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Screening and characteristics of anti-complementary polysaccharides from Chinese medicinal herbs

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Abstract : We conducted screening on Chinese medicinal herbs to examine their anti-complementary activity by hemolytic complementary assay (TCH_{50}). Among 55 kinds of herbs, several herbs showed relatively potent anti-complementary activity which decreased TCH_{50} more than 70% in comparison with control. Then, hot water extracts of the following herbs, *Curcuma aromatica, Areca catechu, Gleditsiae spina, Euonymus alata, Acanthopanax senticous. Lonicera japonica, Aconitum carmichaeli, Curcuma zedoaria and Cinnamoum cassia,* which were shown relatively potent anti-complementary activity were partially purified and analyzed their chemical properties. These activities were resistant to digestion with pronase but decreased by treatment with $NaIO_4$. These results may indicate that the complement activating ability in their herbs is due to polysaccharide. Furthermore, the anti-complementary activity of *Areca catechu* which was showed the most potent activity, was reduced partially in the absence of the Ca^{++} ion. After incubation of the normal human serum with partially purified polysaccharide of *A. catechu* in the absence of Ca^{++} ion, a cleavage of C3 in the serum was found to have occurred through immunoelectrophoresis using rabbit anti-human C3 serum. These results indicate that the mode of complement activation by polysaccharide of *A. catechu* is *via* both the alternative and classical pathway (Received November 30, 1991, accepted February 8, 1992).

It is known that complement system plays the important role in the host defense system, inflammation or alleric reaction. The complement system consists of over 20 serum proteins including 9 complement components (C1~C9) and their regulators. The complement proteins are activated by a cascade mechanism of classical or alternative pathway.[1,2]

Various polysaccharides and polysaccharide containing materials such as endotoxic lipopolysaccharide,[3-5] inulin,[6] water-insoluble β-1.3 glucan,[7] water-insuluble glucan synthesized enzymatically by *Streptococcus mutans* OMZ 176[8] and polysaccharides from Chinese medicinal herbs[9] are known to activate the complement system. Especially, a considerable number of Chinese medicinal herbs has been found to be possesed anti-complementary activity; for example AR-arabinogalactan IIa and IIb-1[10-12] from *Angelica acutiloba* L., LR-polysaccharide IIa[13] from *Lithospermum euchromum* L., AAFIIb-2 and -3[14,15] from *Artemisia princeps* L., and BR-5-I and BR-2-IIb[16,17] from *Bupleurum falcatum* L. These findings present the possibility that Chinese medicinal herbs may contain some kind of regulator of the complement system.

Recently we conducted screening on Chinese medicinal herbs which had used empirically without scientific verification thereof to examine their anti-complementary activity and then, relatively strong anti-complementary polysaccharides could selected. In this paper, the chemical properties and action modes of selected anti-complementary polysaccharides were examined.

Key words : Anti-complementary activity, polysaccharide, Chinese medicinal herb
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Materials and Methods

Materials

Several Chinese medicinal herbs used for experimental materials were purchased at Kyung-Dong market in Korea. Pronase, polymyxin B, rabbit anti-human C3 and lipopolysaccharide from *E. coli* 0127; B8 were purchased from Sigma Co., and IgM haemolysin sensitized sheep erythrocytes (EA) from Nippon Biotest Laboratory Inc. were used in this study. Also normal human sera (NHS) and rabbit red blood cells (RRBC) were prepared freshly in our laboratory.

Analytical procedures

Total carbohydrate and uronic acid contents were determined by the phenol-sulfuric acid[18] and *m*-hydroxybiphenyl methods[19] using arabinose and galacturonic acid as the respective standard material. Protein was assayed by the method of Lowry[20] using bovine serum albumin.

Preparation of hot water extracts from Chinese medicinal herbs

In order to screening of anti-complementary polysaccharides from Chinese medicinal herbs, each of the dried herbs (50g) were boiled with 500 m*l* of H_2O to half volume and the residual materials were extracted, 3 times by same procedure. The extracts were centrifuged (7500 rpm, 30 min) to remove insoluble material. The supernatant was concentrated by vacuum evaporation and lyophilized to give the hot water extract (A-1). A-1 was redissolved in H_2O and dialyzed against H_2O for 4 days to remove the low molecular weight materials. Non-dialyzable portion was centrifuged and supernatant was lyophilized (A-2).

