Preparation and Biological Characterization of Limulus Factor G-activating Substance of Aspergillus spp.

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ABSTRACT

Aspergillus is a medically important fungal genus that causes a life-threatening infection known as aspergillosis in immunocompromised patients. β-1,3-Glucan is detected in the plasma of patients with aspergillosis and appears to be useful for the diagnosis of aspergillosis. In this study, we cultured Aspergillus spp. in a chemically defined liquid medium and prepared an Aspergillus water-soluble fraction (ASWS) from the culture supernatants. ASWS was found to be primarily composed of polysaccharides and proteins. Nuclear magnetic resonance analysis suggested that ASWS is a complex carbohydrate, consisting of α-1,3-glucan, β-1,3-glucan, galactomannan, and protein. The ASWS from Aspergillus fumigatus showed limulus factor G activity, whereas zymolyase-treated ASWS did not. ASWS was eliminated from the blood more rapidly than Aspergillus solubilized cell wall β-glucan. We analyzed the reactivity of human immunoglobulin towards ASWS by an enzyme-linked immunosorbent assay. Anti-ASWS antibodies were detected in human sera, with titers differing among individuals. This study demonstrated that the ASWS corresponds to the limulus factor G-activating substance found in the blood of patients with aspergillosis.

Key words: Aspergillus, glucan, limulus factor G, soluble antigen

Introduction

The number of immunocompromised hosts has increased with the progression of chemotherapy and transplantation therapy. Opportunistic infections can easily occur in these patients, which is a serious problem in clinical settings. Particularly, the number of patients with deep mycosis has increased, an infection that shows a high mortality rate. Aspergillus is a medically important fungal genus that causes a life-threatening infection known as aspergillosis in immunocompromised patients. The rate at which life-saving treatment is provided for invasive aspergillosis is extremely low. Delays in the diagnosis and start of treatment are associated with high mortality. Therefore, early diagnosis and treatment are important. Furthermore, Aspergillus fumigatus, Aspergillus flavus, and Aspergillus niger cause various diseases, such as allergic bronchopulmonary aspergillosis and aspergilloma. The mycelial cell wall of Aspergillus spp. is primarily composed of polysaccharides (galactomannan, α-1,3-glucan, β-1,3-glucan, and chitin). We previously prepared Aspergillus cell wall-solubilized β-1,3-glucan (ASBG) from Aspergillus dried cells using the NaClO-urea-autoclave method. ASBG was capable of activating limulus factor G. We also found that ASBG induces the production of...
interleukin (IL)-8 during human peripheral blood mononuclear cell stimulation. β-1,3-Glucan is an insoluble component of the fungal cell wall. However, a solubilized form of β-1,3-glucan is also produced and released from fungal cells\(^8\). β-1,3-Glucan is detected in the plasma of patients with invasive deep mycosis and fungal febrile episodes. The β-1,3-glucan assay is useful for early diagnosis and contributes to antifungal stewardship\(^9,10\). Therefore, β-1,3-glucan is an effective serological diagnostic parameter in clinical settings.

The structural and physical properties of fungal cell wall β-glucan and other polysaccharides, including their primary structure, conformation, and molecular weight, differ among fungal species. Biological activities, such as cytokine production and limulus factor G activity, depend on the physical properties of the fungi\(^11\). Therefore, determining the structures and physical properties of fungal cell wall polysaccharides is important for understanding their in vivo significance.

We previously cultured Candida spp. in chemically defined medium and prepared a Candida water-soluble fraction (CAWS) from the culture supernatant\(^12\). CAWS showed various activities, such as activation of limulus factor G, acute lethal toxicity, and induction of vasculitis\(^13,14\). The limulus factor G test is effective for early diagnosis of aspergillosis. Aspergillus galactomannan and an Aspergillus lateral-flow device can be used to diagnose invasive aspergillosis\(^15\). Therefore, we aimed to prepare a limulus factor G-activating substance comparable to CAWS using Aspergillus spp.

