Original Article

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Purification of innate immunostimulant from green tea using a silkworm muscle contraction assay

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ABSTRACT: A polysaccharide was purified from a hot water extract of green tea leaves by measuring the immunostimulatory activity in silkworm larvae. Nuclear magnetic resonance and chemical analysis of acid hydrolysates revealed that the purified substance possessed a backbone containing polygalacturonic acids with methyl ester residues. Treatment with β-glucanase attenuated the muscle contraction activity of the purified sample, suggesting that the β-glucan structure, probably as a branched form, was required for its activity. The purified fraction stimulated the production of interleukin-6 by mouse peritoneal macrophages. These results suggest that measuring immunostimulation in silkworm larvae is useful for evaluating innate immunostimulants from various sources.

Keywords: Polysaccharides, pectin, green tea, innate immunity, mice, macrophages, IL-6, insect

1. Introduction

In addition to its use as a popular beverage, tea (*Camellia sinensis*) is also used as medicine to maintain health in humans. The therapeutic effects of green tea components have been studied in several disease models. For example, feeding green tea to mice with lupus-like syndrome improved survival and delayed disease progression (*1*). Laurie *et al*. studied the effect of a green tea extract in human patients with advanced lung cancer (*2*). Several compounds of green tea leaves such as polyphenols are reported to possess antitumor (*3*), antioxidant, and anti-diabetic properties (*4*). Thus we focused on green tea as a source of bioactive substances that stimulate innate immunity.

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The immune system is divided into two categories; adaptive and innate immunity. Innate immunity is the first barrier of host animals against invading pathogens and the system is well conserved among species. In insects, microbial components trigger defensive reactions such as melanization of the blood (*5,6*) and the production of antimicrobial factors (*7,8*). We previously reported that the injection of peptidoglycans and β-glucans, cell wall components of bacteria and fungi, respectively, induces slow paralysis in silkworms (*9*). Peptidoglycans and β-glucans are thought to be recognized by immune cells in silkworms followed by the activation of an insect cytokine, paralytic peptide, which induces muscle contraction and several self-defense reactions (*9-11*). Because peptidoglycan and β-glucan are well-known stimulants of innate immunity in various organisms, we hypothesized that quantitative measurement of silkworm muscle contraction would be useful for assessing the immunostimulatory activities of natural products or compounds. Here, we purified an immunostimulant from a hot water extract of green tea leaves using a silkworm muscle contraction assay. We further observed the stimulatory effect of the extract on murine macrophages. Structural analysis revealed that polygalacturonic acid was responsible for the activity.

2. Materials and Methods

2.1. *Reagents*

Green tea leaves were purchased from a local market. Tea polyphenols [catechin (±), catechin (+), and polyphenon 60], lipopolysaccharides (LPS) from *Escherichia coli*, and thioglycollate medium were purchased from Sigma-Aldrich. RPMI 1640, antibiotics (penicillin and streptomycin), and fetal bovine serum were purchased from Invitrogen. Enzyme-linked immunosorbent assay (ELISA) kits (Ready-Set-go) and interleukin (IL)-6 were purchased from eBioscience.

2.2. *Silkworms*

Eggs of silkworms (*Bombyx mori*, HuYo Tukuba Ne) were purchased from Ehime Sanshu (Ehime, Japan). Silkworm

larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 27°C (*12*).

2.3. *Silkworm muscle contraction assay*

Quantitative measurement of silkworm muscle contraction to evaluate innate immunity activation was described previously (*9*). Briefly, heads of fifth instar silkworm larvae (3-5 g) were cut off and the peritrophic membranes were removed. Each specimen was immobilized and attached with a 27-g load. Samples were dissolved or suspended in sterile saline, and 50 µL of each sample was injected into the body fluid of a specimen with a 1-mL syringe attached to a 27-gauge needle (Terumo, Tokyo, Japan). The muscle contraction value was calculated by measuring the maximum length of each specimen before (x cm) and after (y cm) the injection using the formula (x – y)/x. One unit of activity was defined as that which caused muscle contraction with the value of 0.15. The muscle contraction activity was measured by injecting 50 µL of sample dissolved in saline. The specific activity of the fraction was determined by creating a titration curve with diluted samples.

