Biochemical Properties of Polysaccharides from Black Pepper

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The purified polysaccharides from *Piper nigrum* were prepared as follows: a hot water extract of pepper seeds was fractionated by ultrafiltration with a 5-kDa-membrane cartridge. A fraction with 5 kDa or bigger molecules was successively purified by open column chromatography on DEAE-Toyopearl 650C and Bio-gel P-60 with each active fraction, resulting in PN-Ib and PN-IIa, purified anti-complementary polysaccharides. None of the anti-complementary activity of any polysaccharide was changed by pronase digestion or polymyxin B treatment, but they were decreased by periodate oxidation. Analysis of component sugar and molecular mass determination of the anti-complementary polysaccharides indicated that PN-Ib with an average molecular mass of 21 kDa contained 88.5% glucose and other negligible minor monosaccharides, while PN-IIa showed a different monosaccharide composition, which contained a significant proportion of galactose, arabinose, galacturonic acid and rhamnose. The molar ratio of galactose and arabinose of PN-IIa (48 kDa) was 1.93 : 1. PN-I did not react with β-glucosyl Yariv reagent, however, PN-IIa did react, which indicated that PN-IIa might be an arabinogalactan. Based upon these results, the usefulness of purified anti-complementary polysaccharides from *Piper nigrum* is suggested as a supplement for immune enhancement.

Key words *Piper nigrum*; anti-complementary activity; arabinogalacta; immune potentiator

In general, the complement system composed of over 20 serum proteins, which are activated by classical, alternative and lectin pathways, plays an important role as a primary defense from external invasion.[1)] The peptides produced by the complement activation can mediate several immune reactions, such as opsonization, activation of lymphocytes and mast cell degranulation.[2)] The materials activating the complement system are therefore apparently to enhance the immunity of human body.

Recent studies have been made on BRMs (biological response modifiers) because of their potential to replace chemical drugs in treating human disease.[3)] Hoping to develop new drugs with no adverse effects, many research groups have been studying biologically active substances. Among them, immune stimulating substances, such as anti-complementary polysaccharide,[4)] macrophage-activating polysaccharide,[5)] B-cell proliferation activating materials,[6)] intestinal immunity-stimulating polysaccharide[7)] and anti-cancer substances[8)] have been studied in various parts of the world.

Black pepper (*Piper nigrum* L.) is a major seasoning of the western world, which are belonged to Piperacea, which is cultivated in Southern Asia. Studies on the biological activities of black pepper have been limited to date: pepper juice was found to enhance intestinal absorption of methionine and calcium ions,[9)] and piperine, the best known compound from an alcohol extract of pepper was reported dose-dependently to inhibit fatty acid oxidation in rat liver microsomes.[10)]

In this study, we purified the immune-enhancing polysaccharides from black pepper and investigated their biochemical properties.

MATERIALS AND METHODS

Materials Black pepper was purchased from Hyangwon Spice Co. (Seoul, Korea), which was authenticated by Dr. Sang-In Shim at the Seed Bank for Wild Herbaceous Plant Species, Korea University. The voucher specimen was deposited at the same institute. Resins of Butyl-Toyopearl 650M and Sephadex G-100 were from Pharmacia, Ltd. (Uppsala, Sweden), and DEAE-Toyopearl 650C and Butyl-Toyopearl 650M from Tosoh Co. (Tokyo, Japan). Bio-gel P-60 was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). β-D-Glucosyl Yariv reagent was obtained from Waters Associates (Milford, MA, U.S.A.). EA (sheep red blood cells sensitized by hemolysin) was obtained from Bio-test Co. (Tokyo). All others chemicals were analytical reagent-grade.

