Original Article

DOI: 10.5582/ddt.2017.01044

Structural analysis of an innate immunostimulant from broccoli, *Brassica oleracea* var. *italica*

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Summary **Vegetables are eaten as part of a healthy diet throughout the world, and some are also applied topically as a traditional medicine. We evaluated the innate immunostimulating activities of hot water extracts of various vegetables using the silkworm muscle contraction assay system, and found that broccoli, *Brassica oleracea* var. *italica*, contains a strong innate immunostimulant. We purified the innate immunostimulant from broccoli, and characterized the chemical structure by chemical analyses and NMR spectroscopy. The innate immunostimulant comprised galacturonic acid, galactose, glucose, arabinose, and rhamnose, and had a pectic-like polysaccharide structure. To determine the structural motif involved in the innate immunostimulating activity, we modified the structure by chemical and enzymatic treatment, and found that the activity was attenuated by pectinase digestion. These findings suggest that a pectic-like polysaccharide purified from broccoli has innate immune-stimulating activity, for which the polygalacturonic acid structure is necessary.**

Keywords: Innate immune stimulating activity, pectic polysaccharide, polygalacturonic acid, silkworm muscle contraction assay, structure, vegetable

1. Introduction

Innate immunity is an animal's first line of defense against microbe infection or tumor development. Stimulation of innate immunity may be an effective method of preventing infectious diseases or cancer. An agent that stimulates innate immunity may help to maintain health, especially in aging humans. Medicinal herbs and mushrooms are a potential source of antitumor and immunomodulating agents based on their empirical drug actions, and active substances have been extracted (*1*). Few studies, however, have evaluated the innate immune-stimulating activity of compounds from natural sources by wide screening using a convenient method.

We advocate the use of silkworm larvae, *Bombyx mori*, as an animal model for the discovery of drug candidates (*2*), and previously isolated lysocin E, a bactericidal antibiotic from the cultured supernatant of the soil bacterium *Lysobacter* sp. RH2180-5, using a silkworm model of infection (*3*). Injection of yeast β-glucans and bacterial peptidoglycans into the silkworm *Bombyx mori* induces maturation of the insect cytokine paralytic peptide, which results in muscle contraction of the larvae (*4,5*). We established an assay using the silkworm based on muscle contraction, which is associated with activation of innate immunity (*6*), and purified a polysaccharide with innate immune-stimulating activity from green tea extracts (*7*). We also evaluated the activities of polysaccharides from natural origins using the silkworm muscle contraction assay (*8*). The silkworm muscle contraction assay has several advantages for evaluating innate immunostimulants (*6-8*). The system does not respond to lipopolysaccharide (LPS) due to presence of LPS-absorbing proteins in the hemolymph, and thus potential LPS contamination of the assay is negligible. Furthermore, the method is based on a bioassay using the silkworm body, and compounds with toxic activities or poor pharmacokinetics can be excluded from the candidate compounds.

Vegetables, which contain high amounts of nutrients,

Released online in J-STAGE as advance publication October 11, 2017.

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vitamins, minerals, and dietary fiber, are eaten as part of a healthy diet throughout the world. Some vegetables are also applied topically as a traditional medicine. We hypothesized that vegetables contribute to human health not only through their nutritional value, but also through their innate immunostimulant activity, similar to medicinal herbs. Vegetables are considered harmless to humans, empirically. In the present study, we evaluated the innate immune-stimulating activities of hot water extracts of various vegetables using the silkworm muscle contraction assay system, and found that broccoli, *Brassica oleracea* var. *italica*, contains a strong innate immunostimulant. Furthermore, we purified the innate immunostimulant from a hot water extract of broccoli, and characterized its chemical structure. We also determined the structural motif involved in the innate immunostimulating activity.

2. Materials and Methods

2.1. *Hot water extraction of vegetables*

The vegetables used in this study were purchased from local markets in Japan, and are listed in Table 1. The edible parts of the vegetables were cut into small pieces and autoclaved at 121°C for 15 min in 1 L of water (*7,9*). The sample was cooled and then centrifuged at 8000× g for 10 min at 4°C. The supernatant was lyophilized.