2.4. *Measurement of total sugar*

The amount of total sugar in the fractions was determined using the phenol-sulfuric acid method, a colorimetric test with D-glucose (Nacalai Tesque, Kyoto, Japan) as a standard (*13*). One hundred microliters of the sample was added to an equal volume of 5% phenol solution, and the mixture was vigorously mixed for 1 min. Concentrated sulfuric acid (98%; 500 µL; Nacalai Tesque) was added and the solution was immediately mixed vigorously. The samples were incubated at room temperature for 30 min and the OD_{490} values were measured using an MTP-300 Microplate reader (Corona Electric, Ibaraki, Japan).

2.5. *Preparation and purification of a hot water extract of green tea leave*

Green tea leaves (100 g) were autoclaved at 121°C for 15 min in 1 L of water. The sample was cooled and then centrifuged at 8,000 × g in a Hitachi centrifugation rotor [Himac CR 21E (rotor R10A)] for 10 min at 4°C. The supernatant was filtered through a paper filter (Advantec, pore size 0.45 µm), and the filtrate was freeze-dried for 48 h (Fraction I). The extracted fraction was added to two volumes of ethanol (99.5%; Nacalai, Japan) followed by centrifugation to collect the precipitated active substance (Fraction II).

1st DEAE-cellulose chromatography (pH 7.9) The ethanol precipitate (Fraction II, 610 mg in 8 mL buffer) was slowly applied onto a 20-mL DEAE-cellulose (DE-52, Whatman) column equilibrated with 10 mM Tris-HCl buffer (pH 7.9). After washing with 120 mL Tris buffer, the column was developed with a 0-0.4 M NaCl gradient (250 mL × 2). The eluted fractions (6 mL each) were collected using a CHF fraction collector (Advantec, Japan). The sugar concentrations in the eluted fractions were determined using the phenol-sulfuric acid method as described above.

2nd DEAE-cellulose chromatography (pH 5.6) Active fractions of the 1st DEAE-cellulose chromatography (pH 7.9) were pooled and loaded onto a 20-mL DEAE-cellulose (DE-52, Whatman) column equilibrated with 10 mM MES buffer (pH 5.6). The column was developed with a linear gradient of 0-0.4 M NaCl (250 mL × 2), and 6-mL fractions were collected. Active fractions were pooled and named Fraction III.

1st Mono Q chromatography (pH 7.9) Fraction III (pH 5.6) was loaded onto a Mono Q column equilibrated with 10 mM Tris-HCl, pH 7.9, and fractionated at a flow rate of 0.4 mL/min (2 mL/fraction). After loading the sample, the column was washed with 14 mL of buffer. Two steps of gradient elution, 16 mL of 0-20 mM and 40 mL of 20 mM-1 M NaCl, were performed.

2nd Mono Q chromatography (pH 5.6) The pooled fraction of the 1st Mono Q chromatography (pH 7.9) was loaded onto a Mono Q column (GE Healthcare, Buckinghamshire, UK) equilibrated with 10 mM MES buffer (pH 5.6). Elution by NaCl gradient to elute the bound materials was performed as described above for the 1st Mono Q chromatography (pH 7.9). The fractions were concentrated 6.5-fold by ethanol precipitation, and 50 µL of each sample was subjected to the muscle contraction assay. Active fractions were pooled and named Fraction IV.

Gel filtration with Superdex 200 Five hundred microliters of Fraction IV (14 mg/mL) was loaded on a 24-mL Superdex 200 (GE Healthcare) column equilibrated with 10 mM Tris-HCl (pH 7.9) and 150 mM NaCl. The column was calibrated with compounds of known molecular mass; sodium dextran sulfate (500 kDa), dextran 40 (40 kDa), and D-galacturonic acid monohydrate (0.2 kDa). Fractionation was performed with fast protein liquid chromatography equipment (GE Healthcare) at a flow rate of 0.5 mL/min.