In this study, we prepared an Aspergillus water-soluble fraction (ASWS) from the supernatants of Aspergillus spp. cultured in a chemically defined liquid medium and examined the physical properties of Aspergillus galactomannan and an Aspergillus lateral-flow device can be used to diagnose invasive aspergillosis\(^15\). Therefore, we aimed to prepare a limulus factor G-activating substance comparable to CAWS using Aspergillus spp.

Materials and methods

Materials

All strains of A. fumigatus (NBRC 30870 and 4400), A. niger (NBRC 6342), Aspergillus oryzae (NBRC 30103), and Candida albicans (NBRC 1385) were purchased from the NITE Biological Resource Center (Chiba, Japan), maintained on Sabouraud agar (Difco, Detroit, MI, USA) at 25°C, and transferred once every 3 months. Fungitec G test MK was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Zymolyase was obtained from Seikagaku Corp (Tokyo, Japan). Lipopolysaccharides (LPS) from Escherichia coli O111: B4 were purchased from Sigma (St. Louis, MO, USA). Dextran was purchased from Seikagaku Corp. Polyglobin N was purchased from Bayer AG. Pharmaceuticals (Berlin, Germany). Kenketu glovenin-I was purchased from Nihon Pharmaceutical Co., Ltd. GAMMAGARD was purchased from Baxalta Japan, Ltd. (Tokyo, Japan). Human reference serum was purchased from Sigma and Bethyl Laboratories (TX, USA).

Media

C-limiting medium, originally described by Shepherd and Sullivan\(^16\), was used to grow all strains unless stated otherwise. The C-limiting medium contained (per liter) 10 g of sucrose, 2 g of (NH\(_4\))\(_2\)SO\(_4\), 2 g of KH\(_2\)PO\(_4\), 0.05 g of CaCl\(_2\) 2H\(_2\)O, 0.05 g of MgSO\(_4\) 7H\(_2\)O, 1 mg of ZnSO\(_4\) 7H\(_2\)O, 1 mg of CuSO\(_4\) 5H\(_2\)O, 0.01 g of FeSO\(_4\) 7H\(_2\)O, and 25 µg of biotin, with a final pH of 5.2. The medium (5 L) was placed in a glass jar of a Microferm fermentor (Sakura SI Co., Ltd., Tokyo, Japan) and cultured at 27°C with aeration at 5 L per min and stirring at 400 rpm.

Mice

Specific pathogen-free male 6-7-week-old DBA/2 mice were obtained from Japan SLC (Shizuoka, Japan).

Preparation of ASWS and solubilized cell wall β-glucan

All Aspergillus strains were cultured in a C-limiting medium. To collect ASWS, an equal volume of ethanol was added to the whole culture and the precipitate was collected, which included cells and secreted macromolecules. The precipitate was then suspended in an aliquot of distilled water and the solubilized fraction was collected as ASWS and acetone-dried. ASBG was prepared using NaClO-urea autoclave method as previously described\(^7\).

Carbohydrate analyses

The carbohydrate content was determined using the phenol-sulfuric acid method. Component sugars were determined by capillary gas-liquid chromatography (Ohkura Riken, Tokyo, Japan) of alditol acetate derivatives after complete hydrolysis by 2 M trifluoroacetic acid. A capillary column
of fused silica (J&W Scientific, Folsom, CA, USA; 30 m × 0.262 mm; liquid phase: DB-225, 0.25 µM) was used at 220°C. The molar ratio of mannose, galactose, and glucose (Man/Gal/Glc) was calculated based on the peak area of each component (glucose was set to 100).

Measurement of galactomannan content
Galactomannan was estimated by the Platelia Aspergillus EIA test (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions.

Nuclear magnetic resonance (NMR) analysis
Solubilized fractions and authentic materials were dissolved in D$_2$O, and the $^1$H and $^{13}$C-HSQC spectra were measured at 70°C. A Bruker AV500 instrument equipped with TopSpin software was used (Billerica, MA, USA).

