Purification and Chemical Properties of Anti-complementary Polysaccharide from the Leaves of Artemisia princeps

Haruki Yamada1,4, Katsumi Ohtani2, Hiroaki Kiyohara1, Jong-Chol Cyong1, Yasuo Otsuka1, Yoshio Ueno2 and Satoshi Ōmura3

Received: August 28, 1984; Accepted: December 25, 1984

Abstract: The polysaccharide fraction from the leaves of Artemisia princeps Pamp (Japanese name = Gaiyō) showed a potent anti-complementary activity. Two major active polysaccharides (AAF-IIb-2 and IIb-3) were purified by ion exchange chromatography on DEAE-Sepharose, affinity chromatography on Ricinus communis-agglutinin conjugated Sepharose and gel filtration on Sephadex G-100 and Sepharose CL-4B. The molecular weights of AAF-IIb-2 and IIb-3 were found to be 139 000 and 31 000 by the calibration of gel filtration. AAF-IIb-2 and IIb-3 were composed of rhamnose, xylose, arabinose, galactose and glucose in the molar ratios of 2.5:2.5:4.3:3.6:1.0 and 1.5:1.0:9.4:7.5:1.0, and also contained 59.4% and 49.0% galacturonic acid, respectively. AAF-IIb-2 was shown to have the more potent activity than AAF-IIb-3. Anti-complementary activity of AAF-IIb-3 was almost similar with that of crude AR-arabinogalactan from the root of Angelica acutiloba Kitagawa, but AAF-IIb-2 and IIb-3 showed more potent activity than anti-complementary β-glucan, lentinan.

Introduction

It is known that complement system plays an important role in the host defense system, inflammations and allergic reactions. A considerable number of Chinese herbs have been found to be anti-inflammatory (1). These findings present the possibility that these herbs may contain some kinds of regulators of the complement system. Recently, we have found potent anti-complementary activities in the extracts of some Chinese herbs (2), such as in the extract of the leaves of Artemisia princeps Pamp. Artemisia Argyi Folium, the leaves of A. princeps Pamp (Japanese name = Gaiyō) is a well known crude drug clinically decocted with water (5 l) as above. The extracts were combined and lyophilized to give a water-soluble extract (AAF-O, yield 107.8 g). AAF-O was refluxed with 21 of MeOH for 1 h and centrifuged to give a MeOH-insoluble precipitate. The precipitate was dissolved in water until this volume was reduced by half, and the residue was then further decocted with water (5 l) as above. The extracts were combined and lyophilized to give a water-soluble extract (AAF-O, yield 107.8 g). AAF-O was refluxed with 21 of MeOH for 1 h and centrifuged to give a MeOH-insoluble precipitate. The precipitate was dissolved in water and dialyzed against running water for 3 days. After the non-dialyzable portion was centrifuged at 7,500 r.p.m. for 30 min, the supernatant was lyophilized to obtain crude polysaccharide (AAF-1, yield 12.26 g). The MeOH-soluble fraction was evaporated to dryness, after which the residue was redisolved in water and lyophilized to obtain AAF-MeOH ext. (yield 36.41 g).

Preparation of crude polysaccharide from A. princeps Pamp

The leaves of A. princeps Pamp (500 g) were decoked with water (10 l) until this volume was reduced by half, and the residue was then further decocted with water (5 l) as above. The extracts were combined and lyophilized to give a water-soluble extract (AAF-O, yield 107.8 g). AAF-O was refluxed with 21 of MeOH for 1 h and centrifuged to give a MeOH-insoluble precipitate. The precipitate was dissolved in water and dialyzed against running water for 3 days. After the non-dialyzable portion was centrifuged at 7,500 r.p.m. for 30 min, the supernatant was lyophilized to obtain crude polysaccharide (AAF-1, yield 12.26 g). The MeOH-soluble fraction was evaporated to dryness, after which the residue was redisolved in water and lyophilized to obtain AAF-MeOH ext. (yield 36.41 g).

