Original Article

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Evaluation of innate immune stimulating activity of polysaccharides using a silkworm (*Bombyx mori*) muscle contraction assay

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ABSTRACT: In silkworm larvae, the mature form of paralytic peptide (PP), an insect cytokine, is produced from pro-PP in association with activation of innate immune responses, resulting in slow muscle contraction. We utilized this reaction, muscle contraction in silkworms coupled with innate immunity stimulation, to quantitatively measure the innate immune stimulating activity of various natural polysaccharides. β-Glucan of *Gyrophora esculenta* (GE-3), fucoidan from sporophyll of *Undaria pinnatifida*, and curldan induced silkworm muscle contraction. We further demonstrated that GE-3 had therapeutic effects on silkworms infected by baculovirus. Based on these findings, we propose that the silkworm muscle contraction assay is useful for screening substances that stimulate innate immunity before evaluating therapeutic effectiveness in mammals.

Keywords: Silkworm, innate immunity, polysaccharide

1. Introduction

Innate immunity is the first line of defense against microbe infection. Recent studies indicate that molecular mechanisms of innate immunity are highly conserved among invertebrates and vertebrates (*1,2*). Because innate immunity mechanisms are activated in response to various pathogens, stimulation of innate immunity may be an effective therapy against infectious disease. For example, interferon stimulates the innate immune system and is currently used for hepatitis C antiviral therapy. *In vitro* screening for innate immunity stimulants using immune cells from mammalian animals has been developed (*3-5*). Serious problems, however, are associated with *in vitro* assays using cultured cells to evaluate innate immunity stimulants. For example, most compounds from natural sources are contaminated with lipopolysaccharides (LPS) from Gram-negative bacteria, which stimulate innate immunity at very low concentrations. Further, most candidate compounds obtained by *in vitro* screening are not therapeutically effective because of problems of pharmacokinetics in animal bodies. Various issues with the so-called ADME (absorption, distribution, metabolism, and excretion) properties complicate this process. Therefore, for drug development it is necessary to perform quantitative measurement of therapeutic effects *in vivo*. Efficient methods of evaluating the innate immune stimulating activity of agents *in vivo*, however, are quite limited. To overcome the problems in screening systems of innate immune stimulants, we established an assay using the silkworm *Bombyx mori* based on muscle contraction, which is associated with activation of innate immunity (*6,7*).

Recently, we reported that injection of yeast β-glucans and bacterial peptidoglycans into the silkworm *Bombyx mori* induces maturation of the insect cytokine paralytic peptide (PP), which results in muscle contraction of the larvae (*6,7*). Because the muscle contraction, induced by macromolecules that exist in the outer membrane of the bacteria or cell wall of fungi, is a slow reaction requiring over 5 min (*6,7*), it very clearly differs from the rapid muscle contraction induced by neurotransmitters. Pre-PP is present in the hemolymph of the silkworm larvae (*8*), then processed to mature PP, which causes paralysis of the silkworm larvae (*9*). PP induces activation of cellular and humoral immunity (*7,10*), thus it is likely that the silkworm muscle contraction by PP reflects activation of the innate immune system. The advantages of using the silkworm muscle contraction assay to evaluate immunostimulants include the following: *i*) the system does not respond to LPS due to presence of LPS-absorbing proteins in the

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hemolymph; *ii*) as the method is based on bioassay using the silkworm body, it can be used to exclude compounds with pharmacokinetic problems; and *iii*) compounds that are toxic can be excluded using the bioassay with silkworm muscle specimens (*11-14*).

We previously purified a polysaccharide with innate immune-stimulating activity from green tea extracts that induces silkworm muscle contraction (*15*). In the present study, we evaluated the innate immune stimulating activities of various polysaccharides using the silkworm muscle contraction assay system. To further establish the usefulness of this system for screening therapeutically effective materials, we examined the therapeutic effect of β-glucans from *Gyrophora esculenta* (GE-3) in a silkworm baculovirus infection model (*16*).

2. Materials and Methods

2.1. *Polysaccharides*

GE-3 (*17*), an acid-hydrolyzed product of GE-3, a sulfate of GE-3 (*18*), lichenan (*19*), isolichenan (*19*), and ukonan (*20,21*) were prepared as previously described. Fucoidan (Riken Vitamin, Tokyo, Japan), yeast β-glucans (Sigma-Aldrich, St. Louis, MO, USA or Oriental Yeast Co. Ltd, Tokyo, Japan), curdlan (Sigma-Aldrich), laminarin (Sigma-Aldrich), lentinan (Ajinomoto, Tokyo, Japan), and schizophyllan (Kaken Pharmaceutical Co. Ltd, Tokyo, Japan) were purchased from the indicated vendors. The polysaccharides were dissolved in 0.9% NaCl (2-20 mg/mL). LPS derived from *Escherichia coli*, *Vibrio cholerae* Inaba 569B, *Pseudomonas aeruginosa* 10, *Klebsiella pneumoniae*, and *Shigella flexneri* 1A were purchased from Sigma-Aldrich and dissolved in 0.9% NaCl (250 μg/mL).