Determination of anti-complementary activity

Anti-complementary activity was determined by the methed of Kabat and Mayer[21] with modification. Various dilutions of polysaccharides in water (50 μ*l*) were mixed with 50 μ*l* of NHS and 50 μ*l* of gelatin veronal buffered saline (pH 7.4) containing 500 μM Mg^{++} and 150 μM Ca^{++} (GVB^{++}). The mixtures were preincubated at 37 ℃ for 30 min and 350 μ*l* of GVB^{++} was added. EA cells (250 μ*l*) at 1×10^8 cells/m*l* were added to the mixtures diluted serially (10~160 folds) and then incubated at 37 ℃ for 1.0 hour. After addition of phosphate buffered saline (PBS, pH 7.4) and centrifugation, the absorbance of supernatants were detected at 412 nm. NHS was incubated with water and GVB^{++} as a control. The anti-complementary activity was expressed as the percent inhibition of the total complement hemolysis (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^{--} (Mg^{++}-EGTA-GVB^{--}) by the method of Platt & Ishizaka.[22] A sample was incubated with Mg^{++}-EGTA-GVB^{--} 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^{++}-EGTA-GVB^{--}

Pronase digestion of the crude polysaccharides

A-2 fraction (40 mg) was dissolved in 50 m*l* of 50 mM Tris-HCl buffer, pH 7.9 containing 10 mM $CaCl_2$, and then 10 mg of pronase was added. The reaction mixture was incubated at 37 ℃ for 48 hours with 2~3 drops of toluene. The reaction was terminated by boiling for 5 min. The mixture was then dialyzed against water for 3 days at 4 ℃, and the Non-dialyzable portion was lyophylized to obtain the pronase digested A-2.

Periodate oxidation of the crude polysaccharides

A-2 fraction (40 mg) was dissolved in 50 m*l* of 50 mM acetate buffer, pH 4.5, and then 10 m*l* of 50 mM $NaIO_4$ was added. The reaction mixture was incubated at 4 ℃ in the dark for 3 days. 5 m*l* of ethylene glycol was added to destroy the excess

periodate, and the mixture was dialyzed against water for 3 days. The non-dialyzable solution was concentrated to about 10 ml and 20 mg of sodium borohydride was added to the concentrate with being continuously stirred for 12 hours at room temperature. After the neutralization of the reaction mixture with acetic acid, the boric acid contained in the sample was removed by the repeated addition and evaporation of methanol. Finally, the oxidized A-2 was obtained as the lyophilizate after dialysis.

Effects of anti-complementary polysaccharide on complement in a human serum

Various dilutions of polysaccharide fraction in water were mixed the same volume of NHS and GVB^{++}, and the mixtures were incubated at 37℃ for 30 min. Also the incubated mixtures were sampled at 0, 2, 5, 10, 15 and 30 min for estimation of incubation period effect. EA cells were mixed with 0.8% molten agarose and the mixture allowed to set onto slide glass plate. Wells (φ 2.5 mm) were cut in the agarose and filled with each of the samples. The plate was incubated at 4℃, overnight and warmed to 37℃ for 2 hours, to allow cell lysis to occur.