2.2. *Silkworm muscle contraction assay*

Eggs of silkworms (*Bombyx mori*, HuYo Tukuba Ne) were purchased from Ehime Sanshu (Ehime, Japan), and larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 27°C. The silkworm muscle contraction assay was performed to evaluate innate immunity activation as previously described (*4*). Samples were dissolved in sterile saline, and 100 µL of each sample was injected into the body fluid of a specimen. The muscle contraction value was calculated by measuring the maximum length of each specimen before (x cm) and after (y cm) the injection according to the following formula: (x – y)/x. One unit of activity was defined as that causing muscle contraction with a value of 0.15. The specific activities of the samples were determined by creating a titration curve with diluted samples.

2.3. *Purification of an innate immunostimulant from broccoli*

Before the hot water extraction described above, edible parts (inflorescences and stems) of broccoli were heated in a microwave oven (600 W) for 5 min, and washed with MilliQ water (*9*). Two volumes of ethanol were added to the hot water extract, and the mixture was centrifuged. Precipitate dissolved in MilliQ water was dialyzed against MilliQ water, and lyophilized. The lyophilized sample was dissolved in 10 mM Tris-HCl buffer (pH 8.0) and subjected to DEAE-cellulose column chromatography (DE-52, Whatman). After washing the column with 10 mM Tris-HCl buffer (pH 8.0), it was eluted with a linear gradient (0-0.4 M) of NaCl in the same buffer. Fractions containing carbohydrate were monitored by the phenol-H_2SO_4 method (*10*), combined, dialyzed against MilliQ water, and lyophilized.

2.4. *Monosaccharide analysis*

Samples were hydrolyzed by 4 M trifluoroacetic acid at 100°C for 3 h. Monosaccharides were labeled with aminobenzoic acid ethyl ester (ABEE) using an ABEE labeling kit (Seikagaku Corporation, Tokyo, Japan), and the ABEE-labeled saccharides were separated on an ODS column (Honenpak C18, 75 mm × 4.60 mm, Seikagaku Corporation, Tokyo, Japan) by high-performance liquid chromatography (HPLC; 1500 HPLC system, Waters, Milford, MA) according to the supplier's instructions.

2.5. *Nuclear magnetic resonance (NMR) experiments*

All NMR spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) with an ECA 500 instrument (JEOL Ltd. Tokyo, Japan). Chemical shifts were given in δ units, with acetone (δ ^{1}H 2.23, δ ^{13}C 31.1) used as an internal reference for samples measured in D_2O solutions. Signals were assigned based on two-dimensional homonuclear correlation spectroscopy, total correlated spectroscopy, heteronuclear multiple quantum coherence, and heteronuclear multiple bond coherence experiments. ^{1}H NMR chemical shifts of overlapping signals were obtained from the center of the cross-peaks in the two-dimensional (2D) spectra.

2.6. *Methylation analysis*

Before the methylation reaction, galacturonic acid in the sample was methyl-esterified, and reduced to (6,6-dideutero)galactose using $NaBD_4$ as described previously (*11*). Methylation of the polysaccharide was performed according to the Ciucanu method using sodium hydroxide and CH_3I (*12*). The methylated polysaccharide was then hydrolyzed, reduced, and acetylated before analysis by gas chromatography/mass spectroscopy (GLC-MS). The GLC-MS analysis was performed with a QP-2010 plus instrument (Shimadzu, Kyoto, Japan) fitted with a fused silica capillary column (Rtx-5, 30 m × 0.25 mm × 0.25 µm; Restek, Shimadzu).

2.7. *Structural modifications of the innate immunostimulant*

The arabinofuranose residue in the sample was hydrolyzed with oxalic acid, selectively (*13*). The

hydrolysate was neutralized, dialyzed against MilliQ water, and lyophilized.

The polygalacturonic acid moiety was digested using pectinase from *Aspergillus niger* (Sigma-Aldrich) according to the manufacturer's protocol. The hydrolyzed material was fractionated by gel-filtration column chromatography on a Bio-Gel P-4 (900 × 15 mmϕ) with 0.2 M acetic acid as the eluent. Fractions containing carbohydrate were monitored by the phenol–H_2SO_4 method, combined, and lyophilized.