Pronase digestion

AAF-IIb (200 mg) was dissolved in 50 ml of 50 mM Tris-HCl, pH 7.9, containing 10 mM CaCl2, and then 50 mg of Pronase was added. The reaction mixture was incubated at 37°C for 48 h with a small amount of toluene. The reaction was terminated by boiling for 5 min. The mixture was then dialyzed against H2O for 2 days, and the non-dialyzable portion was lyophilized to obtain the AAF-IIb Pronase digest.

Periodate oxidation

AAF-IIb (50 mg) was dissolved in 30 ml of 50 mM acetate buffer, pH 4.5, and then 50 mM NaOAc (10 ml) was added. The reaction mixture was incubated at 4°C in the dark for 3 days. Ethylene glycol (5 ml) was added to destroy the excess periodate, and the mixture was dialyzed...

1 Oriental Medicine Research Center of the Kitasato Institute, 5-9-1 Shirokanedai, Minato-ku, Tokyo 108, Japan
2 Faculty of Pharmaceutical Sciences, Science University of Tokyo, Shinjuku-ku, Tokyo 162, Japan
3 The Kitasato Institute, Minato-ku, Tokyo 108, Japan
4 Address for correspondence
against H₂O for 2 days. The non-dialyzable solution was concentrated to about 20 ml, and 20 mg of NaBH₄ was added to the concentrate while being continuously stirred for 12 h at room temperature. After the neutralization of the reaction mixture with AcOH, the H₃BO₃ in the sample was removed by the repeated addition and evaporation of MeOH. Finally, the oxidized AAF-IIb was obtained as the lyophilisate after the dialysis.

**Anti-complementary activity**

The anti-complementary activity was measured according to the previously described procedure (12) except distilled water (DIW) was used for dilutions instead of phosphate buffered saline, PBS (pH 7.4).

**Materials**

The leaves of *Artemisia princeps* Pamp (Japanese name = GaiyO) and the seed of *Ricinus communis* were purchased from Uchida Wakanyaku Co. Ltd., Tokyo, Japan. Concanavalin-A (Con-A) Sepharose, CNBr-activated Sepharose 4B, Sephade G-100 and Sepharose CL-4B and CL-2B were obtained from Pharmacia Co. Ltd. Promase was purchased from Kaken-kagaku Co. Ltd. (Tokyo). *Ricinus communis* agglutinin (RCA) was purified from the extract of *R. communis* by the method of Adair and Kornfeld (13). RCA-Sepharose was prepared by the coupling of RCA and CNBr-activated Sepharose (14). Lentinan from *Lentinus edodes* was a gift from Dr. G. Chihara.

**Results**

**Fractionation of polysaccharides from Artemisia princeps Pamp**

The crude polysaccharide fraction was prepared after the reflux of the lyophilized extract with MeOH. The crude polysaccharide fraction showed potent anti-complementary activity dose dependently but the MeOH-soluble fraction did not show the activity even when concentrated materials were used for the assay. The crude polysaccharide fraction was further separated on the column of DEAE-Sepharose (Cl⁻) into unabsorbed fractions (AAF-Ia, b) and absorbed fractions (AAF-IIa, b, c) by the elution with a linear gradient of NaCl (Fig. 1). Furthermore, the remaining absorbed fraction (AAF-IId) was obtained by the elution with 2 M NaCl (data not shown). Fig. 2 shows the anti-complementary activity after the incubation of the different concentrations of the polysaccharide fractions with NHS. The anti-complementary activities are shown to be dose dependent. When 1000 μg/ml of Ia was incubated with an equal volume of NHS about 90% of the TCH₀₅ was reduced. The order of the activities of these fractions was IIa > IIb > Ia > Ic > IId > Ib. The chemical properties of these subfractions are summarized in Table I. The most active fraction, IIa, contained arabinose, galactose, mannose, glucose and uronic acid. The major polysaccharide fraction, Ib, contained arabinose, galactose, and xylose as the major neutral sugar, and a large amount of uronic acid which was estimated to be galacturonic acid by TLC of the acid hydrolyzates.