2.2. *Evaluation of silkworm larvae muscle contraction*

We measured the muscle contraction activity of various samples as previously reported (*6,7*). Briefly, the head of the silkworm larvae (5th instar) was cut off, tied with a string with a weight, and stabilized until autonomous vibration terminated. Samples (50 μL) were injected into the body fluid of decapitated silkworms with a 1-mL syringe attached to a 27-gauge needle (Terumo). The maximum length of each specimen before and after the injection was measured to calculate the contraction ratio (Contraction value; C value). One unit was defined as the activity inducing 15% contraction of the specimen at maximal contraction.

2.3. *Detection of activated PP*

The hemolymph of the silkworm fifth instar larva was collected and an aliquot (75 μL) was mixed with 25 μL of GE-3 suspension (80, 250, 800, or 2,500 μg/mL in 0.9% NaCl). The samples were incubated at 25°C for 3 min, heated at 100°C for 5 min, and then centrifuged at 10,000× g for 10 min. The proteins in the supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblot analysis was performed with anti-PP antibody (a kind gift from Dr. Kamimura (*8*)) as previously described (*7*). Although this antibody also reacts to pre-PP, it can be discriminated from mature PP as their molecular weights differ. Chemically synthesized mature PP (*8*) was used as a positive control.

2.4. *Examination of therapeutic effects of GE-3 in a silkworm nucleopolyhedrosis virus (NPV) infection model*

Bombyx mori nucleopolyhedrosis virus (NPV; 1.28×10^5 pfu/50 μL) (*16*) was injected into the hemolymph of the silkworm larvae (5th instar), and then GE-3 solution or ganciclovir (250 μg/larva) was injected into the hemolymph or into the midgut. Surviving larvae were counted for 5 days post-inoculation. A survival curve was prepared using the Kaplan-Mayer method (GraphPad Prism3, MDF Co. Ltd., Tokyo, Japan).

3. Results

3.1. *Activity of polysaccharides that induce silkworm muscle contraction*

β-Glucans are pathogen cell wall components and are well known to induce activation of innate immunity (*1, 22*). Here, we first examined whether these materials induce silkworm muscle contraction. Curldan of *Alcaligenes faecalis*, GE-3, and fucoidan from the sporophyll of *Undaria pinnatifida* induced muscle contraction (Table 1). On the other hand, lentinan

Table 1. Induction of silkworm muscle contraction by polysaccharides from natural origins

Polysaccharide	Structure	Activity (unit/mg)
Curdlan	β-1,3 Glucan	100
GE-3	β-1,6 Glucan	38
Fucoidan	Sulfated polysaccharide	36
Yeast β-glucan (Sigma)	β-1,3 Glucan (+β-1,6 glucan)	33
Yeast β-glucan (Oriental yeast)	β-1,3 Glucan (+β-1,6 glucan)	20
Lichenan	β-1,3: 1,4 Glucan	6
Isolichenan	α-1,3: 1,4 Glucan	6
Laminaran	β-1,3 Glucan	< 1
Lentinan	β-1,3 Glucan (+β-1,6 glucan)	< 10
Schizophyllan	β-1,3 Glucan (+β-1,6 glucan)	< 2
Ukonan A	Acidic polysaccharide	< 19
Ukonan B	Acidic polysaccharide	< 10
Ukonan C	Acidic polysaccharide	< 10
Ukonan D	Neutral polysaccharide	< 17

Activity (1 unit) was defined as that causing muscle contraction with the value of 0.15 (15% contraction). The inequality sign (<) indicates that the activity was lower than the limits of detection (*i.e.*, the maximum dose tested did not induce 15% muscle contraction).

and schizophyllan, which are reported to activate mammalian immune cells (*23,24*), did not induce muscle contraction. Laminarin, a β-1,3 glucan with low molecular weight, shows little activity for inducing TNF-α production in macrophages (*25*). Laminarin also showed no activity in the silkworm muscle contraction assay. Other plant polysaccharides, such as ukonan A-D (*26,27*), showed little activity to induce silkworm muscle contraction. Thus, various polysaccharides that stimulate mammalian immune cells also stimulated muscle contraction in silkworms.