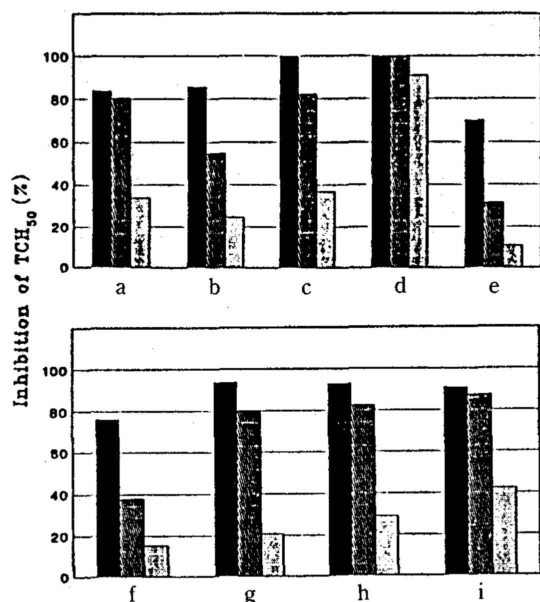

Fig. 1. Anti-complementary activities of hot water extracts from selected Chinese herb sources.
a : *Lonicera japonica*, b : *Aconitum carmichaeli*, c : *Curcuma aromatica*, d : *Areca catechu*, e : *Gleditsiae spina*, f : *Euonymus alata*, g : *Acanthopanax senticosus*, h : *Cinnamoum cassia*, i : *Curcuma zedoaria*

Immunoelectrophoresis (IEP)

NHS was incubated with an equal volume of the solution of the A-2 fraction in GVB^{++}, GVB^{--} containing 10 mM EDTA (EDTA-GVB^{--}) or Mg^{++}-EGTA-GVB^{--} at 37℃ for 30 min. The serum was then subjected to simple or crossed immunoelectrophoresis to locate the C3 cleavage products.[23] 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 a rabbit anti-human C3 serum at a potential gradient of 1 mA/cm for 15 hours. After the electrophoresis, the plate was fixed and stained with bromophenol blue.

Effects of hemolysis by polymyxin B

The effects on hemolysis of polymyxin B were studied by the procedures of Morision & Jacobs.[24] Lipolysaccharide (LPS) or A-2 fraction was treated with an equal weight of polymyxin B in GVB^{++} (1 ml). Fifty μl of the solution was used for the anti-complementary assay. Control samples were treated without polymyxin B.

Results

Screening and isolation of anti-complementary materials from Chinse medicinals herbs

55 crude water soluble polysaccharides isolated from Chinese medicinal herbs have been screened for anti-complementary activity. The crude polysaccharide fractions (A-1) obtained by decoctation with hot water, were examined their anti-complementary activity by hemolytic complement assay (TCH_{50}).

Among them, 9 kinds of extracts showed strong anti-complementary activity which decreased TCH_{50} more than 70% in comparison with control and its proportion was 16% of total numbers of samples.

Anti-complementary activities of these nine extracts are presented in Fig. 1. The order of activity was *Areca catechu*>*Curcuma aromatica*>*Acanthopanax senticosus*>*Cinnamoum cassia*>*Curcuma zedoaria*>*Aconitum carmichaeli*>*Lonicera japonica*>*Euonymus alata*>*Gleditsiae spina* in 1000 μg/ml. Especially, the crude polysaccharide fraction from *A. catechu* showed high anti-complementary activity above 90% in the concentration of 100 μg/ml.

Determination whether activation of C3 had occurred

When NHS is incubated with EA cells as an antigen-antibody complex, the inhibition of complement refers to anti-complementary activity. Therfore, anti-complementary activity includes both activation and inhibition of the complement system.[9]

When complement system is activated, the main complement component, C3 is cleaved into C3a and C3b. In order to determine whether activation of C3 had occurred, the IEP using serum containing anti-human C3 was carried out after incubation of NHS with A-2 fractions of Chinese medicinal herbs.

C3 cleavage products were observed in the serum treated with A-2 fractions. These results indicate that all nine of A-2 fractions activate complement system, directly (Fig. 2).

Chemical properties of active fractions

The chemical composition of nine hot water extracts (A-1) showed relatively potent anti-complementary activity were examined. The contents of neutral sugar, uronic acid and protein were 32.69~81.27, 1.66~8.33%, 13.27~70.62%, respectively. And their yields were 3.43~13.98% (Table 1).

