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Extraction, purification and properties of anti-complementary polysaccharide from Arecae Pericarpium

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Abstract : To examine the characteristics of anti-complementary compounds from Arecae Pericarpium (the pericarps of *Areca catechu*) which showed the highest activity during our screening procedures, the extraction and purification were performed. AC-1 fraction from Arecae Pericarpium was purified by hot water extraction, methanol reflux, ethanol precipitation, dialysis and lyophilization. This compound had total sugar 48.2%, uronic acid 14.6% and protein 36.8%. Rhamnose, arabinose, mannose and galactose were found in sugar components. By cetavlon (cetyltrimethylammonium bromide) treatment AC-1 was fractionated to AC-2, AC-3 and AC-4. Among them, AC-2 showed the highest activity and yield. By periodate oxidation, AC-2 was deactivated, but had no change in activity by pronase digestion. Moreover active fractions, AC-2-IIIa and AC-2-IIIc isolated from AC-2 by two successive column chromatography using DEAE-Toyopearl 650C(Cl^-form) and Sephadex G-100. AC-2-IIIa was mainly made up of rhamnose, mannose, galactose and glucose, and AC-2-IIIc, mannose, galactose and glucose. These both polysaccharides were identified as homogeneous by gel filtration of Sepharose CL-4B and electrophoresis, and molecular weights of them were 120,000 and 15,000, respectively(Received July 2, 1992, accepted July 20, 1992).

Complement is a major defence and clearance system in the bloodstream which can be activated *via* immunoglobins once a foreign particle or organism has been recognized by antibody.[1] Direct activation of the system can also take place if the particle provides a suitable site for the amplified self-activiation of the early acting components (C1, C4, C2 and C3). Complement can be activated by two distinct routes, the classical and alternative pathways. The complement C3 is major plasma glycoprotein and it plays a central role in the system being common to both pathways. Components C5~C9 are designated the terminal components which form the MAC(membrane attack complex), which is common to both pathways and which is responsible for target cell damage and lysis.[2]

Several pharmacological activities, especially immunomodulatory activities have been observed in some polysaccharides derived from Chinese medicinal herbs. These polysaccharide fractions have been shown to possess interferon inducing activity,[3] anti-neoplastic activity,[4] lymphocyte mitogenic activity,[5] anti-complementary activity,[6] anti-inflammatory activity,[7] polyclonal B-cell activating activity[5] and phagocytosis potentiating activity.[4] Among them, anti-complementary activity means activation of complement system.

Recently, we have found potent anti-complementary activities in the extracts of some Chinese medicinal herbs, such as in the extracts of the pericarps of *Areca catechu.* Arecae Pericarpium, the pericarps of *Areca catechu* is a well known crude drug

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clinically used in the treatment of beri-beri, dropsy, sunstroke, and so on.[8] Previously, we reported that the hot water extract of Arecae Pericarpium contained potent anti-complementary activity, and the activity was observed in the non-dialyzable carbohydrate-rich fraction.[9] It was suggested that the active principle might be a kind of polysaccharide molecule. The present paper describes the purification and chemical properties of the major anti-complementary polysaccharides from Arecae Pericarpium.

Materials and Methods

Materials

Arecae Pericarpium used for experimental material was purchased at Kyung-Dong market in Korea. Dowex 1×8 resin, pronase and standard dextran(T-2000, T-500, T-70, T-40 and T-10) were purchased from Sigma Co., and IgM haemolysin sensitized sheep erythrocytes(EA) from Nippon Biotest Lab. Inc., Sephadex G-100 and Sepharose CL-4B from Phamacia Co., DEAE Toyopearl 650C from Toyosoda Co. and cellulose acetate strip from Adventec Co. were used in this study. Also normal human sera were prepared freshly in our laboratory.

