and water-fed flies were statistically analyzed using the log-rank test.

During an assay for fly survival using new preparation of PHA-P, I noticed a lowered motility of flies that had been fed lectin. This could be caused by a contaminant in this lectin preparation. In addition, I was notified that protein determination of lectin at J-Oil Mills, Inc. is done based on optical density at 280 nm. With such information, I decided to determine a protein concentration by the Bradford method and conduct a dose-response experiment, anticipating concentrations below than 2 mg/ml exhibit antiviral activities.

Re-establishment of basal experimental conditions for the examination of antiviral activity of PHA-P

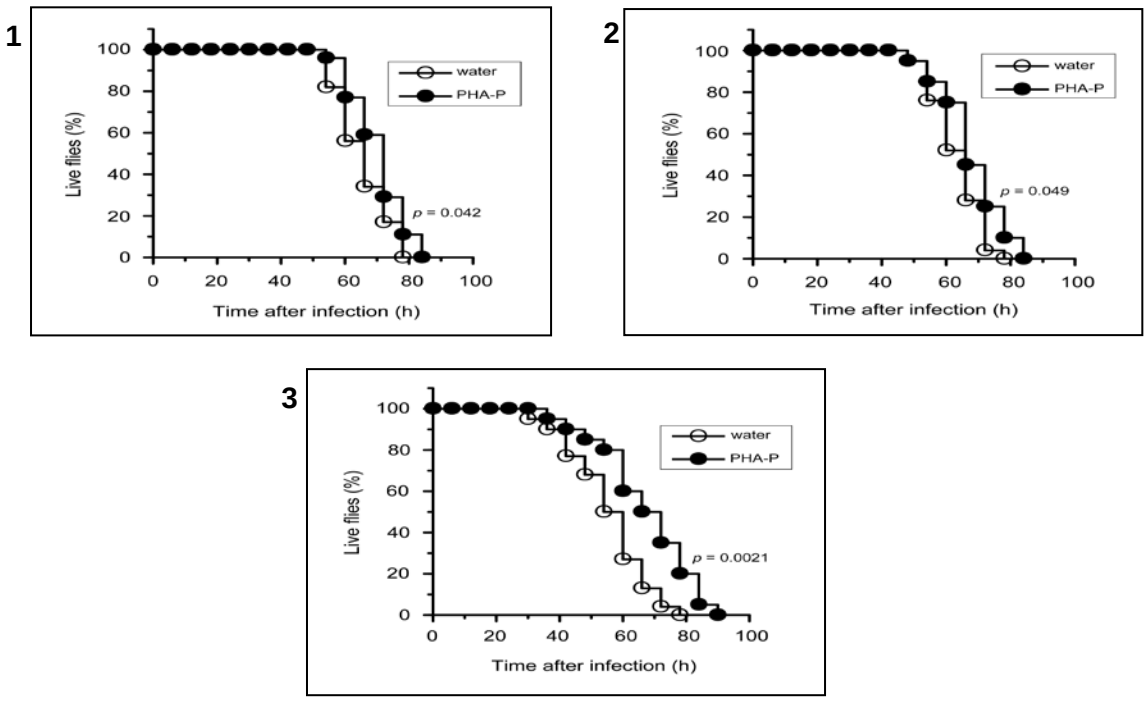
Protein concentrations of two PHA-P preparations, which were dissolved in water at 2 mg/ml based on the optical densitometry data from J-Oil Mills, Inc., were determined by the Bradford method. The results gave the concentrations of 0.48 mg/ml and 0.60 mg/ml for old and new preparations, respectively, suggesting the presence of some contaminations in both preparations. I next conducted a dose-responding experiment using new preparation of PHA-P in

a range of concentrations lower than those used in previous experiments. I found a marginal antiviral activity at 0.06 and 0.12 mg/ml, and lectin at concentrations lower and higher than them was inactive (Table 3, and Figures 6 and 7).

Table 3. Ratio of survival DCV-infected flies pre-administered with new preparation of PHA-P at various concentrations. Flies were infected with DCV at 27,500 $TCID_{50}$. The data were analyzed by the log-rank test.

Hours elapsed	GROUPS											
	I 0.60 mg/ml PHA-P +DCV (p = 0.10)		II 0.30 mg/ml PHA-P +DCV (p = 0.56)		III 0.12 mg/ml PHA-P +DCV (p = 0.044)		IV 0.06 mg/ml PHA-P +DCV (p = 0.042)		V 0.03 mg/ml PHA-P +DCV (p = 0.36)		VI Water + DCV	
	Σ	%	Σ	%	Σ	%	Σ	%	Σ	%	Σ	%
0	22/22	100	25/25	100	25/25	100	27/27	100	22/22	100	23/23	100
6	22/22	100	25/25	100	25/25	100	27/27	100	22/22	100	23/23	100
12	22/22	100	25/25	100	25/25	100	27/27	100	22/22	100	23/23	100
18	22/22	100	25/25	100	25/25	100	27/27	100	22/22	100	23/23	100
24	22/22	100	25/25	100	25/25	100	27/27	100	22/22	100	23/23	100
30	22/22	100	25/25	100	25/25	100	27/27	100	22/22	100	23/23	100
36	21/22	95	25/25	100	25/25	100	27/27	100	21/22	95	23/23	100
42	21/22	95	25/25	100	25/25	100	27/27	100	21/22	95	23/23	100
48	21/22	95	25/25	100	25/25	100	27/27	100	21/22	95	23/23	100
54	20/22	90	23/25	92	23/25	92	26/27	96	19/22	86	19/23	82
60	18/22	81	18/25	72	19/25	76	21/27	77	15/22	68	13/23	56
66	11/22	50	11/25	44	15/25	60	16/27	59	10/22	45	8/23	34
72	6/22	27	4/25	16	8/25	32	8/27	29	5/22	22	4/23	17
78	2/22	9	0/25	0	3/25	12	3/27	11	1/22	4	0/23	0
84	0/22	0			0/25	0	0/27	0	0/22	0		

A PHA-P at 60 μg/mL



B PHA-P at 120 μg/mL

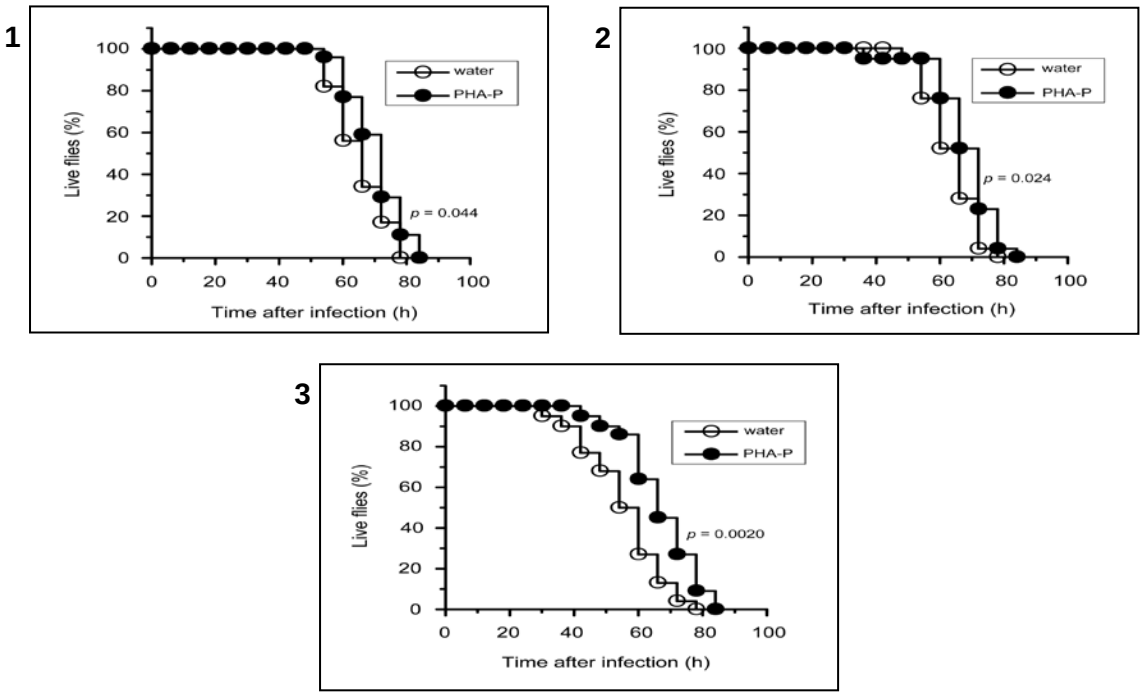


Figure 6. Effect of PHA-P on survival of DCV-infected flies. Flies were administered with PHA-P at 60 μg/mL (**A**) and 120 μg/mL (**B**) or water, followed by the infection with DCV. The ratio of live flies at the indicated time points in 3 independent experiments is shown. The data of lectin- and water-fed flies were statistically analyzed using the log-rank test.