To determine the real moiety of activity, A-2 fractions obtained by dialysis of hot water extracts

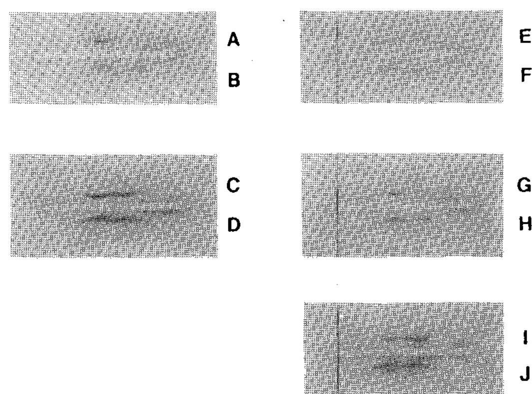

Fig. 2. C3 activation by anti-complementary polysaccharides from selected Chinese medicinal herbs.
Normal human serum was incubated with GVB^{++} and herb-extracts for 30 min at 37℃. The sera were then subjected to immunoelectrophoresis using rabbit anti-human C3 to located C3 cleavage products. The anode is to the right
A : NHS only, B : *Areca catechu*, C : *Acanthopanax senticosus*, D : *Gleditsiae spina*, E : *Curcuma zedoaria*, F : *Cinnamoum cassia*, G : *Euonymus alata*, H : *Curcuma aromatica*, I : *Lonicera japonica*, J : *Aconitum carmichaeli*

Table 1. Yield and chemical properties of A-1, A-2 and its derivatives

Sample	Yield of A-1 (%)	Total sugar (%)	Total protein (%)	Total uronic acid (%)	Yield of A-2 from A-1 (%)	Yield of pronase digest from A-2 (%)	Yield of periodate oxidate from A-2 (%)
Daebogpi (*Areca catechu*)	5.17	32.69	25.90	7.21	29.36	78.00	81.00
Gaepi (*Cinnamoum cassia*)	3.43	35.15	31.24	5.06	13.89	29.00	61.90
Gasiogalpi (*Acanthopanax senticosus*)	13.98	81.27	70.62	8.33	17.45	87.00	55.00
Ulgeum (*Curcuma aromatica*)	5.98	75.49	13.27	2.26	14.81	76.19	32.56
Jogakja (*Gleditsiae spina*)	7.95	57.18	18.29	3.88	8.10	39.58	66.67
Bongchul (*Curcuma zedoaria*)	5.51	44.39	19.49	5.04	22.57	51.22	52.00
Indong (*Lonicera japonica*)	5.24	62.54	34.64	6.41	23.78	72.50	39.44
Buja (*Aconitum carmichaeli*)	5.83	63.21	14.20	1.66	37.99	63.20	76.06
Hwasalnamu (*Euonymus alata*)	4.17	61.30	20.17	4.12	21.33	67.54	48.23

were digested with pronase and oxidated with periodate. Their yields are shown in Table 1. Pronase treatements of A-2 fractions had little effect on anti-complementary activity, however, the activity of periodate oxidate was sharply decreased (Fig. 3).

Therefore, it is supposed that the carbohydrate moieties in A-2 fractions from selected herbs may also contribute to the anti-complementary activity.

Effects of anti-complementary polysaccharide on complement in a human serum

Various dilutions of A-2 fraction from *A. catechu* were mixed the same volume of NHS and GVB^{++}, and the mixtures were incubated at 37℃ for 30 min. These mixtures were filled in the wells of 0.8% agarose plates contained EA cells, and diffused. The plates were incubated at 37℃ for 2 hours, and then the clear zone formed by cell lysis was observed. Fig. 4 shows difference of clear zones formed by NHS treated with the different concentrations of A-2 fraction from *A. catechu*. In case of incubated NHS mixture, the clear zones were not observed because A-2 fraction already activated the complement in NHS. But in not-incubated mixture, the clear zones were formed. Also, in the experiments for effect of incubation time of A-2 fraction on complement, the same results were obtained (data not shown).