2.6. *Monosaccharide analysis*

Purified fractions from green tea and the standard (D-glucose) were treated with 4 M trifluoroacetic acid at 100°C for 3 h. After acid hydrolysis, *N*-acetylation was performed to change amino sugars to their *N*-acetylated forms, by mixing the sample with pyridine methanol (1:9, v/v) and then treating with pyridine/acetic anhydride (4:1, v/v) for 30 min at room temperature. The samples were labeled with aminobenzoic acid ethyl ester (ABEE) by incubating at 80°C for 1 h, and analyzed by reversed phase-high performance liquid chromatography [RP-HPLC; Honenpak C18 (75 mm × 4.6 ID)] with 0.2 M potassium borate: acetonitrile (93:7)

at a flow rate of 1 mL/min. Peaks were detected by UV 305 nm according to retention time.

The standard comprised a mixture of 13 monosaccharides (2 nmol each; L-arabinose, L-fucose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-ribose, D-xylose, D-galacturonic acid, D-glucuronic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine).

2.7. *Nuclear magnetic resonance (NMR) analysis*

Fraction V (11 mg) was dissolved in deuterium oxide (D_2O), and ^{1}H and ^{13}C NMR (ECA-500, JEOL) analyses were performed at 40°C.

2.8. *β-glucanase sensitivity test*

Five milligrams of Fraction V was incubated with or without 11 mg of endo-1,3(4)-β-glucanase from *Apergillus niger* (Sigma) in 0.1 M sodium citrate buffer (pH 5.0) at 25°C for 6 h. After incubation, the enzyme was inactivated by boiling at 100°C for 5 min. Immunostimulation activity of the sample was measured by the silkworm muscle contraction assay as described above.

2.9. *IL-6 production by mouse peritoneal macrophages*

Peritoneal mouse macrophages were collected from C57BL/6 male mice 3 days after the injection of 1 mL of 4% thioglycollate medium. Twenty microliters of each sample was added to tissue culture plates (96 well, flat bottom, Iwaki Science Department, Asahi Glass Corporation, Japan) containing 10^5 cells/well in 200 µL of RPMI 1640 medium. The medium was supplemented with L-glutamine, 25 mM HEPES, 10% fetal bovine serum, and 100 µg/mL each of penicillin/streptomycin. The cells were incubated at 37°C for 24 h. The IL-6 concentration of 100 µL of each culture supernatant was measured using an ELISA kit (eBioscience). The standard curve was drawn using recombinant IL-6 (eBioscience) as the standard.

2.10. *Statistical analysis*

The significance of differences was calculated using the Student's *t*-test. Differences with a p value of less than 0.05 were considered statistically significant.

3. Results

3.1. *Induction of silkworm muscle contraction by a hot water extract of green tea leaves*

Screening of hot water extracts of food samples using the silkworm muscle contraction assay indicated that a hot water extract of green tea leaves had high innate immunostimulation activity. A freeze-dried fraction of the hot water extract of green tea had 6 units/mg of specific activity (Table 1). Activity per weight of dried green tea leaves was 1,300 units/g dry weight. Tea polyphenols [catechin (±), catechin (+), and polyphenon 60] and cellulose, components in green tea leaves, did not induce muscle contraction. Therefore, we concluded that substances other than polyphenols and cellulose were responsible for the innate immunostimulation in the hot water extract of green tea leaves. The fraction was further purified by ethanol precipitation followed by chromatographies with DEAE-cellulose and Mono Q.

3.2. *DEAE-cellulose chromatography*

Acid polysaccharides were reported from plant, such as pectin (*14*). To elucidate whether the substance in tea extract have acid moieties, we performed the DEAE-cellulose chromatography. The elution profile of chromatography (pH 7.9) is shown in Figure 1. A major part of the activity was adsorbed on the column, suggesting the presence of acidic moieties in the active substance. The specific activity of the active fraction recovered from the column was 28 units/mg, which was equivalent to that of the loaded sample. Rechromatography of the fraction with a DEAE-cellulose column at pH 5.6 showed absorbance of the active substance to the column, and there was no increase in specific activity in the salt-eluted fractions (Fraction III).