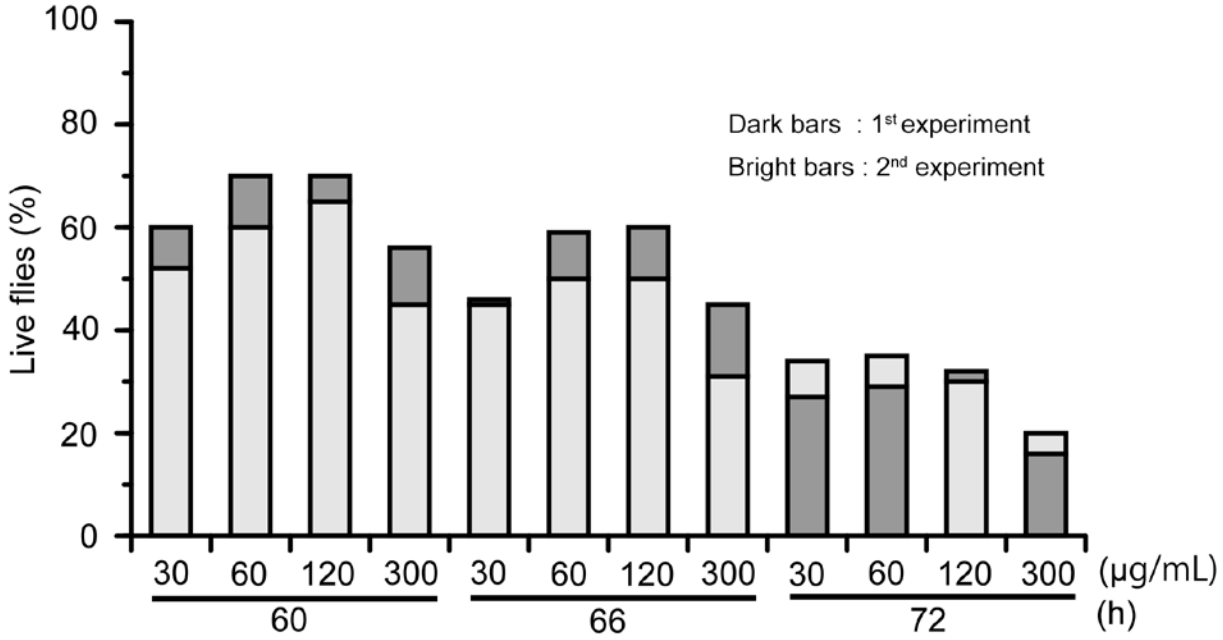


Figure 7. Antiviral effect of new preparation of PHA-P at various concentrations. Flies that had been administered with PHA-P at the indicated concentrations were infected with DCV at 27,500 $TCID_{50}$. The ratio of live flies at the indicated time points is shown.

Aiming at establishing optimal experimental conditions to examine the antiviral actions of PHA-P, I evaluated the period for the recovery from starvation, which was not done previously. I conducted an assay for fly survivorship with recovery periods of 0, 12, and 24 h. In this experiment, flies were pre-administered with new preparation of PHA-P at 0.06 mg/ml. The data indicated that the antiviral actions of lectin were only observed in the experiment with a recovery time of 12 h (Tables 4-6 and Figures 8-10). I therefore, re-set the period of 12 h for the recovery of flies from starvation.

Table 4. Fly survival with recovery period of 0 h

Hours elapsed	Groups			
	I (PHA-P 0.06 mg/ml + DCV)		II (Water +DCV)	
	Fly number (live/total)	% survival	Fly number (live/total)	% survival
0	23/23	100	21/21	100
6	23/23	100	21/21	100
12	22/23	95	21/21	100
18	22/23	95	21/21	100
24	21/23	91	19/21	90
30	20/23	86	17/21	80
36	19/23	82	15/21	71
42	19/23	82	15/21	71
48	17/23	73	14/21	66
54	16/23	69	11/21	52
60	15/23	65	10/21	47
66	13/23	56	9/21	42
72	9/23	39	5/21	23
78	8/23	34	5/21	23
84	6/23	26	2/21	9
90	3/23	13	0/21	0
96	1/23	4		
102	0/23	0		

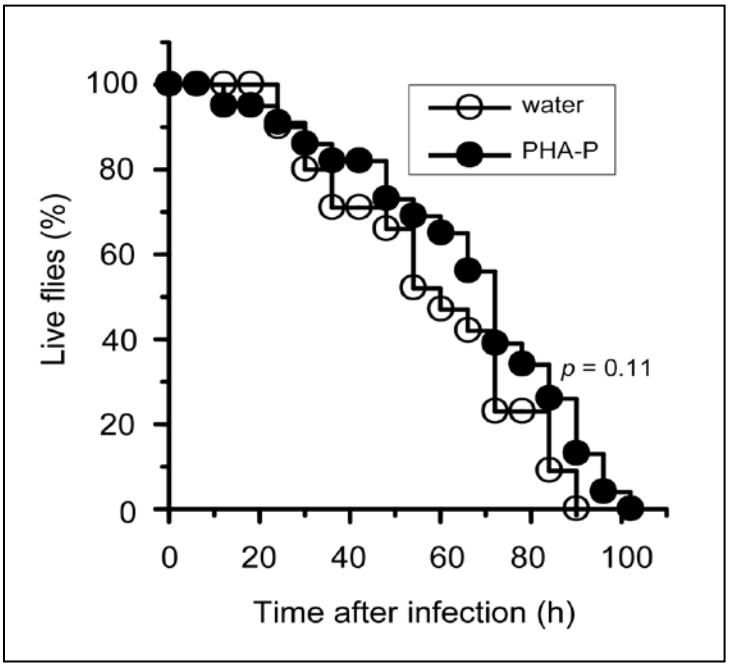


Figure 8. Fly survival with recovery period of 0 h. Flies were administered with PHA-P or water, followed by the infection with DCV. The ratio of live flies at the indicated time points is shown. The data of lectin- and water-fed flies were statistically analyzed using the log-rank test.

Table 5. Fly survival with recovery period of 12 h.

Hours elapsed	Groups			
	I (PHA-P 0.06 mg/ml + DCV)		II (Water +DCV)	
	Fly number (live/total)	% survival	Fly number (live/total)	% survival
0	25/25	100	24/24	
6	25/25	100	24/24	100
12	25/25	100	24/24	100
18	25/25	100	24/24	100
24	25/25	100	24/24	100
30	25/25	100	24/24	100
36	25/25	100	23/24	95
42	25/25	100	22/24	91
48	22/25	88	19/24	79
54	22/25	88	16/24	66
60	17/25	68	10/24	41
66	9/25	36	5/24	20
72	5/25	20	1//24	4
78	3/25	12	0/24	0
84	1/25	4		
90	0/25	0		

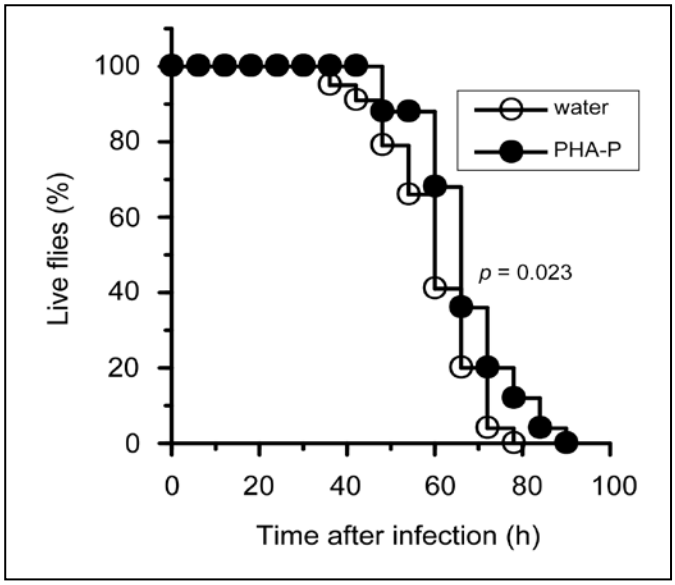


Figure 9. Fly survival with recovery period of 12 h. Flies were administered with PHA-P or water, followed by the infection with DCV. The ratio of live flies at the indicated time points is shown. The data of lectin- and water-fed flies were statistically analyzed using the log-rank test.