Action mode of anti-complementary polysaccharide from *Areca catechu*

The A-2 fraction of *A. catechu* was incubated with

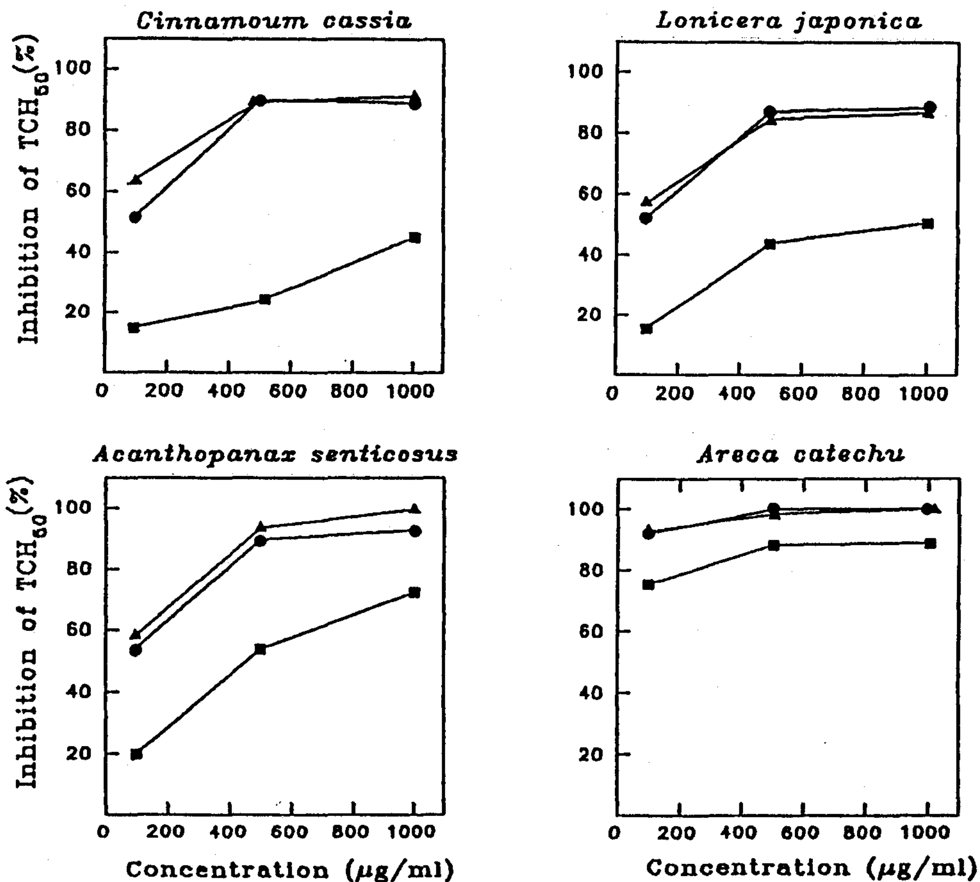

Fig. 3. Effects of pronase digestion and periodate oxidation on selected Chinese medicinal herbs.
●—● : A-2, ▲—▲ : Pronase digested A-2, ■—■ : Periodate oxidized A-2

NHS in GVB^{++}, $EDTA\text{-}GVB^{--}$ or $Mg^{++}\text{-}EGTA\text{-}GVB^{--}$, and then anti-complementary activity (TCH_{50}) was measured with EA cells. The anti-complementary activity in $EDTA\text{-}GVB^{--}$ system was completely decreased in comparison with control whereas in case of $Mg^{++}\text{-}EGTA\text{-}GVB^{--}$ system the activity caused by this polysaccharide was maintained considerably (Fig. 5). Also A-2 fraction was incubated with NHS in $Mg^{++}\text{-}EGTA\text{-}GVB^{--}$, and then a hemolytic assay (ACH_{50}) were carried out using fresh rabbit erythrocytes. A-2 fraction of *A. catechu* showed a dose-dependent anti-complementary activity on ACH_{50} (ACP activity) (Fig. 6). Therefore crossed IEP were carried out after the incubation of NHS with A-2 fraction of *A. catechu* in GVB^{++}, $EDTA\text{-}GVB^{--}$ or $Mg^{++}\text{-}EGTA\text{-}GVB^{--}$ to determine whether C3 activation had occured. A cleavage of the C3 precipitin line was observed in the serum treated with this polysaccharide dissolved in GVB^{++} and $Mg^{++}\text{-}EGTA\text{-}GVB^{--}$ (Fig. 7). These results show that the mode of complement activation by the polysaccharide of *A. catechu* is *via* both the classical and alternative pathway.

