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Action modes of the anti-complementary polysaccharides purified from Arecae pericarpium

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Abstract : Two kinds of complement activating (anti-complementary) polysaccharides, which were expected to be immunomodulators were purified from Arecae Pericarpium (the pericarps of *Areca catechu*), and their action modes have been studied. The active polysaccharides, AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium showed dose-dependent anti-complementary activities on TCH_{50}. The anti-complementary activities of AC-2-IIIa and AC-2-IIIc in metal ion-free condition were completely decreased in comparison with control whereas in case of Ca^{2+}-free condition, these activities were maintained, considerably. Also AC-2-IIIa and AC-2-IIIc showed relatively potent alternative complement pathway activities. Furthermore, after incubation of the normal human serum with polysaccharide of Arecae Pericarpium in the absence of Ca^{2+} ion, a cleavage of C3 in the serum was found to have occurred through immunoelectrophoresis (IEP) with anti-human C3. Also, from the results of IEP using anti-human whole serum, the ratios of the height of 3rd peak to α2-M peak by AC-2-IIIa and AC-2-IIIc proved to be 1.50 ± 0.04 and 1.22 ± 0.08, respectively. These results indicate that the modes of complement activation by AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium are *via* both the classical pathway and the alternative pathway(Received August 28, 1992, accepted October 28, 1992).

It is known that complement system plays the important role in the host defense system, inflammation and allergic reaction.[1,2] Some Chinese medicinal herbs have been found to possess the ability of complement activation (anti-complementary activity).[3] These findings present the possibility that Chinese medicinal herbs may contain some kinds of regulators of the complement system.

Recently, we conducted screening on Chinese medicinal herbs to examine their anti-complementary activities, and then we have found potent anti-complementary activity in the extracts of the pericarps of *Areca catechu*.[4] Arecae Pericarpium, the pericarps of *Areca catechu* have been used in Chinese herbal medicine for the treatment of beri-beri, dropsy, sunstroke, urinary discharges, and so on.[5] Also it is known that *Areca catechu* contains several alkaloids (arecoline, arecaine, arecaidine, isoguvacine, guvacine, etc.), tannins (tannic- and gallic acid), fats (glycerides of palmitic-, stearic-, oleic acid, etc.), carbohydrates and proteins.[5] However studies on polysaccharides from Arecae Pericarpium and their immunomodulating activities have not been undertaken. Previously we reported that the active principle of Arecae Pericarpium might be a kind of polysaccharide molecule.[4] And then the active polysaccharides, AC-2-IIIa and AC-2-IIIc were purified by cetavlon method, ion-exchange chromatography and gel permeation chromatography, and their chemical properties were examined.[6] In

Key words : Complement system, anti-complementary activity, Arecae Pericarpium, action mode, ACP activity
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the present paper, we describe the anti-complementary activities of these two polysaccharides and their modes of action.

Materials and Methods

Materials

Arecae pericarpium used for experimental materials was purchased at Kyung-Dong market in Korea. Polymyxin B, anti-human C3, anti-human whole serum and lipopolysaccharide from *E. coli* 0127;B8 were purchased from Sigma Co., and IgM hemolysin sensitized sheep erythrocytes (EA) from Nippon Biotest Lab. Inc., Sephadex G-100 and Sepharose CL-4B from Pharmacia Co., DEAE-Toyopearl 650C from Toyosoda Co. and PS-K from *Coriolus versicolar* purchased from Kwang-Dong Pharmaceutical Co., Korea. Normal human sera (NHS) and rabbit erythrocytes were prepared freshly in our laboratory.