Activity of immunostimulants for innate immunity has been evaluated based on the induction of cytokine production and phagocytosis by mammalian macrophages (*28,29*). Because LPS from Gram-negative bacteria are active in these assays at extremely low concentrations, the effects of LPS derived from contaminated bacteria in samples is a serious problem for evaluating immunostimulation. We previously reported that *E. coli* LPS do not induce silkworm muscle contraction (*15*). Here, we further examined whether other bacterial LPS fractions induce silkworm muscle contraction. LPS fractions derived from *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Shigella flexneri* did not induce silkworm muscle contraction, even with administration of up to 12.5 μg (data not shown). These findings indicate that the effects of LPS derived from contaminated bacteria in the samples can be ignored in the muscle contraction assay.

We then examined the mechanism of the GE-3 induced silkworm muscle contraction. We previously reported that yeast β-glucan convert pro-PP into mature PP in the silkworm hemolymph, resulting in muscle contraction (*7*). Silkworm hemolymph was incubated with GE-3 and immunoblot analysis was performed to detect mature PP. The increase in mature PP was dependent on the GE-3 concentration (Figure 1), indicating that GE-3 induced PP activation in the silkworm hemolymph. This finding suggests that the observed muscle contraction induced by GE-3 administration was mediated by PP production from pro-PP in the hemolymph of the silkworm sample.

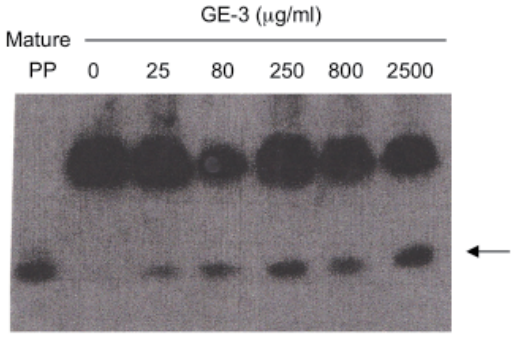

Figure 1. Production of the active form of PP by GE-3 in isolated hemolymph. The silkworm hemolymph (75 μL) and GE-3 solution (25 μL) were incubated and then PP was detected by immunoblotting. The final concentration of GE-3 is shown at the top of each lane. Arrow indicates the band position corresponding to mature PP.

To identify the structural determinants of GE-3 necessary for silkworm muscle contraction, we tested the activity of acid-hydrolyzed or sonicated GE-3. These treatments resulted in decreased muscle contraction (Table 2), suggesting that the high molecular mass of β-1,6 glucans is necessary to induce muscle contraction.

3.2. *Therapeutic effect of GE-3 against viral infection*

We previously reported that the innate immune response activated by PP contributes to defend silkworms from *Staphylococcus aureus* infection (*7*). Microarray analysis revealed that injection of PP alters gene expression patterns in the silkworm hemocytes and fat body cells, resulting in the induction of genes involved in innate immunity (*10*). Stimulants of innate immunity are therapeutically effective against viral infection (*30-34*). Thus, we hypothesized that activation of PP by GE-3 would contribute to the defense against viral infection in the silkworm. To test this, we first searched for genes induced by both PP and baculovirus (*Bombyx mori* nucleopolyhedrosis virus) infection (*35,36*) using microarray analysis. Expression of *Ka11761* (*integrin*), *Ka07712* (*MMP*), *arylphorin*, *promoting protein*, and *actin A3* was induced in the hemocytes, and expression of *Ka07075* (*BmEts*) was induced in the fat body cells by both PP and baculovirus (Supplemental Table 1, *http://www.ddtjournal.com/getabstract.php?id=540*). Ka05805, whose function is unknown, was induced in the two types of cells by both PP and baculovirus infection. In addition, defensin, which is induced during viral infection in *Drosophila* (*37*), was also induced by PP injection in the silkworm fat body cells. Therefore, PP induces the expression of a set of genes whose expression is also induced during viral infection.

We next tested whether GE-3 administration contributes to host defense against viral infection using a silkworm NPV infection model (*16*). Intra-hemolymph administration of GE-3 delayed the NPV killing effect (Figure 2A). The therapeutic effects of GE-3 for NPV-infected silkworms were dose-dependent (Figure 2B). These results suggest that GE-3 contributes to host resistance against viral infection through the activation of innate immunity induced by PP. The extent of the therapeutic effect by GE-3 was comparable to that by ganciclovir, which was used as a positive control (Figure 2C).