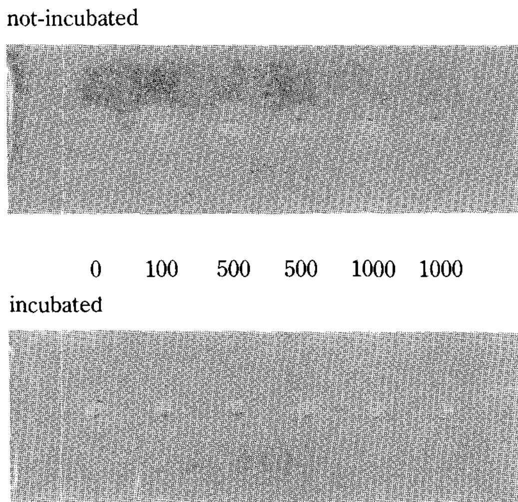

Fig. 4. Effect of crude polysaccharide from *Areca catechu* on complement in a normal human serum. IgM-haemolysin-sensitized sheep erythrocytes were into 0.8% molten agarose and the mixture allowed to set. Wells were cut in the agar plates and filled with the incubated or not-incubated sera. These agar plates were incubated at 37℃ for to 2 hours.

Differentiation between polysaccharide of *A. catechu* and endotoxic lipopolysaccharide (LPS)

Since both the classical and alternative pathway seemed to be involved in the activation of complement by polysaccharide from *A. catechu*, the possibility of contamination by LPS was examined. Be-

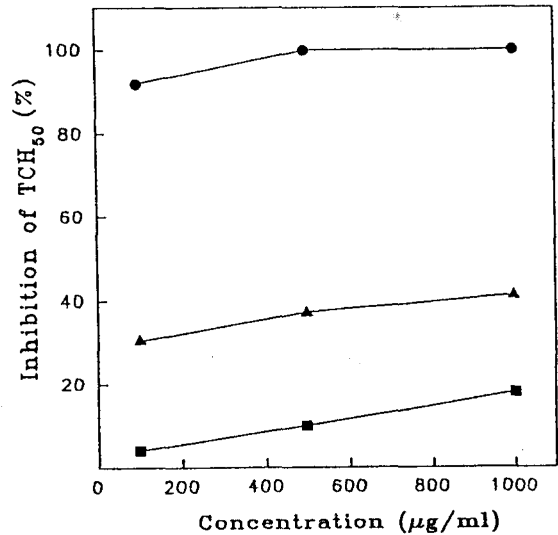

Fig. 5. Changes of TCH_{50} by incubation with the polysaccharide from *A. catechu* in the presence or absence of Ca^{++} and Mg^{++}.
●—● : GVB^{++}, ▲—▲ : $Mg^{++}\text{-}EGTA\text{-}GVB^{--}$, ■—■ : $EDTA\text{-}GVB^{--}$

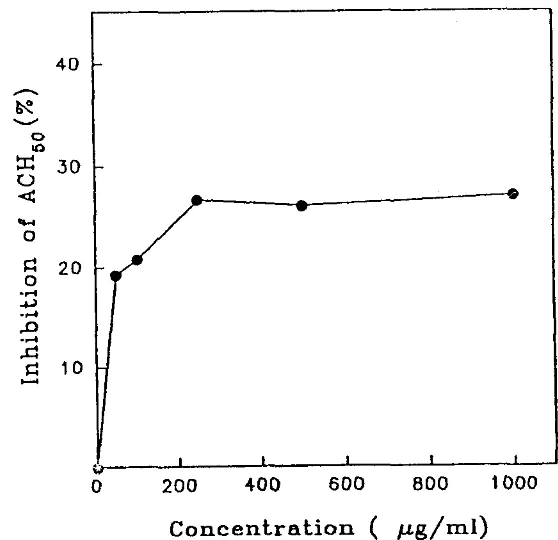

Fig. 6. Alternative complement pathway activity of A-2 from *Areca catechu.*

cause polymyxin B has been reported to inhibit complement activation by LPS,[24] the polysaccharide of *A. catechu* was incubated with an equal amount of polymyxin B, and the residual complement was determined. This treatment largely abrogated the activity of LPS, but it had no effect on the capacity of the polysaccharide of *A. catechu* to activate complement (Fig. 8). These results suggested that the anti-complementary acitivity from *A. catechu* was not due to LPS contamination.