Analytical procedures

The amounts of total carbohydrate and uronic acid were determined by the phenol-sulfuric acid[7] and *m*-hydroxybiphenyl methods[8], respectively. Protein was assayed by the method of Lowry.[9]

Extraction and purification of the anti-complementary polysaccharide from Arecae Pericarpium

Arecae Pericarpium (1 kg) was decocted three times with water (15 *l*), until this volume was reduced by half. And then, AC-1 from Arecae Pericarpium was purified by methanol reflux, ethanol precipitation, dialysis and lyophilization, and this fraction was fractionated to AC-2, AC-3 and AC-4 by cetavlon (cetyltrimethylammonium bromide) treatment. Among them, the active fraction AC-2 was further purified to AC-2-IIIa and AC-2-IIIc by two successive column chromatography of DEAE-Toyopearl 650C (Cl^- form) and Sephadex G-100. The detailed procedures were reported previously.[6]

Determination of anti-complementary activity

The anti-complementary activity was measured according to previously described procedure.[5] Gelatin veronal buffered saline (pH 7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+} (GVB^{2+}) was prepared as described previously.[5] The anti-complementary activity was expressed as the percent inhibition of the total complement hemolysis of 50% (TCH_{50}) of the control.

Determination of the complement hemolysis through the alternative complement pathway (ACH_{50})

ACH_{50} was determined in 10 mM ethylene glycol-bis(β-aminoethylether)*N,N,N′,N′*-tetraacetic acid (EGTA) containing 2 mM $MgCl_2$ in GVB^{2-} (Mg^{2+}-EGTA-GVB^{2-}) by the modified method of Platt & Ishizaka.[10] A sample was incubated with Mg^{2+}-EGTA-GVB^{2-} and NHS at 37℃ for 30 min, and the residual complement mixtures were measured by the hemolysis of rabbit erythrocytes (5×10^7 cells /m*l*) incubated with Mg^{2+}-EGTA-GVB^{2-}.

Crossed immunoelectrophoresis (IEP)

NHS was incubated with an equal volume of the solution of the polysaccharide (1000 μg/m*l*) and GVB^{2+} or Mg^{2+}-EGTA-GVB^{2-} at 37℃ for 30 min. The serum was then subjected to crossed immunoelectrophoresis to locate serum proteins. Shortly after the first run (barbital buffer pH 8.6, ionic strength 0.025 with 1% agarose), the second run was carried out in a gel plate (thickness of layer 1.5 mm) containing 0.5% of an anti-serum at a potential gradient of 1 mA/cm for 10 hours. After the electrophoresis, the plate was fixed and stained with bromophenol blue.[11]

Effects of polymyxin B on anti-complementary activity

The effects of polymyxin B on hemolysis were studied by the procedures of Morison & Jacobs.[12] Lipopolysaccharide (LPS) or the active polysaccharide from Arecae Pericarpium was treated with an equal weight of polymyxin B in GVB^{2+}. Fifty μ*l* of the solution was used for the anti-complementary assay.

Results and Discussion

Anti-complementary activities of AC-2-IIIa and

AC-2-IIIc purified from Arecae Pericarpium

To examine the action modes of anti-complementary polysaccharides from Arecae Pericarpium, the active fractions AC-2-IIIa and AC-2-IIIc were purified by previously described procedures.[6] 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), and their yields were 0.079% and 0.084%, respectively from Arecae Pericarpium. AC-2-IIIa had total sugar 92.3%, uronic acid 7.4% and protein 5.6%, and AC-2-IIIc contained 69.5%, 2.9% and 17.9%, respectively.[6] And then, the anti-complementary activities of AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium were examined by hemolytic complementary assay (TCH_{50}) and compared with that of PS-K from *Coliolus versicolar* which was reported to have anti-tumor[13] and anti-complementary activity.[14] AC-2-IIIa and AC-2-IIIc showed dose-dependent anti-complementary activities on TCH_{50} (Fig. 1). The activities of AC-2-IIIa and AC-2-IIIc were higher than that of PS-K in the tested concentrations. Also the effect of incubation time on the anti-complementary activities of these three polysaccharides was estimated in the concentration of 100, 500 or 1000 μg/m*l*. The anti-complementary activities increased in accordance with increasing of incubation time (Fig. 2).