3. Results

3.1. *Evaluation of innate immune-stimulating activity of hot water extracts of various vegetables*

The edible parts of 17 vegetables purchased from local markets in Japan were extracted by hot water. The innate immune-stimulating activity of hot water extracts was evaluated by the silkworm muscle contraction assay. The activities varied according to the species of vegetables, and the extract from broccoli, *Brassica oleracea* var. *italica*, exhibited the highest activity among them with 7 units/mg of specific activity (Table 1). Next, we tried to purify the active compound from broccoli.

3.2. *Purification of innate immunostimulant from broccoli*

The innate immunostimulant was purified from a hot water extract of the edible parts (inflorescences and stems) of broccoli by monitoring the silkworm muscle contraction activity. The purification of the innate immunostimulant from broccoli is summarized in Table 2. First, the activity was recovered by ethanol precipitation against the hot water extract. This suggests that the active substrate is a high molecular-weight compound, probably a polysaccharide. Next, we performed DEAE-cellulose column chromatography eluted with a linear gradient of NaCl, and carbohydrate-eluted fractions were detected by the phenol-H_2SO_4 method. This procedure produced two major peaks at 0 M (non-absorbed fraction) and ~0.2 M concentration of NaCl (acidic fraction, AF), and the fractions contained in these peaks were combined, dialyzed, and lyophilized (data not shown). The activity was recovered in both peaks. Because the AF may comprise a homogeneous polysaccharide as described below, we characterized this fraction further. Analysis of the non-absorbed fraction may be presented elsewhere. The specific activity was increased in the AF as 130 units/mg (Table 2).

Table 1. Muscle contraction activity of hot water extracts of various vegetables

Vegetables	Specific activity (units/mg)
Allium fistulosum (Leek)	1
Allium sativum (Garlic)	0.0
Brassica oleracea var. *capitata* (Cabbage)	< 0.4
Brassica oleracea var. *italica* (Broccoli)	7
Brassica rapa var. *glabra* (Chinese cabbage)	0.3
Capsicum annuum var. *grossum* (Green pepper)	0.3
Cucumis sativus (Cucumber)	0.8
Cucurbita moschata (Pumpkin)	< 0.1
Daucus carota (Carrot)	0.6
Lycopersicum esculentum (Cherry tomato)	0.5
Petroselinum crispum (Parsley)	0.7
Pisum sativum (Pea)	< 0.2
Raphanus sativus var. *longipinnatus* (Japanese radish)	0.2
Siraitia grosvenorii (Arhat fruit)	0.6
Solanum melongena (Eggplant)	0.5
Spinacia oleracea (Spinach)	< 0.2
Zingiber officinale (Ginger)	< 0.3

3.3. *Structural analysis of the innate immunostimulant from broccoli*

The monosaccharide content of the AF was determined by trifluoroacetic acid hydrolysis followed by HPLC analysis. Consequently, galacturonic acid (GalA), arabinose (Ara), galactose (Gal), rhamnose (Rha), and glucose (Glc) were detected (Figure 1) in molar ratios of 12: 7.3: 4.9: 1.2: 1.0. These results suggest that the structure of the innate immunostimulant in the AF is a pectic-like polysaccharide (*14*).

We performed further structural analysis by NMR. ^{1}H and ^{13}C NMR analysis showed that the pattern of detected signals in the AF was a polysaccharide, not a protein or lipid (Figure 2). Assignments of major signals could be made from 2D NMR experiments (Table 3). The spin system of the GalA residue, the most abundant constituent in AF according to monosaccharide analysis, was strongly detected. The chemical shifts of the anomeric proton and carbon of GalA were detected at 5.07 and 99.8 ppm, respectively, suggesting that the GalA residue has an α configuration. The chemical shifts of this spin system revealed that the GalA residue exists in this fraction as α-1,4-polygalacturonic acid with a pyranose form (*15,16*). Methyl signals of the GalA methyl ester, a characteristic structure of homogalacturonan in a plant pectin, were not detected.

Table 2. Summary of the purification of the innate immunostimulant from broccoli

Fraction	Total activity (units)	Amount (mg)	Specific activity (units/mg)
Hot water extract	50,000	800	63
Ethanol extract	11,000	250	44
DEAE-cellulose chromatography (Acidic fraction)	13,000	100	130

The chemical shift of the carbonyl carbon of GalA detected at 176.2 ppm also suggested that the residue is not methyl-esterified (*15,16*). A small amount of acetyl ester was detected at ~2 ppm in ^{1}H NMR and 20 ppm in ^{13}C NMR analysis (Figure 2). The spin system of the Ara residue, the second-most abundant constituent in the AF, was partially assigned, and the chemical shifts of the detected signals revealed that the Ara residue exists in the AF as α-1,5-arabinan with a furanose form (*15,16*). We could not assign the weak signals belonging to the other sugar residues present in small amounts in the AF.