Analytical procedures

The amounts of total carbohydrate and uronic acid were determined by the phenol-sulfuric acid[10] and *m*-hydroxybiphenyl method.[11] Protein was assayed by the method of Lowry.[12] Gas-liquid chromatography(Shimadsu GC-14A) was equipped a flame ionization detector(FID) and a stainless column(0.2×200 cm) of 3% OV-225 Uniport 100/200 at 210 ℃. Argon was used as a carrier gas at a flow rate of 2 kg/cm^2. After acid hydrolysis of polysaccharides with 2 M trifluoroacetic acid at 121 ℃ for 1 hr, the component aldoses were converted into alditol acetates and analyzed by GC.[13]

Determination of the anti-complementary acitivy

The anti-complementary activity was measured according to previously described procedure.[9]

Preparation of crude polysaccharide from Arecae Pericarpium

Arecae Pericarpium(1 kg) was decocted with water (15 *l*), three times until this volume was reduced by half. The extracts were combined and lyophilized to give a water soluble extract (AC-0). AC-0 was refluxed 5 times with methanol and the precipitate was dissolved in water and 5 volumes of ethanol were added. The precipitate was redissolved in water and dialyzed against running water for 3 days. After the non-dialyzable portion was centrifuged at 4,500×g for 30 min, the supernatant was lyophilized to obtain crude polysaccharide(AC-1). The methanol (AC-M) and ethanol soluble fraction (AC-E) was evaporated to dryness.

Fractionation of the crude polysaccharide, AC-1 with cetavlon

Crude polysaccharide, AC-1 was dissolved in water and treated with an equal volume of 8% cethyltrimethylammonium bromide(cetavlon) by the method of Yamada *et al.*[14] After standing at 20 ℃ for 20 hr, the resulting precipitate was collected by centrifugation and ressolved in 10% NaCl. Five volumes of ethanol were added to the solution, and the resulting precipitate was dissolved in water, followed by dialysis against running H_2O. The acidic polysaccharide fraction(AC-2) was then obtained as the lyophilizate of the non-dialyzable fraction. The supernatant was added to an equal volume of 1% H_3BO_3 solution and the pH was adjusted 8.8 by the addition of 2 M NaOH followed by stirring for 24 hr. The resulting precipitate was washed with 0.5% Na-borate buffer(pH 8.8), and dissolved in 2% acetic acid. Five volumes of ethanol were added to the solution, and the resulting precipitate was dissolved in water, and then dialyzed. The non-dialyzable fraction(AC-3) was obtained as the lyophilizate. The final supernatant of the cetavlon fractionation was acidified with 2% acetic acid, and 5 volumes of ethanol were added. The precipatate was then washed with 2% acetic acid in ethanol and dissolved in H_2O followed by dialysis. The non-dia-

lyzable portion was lyophilized to obtain AC-4.

Ion exchange chromatography

AC-2(200 mg) was applied to a column(3.2×40 cm) of DEAE-Toyopearl 650C(Cl^-) eguilibrated with H_2O. The column was eluted first with H_2O until no sugar was detected, and then the absorbed polysaccharide fractions were eluted with 0.1~2 M NaCl in step wise gradient. The unabsorbed fraction(AC-2-1) and six absorbed fractions after dialysis(AC-2-I~AC-2-VII) were obtained as lyophilizate.

Gel filtration

Gel filtration was carried cut by elution with 0.2 M NaCl solution on Sephadex G-100(2.6×90 cm) and Sepharose CL-4B(1.5×90 cm) at 4 ℃.

Electrophoresis

Electrophoresis on cellulose acetate strip was carried out in 0.08 M pyridine-0.04 M acetate buffer (pH 5.4) at 420 V for 30 min, and the polysaccharide was detected with 0.5 M toluidine blue dissolved in 3% acetic acid.[15]

Determination of molecular weight

Molecular weights of purified polsaccharides were determined by gel filtration on Sepharose CL-4B(1.5×90 cm). Standard dextrans(T-2000, T-500, T-70, T-40, T-10) were used for determination of melecular weight.