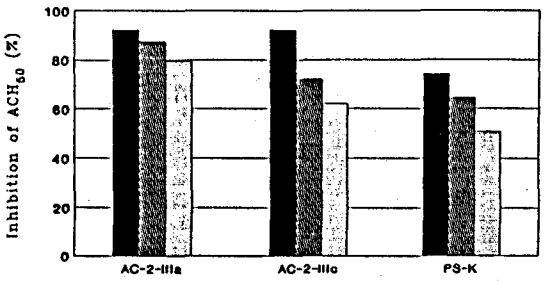

Fig. 1. Anti-complementary activities of AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium.
■: 1000 μg/m*l*, ▨: 500 μg/m*l*, ▩: 100 μg/m*l*

Changes of TCH_{50} by incubation with AC-2-IIIa or AC-2-IIIc in the Ca^{2+} or divalent metal ion free condition

The AC-2-IIIa and AC-2-IIIc purified from Arecae Pericarpium, and PS-K were incubated with NHS in GVB^{2+}, GVB^{2-} containing 10 mM EDTA (EDTA-GVB^{2-}) and Mg^{2+}-EGTA-GVB^{2-}, and then anti-complementary activities (TCH_{50}) were measured with EA cells. The anti-complementary activities in EDTA-GVB^{2-} system were completely decreased in comparison with control, whereas in case of Mg^{2+}-EGTA-GVB^{2-} system the activities caused by these polysaccharides were maintained considerably (Fig. 3). From these results, it is supposed that

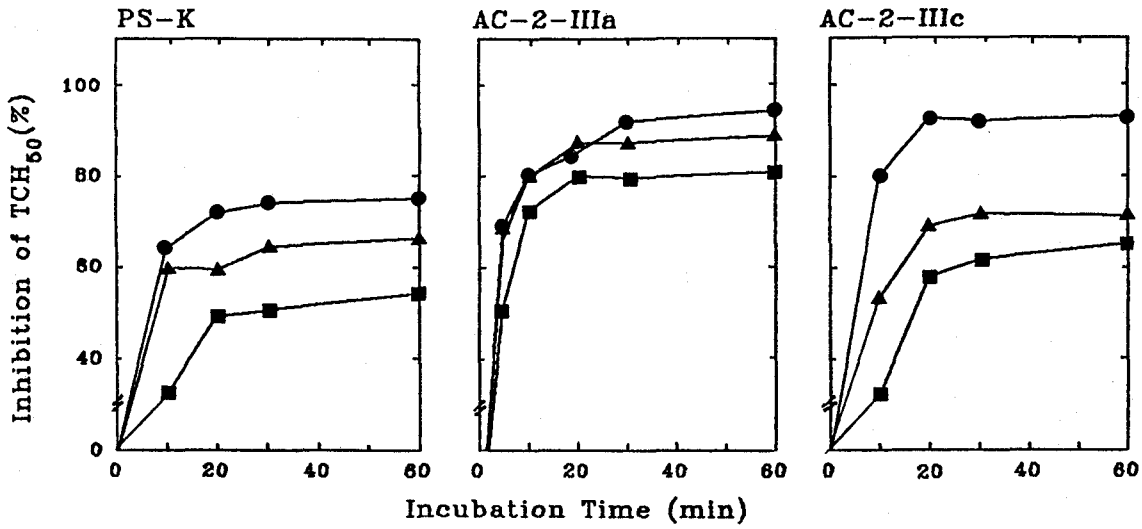

Fig. 2. Effect of incubation time on the anti-complementary activities of AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium and PS-K.
●—●: 1000 μg/m*l*, ▲—▲: 500 μg/m*l*, ■—■: 100 μg/m*l*

these all polysaccharides may also activate the complement system *via* the alternative pathway.