Discussion

Recently, we have found the existence of a potent anti-complementary activity in the extracts from Chinese medicinal herbs. This activity was showed in the crude polysaccharide fraction of high molecular weight. Also, the activity did not change by pronase treatement, but decreased greatly by periodate oxidation of crude polysaccharide. These results indicate that carbohydrate moiety may be related to the anti-complementary activity likewise the leaves of *Artemisia princeps*,[14,15] the roots of *Angelica acutiloba*[10-12] and the roots of *Bupleurum falcatum*.[16,17]

Among the nine crude polysaccharides selected in our screening procedure, the polysaccharide from pericarp of *Areca catechu* (Arechae Pericarpium) showed the highest level of anti-complementary activity. Therefore the anti-complementary polysac-

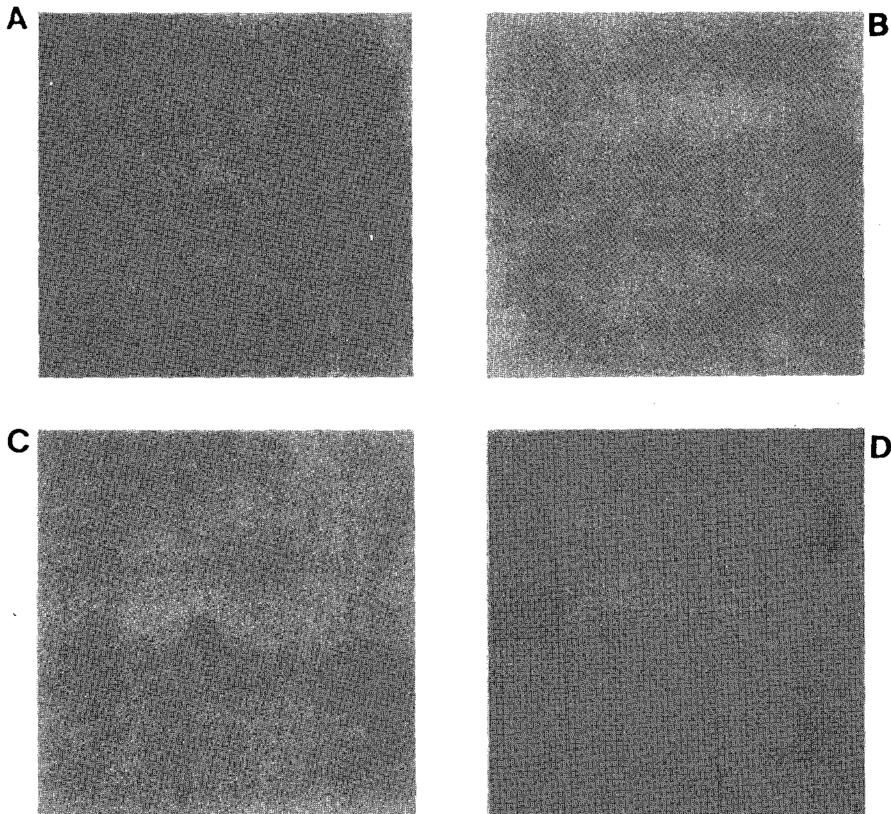

Fig. 7. Crossed immunoelectrophoresis of C3 with A-2 of *Areca catechu* with or without Ca^{++} ion and Mg^{++} ion.
Normal human serum was incubated with *Areca catechu* (A-2) and GVB^{++} or Mg^{++}-EGTA-GVB^{--} or EDTA-GVB^{--} respectively for 30 min at 37℃. The anode is to the left.
A : GVB^{++}+PBS^{-}, B : GVB^{++}+*A. catechu*, C : EDTA-GVB^{--}+*A. catechu*, D : Mg^{++}-EGTA-GVB^{--}+*A. catechu*