Table 6. **Fly survival with recovery period of 24 h.**

Hours elapsed	Groups			
	I (PHA-P 0.06 mg/ml + DCV)		II (Water +DCV)	
	Fly number (live/total)	% survival	Fly number (live/total)	% survival
0	25/25	100	25/25	100
6	25/25	100	25/25	100
12	25/25	100	25/25	100
18	25/25	100	25/25	100
24	25/25	100	25/25	100
30	25/25	100	25/25	100
36	25/25	100	25/25	100
42	25/25	100	24/25	96
48	25/25	100	23/25	92
54	21/25	84	19/25	76
60	17/25	68	14/25	56
66	9/25	36	6/25	24
72	5/25	20	3/25	12
78	3/25	12	1/25	4
84	1/25	4	0/25	0
90	0/25	0		

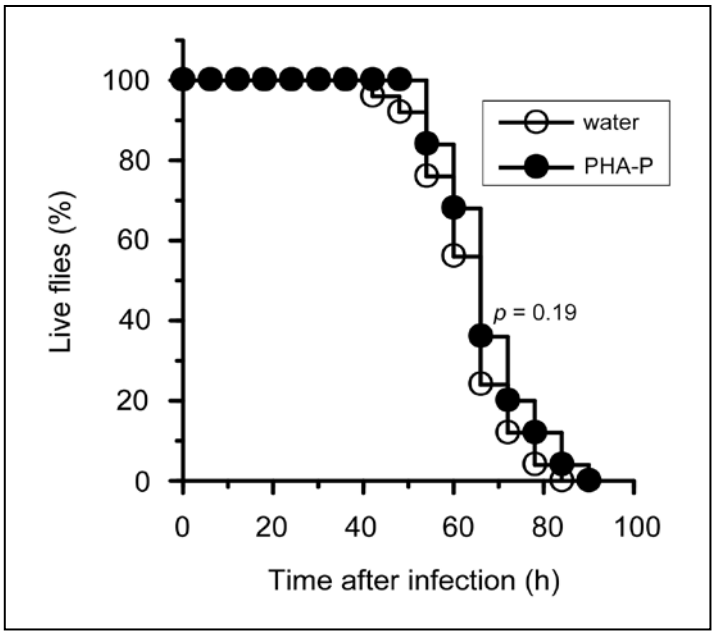


Figure 10. Fly survival with recovery period of 24 h. Flies were administered with PHA-P or water, followed by the infection with DCV. The ratio of live flies at the indicated time points is shown. The data of lectin- and water-fed flies were statistically analyzed using the log-rank test.

Examination of antiviral actions of PHA-L4 in *Drosophila* against DCV infection

The data obtained from experiments conducted so far suggested an antiviral activity of PHA-P, a mixture of isolectins with the subunit compositions of L4, L3E1, and L2E2 (4). Adult flies orally treated with PHA-P survived longer than water-fed control after fatal infection with DCV. However, the analysis of data using the log rank test revealed that a difference between PHA-P- and water-fed groups was marginally significant at certain doses of lectin. Previous studies by other investigators showed that subunit L is responsible for the mitogen actions of *P. vulgaris* lectin against lymphocytes (5). I, thus analyzed PHA-L4 for its antiviral actions.

I first examined if orally administration of lectin was taken up by the flies. For this purpose, flies were fed fluorescence-labeled lectin followed by the fluorescence microscopic analysis of digestive tracts dissected out from flies. I tested PHA-E4 and PHA-L4 for their entry to flies. The data showed that both isolectins were present in the midgut of digestive tracts (Figure 11), indicating a successful treatment of adult flies with *P. vulgaris* lectin under the procedures adopted in this study. I then conducted an assay for fly survival after infection with DCV. Anticipating a higher activity of PHA-L4 than PHA-P, a dose-responding analysis of PHA-L4 was done at concentrations in a range of 0.24 – 30 μg/mL, which is about 10 times lower than that examined for PHA-P. PHA-L4 was found to significantly extend survival periods of DCV-infected flies compared to flies treated with water alone as negative control (Table 7 and Figure 12). As observed in the experiments with PHA-P, there were concentrations of PHA-L4 for optimal antiviral activities. PHA-L4 significantly extended the periods of survival of DCV-infected flies at 1.2 and 6.0 μg/mL (Figure 13), which was 20 – 50 times lower than the effective concentration of PHA-P.

The data obtained above indicated that PHA-L4 protects *Drosophila* from infection with DCV. It is likely that subunit L is responsible for the antiviral actions of *P. vulgaris* lectin. The results from a dose-responding experiment suggested that lectin possesses effective concentration: it becomes less active at lower and higher concentration than them.

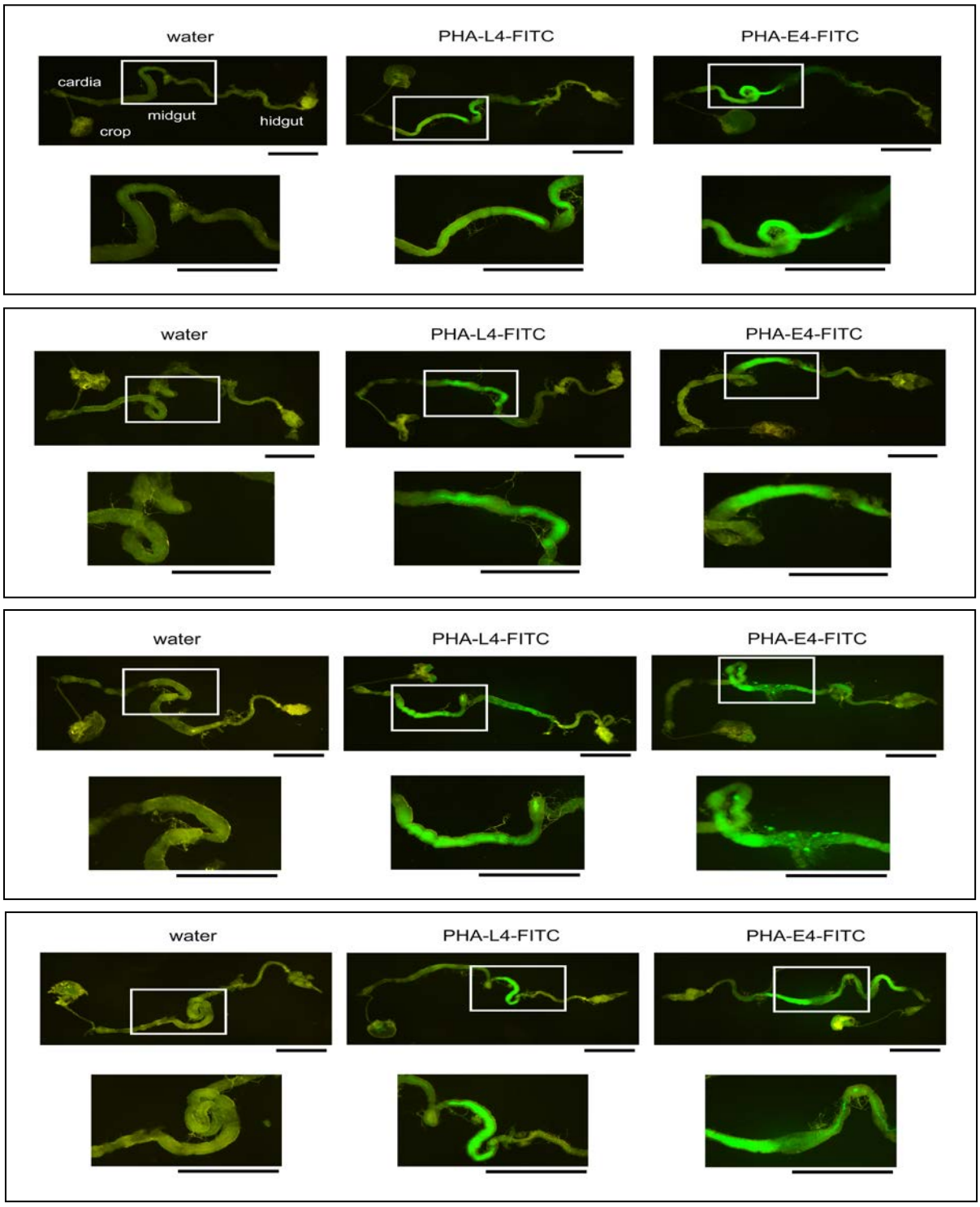
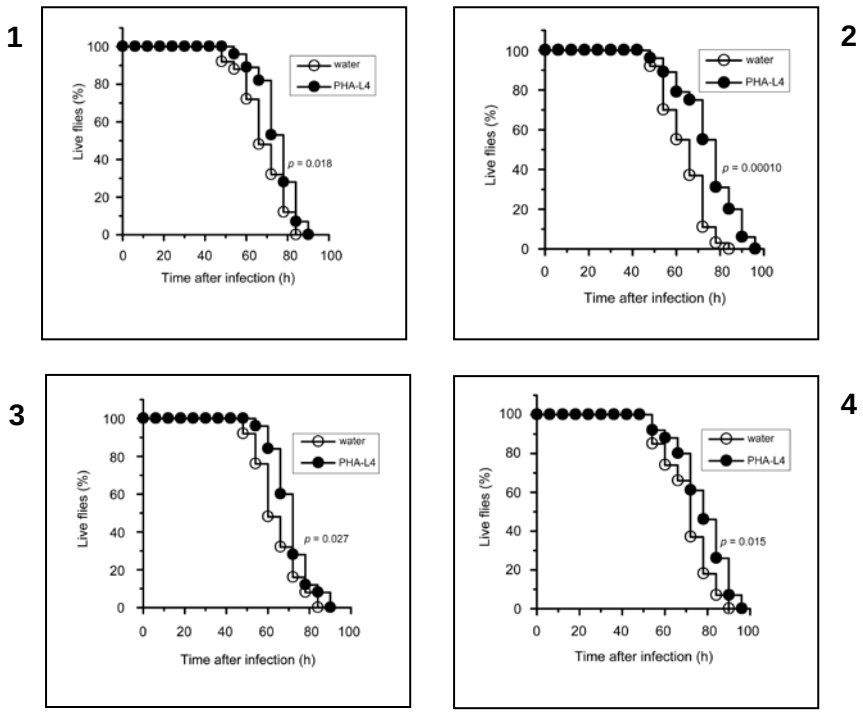


Figure 11. Existance of orally administered *P.vulgaris* lectin in the midgut of flies. Adult flies were fed FITC-labeled PHA-E4 (0.1 μg/ml), FITC-labeled PHA-L4 (0.1 μg/ml), or water for 2 h. Digestive tracts were dissected out and examined by fluorescence microscopy. The bottom panels in each set of the data are magnified views of the squared area in the upper panels. Fluorescence signals derived from FITC are shown in green. Data from 4 independent experiments are presented. Scale bars = 200 μm.