Alternative complement pathway activities of AC-2-IIIa and AC-2-IIIc

Because Ca^{2+} is required for the activation of complement *via* the classical pathway but not the alternative pathway,[1,2] the activation through the alternative pathway was measured in the Ca^{2+}-free conition (Fig. 4). When the active poysaccharides, AC-2-IIIa, AC-2-IIIc and PS-K were incubated with NHS in Mg^{2+}-EGTA-GVB^{2-} at 37℃ for 30 min and a hemolytic assay (ACH_{50}) was carried out using rabbit erythrocytes, AC-2-IIIa and AC-2-IIIc showed dose-dependent anti-complementary activities on ACH_{50} (ACP activity). But PS-K showed the highest activity at a dose of 250 μg/m*l*. In case of AC-2-IIIa, more than 50% of ACP activity was observed when a concentration of 1000 μg/m*l* was used for the assay. The order of ACP activities of polysaccharides was AC-2-IIIa>AC-2-IIIc>PS-K in 1000 μg/m*l*. Also, the effect of incubation time on the ACP activities by these polysaccharides was examined in 100 μg /m*l*. In all cases, ACP activities increased according to increasing of incubation time (Fig. 5). The alternative pathway does not require antibodies and is directly activated by bacteria, viruses, fungi, helminth and protozoan parasites and lymphoblastoid cells.[15] Thus, in general, the alternative pathway constitutes the natural defense mechanism of the non-immune host.[16] Therefore these polysaccharides from Arecae Pericarpium are suggested to have potent non-specific immunopotentiating activities.

Evidence of C3 activation in the Ca^{2+}-free condition

The activation of alternative pathway causes C3 cleavage due to the activation of C3 but does not require the activation of C1, C4 or C2, or presence of Ca^{2+}.[1,2] The crossed IEP was carried out after

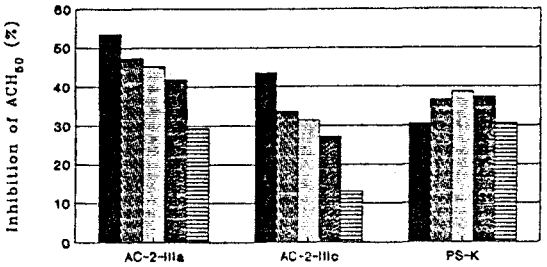

Fig. 4. Alternative complement pathway activities of AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium.
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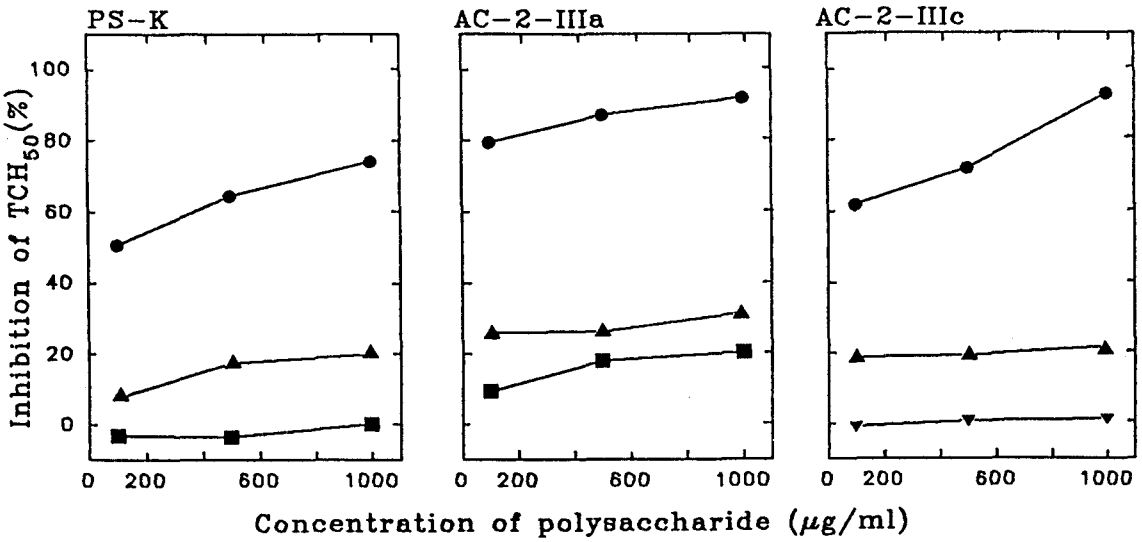