General Analysis Total carbohydrate, uronic acid and protein content were respectively determined by the phenol-sulfuric acid,[11)] *m*-hydroxydiphenyl[12)] and Lowry method[13)] using D-glucose, D-galacturonic acid and bovine serum albumin as the respective standard. Sugar composition of the polysaccharides was analyzed with the alditol acetate derivatives of their acid hydrolysates using gas liquid chromatography (GLC).[14)] Briefly, the monosaccharide alditol acetates were prepared and analyzed by gas chromatography (GC) on a HP-5890 Series II gas chromatograph (Hewlett-Packard, U.S.A.) equipped with a SP-2380 capillary column (0.2 μm film, 0.25 mm i.d.$\times$30 m, Supelco Co., U.S.A.). GC conditions were as follows: injector temperature, 250 °C; splitless mode; carrier gas, He; column temperature, 60 °C for 1 min, 60$\rightarrow$220 °C at 30 °C/min, held for 12 min, 220$\rightarrow$250 °C at 8 °C/min and held for 15 min; and detector temperature, 250 °C. The molar percentage of monosaccharide was calculated from peak areas and the molecular weight of the corresponding alditol acetates. HPLC (Waters 2690 HPLC series) was performed on a column of Shodex OHpak KB-805 (GPC type, 8$\times$300 mm, Toshohaas Co.) eluted with 0.2 M NaCl and monitored with a Waters 410 RI detector. Arabinogalactan moiety was detected by the method of single radial gel diffusion with β-D-glucosyl Yariv reagent.[15)]

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Assay for Anti-complementary Activity To assay the anti-complementary activity by the method of Mayer with some modification,[16] various dilutions of polysaccharides in water (50 μl) were incubated with normal human sera (NHS, 50 μl) and GVB^{2+} (50 μl). The mixtures were incubated at 37 °C for 30 min and the residual total hemolytic complement was determined by using EA cells at 1×10^8 cells/ml. NHS was incubated with water and GVB^{2+} to provide a control. The anti-complementary activity of the polysaccharide was expressed as the percentage inhibition of the TCH_{50} of the control.

Effect of Chemical and Enzymatic Treatment on Anti-complementary Activity The effects of polymyxin B on hemolysis were studied by the method of Morrison and Jacobs.[17] Lipopolysaccharide (LPS) or the polysaccharides were treated with an equal weight of polymyxin B in GVB^{2+} (1 ml) at 37 °C for 30 min. The reaction mixture (50 μl) was used for the anti-complementary assay. To determine an active moiety of the polysaccharides, the anti-complementary fraction (30 mg) was digested with pronase, and oxidized with periodate by the procedure of Yamada *et al*.[18] A control was not treated with polymyxin B, pronase or periodate, respectively.

Isolation of Water-Soluble Polysaccharide from *Piper nigrum* To prepare water-soluble crude polysaccharide from *Piper nigrum* L., pepper seeds (500 g) were refluxed with 10 l of distilled water for 2 h and centrifuged at 5000×***g***. The precipitate was re-extracted by the procedure mentioned above. The supernatant (20 l) was ultra-filtered with a 5-kDa-cellulose membrane catridge by Masterflex prep/scale-tff™ (Millipore Co., Bedford, MA, U.S.A.) to concentrate. The permeate was vacuum-evaporated and lyophilized to obtain PN-5 (molecular mass≦5 kDa). The retentate was precipitated by adding 4-fold volume of ethanol and allowing to stand at 4 °C overnight. Thereafter, ethanol precipitate was redissolved in distilled water and dialyzed against running water for 3 d, and then the non-dialyzable portion (PN-0, 32.4 g) was lyophilized.

PN-0 was purified by successive column chromatographies with active fractions as follows. PN-0 (500 mg) was applied to a column (2.5×20 cm) of DEAE-Toyopearl 650C (Cl^- form). The sample was first eluted with distilled water, and then with a linear gradient of 2.0 M NaCl solution at a flow rate of 2 ml/min. The unabsorbed fraction (PN-I) and three absorbed fractions (PN-II, -III, -IV) were collected, dialyzed and lyophilized. PN-I (5.7 g) and PN-II (6.2 g) with high anti-complementary activity and yield were loaded on a column (2.5×95 cm) of Bio-gel P-60 equilibrated with 50 mM acetate buffer (pH 5.0), and then eluted with the same buffer at a flow rate of 0.2 ml/min. Fractions of 3 ml were collected, and then carbohydrate and UV absorbance of each fraction were analyzed. From size exclusion chromatography, PN-I was divided into PN-Ia and PN-Ia, and PN-II was fractionated into PN-IIa and PN-IIb. The active fractions (PN-Ib, 3.9 g; PN-IIa, 3.2 g) were passed through a respective column of TSK G4000PW (SEC type, 7.5×600 mm) and TSK G5000PW (SEC type, 7.5×600 mm) equipped with a Waters 2690 HPLC system. The molecular mass of each active polysaccharide was determined by comparison with the retention time of standard dextrans under the same conditions.