Measurement of (1 → 3)-β-D-glucan by Fungitec G test MK
The activation of factor G (limulus reactivity) by (1 → 3)-β-D-glucans was measured by a chromogenic method using a (1 → 3)-β-D-glucan-specific reagent (Nissui Pharmaceutical Co., Ltd), which eliminates factor C. Each (1 → 3)-β-D-glucan was dissolved in 0.5 N NaOH (1 mg/mL) and diluted with 0.01 N NaOH. Dilutions were prepared in 0.01 N NaOH, and a sample solution was used directly for the limulus reaction without neutralization. Diluted NaOH was confirmed to be usable for the limulus reaction because of the high buffering action of the reagent. Reactions were performed in a flat-bottomed 96-well Toxipet plate 96F (Seikagaku Corp.) as follows. Samples (50μL) were placed in the wells and Fungitec G test MK reagent (50μL) was added to each well. The plate was incubated at 37°C; during incubation, the absorbance at 405 nm (reference 492 nm) was measured kinetically using a microplate reader (Wellreader SK603, Seikagaku Corp.). Disposable plastic materials for tissue culture or clinical use were employed, and all glassware was sterilized at 260°C for 3 h. All operations were performed in triplicate under aseptic conditions.

Zymolyase digestion of ASWS
ASWS (20 mg) suspended in 10 mL of acetate buffer (50 mM, pH 6.0) was mixed with 1 mg of zymolyase 100T (Seikagaku Corp.). After overnight incubation at 45°C, the reaction mixture was boiled for 3 min to inactivate the enzyme. The resulting solution was diluted and applied to Fungitec G test MK.

Preparation of plasma for blood clearance assay
Each ASWS prepared from A. fumigatus NBRC 30870 and ASBG was diluted in physiological saline and autoclaved. ASWS and ASBG (1,000μg) were administered to mice (n = 3, each group) by intravenous injection. An aliquot of blood was collected from the tail vein at appropriate intervals using heparinized capillaries. After centrifugation, plasma samples were stored at 4°C until the G test (Fungitec G test MK).

Enzyme-linked immunosorbent assay for anti-ASWS antibody
A 96-well Nunc plate was coated with ASWS prepared from A. fumigatus NBRC 30870 and ASBG (25 μg/mL) in 0.1 M carbonate buffer (pH 9.6) by incubation at 4°C overnight. The plate was washed with phosphate-buffered saline buffer (PBS) containing 0.05% Tween 20 (Wako Pure Chemical Co., Osaka, Japan) (PBST) and blocked with 0.5% bovine serum albumin (BSA, Sigma) at 37°C for 60 min. After washing with PBST, the plate was incubated with diluted human immunoglobulin preparation (Polyglobin N, Bayer Schering Pharma, Berlin, Germany, Kenketsu glovenin-Ⅰ, Nihon Pharmaceutical Co., Ltd., GAMMAGARD, Baxalta Japan, Ltd.) or human reference serum at 37°C for 60 min. The plate was then washed with PBST and treated with an antibody for peroxidase-conjugated anti-human IgG + M + A (Sigma) in PBST containing 0.1% bovine serum albumin and developed with a tetramethylbenzidine substrate system (KPL, Inc., Gaithersburg, MD, USA). Color development was stopped with 1 N phosphoric acid and optical density was measured at 450 nm.

Results

Preparation and physical properties of ASWS
The limulus factor G-positive substance is found in the blood of patients infected with Aspergillus. We cultured Aspergillus spp. in a chemically defined liquid medium and prepared ASWS from the culture supernatant. We prepared the ASWS of each Aspergillus spp. (Table 1). Composition analysis revealed that ASWS was primarily composed of polysaccharides and proteins (Table 2). These results were supported by elemental analysis (C, H, and N). We analyzed the saccharide composition of ASWS by gas liquid chromatography. We found that glucose was the main component; while mannose and galactose were also detected. We measured the content of...
galactomannan (Aspergillus antigen) by using Plateria Aspergillus (Aspergillus antigen detection kit). Galactomannan was detected in all preparations of ASWS used in this study. These results suggest that ASWS is a glycoprotein containing galactomannan.