3.3. *Mono Q chromatography*

To further purify the immunostimulant from green tea leaves, we performed two rounds of Mono Q column chromatography. The active substance was adsorbed to the column (Figure 2). A major part of the activity was eluted by the salt gradient. Quantification of total sugar by the phenol-sulfuric acid method revealed that the activity peak coincided with the presence of polysaccharide. The specific activity of the active fraction was 40 units/mg. There was

Table 1. Summary of purification of innate immunity stimulating fraction from 100 g of green tea leaves

Fraction	Total activity (units)	Yield (%)	Amount (mg)	Specific activity (units/mg)
I Hot water extract	132,000	100	22,000	6
II Ethanol extract	75,600	57	2,700	28
III DEAE-cellulose chromatographies (pH 7.9 and 5.6)	16,200	12	810	20
IV Mono Q chromatographies (pH 7.9 and 5.6)	7,400	6	185	40
V Superdex 200 chromatography	4,000	3	100	40

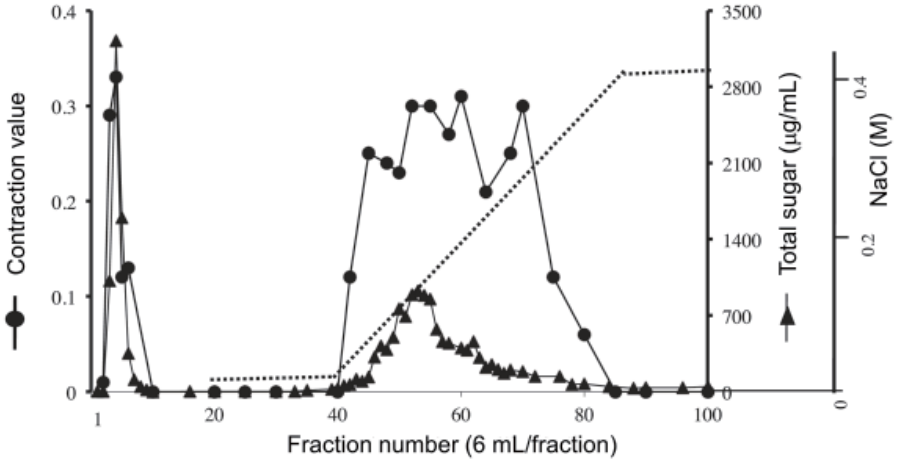

Figure 1. Elution profile of 1st DEAE-cellulose chromatography (pH 7.9). Silkworm muscle contraction assay was performed by injecting 50 μL of each sample (concentrated 6.5-fold). Total sugar concentration was measured by the phenol-sulfuric acid method. Active fractions (43-80) were pooled for further procedures.

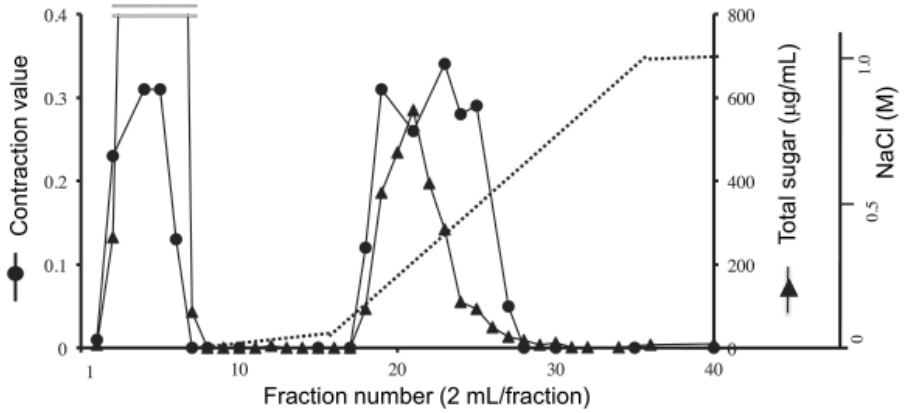

Figure 2. Elution profile of Mono Q chromatography (pH 7.9). Silkworm muscle contraction assay was performed by injecting 50 μL of each sample (concentrated 6.5-fold). Active fractions (18-27) were pooled and used for further purification.

no increase in the specific activity by rechromatography with Mono Q at pH 5.6 (Fraction IV).