RESULTS

Isolation of Crude Anti-complementary Polysaccharides The seeds of *Piper nigrum* were refluxed with 10 l of distilled water twice and ultra-filtrated by a 5-kDa-cellulose membrane cartridge to obtain a water-soluble fraction of molecular mass of more than 5 kDa. The filtrated fraction of molecular mass less than 5 kDa was evaporated and lyophilized (PN-5). The non-filtrated fraction was precipitated with 4-fold volume of ethanol and the precipitate was dialyzed and lyophilized (PN-0). PN-0 expressed higher anti-complementary activity than PN-5, although PN-5 showed a higher anti-complementary activity than PSK at 1000 μg/ml (Table 1).

Effect of Chemical and Enzymatic Treatments Because PN-0 was composed of carbohydrate and protein, it was digested with pronase and oxidized with sodium periodate to investigate the real anti-complementary moiety. As shown in Fig. 1, the anti-complementary activity of PN-0

Table 1. Anti-complementary Activity of Subfractions Fractionated from Each Purification Step

	Anti-complementary activity ($ITCH_{50}$ %)[a]
PSK[b]	60.2±1.5
PN-5	56.5±2.5
PN-E	2.2±1.7
PN-0[c]	87.0±3.5*
A. Ion exchange chromatography	
PN-I	92.0±1.3*
PN-II	94.5±2.9*
PN-III	80.5±0.5
PN-IV	71.3±0.1
B. Size exclusion chromatography	
PN-Ia	81.0±1.7
PN-Ib	96.5±2.2*
PN-IIa	98.7±1.9*
PN-IIb	76.8±1.7

a) $ITCH_{50}$ (%): 50% inhibition of total complement hemolysis at 1000 μg/ml. *b*) PSK: polysaccharide-K as a positive reference. *c*) PN-0: Crude polysaccharide isolated from hot-water extract of *Piper nigrum* L. $*p<0.05$: Significantly different from each active fraction of the previous purification step.

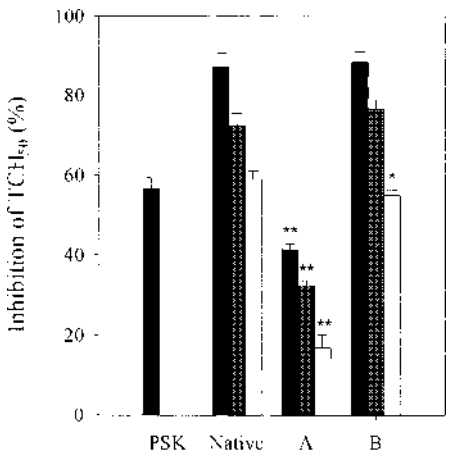

Fig. 1. Anti-complementary Activity of Crude Polysaccharide from *Piper nigrum* after Treatments of Periodate Oxidation and Pronase Digestion

$*p<0.05$ and $**p<0.01$: significant difference between the native sample and the sample treated with periodate or pronase at the same concentration. PSK: polysaccharide-K as a positive reference. A: Periodate; B: Pronase; Native: untreated sample. ■: 1000 μg/ml; ▩: 500 μg/ml; □: 100 μg/ml.

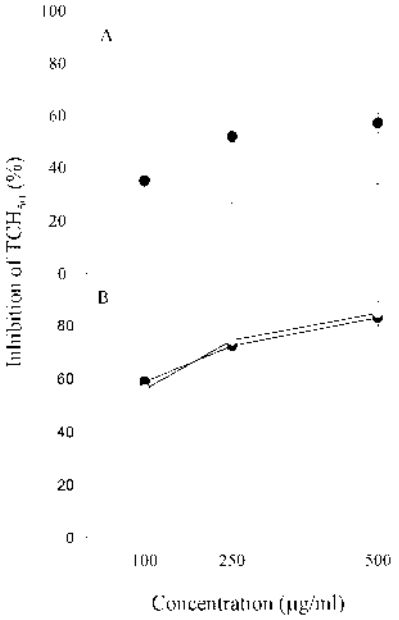

Fig. 2. Effect of Polymyxin B on Anti-complementary Activity of Crude Polysaccharides

LPS (A) and PN-0 (B) were incubated with an equal volume of polymyxin B and the mixture was evaluated for its anti-complementary activity. ○: treated with polymyxin B, ●: control.

was not affected by pronase digestion, but was markedly decreased by periodate oxidation. Therefore, the periodate labile part of the fraction was suggested to be responsible for the anti-complementary activity. The crude fraction (PN-0) was thus considered as a polysaccharide.