Table 7. Survival of DCV-infected flies pre-administered with PHA-L4 at various concentrations. Flies were infected with DCV at 27,500 $TCID_{50}$. The data were analyzed by the log-rank test.

	Groups									
	I (PHA-L 30 µg/ml +DCV) $p = 0.90$		II (PHA-L 6.0 µg/ml + DCV) $p = 0.000040$		III (PHA-L 1.2 µg/ml + DCV) $p = 0.018$		IV (PHA-L 0.24 µg/ml + DCV) $p = 0.73$		V (Water + DCV)	
	Fly number (live/total)	% survival	Fly number (live/total)	% survival	Fly number (live/total)	% survival	Fly number (live/total)	% survival	Fly number (live/total)	% survival
0	28/28	100	28/28	100	28/28	100	29/29	100	25/25	100
6	28/28	100	28/28	100	28/28	100	29/29	100	25/25	100
12	28/28	100	28/28	100	28/28	100	29/29	100	25/25	100
18	28/28	100	28/28	100	28/28	100	29/29	100	25/25	100
24	28/28	100	28/28	100	28/28	100	29/29	100	25/25	100
30	28/28	100	28/28	100	28/28	100	29/29	100	25/25	100
36	28/28	100	28/28	100	28/28	100	28/29	96	25/25	100
42	27/28	96	28/28	100	28/28	100	28/29	96	25/25	100
48	26/28	92	28/28	100	28/28	100	28/29	96	23/25	92
54	24/28	85	28/28	100	27/28	96	27/29	93	22/25	88
60	16/28	57	27/28	96	25/28	89	20/29	68	18/25	72
66	13/28	46	26/28	92	23/28	82	16/29	55	12/25	48
72	6/28	21	19/28	67	15/28	53	7/29	24	8/25	32
78	6/28	21	15/28	53	8/28	28	2/29	6	3/25	12
84	1/28	3	7/28	25	2/28	7	0/29	0	0/25	0
90	0/28	0	2/28	7	0/28	0				
96			0/28	0						

A PHA-L4 at 1.2 µg/mL



B PHA-L4 at 6.0 µg/mL

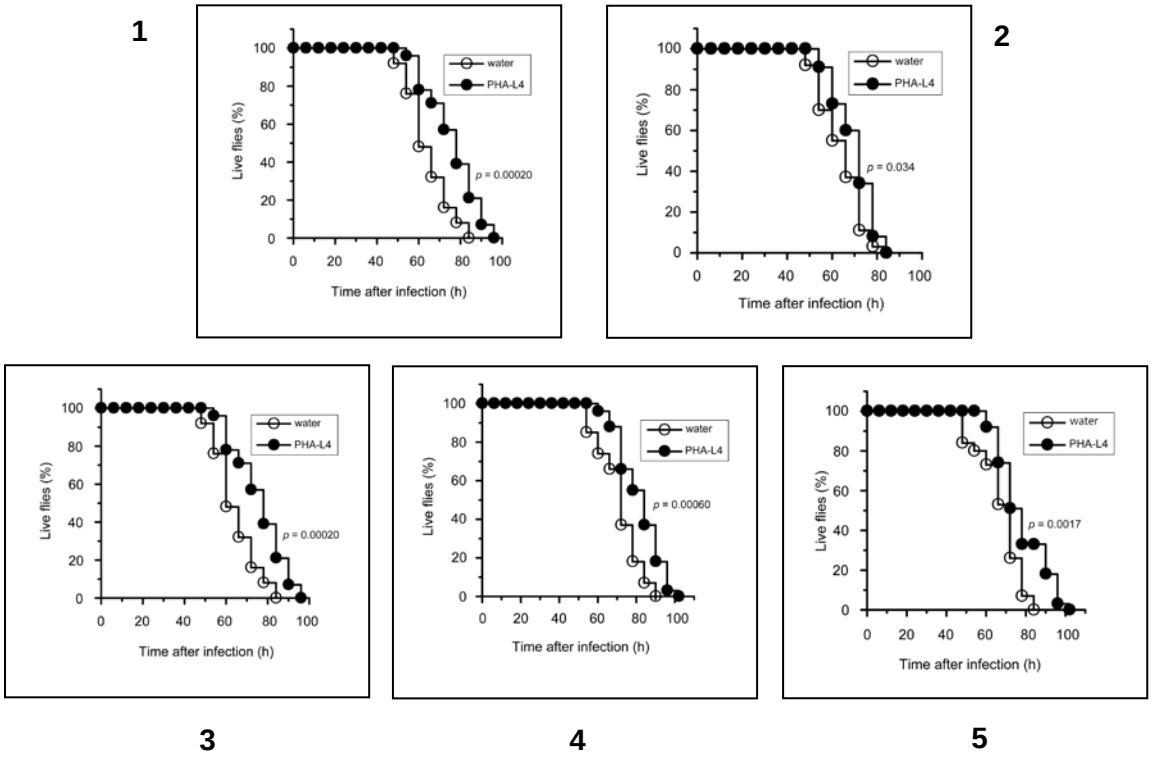


Figure 12. Survival of DCV-infected flies pre-administered with PHA-L4. Flies were administered with PHA-L at 1.2 µg/ml **(A)** and 6 µg/ml **(B)** or water, followed by the infection with DCV at 27,500 $TCID_{50}$. Data from 4 (**A**) and 5 (**B**) independent experiments are shown. The data of lectin- and water-fed flies were statistically analyzed using the log-rank test.

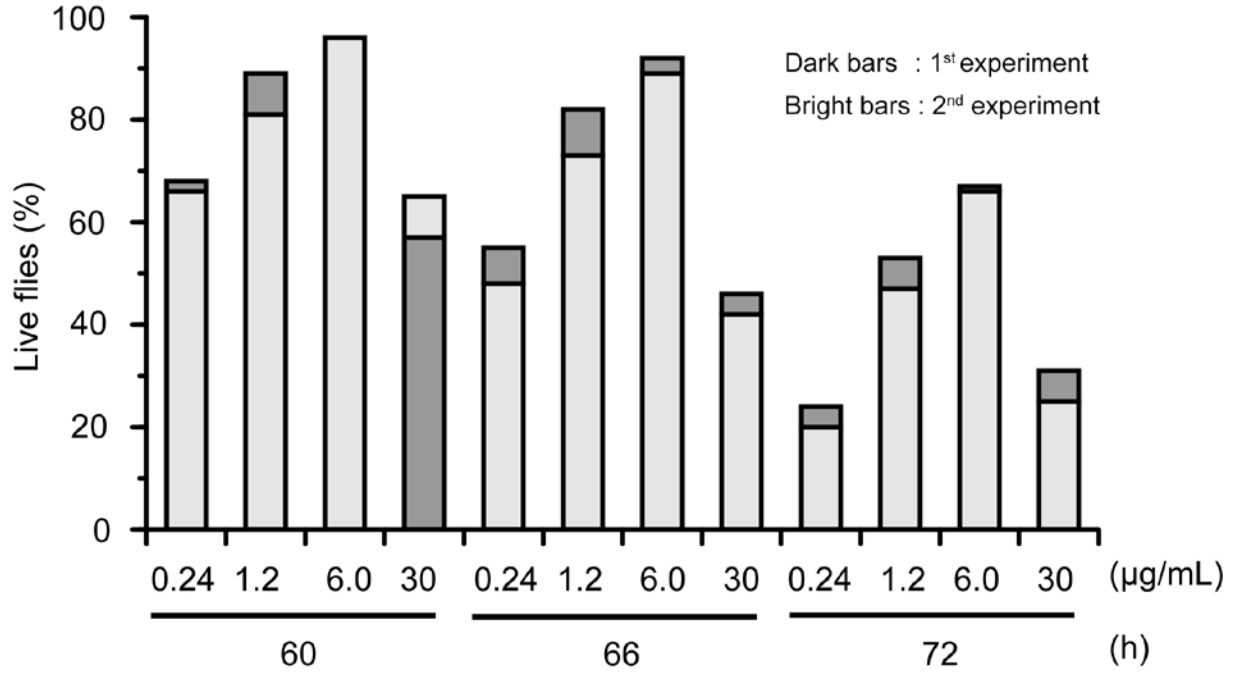


Figure 13. Antiviral actions of PHA-L4 at various concentrations. Flies that had been administered with PHA-L4 at the indicated concentrations were infected with DCV at 27,500 $TCID_{50}$. The ratio of live flies at the indicated time points is shown.