Fig. 3. Changes of TCH_{50} by incubation with the polysaccharides from Arecae Pericarpium in the presence or absence of Ca^{2+} and Mg^{2+}.
●—●: GVB^{2+}, ▲—▲: Mg^{2+}-EGTA-GVB^{2-} (Ca^{2+}-free condition), ■—■: EDTA-GVB^{2-} (metal ion-free condition)

the incubation of NHS with AC-2-IIIa or AC-2-IIIc in GVB^{2+} or Mg^{2+}-EGTA-GVB^{2-} to determine whether C3 activation had occurred. Activation of the complement pathway cleaves the main complement component, C3, into the fragments C3a and C3b. The C3 in its inactive state has a β_{1C} electrophoretic mobility (1st arc from well) which changes to a β_{1A} mobility (2nd arc from well) after activation. It is therefore possible to show the disappearance of the 1st arc and the appearance of the 2nd arc.[17] At this, two rocket arcs of C3a and C3b are fused because of shared antigenic determinants. When crossed IEP was carried out after incubation of NHS with these polysaccharides in Mg^{2+}-EGTA-GVB^{2-} (Fig. 6 C and E), a cleavage of C3 was apparent, but more significant cleavage of C3 was obtained in the serum treated with AC-2-IIIa or AC-2-IIIc in GVB^{2+} (Fig. 6 B and D). Especially, the potent ACP active AC-2-IIIa caused the greatest cleavage. The results of tests on anti-complementary activity in the absence of Ca^{2+}, IEP and ACP activity with rabbit erythrocytes indicate that the modes of complement activation by AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium are *via* both the classical and alternative pathway and that are simi-

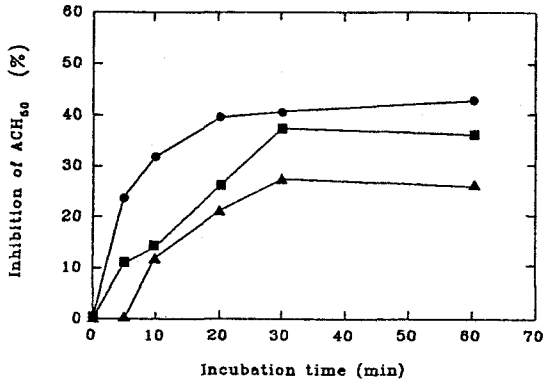

Fig. 5. Effect of incubation time on the ACP activities of AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium. ●—●: AC-2-IIIa, ▲—▲: AC-2-IIIc, ■—■: PS-K