Effect of Polymyxin B on the Anti-complementary Polysaccharide Bacterial LPS activates the complement system.[19] LPS is composed of the lipid A region, a core structure and O-specific side chain. It is reported that the lipid A region activating the classical complement pathway is blocked by incubation with polymyxin B, which causes a decrease in the anti-complementary activity of LPS.[17] PN-0 and LPS were incubated with an equal volume of polymyxin B at 37 °C for 30 min, and their activities were evaluated. A remarkable decrease of anti-complementary activity was observed in LPS treated with polymyxin B (Fig. 2A), while there was no change of the activity in PN-0 treated with polymyxin B (Figs. 2B, C). This suggested that the crude polysaccharide had an anti-complementary activity independent of LPS. There is thus no presence of a microbial contamination in the crude polysaccharide.

Purification of Anti-complementary Polysaccharides PN-0 was fractionated by DEAE-Toyopearl 650C and PN-I, -II, -III and -IV were obtained from PN-0 (Fig. 3A). PN-I eluted with distilled water presented only a carbohydrate peak, however, PN-II showed almost the same high peaks of carbohydrate and protein. Later eluted peaks (PN-III and PN-IV) were mainly detected by UV absorbance. Among these fractions, PN-I and PN-II expressed higher anti-complementary activities (>90%) than PN-III and PN-IV, although the latter two showed more potent activity (>70%) than PSK at 1000 μg/ml. The anti-complementary activity of the fractions eluted with low ionic strength (<0.5 M NaCl) expressed the higher activity than those with high ionic strength (Table 1A). The active fractions obtained by ion exchange chromatography, PN-I and PN-II, were further purified by size exclusion chromatography. When PN-I was fractionated by size exclusion chromatography on Bio-gel P-60, a small peak of PN-Ia and a large peak of PN-Ib with low molecular mass were obtained (Fig. 3B). The major fraction, PN-Ib, showed a potent anti-complementary activity, but PN-Ia had a weak activity at 100 μg/ml (Table 1), which was different from the report that the molecular aggregation of the polysaccharide showed higher anti-complementary activity.[20]

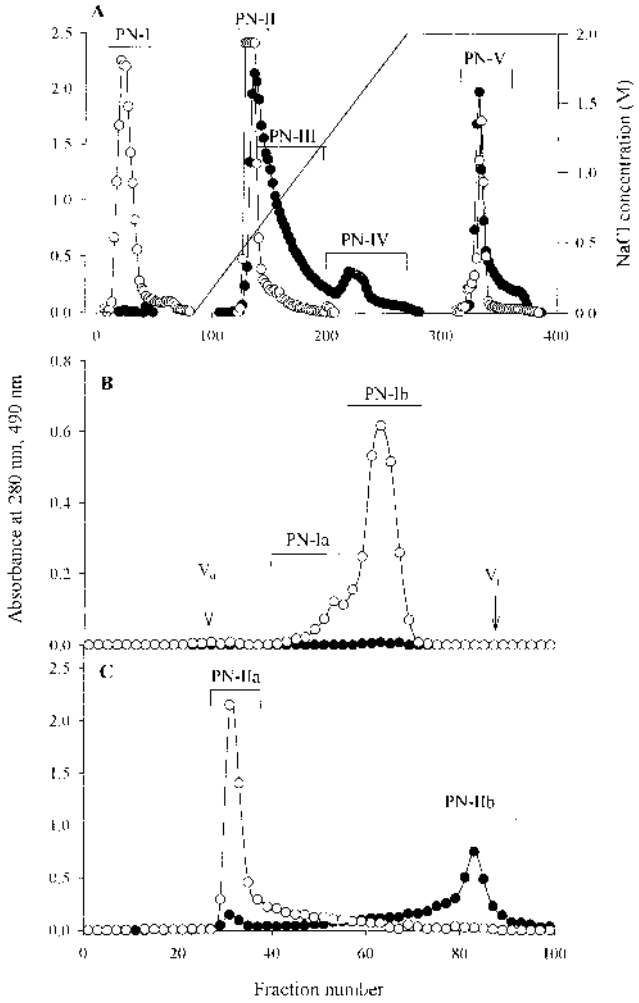

Fig. 3. Chromatograms of Each Subfraction of PN-0 from *Piper nigrum* L.