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

The extension of fly survival after DCV infection by pre-administration with *P. vulgaris* lectin could be due to the inhibition of viral growth and/or enhancement of viral elimination. In order to gain a clue to clarifying the mechanism of lectin actions, I next examined changes of viral load in flies during infection. Adult flies were pre-administered with PHA-L4 at 6 μg/ml, infected with DCV at 27,500 $TCID_{50}$, and disrupted for lysate preparation at certain time points after infection. The levels of DCV in the lysates were determined using the *Drosophila* cell line S2 as a host. I found that an increase in the level of viral load after 30 h post infection seen in control flies, which was the timing for flies to start dying, was kept low in lectin-fed flies (Table 8 and Figure 14). To further confirm the inhibitory effects of PHA-L4 on viral load, I conducted a similar experiment with infection at a viral dose 10 times lower than that in the previous experiment. The pre-administration with PHA-L4 decreased viral load in flies at early stages of infection: an initial

increase of virus at 12-24 h post infection was kept low (Table 9 and Figure 15). However, a maximum level of viral load was not affected by lectin. These results indicated that PHA-L4 decreases the level of viral load in flies before they start to die.

Table 8. Changes of viral load in flies pre-administered with PHA-L4 during infection with DCV at 27,500 $TCID_{50}$. The values in $TCID_{50}$ at the indicated time points after infection in three independent experiments are shown.

Hours after infection	Water-fed group				PHA-L4-fed group			
	1	2	3	Average ± SD	1	2	3	Average ± SD
0	$2.0x10^6$	$2.0x10^6$	$1.3x10^6$	$1.8x10^6 \pm 4.0x10^5$	$2.0x10^6$	$1.0x10^6$	$9.3x10^5$	$1.3x10^6 \pm 6.0\ x10^5$
12	$9.3x10^8$	$1.6x10^9$	$9.3x10^8$	$1.2x10^9 \pm 3.9x10^8$	$6.3x10^8$	$2.5x10^9$	$1.3x10^9$	$1.5\ x10^9 \pm 9.5x10^8$
24	$6.1x10^9$	$8.8x10^9$	$7.8x10^9$	$7.6\ x10^9 \pm 1.4x10^9$	$6.3x10^9$	$7.5x10^9$	$7.5x10^9$	$7.1\ x10^9 \pm 6.9\ x10^8$
30	$2.0x10^{10}$	$4.6x10^{10}$	$5.1x10^{10}$	$3.9\ x10^{10} \pm 1.7x10^{10}$	$7.8x10^9$	$1.3x10^{10}$	$1.3x10^{10}$	$1.1\ x10^{10} \pm 3.0\ x10^9$
36	$6.3x10^{10}$	$8.9x10^{10}$	$9.0x10^{10}$	$8.1x10^{10} \pm 1.5x10^{10}$	$2.6x10^{10}$	$2.0x10^{10}$	$1.6x10^{10}$	$2.1\ x10^{10} \pm 5.0\ x10^9$
42	$3.6x10^{11}$	$2.0x10^{10}$	$1.4x10^{11}$	$2.3x10^{11} \pm 1.1x10^{11}$	$4.3x10^{10}$	$3.2x10^{10}$	$3.2x10^{10}$	$3.6\ x10^{10} \pm 6.4\ x10^9$
48	$3.2x10^{10}$	$3.2x10^{10}$	$3.9x10^{10}$	$3.4x10^{10} \pm 4.0x10^9$	$3.2x10^{10}$	$3.6x10^{10}$	$4.3x10^{10}$	$3.7\ x10^{10} \pm 5.6\ x10^9$
60	$4.3x10^{10}$	$4.3x10^{10}$	$3.2x10^{10}$	$3.9\ x10^{10} \pm 6.4x10^9$	$5.4x10^{10}$	$5.1x10^{10}$	$3.9x10^{10}$	$4.8\ x10^{10} \pm 7.9\ x10^9$

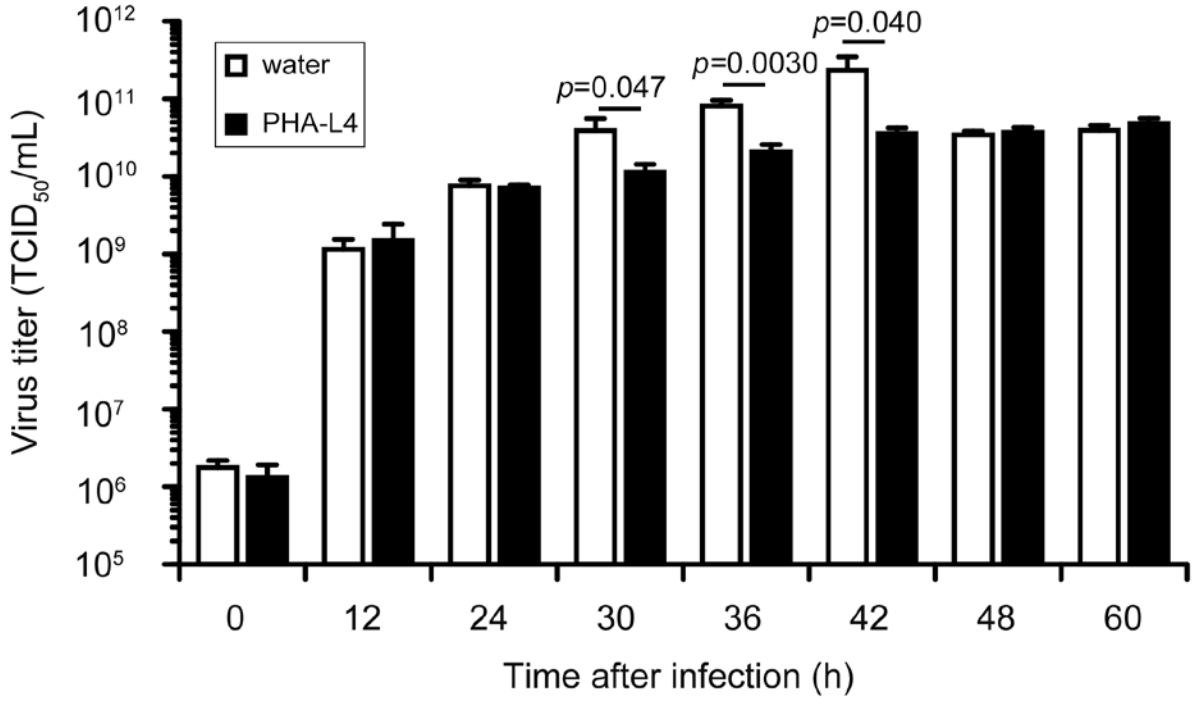


Figure 14. Changes of viral load in flies pre-administered with PHA-L4 or water alone after infection with DCV at 27,500 $TCID_{50}$. Mean ± SD of the data obtained from three independent experiments are presented. The values in two groups were statistically analyzed by the two-tailed Student *t* test, and *p* values greater than 0.05 are indicated.

Table 9. Changes of viral load in flies pre-administered with PHA-L4 during infection with DCV at 2,750 $TCID_{50}$. The values in $TCID_{50}$ at the indicated time points after infection in three independent experiments are shown.

Hours after infection	Water-fed group				PHA-L4-fed group			
	1	2	3	Average ± SD	1	2	3	Average ± SD
0	$1.3x10^5$	$6.3\ x10^4$	$2.0\ x10^4$	$7.1\ x10^4 \pm 5.5x10^4$	$1.0x10^5$	$3.4x10^4$	$1.3x10^4$	$4.9x10^4 \pm 4.5x10^4$
12	$2.0x10^7$	$6.3\ x10^6$	$5.4x10^6$	$1.1\ x10^7 \pm 8.2x10^6$	$5.0x10^6$	$1.3x10^6$	$1.9x10^6$	$2.7x10^6 \pm 2.0x10^6$
18	$1.3x10^7$	$4.9\ x10^6$	$9.3x10^7$	$3.7\ x10^7 \pm 4.9x10^7$	$4.3x10^6$	$1.3x10^6$	$3.2x10^7$	$1.3x10^7 \pm 1.7x10^7$
24	$6.3x10^7$	$5.1x10^8$	$5.1x10^8$	$3.6\ x10^8 \pm 2.6x10^8$	$1.3x10^7$	$8.8x10^7$	$4.3x10^7$	$4.8x10^7 \pm 3.8x10^7$
30	$1.1x10^9$	$1.1x10^9$	$3.2x10^9$	$1.8\ x10^9 \pm 1.2x10^9$	$5.1x10^8$	$7.5x10^8$	$6.3x10^9$	$2.5x10^9 \pm 3.3x10^9$
36	$2.0x10^9$	$3.9x10^9$	$1.6x10^9$	$2.5\ x10^9 \pm 1.2x10^9$	$1.6x10^9$	$3.4x10^9$	$4.3x10^9$	$3.1x10^9 \pm 1.4x10^9$
42	$4.3x10^9$	$5.4x10^9$	$7.5x10^9$	$5.7\ x10^9 \pm 1.6x10^9$	$1.5x10^9$	$4.3x10^9$	$4.3x10^9$	$3.4x10^9 \pm 1.6x10^9$
48	$9.3x10^9$	$3.2x10^{10}$	$5.1x10^9$	$1.6\ x10^{10} \pm 1.5x10^{10}$	$3.9x10^9$	$4.3x10^{10}$	$3.2x10^9$	$1.7x10^{10} \pm 2.3x10^{10}$
60	$9.3x10^9$	$2.6x10^{10}$	$3.2x10^{10}$	$2.2\ x10^{10} \pm 1.2x10^{10}$	$7.5x10^9$	$3.9x10^{10}$	$2.0x10^{10}$	$2.2x10^{10} \pm 1.6x10^{10}$
72	$1.6x10^{10}$	$6.3x10^9$	$3.2x10^{10}$	$1.8\ x10^{10} \pm 1.3x10^{10}$	$7.5x10^9$	$6.3x10^9$	$6.3x10^{10}$	$2.6x10^{10} \pm 3.2x10^{10}$