Table 2. Structure of GE-3 necessary for inducing silkworm muscle contraction

Treatment to GE-3	Activity (units/mg)
None	38 ± 6 (*n* = 23)
Sonication	11 ± 4 (*n* = 3)
Hydrolysis by sulfuric acid	< 2 (*n* = 5)

n: number of experiments.

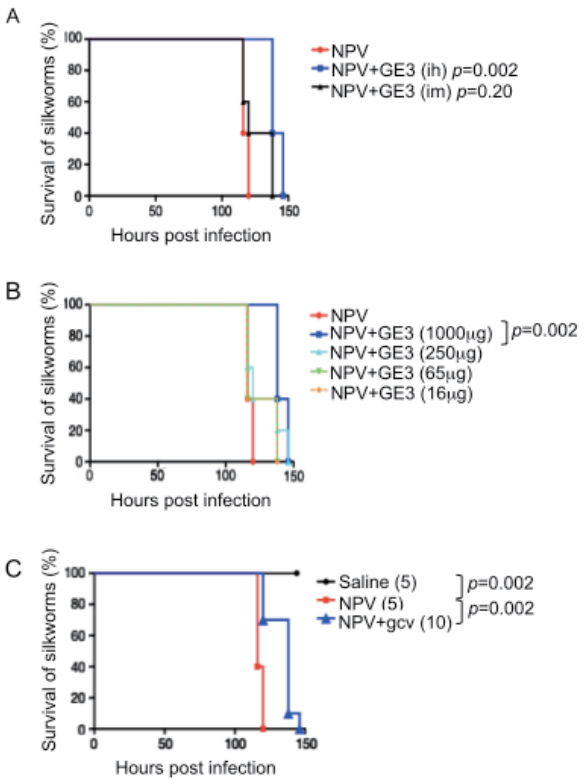

Figure 2. Therapeutic effects of GE-3 on silkworm larvae survival after NPV infection. (A) GE-3 (1 mg/larva) was injected into the hemolymph (ih) or midgut (im) of NPV-infected larvae. Survival curves were compared between non-treated and GE-3 injected groups using the log-rank test. N = 5 for each group. **(B)** Various concentrations of GE-3 solution were injected into the hemolymph of NPV-infected larvae. N = 5 for each group. p; log-rank test. **(C)** NPV or saline was injected into the hemolymph and then ganciclovir (250 μg/larva) was injected into the hemolymph. Silkworm larvae survival rate was examined. Number of tested larvae is shown in parentheses.

4. Discussion

The findings of the present study demonstrated that yeast β-1,6 glucans, GE-3 from Gyrophora esculenta, fucoidan, and curdlan (β-1,3 glucan) induced silkworm muscle contraction. These polysaccharides may activate immune cells in the hemolymph to produce mature PP, resulting in silkworm muscle contraction (*7*). Some β-glucans, such as laminarin, lentinan, and schizophyllan showed little activity in the silkworm muscle contraction system. One possible explanation for this is that receptors for silkworm muscle contraction have different specificities from those of immune cells in mammals. Another possibility is that β-glucans that did not induce silkworm muscle contraction may have ADME problems in the animal body. Further evaluation is needed to address these possibilities.

LPS does not induce silkworm muscle contraction. Therefore, contamination by LPS in the samples can be ignored in this screening system. The limited action of LPS in the silkworm muscle specimen can be explained by the presence of LPS binding proteins that neutralize LPS in the insect hemolymph (*38,39*). Furthermore, using the silkworm model, ADMET (absorption, distribution, metabolism, excretion, and toxicity) of compounds can be easily evaluated (*13,14, 40*). We propose that screening substances that activate innate immune systems using the silkworm muscle contraction assay system is an efficient method for identifying therapeutically effective compounds for infectious diseases, as a pre-screening system prior to more advanced stages of evaluation with mammals.

In the present study, we examined the therapeutic effectiveness of β-1,6 glucans from GE-3 in a silkworm model of NPV infection. Whereas several lines of evidence suggest the effectiveness of polysaccharides in the treatment for viral infectious diseases (*30-32, 41*), evidence for a relationship between therapeutic effectiveness and stimulation of immunity by polysaccharides is limited. Our results provide new evidence for this relationship. We showed that genes induced during NPV infection (*35,36*) were also induced in the hemocytes and fat body cells of the silkworm when PP was activated. These findings suggest that various molecules involved in the defense system against NPV infection may be induced by PP activation. For example, tetraspanin and integrin, which are induced in hemocytes after PP activation, are induced during NPV infection (*35*). Association of these molecules contributes to the cellular immune response (*42*), suggesting that they are involved in cellular immune responses to exclude virus-infected cells during NPV infection. Mechanism of which PP induces these gene expressions will be interesting subjects for future.

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