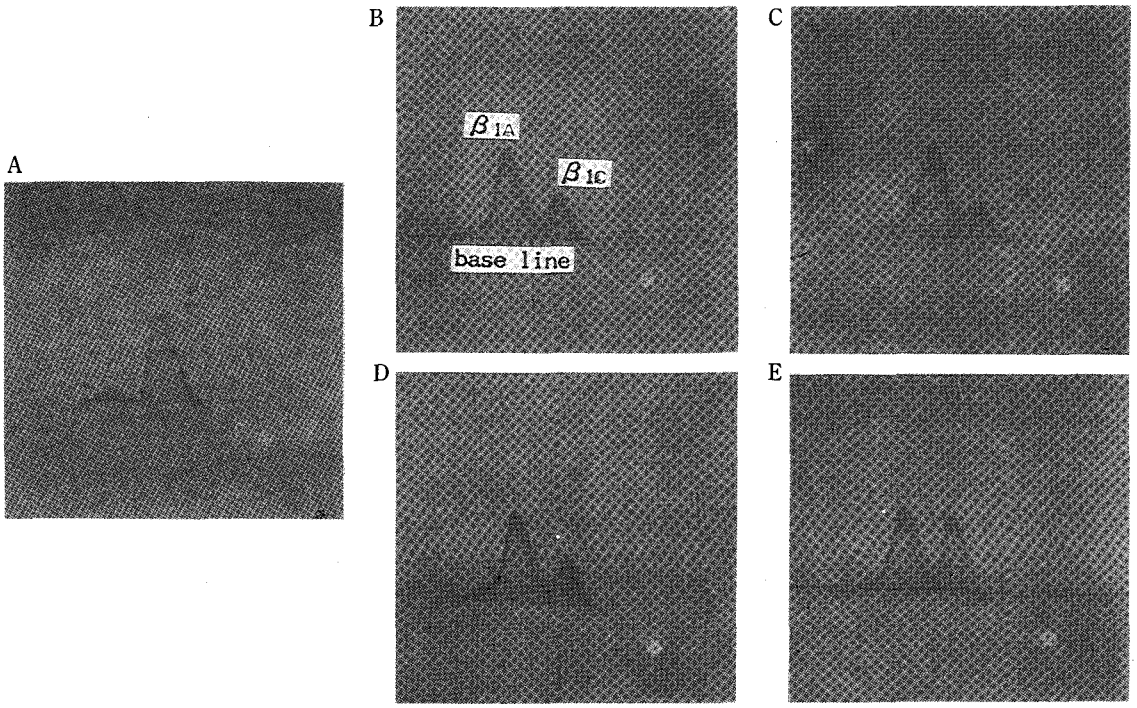

Fig. 6. C3 activation by AC-2-IIIa and AC-2-IIIc with or without Ca^{2+} ion.
Normal human serum was incubated with PS-K and GVB^{2+} or Mg^{2+}-EGTA-GVB^{2-} at 37℃ for 30 min. The sera were then subjected to immunoelectrophoresis using anti-human C3 sera to located C3 cleavage products. The anode is to the left. A : GVB^{2+} + PBS^{-}, B : GVB^{2+} + AC-2-IIIa, C : Mg^{2+}-EGTA-GVB^{2-} + AC-2-IIIa, D : GVB^{2+} + AC-2-IIIc, E : Mg^{2+}-EGTA-GVB^{2-} + AC-2-IIIc

lar to those of AR-arabinogalactan from *Angelica acutiloba*,[18] Zizyphus arabinan from *Zizyphus jujuba*, Plantago-mucilage A from *Plantago asiatica*,[19] and AAFIIb-2 and IIb-3 from *Artemisia princeps*.[20]

On the other hand, the crossed IEP patterns of polysaccharide treated NHS with anti-human whole sera are shown in Fig. 7. Shimura *et al.*[14] reported that on IEP, complement component C3 converted by host mediated anti-tumor polysaccharide moved faster than native C3 appearing as the 3rd peak and the ratio of the height of the 3rd peak to the α2-macroglobulin (α2-M) peak was proportional to the dose of anti-tumor polysaccharide. As shown Fig. 7, the height of the 3rd peak was increased with decreasing height of the native C3 peak. The ratio of the height of the 3rd peak to the α2-M peak of NHS treated with AC-2-IIIa (1.50± 0.04) or AC-2-IIIc (1.22± 0.08) in 1000 μg/ml was higher than that of PS-K (1.06± 0.06). We have not yet examined whether AC-2-IIIa and AC-2-IIIc have other immunomodulating activities such as anti-tumor activity, but the present results suggested that these polysaccharides may be responsible, at least in part, for the immunomodulating activities of Arecae Pericarpium.