(A) PN-0 was applied on a column of DEAE-Toyopearl 650C (Cl^- form, 2.5×20 cm) and then eluted with a linear gradient of NaCl solution with a fraction size of 8.0 ml at a flow rate of 2 ml/min. PN-I (B) and PN-II (C) were loaded on a column of Bio-gel P-60 (2.0×90 cm) and eluted with 50 mM acetate buffer (pH 5.0) at a flow rate of 0.2 ml/min. Fractions of 3 ml were collected. V_o: void volume, V_i: inner volume, ○: 490 nm, ●: 280 nm.

Another polysaccharide from *Piper nigrum*, PN-II was also further purified by size exclusion chromatography on Bio-gel P-60 by the above described purification method of PN-I, and two fractions (PN-IIa, PN-IIb) were gained (Fig. 3C). Evaluation of the anti-complementary activities of these fractions showed that PN-IIa, which was eluted near the void volume of the column, had higher anti-complementary activity than PN-IIb at 1000 μg/ml (Table 1B).

Properties of Anti-complementary Polysaccharides Alditol acetate derivatives of anti-complementary polysaccharides were analyzed by GC on a column of SP-2380 using the alditol acetate method to determine structural monosac-

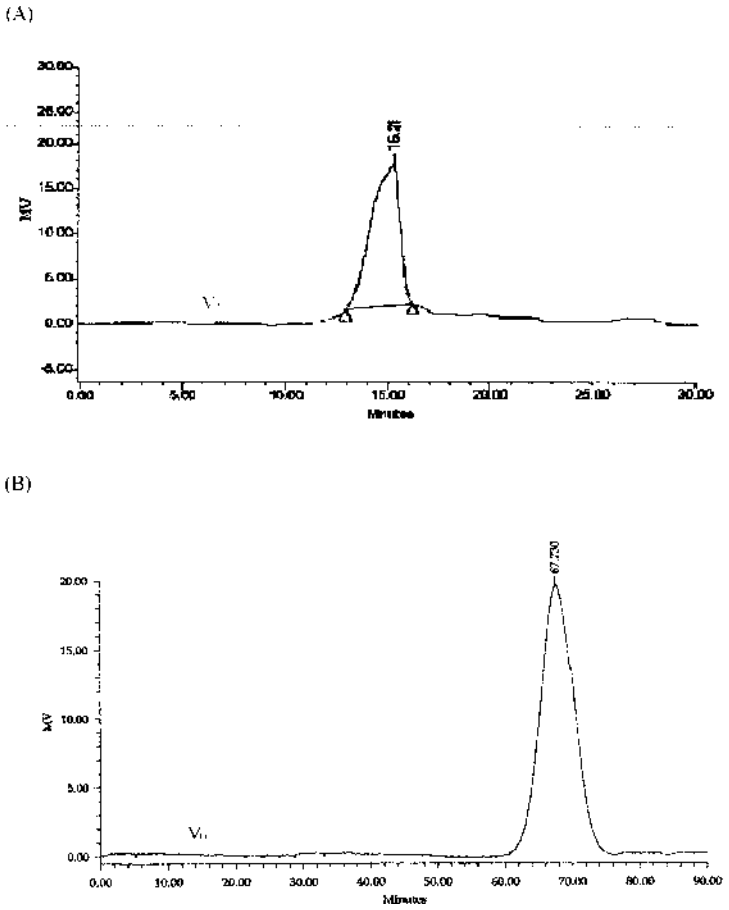

Fig. 4. HPLC Profile of the Purified Polysaccharide from *Piper nigrum*

PN-Ib (A) and PN-IIa (B) were injected into a column of TSK G4000PW (SEC type, 7.5×600 mm) and TSK G5000PW (SEC type, 7.5×600 mm), respectively, and then eluted with 0.2 M $NaNO_3$. The HPLC system was Waters 2690 series equipped with a reflective index (RI) detector.