Next, we analyzed the structure of ASWS derived from A. fumigatus NBRC 30870 by 2D-NMR analysis (Fig. 1). The peaks corresponding to α-1, 3-glucan, β -1, 3-glucan, and β -1, 5-galactofuranose linkages were identified by NMR analysis. Major signals were assigned by comparing the published spectra of the Aspergillus cell wall galactomannan and glucan. The peaks of α-1, 3-glucan, β -1,3-glucan, α -mannan, and β -1,5-galactofuranose linkages were identified by NMR analysis. ASWS was predicted to be a glycoprotein, consisting of α-1,3-glucan, β-1,3-glucan, and galactomannan. This suggests that ASWS corresponds to the material released in the blood of patients with aspergillosis.

Activation of limulus factor G by ASWS

Examining the activation of limulus factor G by ASWS is of considerable clinical importance as ASWS is thought to correspond to the material in the blood of patients with aspergillosis. We examined the activation of limulus factor G by ASWS from A. fumigatus, a clinically important strain (Fig. 2a). ASBG and CAWS were used as controls. The activation of limulus factor G of ASWS from A. fumigatus NBRC 30870 and NBRC 4400 was one-tenth that of CAWS. In addition, decreased activation was observed at high concentrations of ASWS.

To demonstrate that activation of ASWS limulus factor G depends on β -1,3-glucan in ASWS, we compared the activity of ASWS limulus factor G before and after treatment with zymolyase 100T (β -1,3-glucanase) (Fig. 2b). The activity of ASWS limulus factor G treated with zymolyase was significantly reduced. This indicates that ASWS contains β -1,3-glucan, and further supports the

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Table 1. Strains, culture conditions, and yield of ASWS

| Strain      | Culture | Yield |
|-------------|---------|-------|
|             | Temp (℃) | Days | Mycelium (g/L) | ASWS (mg/L) |
| A. fumigatus| 27       | 2    | 2.3            | 62.5        |
| A. fumigatus| 27       | 4    | 2.4 ± 1.0      | 86.7 ± 28.4 |
| A. fumigatus| 37       | 4    | 3.2            | 110         |
| A. fumigatus| 27       | 4    | 1.7 ± 0.1      | 95.5 ± 57.2 |
| A. niger    | 27       | 4    | 5.2 ± 1.6      | 135.5 ± 45.9|
| A. oryzae  | 27       | 4    | 3.5 ± 1.0      | 42.5 ± 10.6 |
| C. albicans| 27       | 2    | 3.3 ± 0.1      | 142.4 ± 14.3|

a) This preparation is CAWS.

Table 2. Properties of ASWS

| Strain      | Carbohydrate a) (%) | Protein b) (%) | Galactomannan c) (%) | Man/Gal/Glc |
|-------------|----------------------|----------------|-----------------------|-------------|
| A. fumigatus| 53.5 ± 3.5           | 13.5 ± 2.1     | 10.3 ± 2.0            | 26.4/ 11.5/ 100 |
| A. fumigatus| 36.5 ± 0.7           | 13.0           | 11.9 ± 3.3            | 9.1/ 25.3/ 100 |
| A. niger    | 54.0 ± 2.8           | 11.0           | 48.8 ± 1.7            | 153.3/ 18.4/ 100 |
| A. oryzae  | 67.5 ± 2.1           | 5.0            | 2.5 ± 0.6             | 7.9/ 5.1/ 100 |
| C. albicans| 78.0 ± 6.6           | 15.0 ± 6.3     | 630/ 0/ 100           |             |

a) Carbohydrate levels were measured using the phenol-sulfuric acid assay.
b) Protein concentration was measured using the BCA assay.
c) Galactomannan was measured using the Plateria® Aspergillus EIA test.
d) This preparation is CAWS.
**Fig. 1.** $^1$H, $^{13}$C-HSQC spectra of ASWS in D$_2$O.