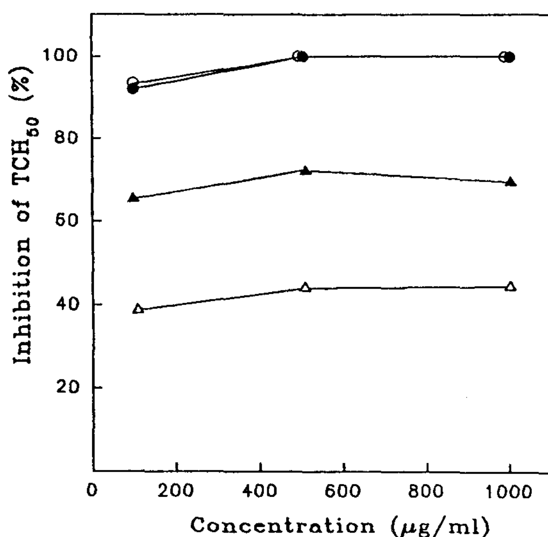

Fig. 8. Liability of anti-complementary activity caused by A-2 from *A. catechu* or lipopolysaccharide to polymyxin B treatment.
●—● : A-2, ○—○ : Polymyxin B treated A-2, ▲—▲ : Lipopolysaccharide, △—△ : Polymyxin teated LPS

charide from *A. catechu* has been studied for its mode of action. The complement system is activated through either or both of the alternative and classical pathway, and both Ca^{++} and Mg^{++} ions are needed for the activation of classical pathway but only Mg^{++} ion is needed for the activation of alternative pathway. If Ca^{++} is removed from the reaction mixture, the classical complement pathway can be suppressed. Each anti-complementary polysaccharide was incubated with NHS in Ca^{++} free GVB^{++} (Mg^{++}-EGTA-GVB^{--}), and then hemolytic assay (ACH_{50}) of complement was carried out using rabbit erythrocytes, an activator of the alternative pathway. This polysaccharide from *A. catechu* considerably inhibited ACH_{50}, and under same condition, the value of inhibition of TCH_{50} was also decreased in comparison with control. Activation of the alternative pathway cleaves the first complement component, C3, into the fragments C3a and C3b. When crossed IEP using anti human C3 serum was carried out after incubation of NHS with the anti-complementary polysaccharide in Mg^{++}-EGTA-GVB^{--} to determine whether activation of C3 had occured, a cleavage of C3 precipitation line was observed in the serum treated with A-2 fraction. These results also indicate that this polysaccharide activates the complement system *via* not only classical but also alternative pathway, and that the mode of complement activation of A-2 fraction is similar to that of AR-arabinogalactan IIa[11] from *Angelica acutiloba*, IR-polysaccharide IIa[13] from *Lithospermum euchromum* and LPS.[25] However activation of the complement by the action of A-2 fraction does not result in contaminating LPS because the activity by the polysaccharide was resistant to polymyxin B treatment.

But the anti-complementary polysaccharide from *A. catechu* is required further purification and a more detailed study on the structure and its property. Further studies of this aspect are in proress.

Acknowlegement

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한약재로부터 항보체 활성 다당의 검색 및 특성

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초록 : 한약재를 대상으로 용혈 보체 분석법(TCH_{50})을 이용, 항보체 활성물질에 대한 전반적인 검색을 실시하였다. 총 55종의 시료의 열수 추출물을 대상으로 동 활성을 측정한 결과, 대조구에 비해 70%이상의 TCH_{50}(total complement hemolysis of 50%)의 감소를 일으키는 비교적 강력한 항보체 활성을 소유한 울금, 대복피, 조각자, 화살나무, 가시오갈피, 인동, 부자, 계피 등 9종의 시료를 선택할 수 있었다. 이들은 pronase 소화 후 활성을 유지한 반면, $NaIO_4$ 산화에 의해 급격한 활성의 감소를 나타냄으로써, 그 활성의 본체가 다당에 기인함을 알 수 있었다. 또한 가장 높은 활성을 보였던 대복피(*Areca catchu*) 다당의 경우 Ca^{++} 이온 부재시 부분적으로 활성의 감소를 보였으며, 정상인의 혈청과 반응시 C3의 분해산물을 면역 전기영동법에 의해 확인할 수 있었는 바, 동 사실로부터 대복피의 보체 활성화 기구는 classical pathway뿐만 아니라 alternative pathway도 경유함을 알 수 있었다.