Pronase digestion and periodate oxidation of active fraction

Pronase digestion and periodate oxidation of AC-2 were carried out according to the previously described procedure.[9]

Results and Discussion

Extraction of water soluble crude polysaccharide

In order to examine the optimal extraction condition, Arecae Pericarpium were extracted 10 times with hot water. The anti-complementary activity in 100 μg/ml and yield were decreased according to the number of times(Fig. 1), and therefore 3 times of extraction were carried out after this. And then, the crude polysaccharide fraction, AC-1 was obtained by ethanol precipitation and dialysis after methanol reflux of hot water extract(AC-0). The yield of AC-1 was 1.5g from Arecae Pericarpium (1 kg). AC-1 had potent anti-complementary activity, but methanol- and ethanol-soluble materials obtained during the extraction process did not show the anti-complementary activity (Fig. 2). AC-1 had galacturonic acid 14.6%, protein 36.8% and neutral sugar 48.2% which was composed of rhamnose, arabinose, xylose, mannose, galactose and glucose(molar ratio, 0.96 : 1.00 : 0.72 : 0.99 : 1.13 : 0.58)(Table 1).

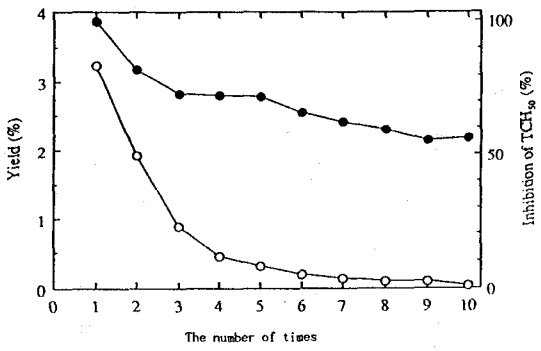

Fig. 1. Anti-complementary activities and yields of hot water extracts from Arecae Pericarpium.
●—●: Anti-complementary activity, ○—○: Yield

Table 1. Yields and chemical properties of polysaccharide fractions on cetavlon treatment of AC-1

(Unit: %)

	AC-1	AC-2	AC-3	AC-4
Total sugar	48.2	40.7	61.2	81.5
Uronic acid	14.6	17.3	9.7	8.9
Protein	36.8	47.8	25.6	9.1
Yield	1.5[a]	57.4[b]	4.8[b]	18.0[b]
Component sugar				(Unit: Molar ratio)
Rhamnose	0.96	1.66	0.81	0.80
Arabinose	1.00	1.00	1.00	1.00
Xylose	0.72	0.52	0.50	0.62
Mannose	0.99	1.19	2.59	0.91
Galactose	1.13	1.81	1.70	1.15
Glucose	0.58	0.94	–	0.64

[a] From Arecae Pericarpium, [B] From AC-1

Fractionation of crude polysaccharide

The crude polysaccharide fraction, AC-1 was fractionated into three fractions, AC-2, AC-3 and AC-4 by cetavlon treatment. Anti-complementary activities of these fractions are presented in Fig. 3. The order of activities of them was AC-2>AC-4>AC-3 in 50 μg/m*l*. The chemical properties of these fractions are summarized in Table 1. High anti-complementary polysaccharide fraction, AC-2 showed highest yield and had a 40.7% neutral sugar, and composed arabinose, galactose, and glucose in the relatively high molar ratio. Also AC-2 contained a large amount of galacturonic acid(17.3%).

Especially, AC-2 was composed of sugar and protein in the approximately same ratio. To determine the real moiety of activity, AC-2 was digested with pronase and oxidized with periodate. The anti-complementary activity of the deproteinized AC-2 by pronase did not change compared with that of AC-2, but the activity of periodate oxidate was found to decrease to 40~60% of that of AC-2(Fig. 4). These results accorded with those of polysaccharides from the leaves of *Artemisia princeps* and the roots of *Angelica acutiloba* L.[16] It is supposed that the carbohydrate moieties in AC-2 may also contribute to anti-complementary activity.

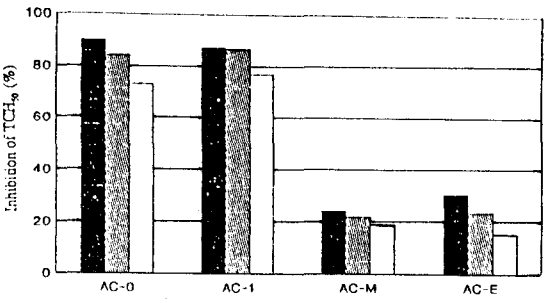

Fig. 2. Anti-complementary activities of crude extracts from Arecae Pericarpium.
■: 1000 μg/m*l*, ▨: 500 μg/m*l*, □: 100 μg/m*l*