3.4. *Gel filtration with Superdex 200*

To further evaluate the purity of the fraction obtained by rechromatography with the Mono Q column (Fraction IV), gel filtration was performed using Superdex 200 (GE Healthcare). The activity co-migrated with the polysaccharide peak (Figure 3). The specific activity of the pooled fraction (Fraction V) was 40 units/mg, equivalent to that of the loaded fraction. The active substance was eluted in fractions between those of sodium dextran sulfate (500 kDa) and dextran 40 (40 kDa), suggesting that the innate immunostimulants in green tea leaves possessed heterogeneous molecular masses.

3.5. *Monosaccharide analysis of acid hydrolysate*

Purified fraction (Fraction V) was hydrolyzed by acid treatment, and the monosaccharide composition was determined. RP-HPLC analysis of the ABEE-labeled sample revealed the presence of galacturonic acid, galactose, glucose, and rhamnose (Figure 4). The molar ratio of each monosaccharide was determined by measuring the peak area. The ratio of galacturonic acid: galactose:glucose:rhamnose was 22:4:5:1, respectively. These findings suggested that a major component in the fraction was D-galacturonic acid.

3.6. *NMR analysis*

Four kinds of anomeric proton signals were detected by ^{1}H NMR at 5.12, 5.15, 5.16, and 5.19 parts per million (ppm). The relative intensity of the signal detected at 5.12 ppm was two times higher than that of the other anomeric signals. Based on the observed chemical shifts of anomeric protons, these saccharide residues might have an alpha anomeric configuration. A methyl proton signal characteristic to galacturonic acid methyl ester was also detected at 3.87 ppm. Based on comparisons with the relative intensity of the methyl proton signal to that of the anomeric proton signals, approximately half of the galacturonic acid was the methyl ester (Figure 5).

The chemical shifts obtained by ^{13}C NMR analysis of Fraction V were similar to that of polygalacturonic acid (*15,16*). Four kinds of anomeric carbon signals were detected at 99.2, 99.6, 100.3, and 100.6 ppm. The intensity of the signal detected at 99.2 ppm was higher than that of the other anomeric signals. Based on the observed chemical shifts of anomeric carbons, saccharide residues were assigned an alpha anomeric configuration. Three kinds of methyl carbon signals characteristic of galacturonic acid methyl ester were detected at 52.5, 52.8, and 53.1 ppm. Signals of carbons thought to be of carboxylic acid (around 175 ppm) and methyl esterified carboxylic acid (around 171 ppm) were also detected (Figure 5).

3.7. *Effect of β-glucanase on the activity of green tea*

β-glucans are major cell wall components of plants and fungi. We previously reported that yeast β-glucans induce muscle contraction in silkworms (*9*). To test whether the purified fraction from green tea leaves contained a β-glucan structure that contributes to its activity, we

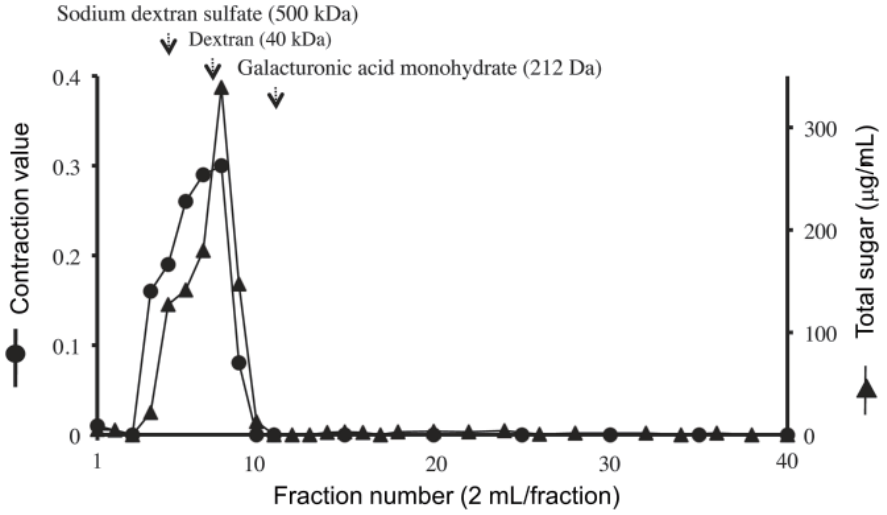

Figure 3. Elution profile of gel filtration with Superdex 200. Silkworm muscle contraction assay was performed by injecting 50 μL of each sample (concentrated 6.5-fold).