Differentiation between the polysaccharides of Arecae Pericarpium and the endotoxic lipopolysaccharide (LPS)

Since both the classical and alternative pathway seemed to be involved in the activation of complement by AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium, the possibility of contamination by LPS was examined. Because poymyxin B has been reported to inhibit complement activation by LPS,[12] the polysaccharide from Arecae Pericarpium was treated with an equal amount of polymyxin B, and the residual activity was assayed. This treatment

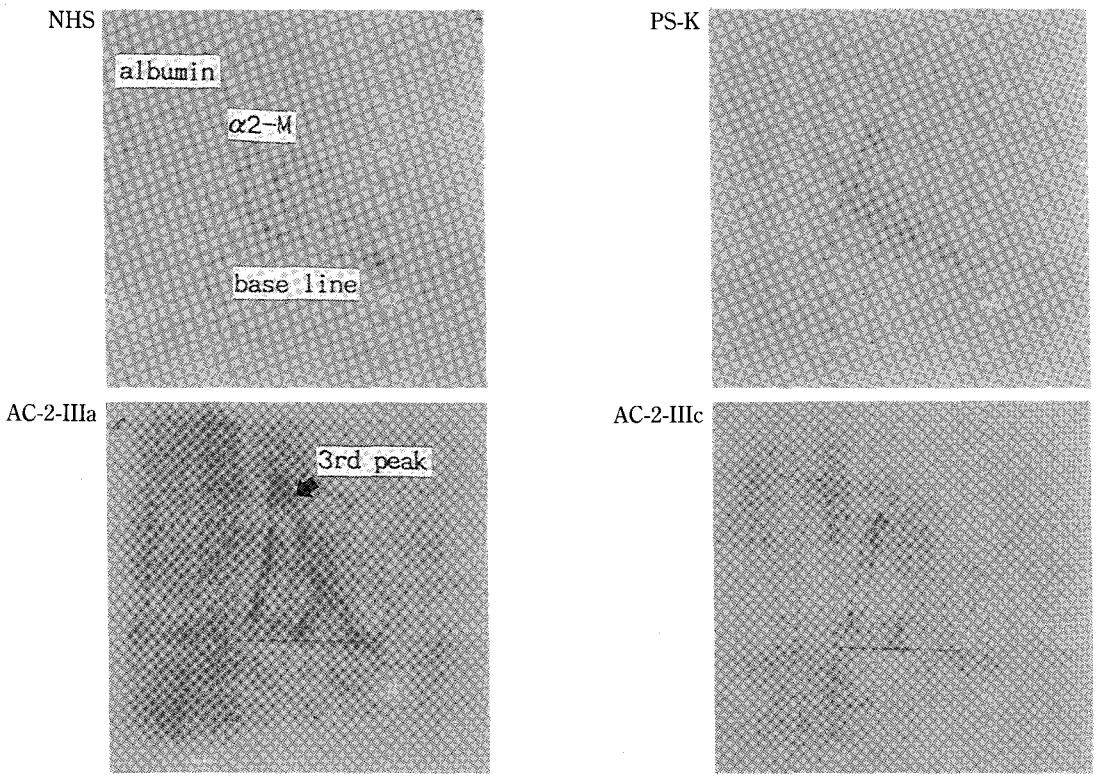

Fig. 7. Crossed immunoelectrophoretic patterns of C3 converted by AC-2-IIIa, AC-2-IIIc and PS-K.
Normal human serum was incubated with each polysaccharide and GVB^{2+} at 37℃ for 30 min. The sera were then subjected to immunoelectrophoresis using anti-human whole sera. The arrow designates the 3rd peak. The anoe is to the left.