The ASWS of *Aspergillus fumigatus* NBRC 30870 was dissolved in D$_2$O and measured using $^1$H, $^{13}$C-HSQC as described in Materials and Methods.

**Fig. 2.** Limulus activity of ASWS derived from *A. fumigatus*.

(a) ASWS derived from various *Aspergillus* spp. and CAWS were dissolved in 0.5 N NaOH and dilutions were prepared in distilled water. The Fungitec G test MK reactivities of these solutions were determined as described in Materials and Methods. ASBG was used as a standard material.

(b) ASWS from *A. fumigatus* NBRC 30870 was dissolved in acetate buffer and digested with zymolyase 100T as described in Materials and Methods. The resulting solutions were treated with 0.5 N NaOH, diluted with distilled water and subjected to the Fungitec G test MK as described in Materials and Methods.
conclusion that ASWS is the material in the blood of patients with aspergillosis.

**Concentration of ASWS in blood after intravenous administration of ASWS in DBA/2 mice**

The blood clearance of ASWS in the sera of DBA/2 mice administered with ASWS intravenously was examined by the limulus G test to measure β-1,3-glucan concentration (Fig. 3). First, it was found that ASWS was immediately cleared from the blood. The half-period of ASWS clearance was approximately 14 min. The clearance of ASWS was faster than that of ASBG. This suggests that the rapid clearance of ASWS is attributable to a component other than β-1,3-glucan.

**Antibody titer in human sera against ASWS**

An antibody against ASBG was detected in human sera. The titer of anti-β-glucan antibody changed in the sera of patients with deep mycosis depending on disease progress. The reactivity of antibodies to ASWS in human sera was examined by ELISA using an ASWS-coated plate. The reactivity of Polyglobin N, a human immunoglobulin preparation, increased in a dose-dependent manner (Fig. 4a). These results suggest that an anti-ASWS antibody is present in human sera. Next, we examined the reactivity of the anti-ASWS antibody by competitive ELISA with ASWS, ASBG, and LPS as soluble antigen to the ASWS antigen (Fig. 4b). When ASWS was added, binding was significantly inhibited. On the other hands, the inhibition of ASBG was partial. This result suggested the presence of antibodies to components of ASWS other than β-glucan. Also, we compared the reactivity to antigens: ASWS, ASBG, LPS from *E. coli*, and dex-tran in the human sera (n = 2) and immunoglobulin preparation (n = 3) (Fig. 4c). The titer differed between samples. Also, the ratio of anti-ASAG to anti-ASBG varied in each sample.

**Discussion**

β-glucan (a limulus factor G-positive substance) is known to be released in the blood of patients infected with *Aspergillus*. The limulus factor G test is effective for the early diagnosis of aspergillosis. However, the detailed chemical properties of the limulus factor G-positive substance remain unknown because of its low abundance and the impossibility of its isolation. Previously, we cultured *Candida* spp. in chemically defined medium and prepared CAWS (limulus factor G-positive analogical material of β-glucan). Hence, we predicted that it was possible to prepare analogical materials similar to CAWS from *Aspergillus* spp. Therefore, we cultured *Aspergillus* spp. in chemically defined complete media and prepared ASWS from the culture supernatants. Next, we examined the structure, activity for limulus factor G, and blood clearance. We compared the physical properties and activities between ASWS from each *Aspergillus* strain and CAWS.