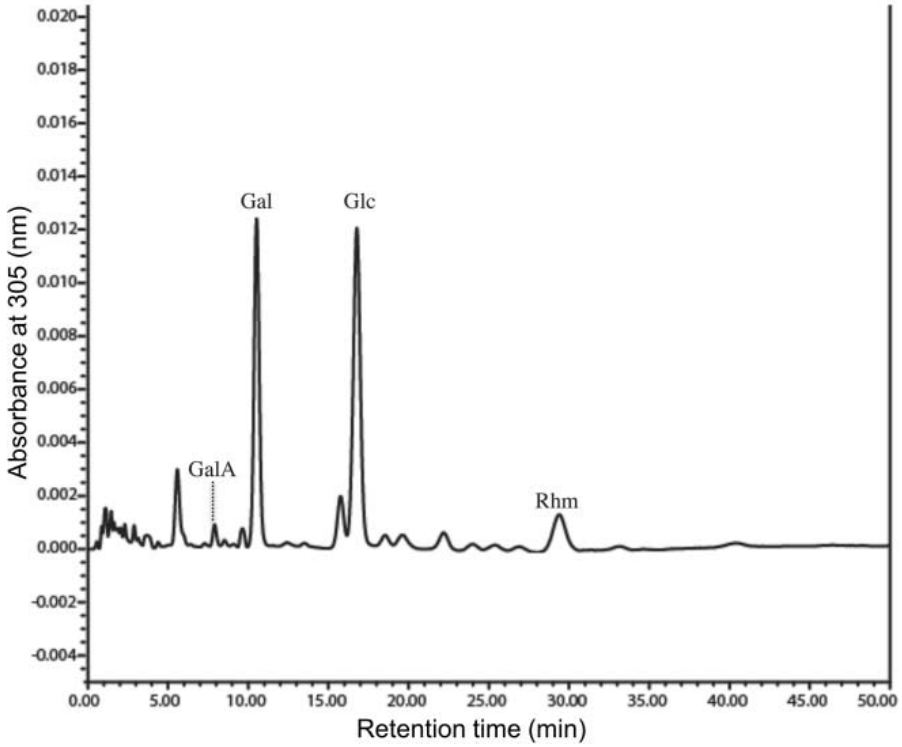

Figure 4. HPLC analysis of acid hydrolyzed monosaccharides from green tea. Acid hydrolyzed monosaccharides labeled with aminobenzoic acid ethyl ester were analyzed by RP-HPLC.