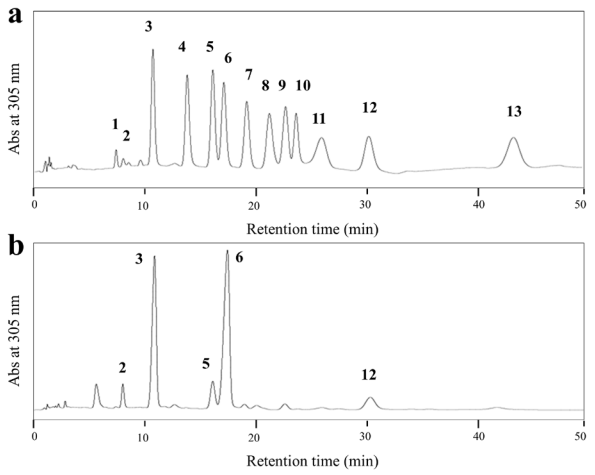

Figure 1. HPLC elution profile of acid hydrolysate of the acidic fraction. Panel **(a)**, standard samples; and **(b)**, AF. Standard samples: 1, D-glucuronic acid; 2, D-galacturonic acid; 3, D-galactose; 4, D-mannose; 5, D-glucose; 6, L-arabinose; 7, D-ribose; 8, *N*-acetyl-D-mannosamine; 9, D-xylose; 10, *N*-acetyl-D-glucosamine; 11, L-fucose; 12, L-rhamnose; and 13, *N*-acetyl-D-galactosamine.

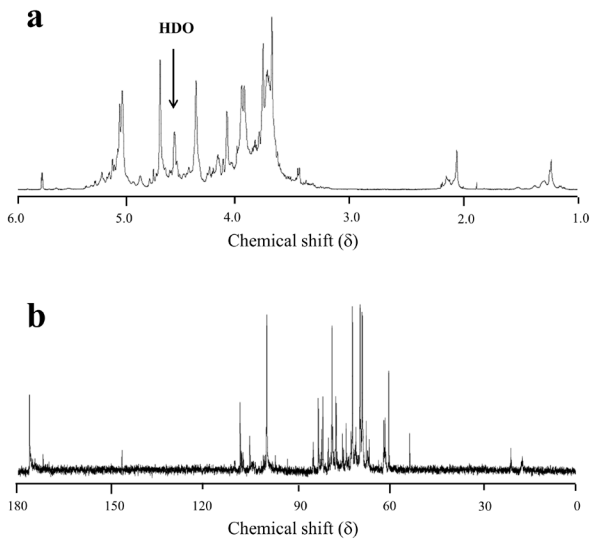

Figure 2. NMR spectra of the acidic fraction. (a), 500 MHz ^{1}H NMR; **(b),** 125 MHz ^{13}C NMR spectrum recorded in D_2O at 40˚C.

We then performed methylation analysis to elucidate the detailed connectivity of sugar residues detected by the monosaccharide analysis. Thirteen peaks of the partially methylated alditol acetate derivatives were detected by gas liquid chromatography-mass spectrometry analysis (Supplementary Figure 1). According to the retention times and mass fragmentation patterns of the detected peaks, the connectivity of the sugar residues was determined (Table 4). The datasets of the detected sugar connectivity suggest that the structure of the innate immunostimulant in AF is a pectic-like polysaccharide, composed of a homogalacturonan with a rhamnogalacturonan I (RG-I) structure, as described below. Based on the pectin structure reported previously (*14*), we propose the structural model of the innate immunostimulant from broccoli shown in Figure 3. The abundance of 4-Gal*p*A and 5-Ara*f* residues was consistent with the results of the NMR analysis, and these results suggest that the 4-Gal*p*A residue has an α-1,4-polygalacturonic acid structure, and the 5-Ara*f* residue has an α-1,5-arabinan structure. Detection of the 2,4-Rha*p* residue suggests that the AF has an RG-I structure; *i.e.* →4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→, with the Rha residue substituted for by galactan and arabinan chains at the C-4 position (*14,17*). The presence of T-Ara*f*, 3-Ara*f*, and 3,5-Ara*f* residues suggest that the α-1,5-arabinan chains are partially branched at the C-3 position (*14,17*). Detection of T-Gal*p*, 4-Gal*p*, 3-Gal*p*, 6-Gal*p*, and 3,6-Gal*p* suggest the presence of β-1,4-galactan and β-1,3/6-galactan structures in the AF (*14,17*). We cannot propose the binding sites of the T-Rha*p* and T-Glc*p* residues detected as a minor components based on the methylation analysis.