**Table I. Chemical Properties of Polysaccharide Fraction from *A. Princeps* (%)**

<table>
<thead>
<tr>
<th>Component sugars</th>
<th>Ia</th>
<th>Ib</th>
<th>IIa</th>
<th>Iib</th>
<th>Iic</th>
<th>IId</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>-</td>
<td>-</td>
<td>0.7</td>
<td>8.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>54.1</td>
<td>30.5</td>
<td>38.6</td>
<td>36.4</td>
<td>78.0</td>
<td>30.7</td>
</tr>
<tr>
<td>Xylose</td>
<td>2.3</td>
<td>2.5</td>
<td>3.9</td>
<td>14.8</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>12.5</td>
<td>19.5</td>
<td>16.2</td>
<td>6.5</td>
<td>3.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Galactose</td>
<td>11.9</td>
<td>14.4</td>
<td>26.5</td>
<td>29.3</td>
<td>11.3</td>
<td>30.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.7</td>
<td>33.2</td>
<td>13.8</td>
<td>4.9</td>
<td>5.7</td>
<td>25.3</td>
</tr>
</tbody>
</table>

* High value was given by phenol-sulfuric acid assay using arabinose as the standard because hexose is more sensitive than arabinose in this assay.
This fraction was used for further purification. The anti-complementary activity of the deproteinized IIb did not change significantly, compared with that of IIb, but its activity was found to decrease with the periodate oxidation of IIb (Fig. 3). The yield of AAF-IIb was 25.5% from the crude polysaccharide fraction.

Purification of the anti-complementary polysaccharide, AAF-IIb

AAF-IIb was found to contain a remarkable amount of galactose and was further purified by affinity chromatography on RCA-Sepharose. The RCA unbound fractions showed higher activity than the bound fraction (data not shown). The RCA unbound fraction (yield; 92.5% from AAF-IIb) was further fractionated by Con-A Sepharose because the RCA-unbound fraction was found to contain mannose. However, the amount of the Con-A bound fraction obtained by this second affinity chromatography was insignificant. The RCA-unbound fraction was further purified by gel filtration on Sephadex G-100 as shown in Fig. 4. The anti-complementary active polysaccharide fraction was eluted in the void volume. The fraction eluted in the void volume (yield; 13% from RCA-unbound fraction) yielded galactose, xylose, rhamnose, arabinose, galacturonic acid and a trace of mannose on hydrolysis whereas the fraction of smallest molecular weight contained a large amount of galac-turonic acid. When the RCA-unbound fraction was subjected to gel filtration on Sepharose CL-2B by the elution with water, broad carbohydrate fractions were obtained but the active fraction was eluted near in the void volume (Fig. 5). This result suggests that the active polysaccharide molecules tend to form an aggregate. Therefore the fraction eluted in the void volume by Sephadex G-100 gel filtration was applied on Sepharose CL-4B chromatography in the 0.2 M NaCl solution to determine whether the apparent large molecular weight of the active polysaccharide resulted from aggregation of smaller polysaccharides. Under this condition, small amount of polysaccharide (AAF-IIb-1) voided the column but major polysaccharides (AAF-IIb-2 and 3) were included in the gel (Fig. 6). The colormetrical assay elution profile of these main polysaccharides (AAF-IIb-2 and 3) showed that there are fractions of relatively enriched in uronosyl residues (AAF-IIb-2) and fractions enriched in neutral sugars (AAF-IIb-3). AAF-IIb-1 and IIb-2 were shown to have the more potent activity than AAF-IIb-3 (Fig. 7). AAF-IIb-2 and IIb-3 gave a single spot on glass-fiber paper electrophoresis in the acetate buffer and borate buffer, respectively.

**Fig. 3.** Lability of polysaccharide anti-complementary activity to periodate and Pronase treatment.

- O AAF-IIb, □ AAF-IIb Pronase digest, ● NaIO₄ oxidized AAF-IIb.