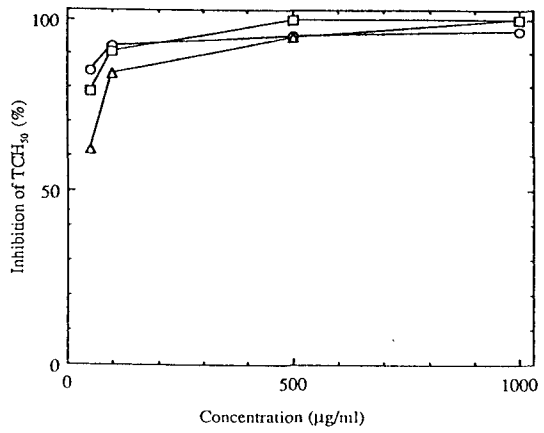

Fig. 3. Anti-complementary activities of polysaccharide fractions on cetavlon treatment of AC-1.
○—○: AC-2, △—△: AC-3, □—□: AC-4

Ion exchange chromatography of AC-2

The acidic polysaccharide fraction(AC-2), showed high anti-complementary activity, was further fractionated on the column of DEAE-Toyopearl 650C (Cl^-)(Fig. 5). One unabsorbed fraction(AC-2-I) and six absorbed fractions(AC-2-II~VII) were obtained as lyophilizates. Fig. 6 shows the anti-complementary activity after the incubation of the different concentrations of the polysaccharide fractions with normal human sera. Among them, the AC-2-III showed marked anti-complementary activity and yield. And AC-2-III had 8.4% of protein and 54.2% of total sugar which was mainly composed of rhamnose and mannose(Table 2).

Purification of anti-complementary polysaccharide, AC-2-III

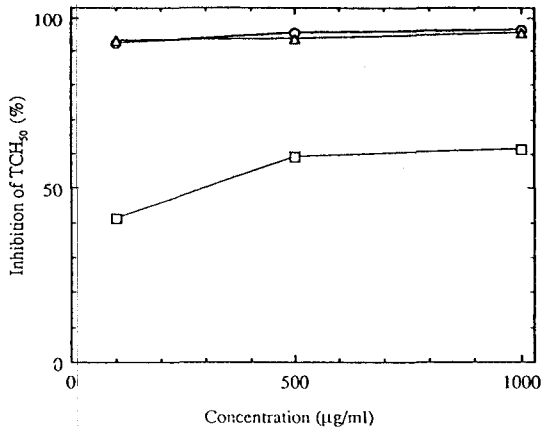

Fig. 4. Anti-complementary activities of pronase digested and periodate oxidized AC-2s.
○—○: AC-2, △—△: Pronase digested AC-2, □—□ Periodate oxidized AC-2

AC-2-III was further purified by gel filtration on Sephadex G-100 under described condition. AC-2-III was fractionated to AC-2-IIIa, IIIb, IIIc and IIId (Fig. 5), and especially AC-2-IIIa and AC-2-IIIc were shown to have the more potent activity and higher yields than AC-2-IIIb and AC-2-IIId(Fig. 7). The colorimetrical assay elution profile of these main polysaccharides showed that there are the fraction of relatively enriched in uronosyl residues(AC-2-IIIc) and enriched in neutral sugars(AC-2-IIIa)(Fig. 5). These two active fractions were eluted as a single symmetrical peak as shown by gel filtration of Sepharose CL-4B and gave a single spot on cellulose acetate membrance electrophoresis(data not shown). These results suggested that two fractions were homogeneous and pure enough for structural studies.