3.4. *Effects of structural modifications on the activity of the innate immunostimulant from broccoli*

To determine the structural motif involved in the innate immunostimulating activity, we modified the structure of the innate immunostimulant chemically or enzymatically. The strategy used to modify the polysaccharide

Table 3. ^{1}H and ^{13}C NMR chemical shifts (ppm) of the acidic fraction recorded in D_2O at 40˚C

Glycosyl residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6
→4)-α-Gal*p*A-(1→	5.07	3.76	3.98	4.41	4.73	-
	99.8	69.1	69.8	78.8	72.2	176.2
→5)-α-Ara*f*-(1→	5.09	4.13	4.02	4.21	3.84	-
	108.3	NA	NA	NA	61.7	-

NA, not assigned.

Table 4. Methylation analysis of the acidic fraction

Peak[a]	Derivatives	Structural feature	Mol %
1	1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl arabitol	T-Ara*f*	5.7
2	1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol	T-Rha*p*	1.3
3	1,3,4-tri-*O*-acetyl-2,5-di-*O*-methyl arabitol	3-Ara*f*	1.0
4	1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl arabitol	5-Ara*f*	16
5	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	T-Glc*p*	3.7
6	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl galactitol	T-Gal*p*	3.6
7	1,3,4,5-tetra-*O*-acetyl-2-mono-*O*-methyl arabitol	3,5-Ara*f*	4.4
8	1,2,4,5-tetra-*O*-acetyl-3-mono-*O*-methyl rhamnitol	2,4-Rha*p*	3.2
9	1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl (6,6-dideutero)galactitol[b]	4-Gal*p*A	46
10	1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol	4-Gal*p*	5.1
11	1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol	3-Gal*p*	3.7
12	1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl galactitol	6-Gal*p*	1.5
13	1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl galactitol	3,6-Gal*p*	4.5

[a]Detected peaks were numbered in Supplementary Figure 1. [b]Before the methylation reaction, galacturonic acid residue in the acidic fraction was methyl-esterified, and reduced to (6,6-dideutero)galactose using $NaBD_4$.