We prepared ASWS from each *Aspergillus* sp. The yield of ASWS from *A. fumigatus* and *A. niger* was approximately 100 mg/L, while that from *A.
oryzae was as low as 42.5 mg/L. Additionally, the content of galactomannan and sugar composition of ASWS in *A. oryzae* differed from those of ASWS derived from the other two species. These differences may be related to the pathogenicity of each *Aspergillus* sp. We focused on ASWS derived from *A. fumigatus*, which is the most commonly isolated species from patients with aspergillosis. ASWS was found to be primarily composed of saccharides and proteins. We measured galactomannan (*Aspergillus* antigen) content using Plateria *Aspergillus* antigen test. ASWS contained approximately 10% of the glycoprotein galactomannan. The β-1,5-galactofuranose linkage was also identified by NMR analysis. We analyzed the composition of saccharides in ASWS and found that glucose was the major saccharide present. The *Aspergillus* cell wall is known to contain β-1,
3-glucan and α-1,3-glucan. Based on the NMR results, the major glucan present in ASWS was α-1,3-glucan. Recent reports showed that α-1,3-glucan contributes to biofilm formation as a structural factor of the extracellular matrix. Thus, ASWS may contribute to biofilm formation. In addition, a β-1,3-glucan peak was identified. These results suggest that ASWS is a limulus factor G activator.

We compared the activation of limulus factor G of ASWS and CAWS. ASWS from A. fumigatus NBRC 30870 and 4400 showed one-tenth of the activity of CAWS. We prepared Candida and Aspergillus cell wall β-1,3-glucan and compared the activation of limulus factor G in each sample. Each glucan showed the same limulus factor G activity. The difference in limulus factor G activity of the water-soluble fractions may be related to the β-1,3-glucan content. The limulus factor G activity of ASWS decreased at high concentrations. These results indicate that ASWS has glucan inhibitor (GI) activity because it contains low molecular weight β-1,3-glucan. The activity of ASWS limulus factor G was lost after zymolase treatment, indicating that ASWS contains β-1,3-glucan and corresponds to the limulus factor G-activating substance in the blood of patients with aspergillosis.

To examine the blood clearance of ASWS, we intravenously administered ASWS into DBA/2 mice and measured the concentration of β-glucan in blood samples using the limulus G test. Aspergillus activated the classical and lectin complement pathways. The production of anaphylatoxins by ASWS may result in lethal toxicity, such as in CAWS. Therefore, we examined the blood clearance of ASWS in DBA/2 mice, which are C5a-deficient. ASWS was immediately eliminated from the blood with an elimination half-life of 14 min. ASWS was more rapidly eliminated from the blood than was ASBG. This suggests that there are structural components involved in the blood clearance of ASWS other than β-glucan. Additionally, a previous study showed that the conformation of β-glucan affects its blood clearance. Differences in physical properties such as conformation and molecular weight between β-glucan contained in ASWS and ASBG may affect their blood clearance. If the antigen released from Aspergillus cells in the host is similar to ASWS, the values of β-1,3-glucan and galactomannan in clinical samples would indicate the presence of Aspergillus cells and be released without a lag time.

Few studies have examined the biological activity of each Aspergillus cell wall component and the host recognition molecule that interacts with the Aspergillus cell wall polysaccharide. We previously reported that the anti-β-glucan antibody is a β-glucan-recognizing molecule that mediates acquired immunity. We examined the anti-ASWS antibody titer and detected an anti-ASWS antibody in human immunoglobulin preparations and sera. The anti-ASWS antibody titer differed among individuals; the anti-ASWS antibody showed a specific titer that differed from that of the anti-ASBG antibody titer. This result suggests the presence of antibodies that recognize components other than β-glucan, such as α-glucan and galactomannan. Several reports demonstrated the contribution of antibodies to the defense against fungal infection. Thus, the anti-ASWS antibody may also participate in the defense against Aspergillus infection.

In this study, we prepared water-soluble saccharide fractions released from Aspergillus and determined their characteristics. ASWS may correspond to materials in the blood of patients with aspergillosis. In order to better understand their functions in the host and fungi, further studies should be conducted to identify and characterize the antigens derived from pathogenic fungi.

**Conflict of Interest**

None declared.

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