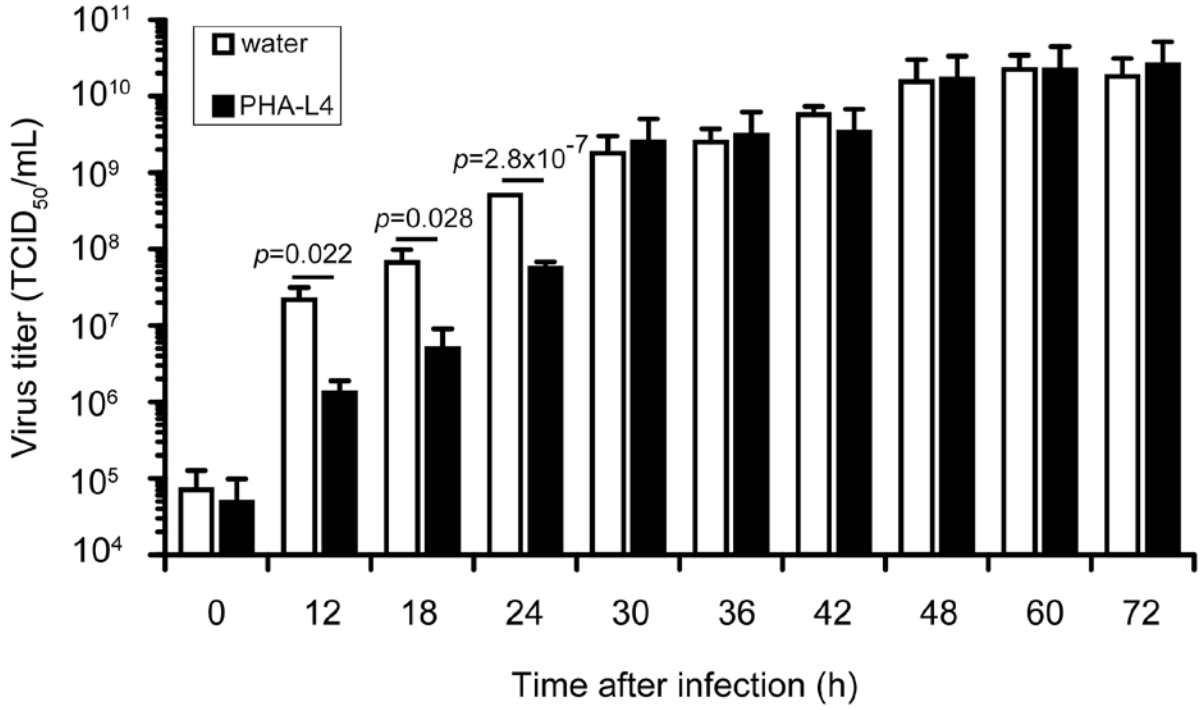


Figure 15. Changes of viral load in flies pre-administered with PHA-L4 or water alone after infection with DCV at 2,750 $TCID_{50}$. Mean ± SD of the data obtained from three independent experiments are presented. The values in two groups were statistically analyzed by the two-tailed Student *t* test, and *p* values greater than 0.05 are indicated.

Two timings of fly death, when flies started to die and all flies died, were summarized using data from three independent experiments (Table 10). The pre-administration of flies with PHA-L4 appeared not only to extend the period of fly survival but also make a delay of the onset of fly death. It is most likely that lectin mitigates the pathogenic effects of virus through reducing the level of virus in flies.

Table 10. Timings of fly death. 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.

Infection with DCV	2,750 $TCID_{50}$		27,500 $TCID_{50}$	
Timing	Onset	Extinct	Onset	Extinct
Water-fed flies	42 – 48	90	42 – 54	78 – 90
PHA-L-fed flies	48 – 66	96	54 – 60	84 – 102

Effects of PHA-L4 on the levels of mRNA of proteins related to fly immunity

Unlike mammals that are equipped with both innate and adaptive immunity, *Drosophila* fights against a variety of microbial invaders with only the former type of immunity (10,25). Antiviral mechanisms in *Drosophila* rely on several modes of immune responses categorized into either humoral or cellular immunity (22,26). The most intensely studied one is based on the mechanism of RNA interference, by which the genomes and mRNA of virus are degraded in host cells in a sequence-specific manner (27-30). In addition, the Toll-Dorsal pathway that leads to the production of antimicrobial peptides (10) as well as the apoptosis-dependent phagocytosis of virus-infected cells (22) are known to protect host organisms from viral attack. I, thus, next determined changes in the levels of antimicrobial peptides and phagocytosis receptors after the treatment of flies with *P. vulgaris* lectin.

To this end, I conducted reverse transcription-mediated PCR for the determination of the levels of mRNA of target proteins. In this method, the use of cDNA prepared from RNA specimens within a range of quantification is necessary. I first carried out PCR using increasing amounts of cDNA prepared from various RNA samples as templates and oligoDNA possessing the sequences of target mRNAs as primers. After visualizing PCR products by gel electrophoresis and ethidium bromide staining, ranges for quantifiable cDNA amounts with each primer were determined (data not shown). Another important issue in this series of experiment is to compare, in an appropriate way, the levels of target mRNAs contained in two RNA samples prepared from flies administered with lectin or water. For this purpose, I analyzed the mRNA of the ribosomal protein rp49 as an internal control. With the anticipation that the expression of rp49 mRNA is not affected in flies by the treatment with lectin, I determined the ratio of two cDNA preparations that give a similar level of rp49 mRNA (Figure 16) and used that ratio to analyze all other mRNAs.

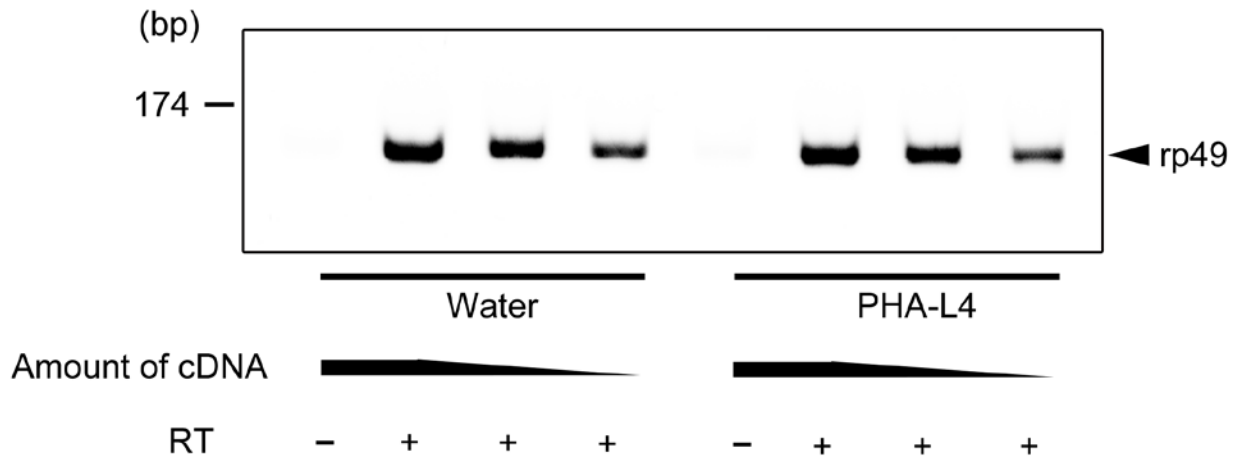


Figure 16. Reverse transcription-mediated PCR analysis of rp49 mRNA. Total RNA extracted from flies that had been administered with PHA-L4 (6 μg/ml) or water were subjected to reverse transcription. The resulting cDNAs at three different amount were used as template in PCR with a primer specific to rp49 mRNA. PCR product were separated on a 6% polyacrylamide gel and visualized by the staining with ethidium bromide.

There exist two signaling pathways in *Drosophila* that induce the expression of antimicrobial peptides upon infection with microbial pathogens, the Toll and Imd pathways (10). The former pathway is activated after infection with Gram-positive bacteria and fungi while the latter with Gram-negative bacteria. I analyzed the mRNAs of attacin and diptericin in the Toll pathway as well as those of defensin, metchnikowin, and drosomycin in the Imd pathway. The data indicated that the levels of mRNAs of defensin and drosomycin were raised in adult flies after the oral administration with PHA-L4 (Figure 17), suggesting the activation of the Imd pathway by lectin.