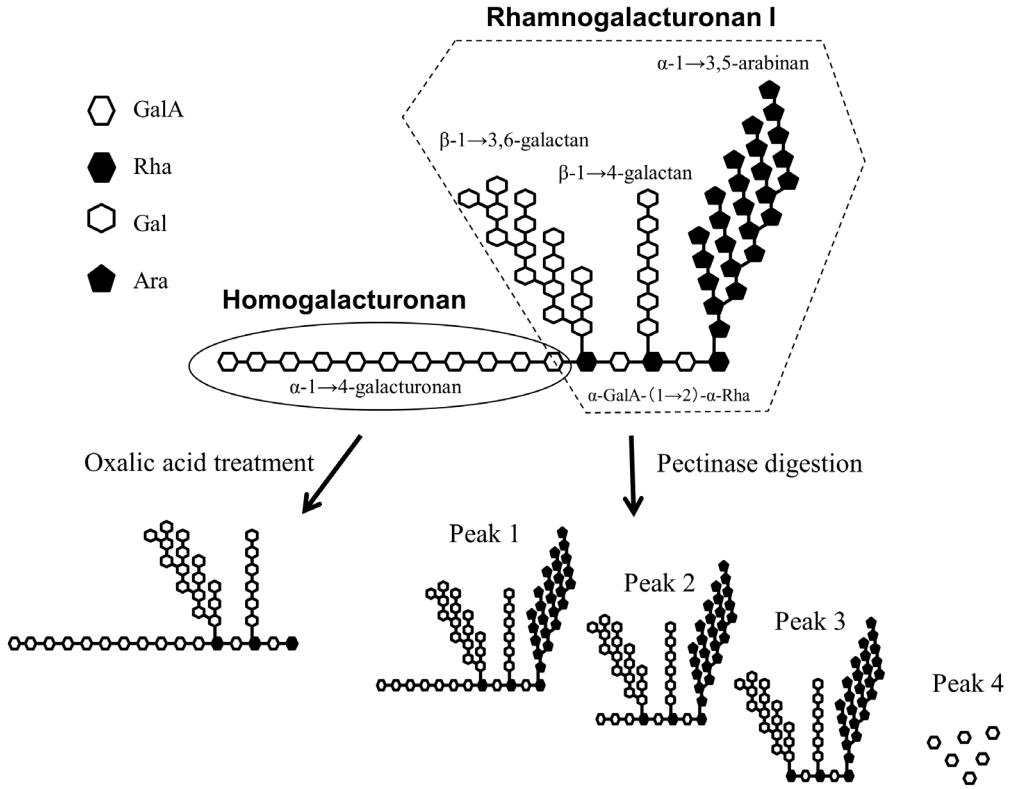

Figure 3. Structural model of the acidic fraction and scheme of the structural modifications. A schematic model of the structure of the acidic fraction and its structural modifications.

structure is shown in Figure 3. α-1,4-Polygalacturonic acid and arabinan are the main structures of the native polysaccharides, and thus we attempted to remove that moiety. Firstly, oxalic acid treatment was performed to remove the arabinan chain from this polysaccharide by specifically hydrolyzing the arabinofuranose bond. NMR analysis of the oxalic acid-treated fraction revealed that the signals of the Ara residue detected in the native polysaccharide (Table 3) were completely abolished (Figure 4). Monosaccharide analysis of this fraction also revealed that the Ara residue in the fraction was drastically reduced compared to the native polysaccharide (Table 5). The innate immunostimulating activity of the fraction was evaluated using the silkworm muscle contraction assay, and the activity was not affected by oxalic acid treatment (Table 5). These findings suggest that the arabinan structure existing in the AF does not contribute to the innate immunostimulating activity.

Next, pectinase digestion was performed against the native polysaccharide to degrade the α-1,4-polygalacturonic acid structure. The digested sample was fractionated by gel filtration chromatography (Bio-Gel P-4) and carbohydrate-containing fractions were yielded as four peaks numbered from the first eluted one

shown in Figure 5. Monosaccharide analysis of each peak indicated that the molar ratio of the GalA residue contained in peaks 1 and 2 was reduced compared to that of native polysaccharide (Table 5). The GalA residue contained in peak 3 was undetectable. Peak 4 was a monosaccharide-containing fraction, and because the peak only consisted of GalA, it was released by pectinase digestion. Compared with the native polysaccharide, the innate immunostimulating activity was attenuated along with a decrease in the molecular weight by pectinase digestion, and that of peaks 3 and 4 was undetectable. These results suggest that the α-1,4-polygalacturonic acid structure in the AF is necessary for the innate immunostimulating activity.

4. Discussion

In the present study, we screened an innate immunostimulant from hot water extracts of 17 vegetables using the silkworm muscle contraction assay, and found that broccoli extracts had the highest immunostimulant activity among them. Broccoli is an attractive vegetable for human health, and other bioactive compounds have been extracted, such as sulforaphane, which has anticarcinogenic activities (*18*). Recently, a polysaccharide having anti-cancer cell proliferation properties was also purified from the broccoli stem (*19*). Innate immune-stimulating compounds from broccoli, however, have not been reported. White cabbage (*Brassica oleracea* var. *capitata*, cultivar Bartolo), kale (*B. oleracea* var. *Sabellica* cultivar green Moskruset), and red kale (*B. oleracea* var. *Sabellica* cultivar Redbor), related subspecies of broccoli, contain a pectic polysaccharide that activates the complement system, which plays an important role in innate immunity (*20*). In the present study, hot water extract of cabbage cultivated in Japan (*B. oleracea* var. *capitata*), which is also a related subspecies of broccoli, did not exhibit innate immune-stimulating activity in the silkworm muscle contraction assay. The activities varied according to the vegetable subspecies.