**Fig. 4.** Gel filtration of AAF-IIb/RCA unbound fraction on Sephadex G-100. The Sephadex G-100 column (3 × 87 cm) was equilibrated with H₂O and elution was performed with H₂O. Carbohydrate, 490 nm (O); protein, 280 nm (●); uronic acid, 520 nm (▲); anti-complementary activity (△).

**Fig. 5.** Gel filtration of AAF-IIb/RCA unbound fraction on Sepharose CL-2B. The Sepharose CL-2B column (3 × 90 cm) was equilibrated with distilled H₂O and elution was performed with H₂O. Symbols expressed same as Fig. 4.

**Fig. 6.** Gel filtration of Sephadex G-100 void volume fraction from Fig. 4 on Sepharose CL-4B. The Sepharose CL-4B column (3 × 92 cm) was equilibrated with 0.2 M NaCl and elution was performed with 0.2 M NaCl. Carbohydrate, 490 nm (O); uronic acid, 520 nm (▲).
The molecular weights of AAF-IIb-2 and IIb-3 were estimated to be 139000 and 31000 by the calibration of gel filtration on Sepharose CL-4B, but AAF-IIb-3 contained at least three kinds of anti-complementary polysaccharides which have a different molecular weight. The present results indicate that the major anti-complementary polysaccharides from AAF-IIb did not have affinities to RCA and Con-A. This suggests that the significant amounts of galactosyl and glucosyl residues are not present at the non-reducing ends. The anti-complementary polysaccharide fraction from AAF-IIb showed a broad single peak by gel filtration on Sepharose CL-4B, but AAF-IIb-3 contained at least three kinds of anti-complementary polysaccharides which have a different molecular weight. In a previous study it was shown by this research group that an anti-complementary arabinogalactan was isolated from the root of Angelica acutiloba Kitagawa (12, 18). Crude AR-arabinogalactan IIa showed almost similar activity with AAF-IIb-3. It was expected that arabinogalactan moiety may participate in the anti-complementary activity.

Some anti-complementary polysaccharides have already been isolated from bacteria, fungi and plants, but not yet from Chinese crude drugs except β-1,3-glucan from Poria cocos (19), Lentinas edodes (19) and Coriolus versicolor (20) or AR-arabinogalactan IIa from A. acutiloba Kitagawa (12, 18). The major anti-complementary polysaccharides from A. princeps Pamp showed more potent activity than anti-complementary β-1,3-glucan from L. edodes. AAF-IIb-2 and IIb-3 contained remarkable amounts of galacturonic acid. But uronic acid containing anti-complementary polysaccharide has not yet been reported. Another acidic polysaccharide, pectin from apple or A. acutiloba did not show the significant anti-complementary activity in comparison with AAF-IIb-2 and IIb-3 (unpublished). These facts indicate that the anti-complementary acidic heteroglycan from A. princeps Pamp 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 activity of this unique acidic heteroglycan are now in progress.

Acknowledgements

We are grateful to Dr. G. Chihara (National Cancer Center, Japan) for a kind gift of lentinan. An equipment fund from Tsumura-Juntendo Co., Ltd., Tokyo, Japan supported a part of this work.

References


Introduction

A number of protein amino acids and some other closely related compounds i.e. ornithine, nicotinic acid and anthranilic acid are essential precursors in alkaloid biosynthesis.

Usually amino acids are decarboxylated during the course of the formation of various nitrogen-containing heterocyclic ring systems. In this context anthranilic acid occupies an unique position. In anthranilate-derived alkaloids, the carboxyl group is retained and takes part in the biosynthesis of the different heterocycles. To this particular group of secondary metabolites belong the acridone alkaloids which are exclusively found in some genera of the Rutaceae. It was argued for thermodynamical reasons that “activated” anthranilic acid might react with malonyl-CoA in the course of acridone biosynthesis (1). Anthraniloyl-coenzyme A (CoA) thioester seemed to be the most likely candidate. It is now well established that the N-methylation of anthranilic acid catalyzed by a particular N-methyltransferase is the first pathway-specific step in acridone biosyn-