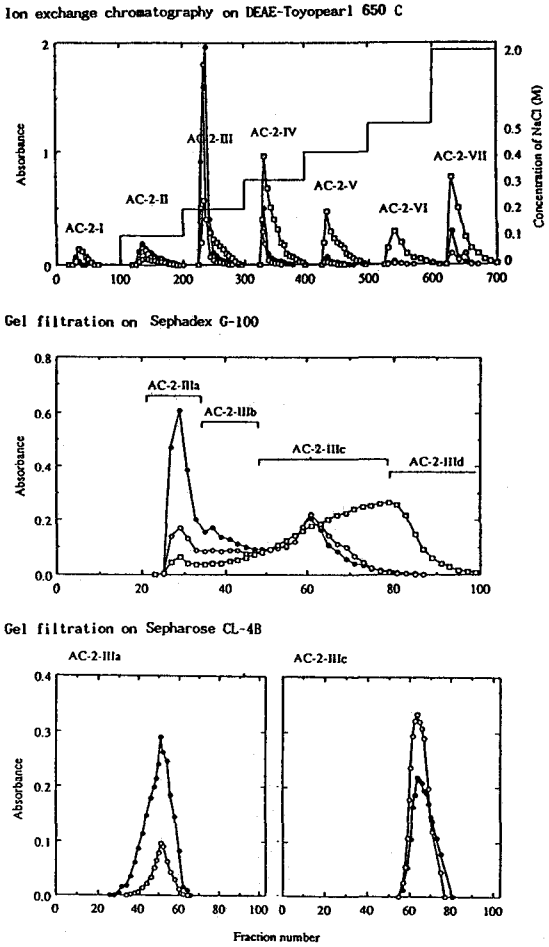

Fig. 5. Purification steps and chromatographies of anti-complementary polysaccharides from Arecae Pericarpium.
●—●: Total sugar, ○—○: Uronic acid, □—□: Protein

Properties of purified anti-complementary polysaccharides

The molecular weights of AC-2-IIIa and AC-2-IIIc were estimated to be 120,000 and 15,000 by calibration of gel filtration on Sepharose CL-4B(0.2 M NaCl)(Fig. 8). AC-2-IIIa contained a more substan-

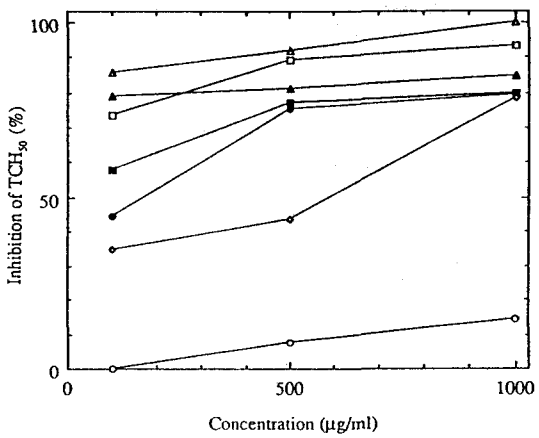

Fig. 6. Anti-complementary activities of AC-2 polysaccharide fractions from ion-exchange chromatography on DEAE-Toyopearl 650C.
○—○: AC-2-I, ●—●: AC-2-II, △—△: AC-2-III, ▲—▲: AC-2-IV, □—□: AC-2-V, ■—■: AC-2-VI, ◇—◇: AC-2-VII

Table 1. Yields and chemical properties of polysaccharide fractions on cetavlon treatment of AC-1

(Unit: %)

	AC-2-III	AC-2-IIIa	AC-2-IIIc
Total sugar	54.2	92.3	69.5
Uronic acid	10.1	7.4	2.9
Protein	8.4	5.6	17.9
Yield	30.5[a)]	30.0[b)]	32.0[b)]
Component sugar			(Unit: Molar ratio)
Rhamnose	1.38	4.04	0.58
Arabinose	1.00	1.00	1.00
Xylose	0.50	1.44	1.43
Mannose	1.11	7.49	2.58
Galactose	1.57	13.70	3.67
Glucose	0.57	9.63	3.49

[a)] From AC-2. [b)] From AC-2-III

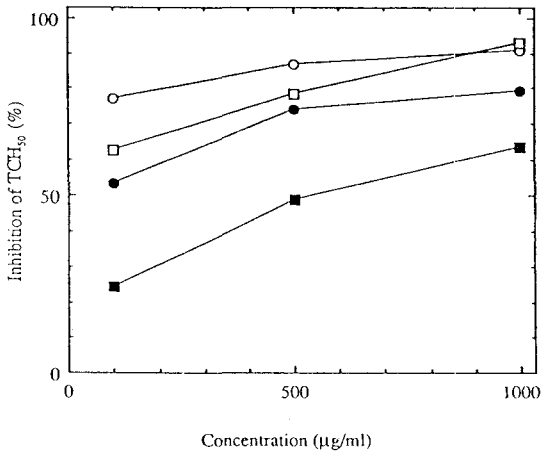

Fig. 7. Anti-complementary activities of AC-2-III polysaccharide fractions from gel filtrantion on Sephadex G-100.
○—○: AC-2-IIIa, ●—●: AC-2-IIIb, □—□: AC-2-IIIc, ■—■: AC-2-IIId