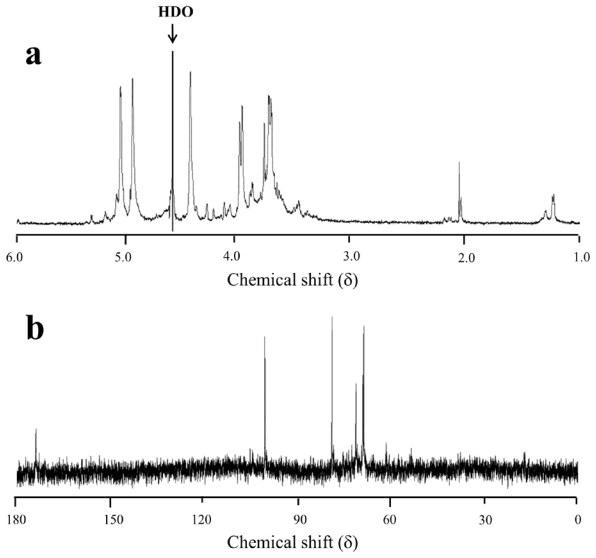

Figure 4. NMR spectra of the oxalic acid-treated acidic fraction. (a), 500 MHz ^{1}H NMR; **(b)**, 125 MHz ^{13}C NMR spectrum recorded in D_2O at 40˚C.

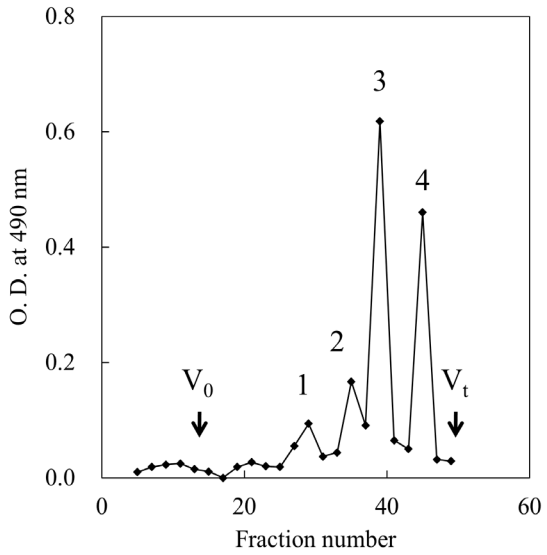

Figure 5. Gel filtration column chromatography of the pectinase-digested acidic fraction. The sample was applied to a Bio-Gel P-4 gel filtration column (1150 mm × 15 mm ϕ), with 0.2 M acetic acid as an eluate. Blue dextran 2000 was used as a size marker to estimate the void volume (V_0), and Vt indicates the total volume of the gel. Fractions containing saccharide were monitored by the phenol–H_2SO_4 method.

Table 5. Effects of structural modifications on the muscle contraction activity of the acidic fraction

Samples	Sugar composition (mol %)						Activity (units)
	GalA	Gal	Glc	Ara	Xyl	Rha	
Acidic fraction	46	18	3.7	27	ND	4.5	630
Oxalic acid treatment	30	45	5.5	3.2	3.6	12	670 <
Pectinase							
Peak 1	23	22	5.0	28	5.0	18	370
Peak 2	24	24	6.0	17	5.5	24	240
Peak 3	ND	36	5.7	47	ND	12	< 160
Peak 4	100	ND	ND	ND	ND	ND	< 160

Ara, arabinose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; Rha, rhamnose; Xyl, xylose. ND, not detected.

We purified the innate immunostimulant from hot water extract of broccoli, and characterized the chemical structure as a pectic-like polysaccharide, comprising homogalacturonan with an RG-I structure (structural model shown in Figure 3). Pectin is a structurally complex polysaccharide in plant cell walls that comprises mainly homogalacturonan, RG-I, and rhamnogalacturonan II (RG-II), and the distribution of each type of polysaccharide varies by the plant species (*14*). RG-II has a highly substituted α-1,4-polygalacturonic acid at the C-2 and C-3 positions with various oligosaccharide chains (*14*). Methylation analysis revealed a GalA residue as only a 4-substituted form in the innate immunostimulant purified from broccoli. This finding suggests that this polysaccharide did not contain the RG-II structure.