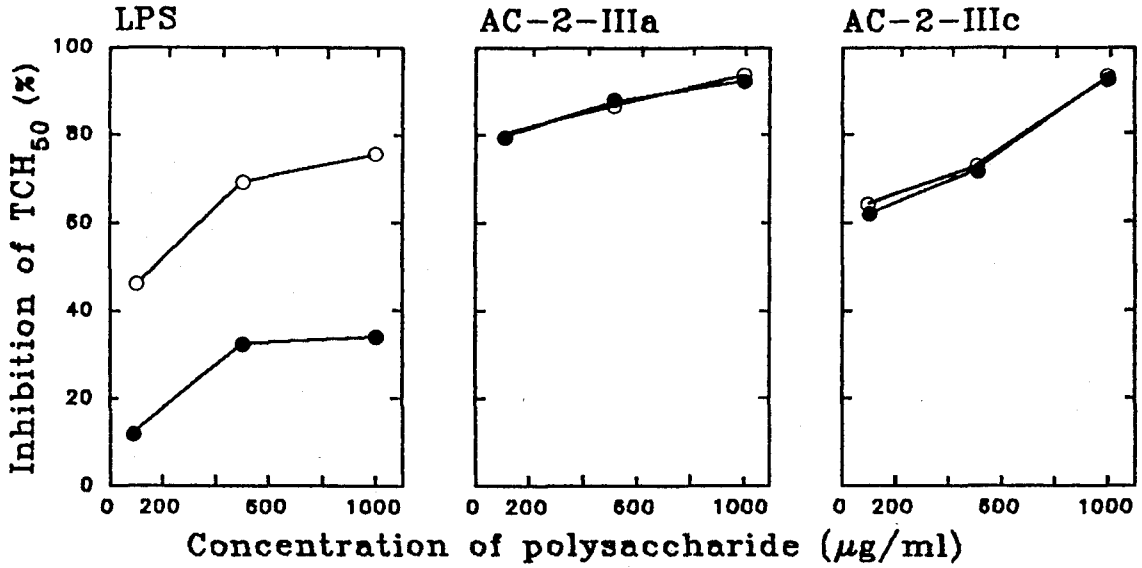

Fig. 8. Effect of polymyxin B on the anti-complementary activities of lipopolysaccharide (LPS) and purified polysaccharide fractions from Arecae Pericarpium.
●—●: Polymyxin treated, ○—○: Polymyxin not treated

largely abrogated the activity of LPS, but it had no effect on the capacities of the AC-2-IIIa and AC-2-IIIc to activate complement (Fig. 8). These results suggested that the anti-complementary activities of AC-2-IIIa and AC-2-IIIc from Arecae Pericarpium were not due to LPS contamination and were possessed in their own molecules.

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대복피로부터 정제된 보체활성화 다당의 작용양식

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초록 : 대복피(빈랑나무의 과피)로부터 면역조절 역할이 기대되는 2종의 보체활성화(항보체) 다당을 정제하고 그 작용양식에 대해 검토하였다. 활성다당 AC-2-IIIa 및 AC-2-IIIc는 농도가 증가함에 따라 항보체 활성이 증가하는 양상을 보였으며, 2가 금속이온 부재시 대조구에 비해 거의 완전한 활성의 감소를 보인 반면, Ca^{2+} 이온만을 선택적으로 제거한 경우 활성이 상당량 유지되었다. 또한 이들 활성다당들은 비교적 강력한 보체 제2경로 활성화능을 나타내었으며, Ca^{2+} 이온 부재 상태에서 정상인의 혈청과 반응시, C3의 분해산물을 anti-human C3를 이용한 면역 전기영동법에 의해 확인할 수 있었다. 또한 anti-human whole serum를 이용한 면역 전기영동 결과, α2-M peak 대비 3rd peak의 높이 비율은 AC-2-IIIa가 1.50± 0.04, AC-2-IIIc가 1.22± 0.08이었는바, 이러한 사실로부터 대복피 유래 AC-2-IIIa 및 AC-2-IIIc의 보체 활성화 양식은 classical 및 alternative pathway 양경로를 모두 경유함을 알 수 있었다.