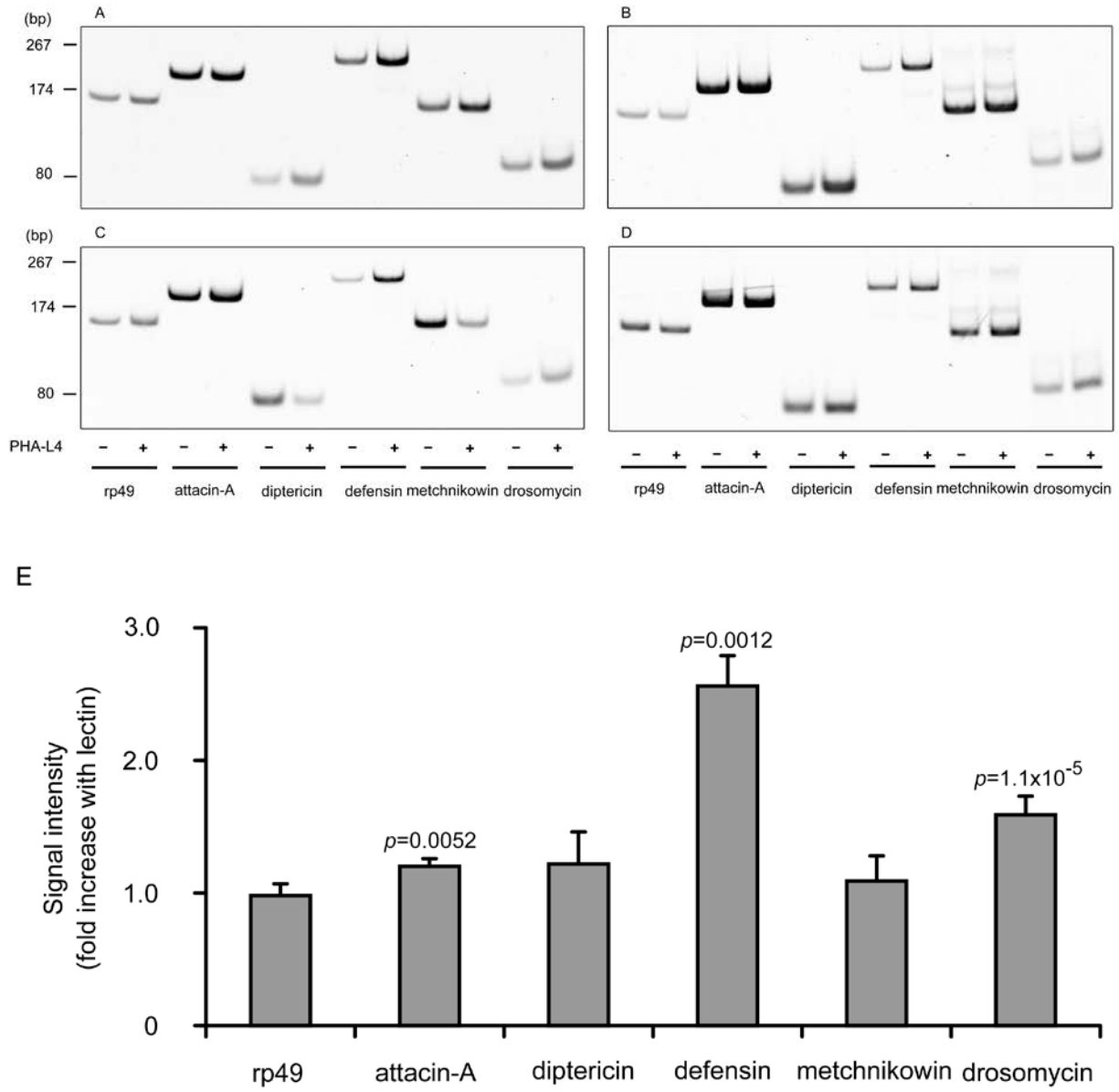
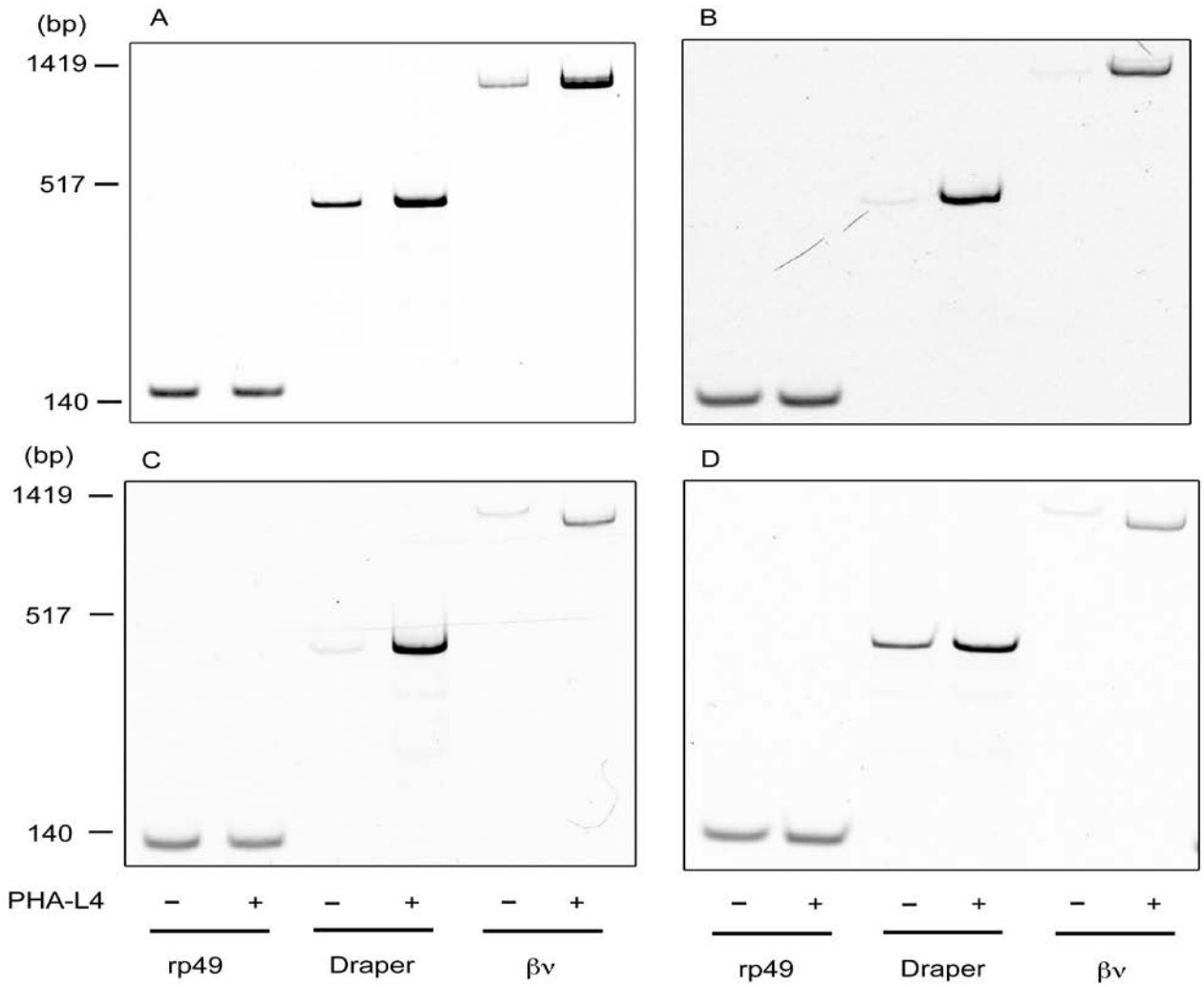


Figure 17. Reverse transcription-mediated PCR analysis of antimicrobial peptide mRNA. Adult flies were fed PHA-L4 (6 µg/ml) or water alone. Total RNA was analyzed by reverse transcription-mediated PCR for the mRNAs of the indicated antimicrobial peptides and rp49 as an internal control. (**A-D**) Products in PCR were separated on a 6% polyacrylamide gel and visualized by the staining with ethidium bromide. The positions and sizes, in base pairs, of standard DNA are shown on the left. The data from four independent experiments are shown. (**E**) The intensities of the signals were digitized and shown relative to those obtained with RNA of water fed flies, taken as 1.0. The data from four independent experiments were analyzed by the Student's *t* test, and the means +/- standard deviations are shown. *p* values greater than 0.05 are indicated.

I next analyzed the mRNAs of phagocytosis receptors Draper and integrin αPS3-βν, which are responsible for the apoptosis-dependent phagocytosis of DCV-infected cells in *Drosophila* (22). The mRNA of αPS3, an α subunit of integrin αPS3-βν, was not detectable under the experimental conditions adopted in this study (data not shown). The data in the analysis of Draper and βν, a β subunit of integrin αPS3-βν, showed that the levels of the two mRNA were dramatically increased in flies after the treatment with PHA-L4 (Figure 18). This suggests the augmentation of the phagocytic activity of *Drosophila* phagocytes such as plasmatocytes, equivalent to mammalian macrophages.