charides. During the purification steps, PN-I consisted of 92.6% carbohydrate, 7.4% protein, and no uronic acid. Component sugar analysis of PN-I showed that its carbohydrate moiety was composed of 78.2 mol% glucose, 7.1 mol rhamnose and 6.5 mol% arabinose; galactose, mannose, fucose, xylose occupied only a minor portion of PN-I. After size exclusion chromatography, the carbohydrate content increased to 98.4%, and the structural monosaccharides of PN-Ib were primarily 88.5 mol% glucose; the contents of other monosaccharides were negligibe. From the significant increase of glucose content, it appeared that the purified polysaccharide, PN-Ib, might be an anti-complementary glucan (Table 2). On the HPLC profile, the purified polysaccharide appeared as a single peak (Fig. 4A), and the average molecular mass was calculated to be 21 kDa, which was relatively smaller than the previously reported anti-complementary polysaccharides which ranged from 80 to 1000 kDa.[21)]

Contrary to the case of PN-I, PN-II obtained from ion exchange chromatography contained 63.8% carbohydrate and a large amount of protein as well. The significant proportion of galactose, arabinose, galacturonic acid and rhamnose was detected in the carbohydrate portion of PN-II; particularly, the molar ratio of galactose and arabinose was 1.93 : 1. By size exclusion chromatography, the carbohydrate and uronic acid contents of PN-IIa increased to 97.3% and 17.4%, respectively. From these structural monosaccharide contents, it was assumed that PN-IIa might be an arabinogalactan containing acidic monosaccharides (Table 2). PN-I did not show any precipitation line with the reaction to β-glucosyl Yariv reagent, but PN-IIa displayed a distinguished precipitation line, so it was thought that PN-IIa might consist, in part, of arabinogalactan portion (Fig. 5). The purified anti-complementary polysaccharide, PN-IIa, showed a symmetric peak

Table 2. Chemical Properties and Molecular Mass of Each Active Fraction Obtained from *Piper nigrum* L.

	PN-I	PN-II	PN-Ib	PN-IIa
Molecular mass (kDa)	n.d.	n.d.	21.0	48.0
Carbohydrate (%)[a)]	92.6	63.8	98.4	97.3
Uronic acid (%)[b)]	—	6.7	—	17.4
Protein (%)[c)]	7.4	34.4	1.6	2.7
Structural monosaccharide (mol %)				
Rhamnose	7.1	13.1	2.6	12.0
Fucose	1.5	5.8	0.7	5.1
Arabinose	6.5	18.0	2.1	16.5
Xylose	0.8	1.2	0.8	1.1
Mannose	2.2	7.4	1.6	6.8
Galactose	3.6	34.7	3.7	31.9
Glucose	78.2	9.9	88.5	9.1
Galacturonic acid	—	8.5	—	14.9
Glucuronic acid	—	1.6	—	2.6

a) Carbohydrate: α-D-glucose as a reference. *b*) Uronic acid: α-D-galacturonic acid as a reference. *c*) Protein: bovine serum albumin as a reference. —: not determined. n.d.: not determined.

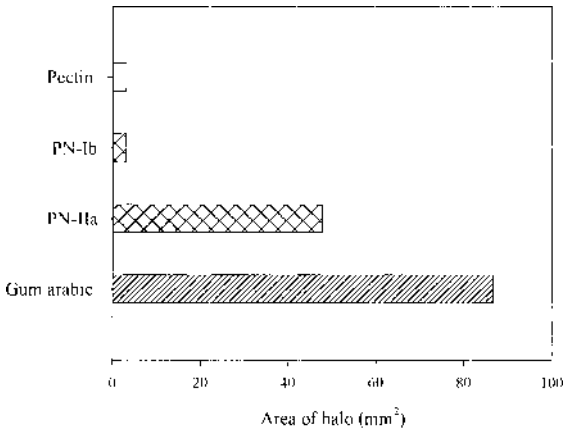

Fig. 5. Reactivity of the Purified Anti-complementary Polysaccharide to β-Glucosyl Yariv Reagent

β-Glucosyl Yariv reagent has specific binding affinity to 3,6-arabinogalactan. Gum arabic and pectin were used as a positive and negative reference, respectively.

on HPLC, and its molecular mass was determined as 48 kDa by comparison with the retention time of the standard dextrans (Fig. 4B).