Pectin exhibits various types of bioactivity, such as immunomodulating activity, and the active moiety necessary to exert the activities is known for some of the types (*21,22*). Almost all active moieties of plant pectin having immunostimulating activity have a galactan or arabinan structure (*21,22*). Because α-1,4-polygalacturonic acid and arabinan were the main structures in the polysaccharide purified in our study, we removed each moiety by chemical and enzymatic treatment, and found that the activity was attenuated by degradation of the α-1,4-polygalacturonic acid structure. To our knowledge, this is the first report that the α-1,4-polygalacturonic acid motif in the pectic-like polysaccharide structure is necessary for the innate immune-stimulating activity. The pectic-like polysaccharide from broccoli may have a novel mode of action to stimulate innate immunity.

Although homogalacturonan is a common structure of plant pectins, it is unclear how the α-1,4-polygalacturonic acid in the broccoli polysaccharide stimulates innate immunity. Previously, we purified a polysaccharide with innate immune-stimulating activity from green tea extracts using the silkworm muscle contraction assay (*7*). This polysaccharide had a pectic-like structure composed of GalA, Gal, Glc, and Rha in a molar ratio of 22: 4: 5: 1. The main structure of this polysaccharide was α-1,4-polygalacturonic acid, like that of the innate immunostimulant from broccoli. Further studies are necessary to determine the structure-activity relations of the α-1,4-polygalacturonic acid in the silkworm muscle contraction assay. Additional studies are needed to determine whether α-1,4-polygalacturonic acid, which induces silkworm muscle contraction activity, exhibits innate immunostimulating activity in a mammalian system.

Acknowledgements

We acknowledge K. Imamura for technical assistance. This study was supported by a grant from the Genome Pharmaceutical Institute, Ltd.

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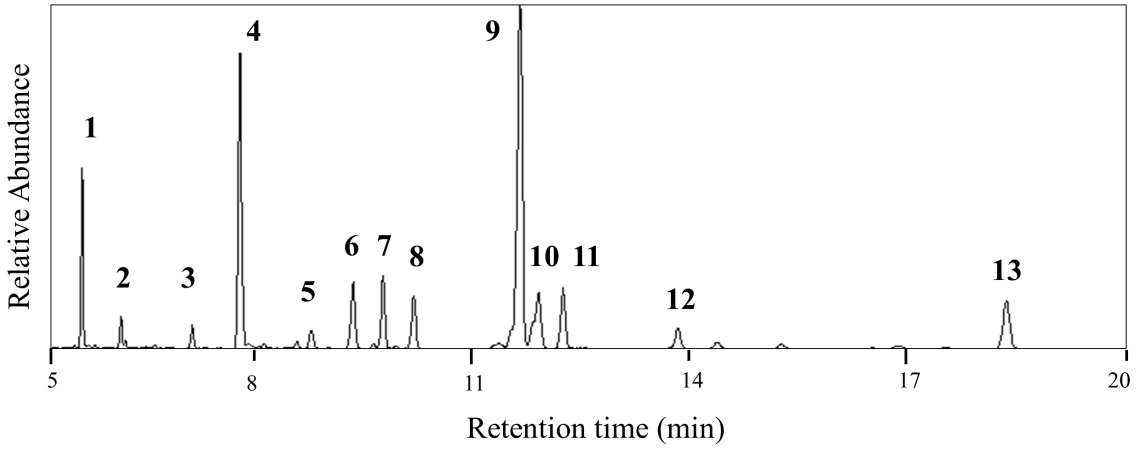
(Received August 14, 2017; Accepted September 10, 2017)

Supplemental Data



Supplementary Figure S1. GLC patterns of partially methylated alditol acetate derivatives from the acidic fraction. 1, 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl arabitol; 2, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol; 3, 1,3,4-tri-*O*-acetyl-2,5-di-*O*-methyl arabitol; 4, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl arabitol; 5, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol; 6, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl galactitol; 7, 1,3,4,5-tetra-*O*-acetyl-2-mono-*O*-methyl arabitol; 8, 1,2,4,5-tetra-*O*-acetyl-3-mono-*O*-methyl rhamnitol; 9, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl (6,6-dideutero)galactitol; 10, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol; 11, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol; 12, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl galactitol; and 13, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl galactitol.