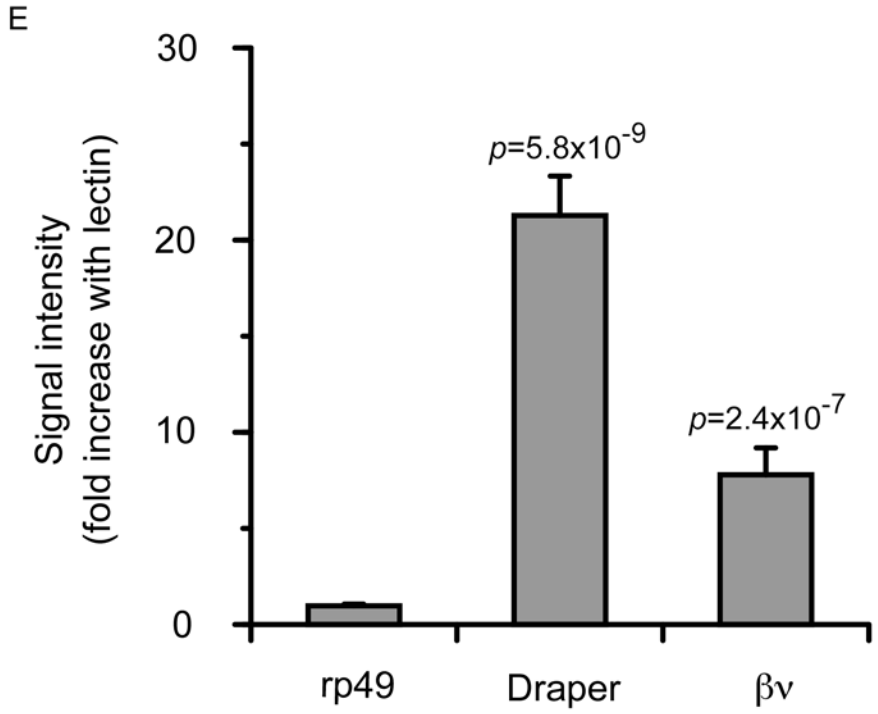


Figure 18. Reverse transcription-mediated PCR analysis of phagocytosis receptors mRNA. RNA of adult flies fed PHA-L4 (6 µg/ml) or water was analyzed similarly to the experiments shown as Figure 18 for the mRNAs of the indicated phagocytosis receptors. The data from four independent experiments (**A**-**D**) were analyzed by the Student's *t* test, and the means +/- standard deviations are shown (**E**). *p* values greater than 0.05 are indicated.

DISCUSSION

Pre-feeding with lectin isolated from *P. vulgaris* beans extended the survival period of *Drosophila* adults after an infection with DCV, indicating that *P. vulgaris* lectin showed antiviral activity in *Drosophila*. This is, to my knowledge, the first to show the antiviral effects of this type of lectin in vivo. Lectin-fed flies started to die later and survived longer than control water-fed 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 (31).

The antiviral defenses of *Drosophila* rely on RNA interference, the Toll-Dorsal pathway, the JAK-STAT pathway, the phagocytosis of virus particles, and the phagocytosis of virus-infected cells (17-19,28,32,33). Therefore, humoral and cellular innate immune responses appear to participate in the protection of *Drosophila* against viral infection. The data I obtained suggest the enhancement of both types of immunity in flies after the treatment with *P. vulgaris* lectin: the mRNA levels of antimicrobial peptides and phagocytosis receptors were raised. An increase in the level of the phagocytosis receptors Draper and integrin could be a mechanistic interpretation of the antiviral actions of *P. vulgaris* lectin, because those receptors are necessary for the phagocytic elimination of DCV-infected cells by *Drosophila* phagocytes in flies (22). In contrast, the involvement of antimicrobial peptides such as defensin and drosomycin, of which mRNAs were increased by lectin, in the defense against DCV infection remains to be investigated.

PHA-L4 consisting of a tetramer of subunit L was 10 time more active than PHA-P, a mixture of L4, L3E1, and L2E2, suggesting an importance of subunit L in the antiviral actions of lectin. This subunit was previously shown to be responsible for the mitogenic actions of *P. vulgaris* lectin against lymphocytes (5). It is, thus, possible that *P. vulgaris* lectin stimulates mammalian lymphocytes and *Drosophila* immune cells through molecular interactions between subunit L and corresponding receptors present at the surface of those cells. However, results in this study suggest

an indirect mechanism for the stimulation of immune cells of *Drosophila* by lectin. Although an analysis with fluorescence-labeled lectin indicated the presence of lectin in the digestive tracts of flies, I 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 (34). Therefore, the antiviral effects of *P. vulgaris* lectin probably do not occur through a direct interaction with the virus. If lectin remains inside the digestive tracts, it could stimulate epithelial cells of the midgut to induce a systemic increase in the level of immunity (Figure 19). Further investigation is required for the clarification of the actions of *P. vulgaris* lectin to protect *Drosophila* from DCV infection. Another issue to be solved is given from the data of dose-responding experiments of lectin: both PHA-P and PHA-L4 gave a bell-shaped dose response curve in an assay for fly survival. There seem to exist a range of lectin concentrations valid in antiviral actions; doses below and beyond that range are less effective. A pharmacological examination of *P. vulgaris* lectin could help to solve how it exhibits antiviral actions.

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

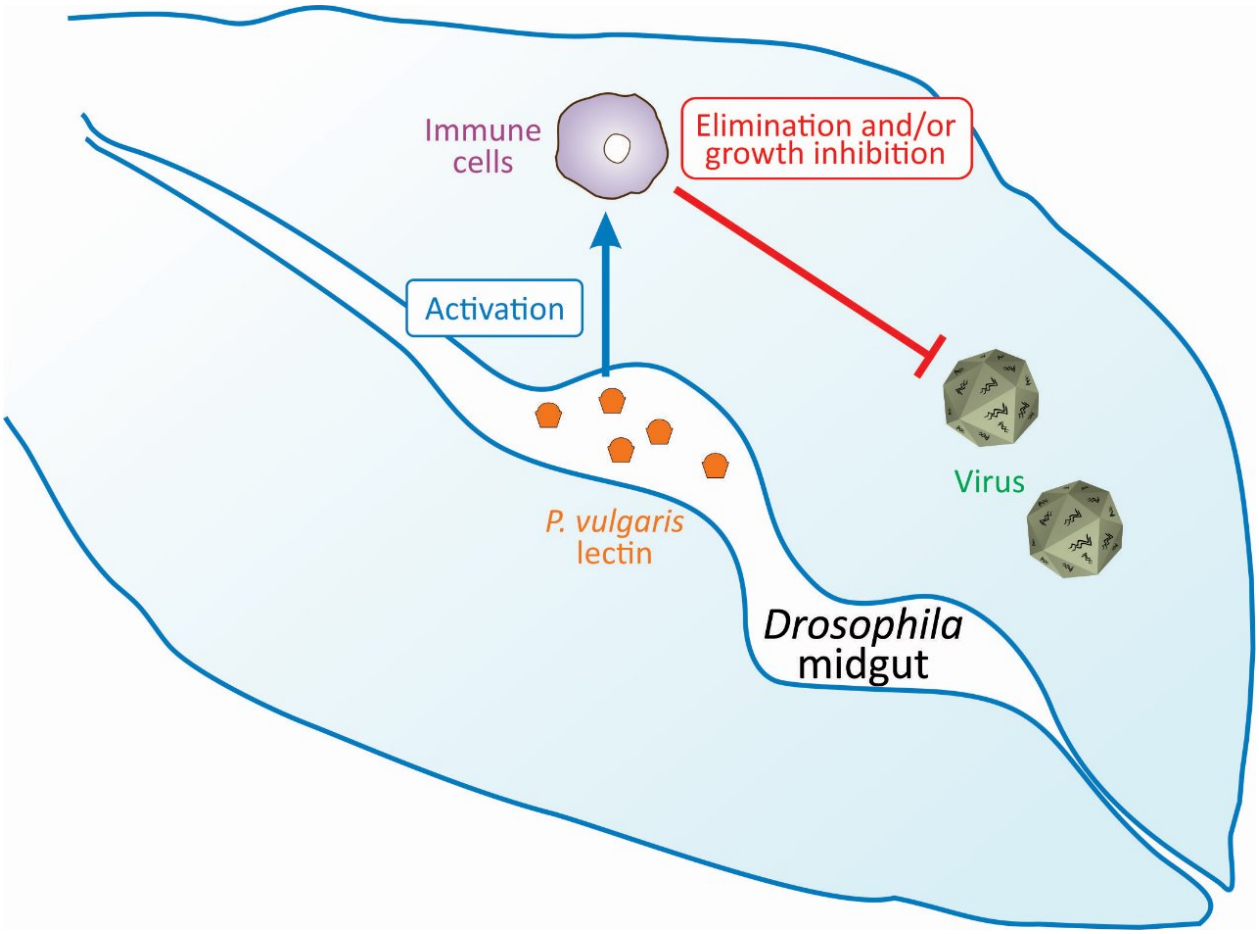


Figure 19. A model for antiviral actions of *P. vulgaris* lectin. Orally administered lectin remains in the midgut of *Drosophila* and indirectly activates immune cells so that the level of viral load is reduced.

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