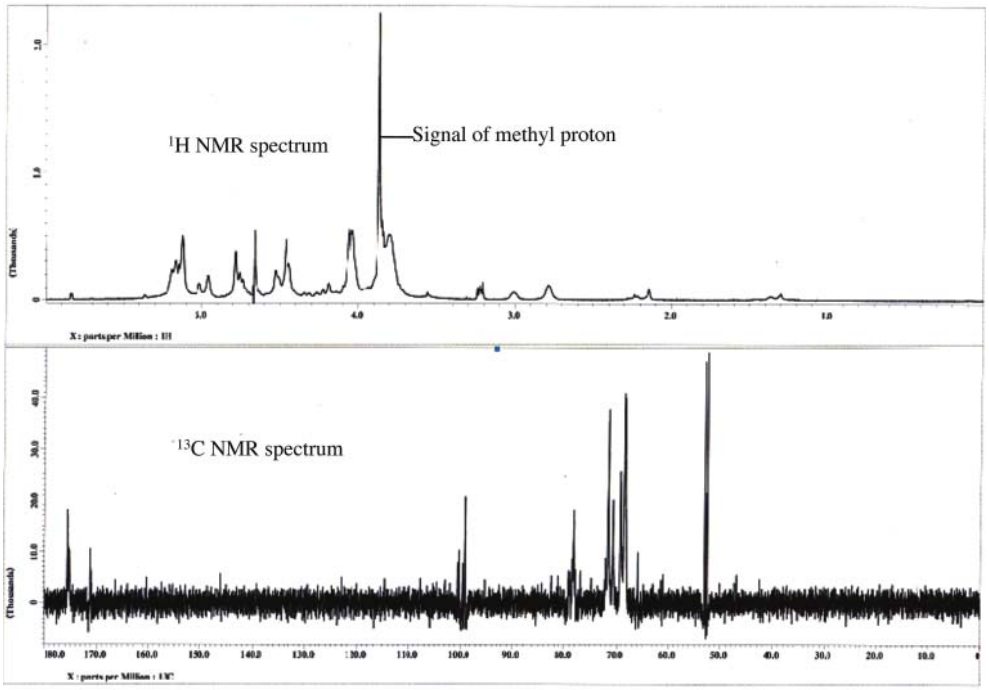

Figure 5. ^{1}H NMR and ^{13}C NMR spectrum of the purified substance from green tea. Fraction V (11 mg) dissolved in deuterium oxide (D_2O), was analyzed by ^{1}H and ^{13}C NMR (ECA-500, JEOL).

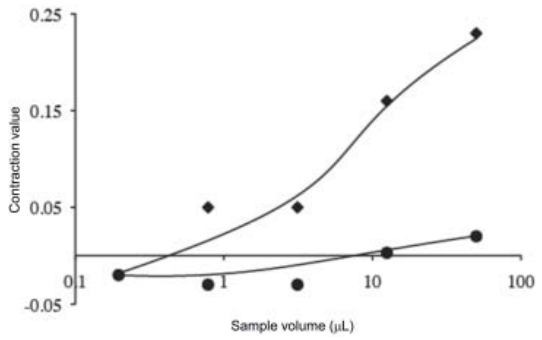

Figure 6. Effect of 1,3(4)β-glucanase on green tea Fraction IV. Fraction IV (5 mg) was incubated with (closed circles) or without (closed diamonds) 11 mg of 1,3(4)β-glucanase from *A. niger* in a sodium citrate buffer (pH 5.0) at 25°C for 6 h. After the incubation, the enzyme was inactivated by boiling at 100°C for 5 min. Immunostimulation activity was measured using the silkworm muscle contraction assay.

examined the sensitivity of the fraction to β-glucanase. The result of the muscle contraction assay indicated that β-glucanase treatment of the purified fraction (Fraction IV) of green tea abolished the innate immunostimulation activity in silkworm. In contrast, the sample treated under the same condition without β-glucanase induced muscle contraction of the larval specimen (Figure 6). These findings demonstrated that the immunostimulation activity of the purified material was sensitive to β-glucanase from *A. niger*.

3.8. *IL-6 production by mouse macrophages stimulated by polysaccharide purified from green tea*

Silkworm larvae muscle contraction was used to evaluate the innate immunostimulating activity of the samples

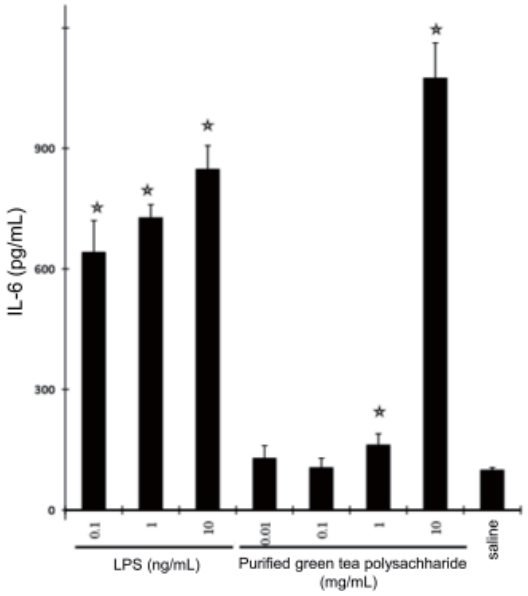

Figure 7. Induction of IL-6 production by mouse peritoneal macrophages incubated with purified compounds from green tea. Mouse peritoneal macrophages stimulated with thioglycollate (4%) from C57BL/6 male mice were incubated with different concentrations of samples for 24 h. The amount of IL-6 in the supernatant of culture medium was measured by ELISA. Data represent mean ± S.D. of 3 experiments (★, $p < 0.05$ compared with saline).