DISCUSSION

To investigate anti-complementary activity from plants, three hundred extracts from edible plants were examined by 50% inhibition of total complement hemolysis. Black pepper is the most commonly used to be seasoning in the world. In pepper extracts, piperine, a water-insoluble compound, has been reported to be an anti-cancer agent,[21] anti-oxidant and amino acid uptake promoter in intestinal cells.[23]

To optimize the extraction conditions for anti-complementary substances, several solvent extracts were prepared to compare the activity, and hot-water extraction was selected as the optimal extraction condition in spite of the observation of potent anti-complementary activities in organic solvent extracts. Organic solvents are generally used for low-molecular compounds while water is used for high-molecular materials, the type most commonly used for foods. Extracts with organic solvents were suggested to be low molecular compounds: anti-complementary phytosterols,[24] stigmastane, cholestane and pregnone.[25] On the contrary, the hot-water extracts of pepper were postulated to be macromolecules such as polysaccharides. The hot-water extract of *Piper nigrum* was fractionated by ethanol precipitation and ultrafiltration, obtaining crude materials of PN-0. In these material, the protein content was significantly high, thus their active moiety was investigated. Generally, the anti-complementary activities were expressed by the polysaccharide portion of plant extracts. Periodate oxidation is a valuable analytical technique for determination of the active moiety by degradation due to the polysaccharide oxidation. Carbohydrate residue contains glycol groups on adjacent carbon atoms, which are oxidized to dialdehydes. The newly formed aldehyde groups may form hemiacetal bonds with non-oxidized hydroxyl groups.[26] When the crude material was treated with periodate and pronase, the crude polysaccharide showed a reduction in the activity with periodate oxidation, and no changes in the activity digested by pronase. Therefore, PN-0 was considered to be the anti-complementary polysaccharide.

Bacterial LPS easily contaminated the crude materials isolated from natural origins. This lipopolysaccharide can strongly inhibit the complement hemolysis through the alternative and classical pathway. The active moieties of LPS were known to be the lipid A region and O-specific side chain.[19] Prior to purification of the anti-complementary polysaccharides, the crude polysaccharide was incubated with polymyxin B to check for confirming the presence of bacterial LPS in the former. PN-0 showed no change of the activity, confirming that no bacterial contamination was present in the polysaccharide. To purify the anti-complementary polysaccharides, column chromatography such as ion exchange chromatography and size exclusion chromatography were performed with the crude polysaccharide. By sequential column chromatographies, two purified polysaccharides (PN-Ib, PN-IIa) were obtained from *Piper nigrum*.

In discussing the feature of each purified polysaccharide, PN-Ib with an average molecular mass of 21 kDa purified from *Piper nigrum* showed a remarkable increase of glucose content to reach 88.5% after size exclusion chromatography. Other monosaccharides of PN-Ib were negligibe. The biologically active glucans found in nature are primarily β-glucans, which show potent anti-tumor, macrophage-stimulating and anti-complementary activity. According to the report of Kitamura *et al.*[27], β-glucan with an average molecular mass of $<$20000 and no ordered structure shows great anti-tumor activity if the degree of branch is $<$0.25. Yamada *et al.* insisted that the high molecular weight polysaccharides had relatively more potent anti-complementary activity than the low molecular weight ones.[28] PN-Ib in this work agreed with the features of Kitamura, but showed a different result from Yamada, being relatively smaller than the previously reported anti-complementary polysaccharides which ranged from 80 to 1000 kDa.

PN-IIa contained a significant proportion of galactose, arabinose, galacturonic acid and rhamnose; particularly, the molar ratio of galactose and arabinose of PN-IIa was 1.93 : 1. These monosaccharide contents of PN-IIa were comparable to the anti-complementary polysaccharides isolated from *Angelica acutiloba* KITAGAWA, which were grouped in arabinogalactans.[29] The average molecular mass of PN-IIa was measured as 48 kDa, thus the size of molecular mass of the polysaccharides was not considered an essential factor in expressing the anti-complementary activity. The different structural characteristics of the two polysaccharides such as difference of the branch frequency or three-dimensional net work might be involved in showing and differentiating the anti-complementary activity. The component sugar analysis and the reaction to β-glucosyl Yariv reagent indicated that PN-IIa might contain arabinogalactan and acidic polysaccharides. These results were typical in anti-complementary pectic polysaccharides found in *Bupleurum falcatum*,[30] *Glycyrrhiza uralensis*[31] and *Panax ginseng*.[32]

Although anti-complementary polysaccharides of PN-Ib and PN-IIa were isolated from the hot-water extract of *Piper nigrum* at the same time, their constituent sugar composition and molecular mass differed. Therefore, deeper research on the structure and the relationship between structure and activity of the purified polysaccharides from *Piper nigrum* is required, and is in progress.

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