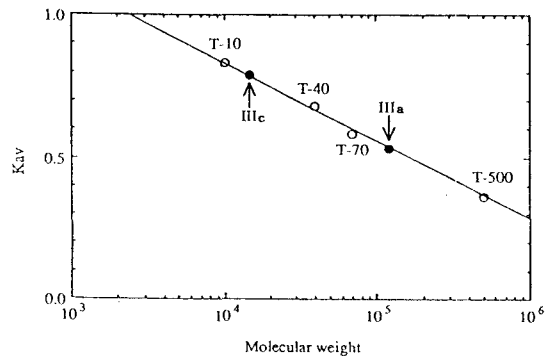

Fig. 8. Determination of molecular weights of AC-2-IIIa and AC-2-IIIc by gel filtration on Sepharose CL-4B.
T-500, T-70, T-40 and T-10 are standard dextrans of 5×10^5, 7×10^4, 4×10^4 and 1×10^4 molecular weight respectively.

$$Kav = \frac{Ve-Vo}{Vt-Vo}$$

(Vo: Void volume, Vt: Total volume, Ve: Elution volume)

tial amount of hexose than AC-2-IIIc. AC-2-IIIa was composed of rhamnose, mannose, galactose, glucose and small amounts of arabinose and xylose, while AC-2-IIIc was mainly composed of mannose, galactose and glucose(Table 2).

The anti-complementary activities of AC-2-IIIa and AC-2-IIIc were more potent than anti-complementary β-1,3 glucan from *Lentinus edodes*(Lentinan), crude AR-arabinogalatan from *Angelica acutiloba* Kitagawa and AAF-IIIb-1, 2 and 3 from *Artemisia princeps*.[16] These facts indicate that the anti-complementary heteroglycan from the pericarps of *A. catechu* plays important role in the effect of Chinese crude drugs, and is also useful for the study of complement system.

Further studies of the structural analysis and mode of anti-complementary acitivity of the unique heteroglycan are now in progress

Acknowledgement

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대복피로부터 항보체 활성다당의 추출, 정제 및 그 특성

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초록 : 선별과정 중 가장 높은 항보체 활성을 보였던 대복피를 대상으로 활성물질의 특성을 조사하기 위하여 추출 및 정제를 행하였다. 대복피를 열수추출, methanol 환류 및 ethanol 침전을 행한 후, 투석 및 동결건조를 거쳐 활성획분 AC-1을 얻었는 바, 이들은 총당 48.2%, 산성당 14.6%, 단백질 36.8%로 구성되어 있었고 주구성당으로 rhamnose, arabinose, mannose 및 galactose를 함유하고 있었다. 이들은 다시 cetavlon(cetyltrimethylammonium bromide) 처리에 의해 AC-2, AC-3, AC-4로 분획되었으며 특히 AC-2의 경우 활성과 수율면에서 우수하였다. 이들은 periodate 산화에 의해 활성의 감소를 일으킨 반면 pronase 소화에 의해서는 활성의 변화가 없는 특성을 보여 주었다. 더우기 AC-2는 DEAE-Toyopearl 650C(Cl^-형) 및 Sephadex G-100을 이용한 연속적인 column chromatography를 통하여 활성이 우수한 AC-2-IIIa와 AC-2-IIIc로 분획되었으며 전자의 경우 rhamnose, mannose, galactose 및 glucose가, 후자의 경우 mannose, galactose 및 glucose가 주구성당이었다. 이들 활성다당들은 gel 여과 및 전기영동 결과, 순수한 물질임이 확인되었으며 이들의 분자량은 각각 120,000 및 15,000 이었다.