during the purification process. Whether the purified fraction stimulates innate immunity in the mammalian system remained to be clarified. To address this question, we examined the effect of the purified fraction (Fraction V) on mouse macrophages. The amount of IL-6 produced by mouse peritoneal macrophages was significantly increased by incubation with Fraction V (Figure 7). These results suggest that the innate immunostimulants purified

using the silkworm system also stimulate innate immunity in mammalian systems. The specific activity of Fraction V in terms of IL-6 production by mouse macrophages, however, was much less than that of LPS.

4. Discussion

The innate immune system has a vital role in eliminating microbial invaders in insects that lack an adaptive immune system. Based on our previous report demonstrating that an insect cytokine paralytic peptide induced both muscle contraction and defensive reactions in immune cells (*9*), we hypothesized that the immunostimulatory activities of foreign compounds could be assessed using a silkworm muscle contraction assay. Here, using green tea (*C. sinensis*) leaves as a starting material, we demonstrated that this silkworm muscle contraction assay system could be utilized for the purification of immunostimulants.

The active immunostimulating substance was fractionated by successive chromatographies of DEAE-cellulose, Mono Q, and gel filtration. The results indicated that these procedures produced little increase in the specific activities of the substances, suggesting that the active materials were almost pure. Since DEAE-cellulose column chromatography did not increase the specific activity, this step could omit for the purification of innate immunity stimulant of green tea. The results further indicated that the innate immunostimulating factor in green tea had an acidic nature, since the substance was absorbed on DEAE-cellulose column. Previous studies demonstrated that acidic polysaccharides derived from plants activate innate immunity in mice. Yang *et al*. reported that an acidic polysaccharide isolated from the roots of *Angelica sinensis* activated macrophages, resulting in the increased production of nitric oxide (*14*). Further comparative analysis is required to identify the relevance of the acidic nature to immunostimulatory activities of the acidic polysaccharides from different plant sources.

Structural analysis of the innate immunostimulant purified from green tea extract was performed by monosaccharide composition analysis and NMR. The ^{1}H and ^{13}C NMR results allowed us to identify the chemical-shift of each atom. The main structures of the purified green tea fraction were polygalacturonic acid and methyl ester residues. The sensitivity of this fraction to β-glucanase (*A. niger*) is likely due to the presence of an endo-1,3(4)β-glucanase-sensitive structure with the main polygalacturonic acid backbone, which is responsible for the biologic activity to stimulate silkworm innate immunity. Apple pectin, another acidic polysaccharide with a polygalacturonic acid backbone, had a low stimulatory effect in the silkworm muscle contraction assay (unpublished data). Based on these findings, we propose for the first time that modification of polygalaturonic acid backbones with a β-glucan structure is critical for the immunostimulatory activity.

Purified substances from green tea stimulated mouse macrophages to produce IL-6, suggesting that the purification method using silkworms could be applied to search for immunostimulants that act on mammalian cells. Because LPS do not cause silkworm muscle contraction even at high doses (unpublished data), we assumed that the activity of the purified sample observed in the silkworm system was not due to LPS contamination. On the other hand, because of the high sensitivity of mouse macrophages, we could not exclude the possibility that a trace amount of LPS contamination stimulated the IL-6 production.

β-glucans are recognized by several immune receptors such as Toll-like receptor 4, Toll-like receptor 2/6, Dectin-1, specific intercellular adhesion molecule-3-grabbing non-integrin 1, complement receptor 3, lactosylceramide, and β-glucan recognizing proteins. β-glucans trigger the activation of a group of immune cells, including macrophages, neutrophils, monocytes, natural killer cells, and dendritic cells, in mammals (*17*). Because some of the microbial recognition mechanisms are conserved among species, we propose that the purified fraction from green tea leaves might stimulate innate immunity in both insects and mammals *via* common mechanisms. Future studies to identify the receptors and signaling molecules involved in stimulating innate immunity will help to clarify this point.

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