Isolation of Mitogenic Substance from Sclerotia of *Sclerotinia sclerotiorum* IFO 9395 Extracted with Phosphate Buffer

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The buffer extracts (3S) of sclerotia of *Sclerotinia sclerotiorum* IFO 9395 contained mitogenic substance(s) to murine splenocytes (Shinohara *et al.* Chem. Pharm. Bull., 38, 2219 (1990)). Although the native 3S was slightly mitogenic, heating of 3S induced significant mitogenic activity. To isolate the mitogen, we separated 3S by ion-exchange and gel filtration chromatographies. The isolated mitogen, named sclerogen, has a molecular mass of 32 kilodaltons (kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the isoelectric point (pI) of 5.9 by chromatofocusing. Sclerogen was significantly mitogenic *in vitro* against murine splenocytes after heat denaturation, and also showed the augmentation of the primary mixed lymphocyte reaction (MLR) *in vitro*. However, sclerogen did not show the activation of an alternative pathway of complement and hemagglutination activity. These results suggest that sclerogen is a unique mitogen which differs from lectins and shows mitogenicity after heat denaturation.

Keywords *Sclerotinia sclerotiorum*; sclerotia; mitogenic activity; fungal mitogen; phosphate buffer extract; heat denaturation; mixed lymphocyte reaction

Previously, we have demonstrated the existence of immunomodulator(s) in hot water extracts from sclerotia of *Sclerotinia sclerotiorum* IFO 9395.¹⁾ The extracts, named TSHW, showed mitogenicity, polyclonal B cell activation, reticuloendothelial system stimulation, and antitumor activities.¹⁾ In these activities, (1→3)- β -D-glucan parts and water insoluble-protein fractions played a part in the antitumor and mitogenic activities in TSHW, respectively.²⁾ Preliminary characterization of TSHW suggested the charge and molecular mass heterogeneities of the mitogen(s) in TSHW and resulted in difficulty in isolating the mitogenic substance. We concluded that this heterogeneity must result from some post-translational modifications during extraction.

Recently, we overcame this difficulty by extracting sclerotia with milder conditions. Interestingly, the phosphate buffer (P.B.) extracts, referred to as 3S, showed significant mitogenic activity after heat denaturation.³⁾ Treatment of native 3S for only 1 min in boiling water or 10 min at 70°C generated an obvious mitogenicity. The activity was also observed against the splenocytes from C3H/HeJ, a lipopolysaccharide low-responder strain, and athymic (BALB/c Nu/Nu) mice. Accompanied with heat denaturation, the apparent physicochemical changes (probably polymerization) were observed in normal-polyacrylamide gel electrophoresis (PAGE).

In this study, we tried to isolate a mitogenic substance from 3S, and some biochemical characteristics were examined. The isolated mitogenic substance, named sclerogen, was a 32 kilodaltons (kDa) protein having no hemagglutinating activity against erythrocytes, showed mitogenicity after heat denaturation and augmented the primary mixed lymphocyte reaction (MLR) of splenocytes *in vitro*.

Materials and Methods

Mice ICR, C3H/He, C3H/HeJ and C57BL/6 mice (6—12-wk-old, male) were from SLC, Japan, and were maintained under specific pathogen-free conditions for the duration of the experiments.

Materials and Microorganisms DEAE-Sephadex A-25, Polybuffer 74, and PBE 94 were obtained from Pharmacia Fine Chemicals, Sweden. Toyopearl TSK-gel HW-55F was from Tosoh, Japan. The prestained sodium dodecyl sulfate (SDS) molecular weight markers were from Sigma

Chemical Co., Ltd., U.S.A. Silver stain kit Wako was from Wako Pure Chemical Industries, Japan. Tritiated thymidine (³H-TdR), specific activity 38 Ci/mmol, was from ICN Biomedicals Inc., U.S.A. Sheep whole blood in Alsever's solution was from Japan Material Center, and human whole blood groups (A, B, O, and AB) were from healthy volunteers. Mouse whole blood was from C3H/HeJ. The mycelia of *Sclerotinia sclerotiorum* IFO 9395 were from the Institute for Fermentation, Osaka, Japan (IFO).

Production of Sclerotia from *S. sclerotiorum* IFO 9395 The sclerotia were obtained by culturing on potato-sucrose agar media at 25°C in our laboratory.⁴⁾ Sclerotia on the agar media were picked up after 3—4 weeks and lyophilized.

Preparation of the Buffer Extracts from Sclerotia The buffer extracts (3S) from sclerotia was prepared by the method previously described.³⁾

Assay for Mitogenic Activity The measurement of mitogenic activity was carried out by the method previously described.⁵⁾ Briefly, the splenocytes from ICR or C3H/HeJ were prepared at a concentration of 5×10^6 cells/ml in ice cold RPMI 1640 medium with or without fetal calf serum (FCS) at a final concentration of 5%. Fifty μ l of osmotic stabilized native sclerogen (sclerogen-M) or heat denatured-sclerogen (sclerogen-MB) were placed in a flat-bottomed 96-well microplate, and the cell suspension was added in 100 μ l volumes. Cultures were incubated for 28 h at 37°C in a 5% CO₂ incubator and pulsed with 0.5 μ Ci of ³H-TdR for a further 20 h. After harvesting cells, radioactivity incorporated into the cells was measured by a liquid scintillation counter. Data are expressed as mean cpm $\pm$ S.D. of triplicate cultures.

Primary Mixed Lymphocyte Reaction (MLR) Splenocytes from C3H/He were used as a responder and C57BL/6 spleen cells were used as a stimulator. Responder cells were prepared at 5×10^6 cells/50 μ l in RPMI-1640 containing 10% FCS and 5×10^{-5} M β -mercaptoethanol. Stimulators from C3H/He and C57BL/6 prepared at 3×10^7 cells/ml in Hanks' balanced salt solution (HBSS) were treated with mitomycin C (25 μ g/ml final concentration) at 37°C in a water bath for 30 min with gentle stirring every 10 min.⁶⁾ After washing with HBSS (three times), the cells were prepared at 5×10^6 cells/50 μ l in RPMI-1640 containing 10% FCS and 5×10^{-5} M β -mercaptoethanol. An osmotic stabilized-sample (sclerogen-M and -MB) in RPMI-1640 (50 μ l) was added to each well in a flat-bottomed 96-well microplate, and then 50 μ l of each prepared cell suspension was added. After 76 h of incubation at 37°C in a 5% CO₂ incubator, 0.5 μ Ci of ³H-TdR was added and further incubated for 20 h. After harvesting cells, radioactivity incorporated into the cells was measured by a liquid scintillation counter. Data are expressed as mean cpm $\pm$ S.D. of quadruplicate cultures.

Assay for Alternative Pathway of Complement Twenty five μ l of human serum from healthy volunteers were mixed with 5 μ l of VBS-(EGTA)-MgCl₂ (VEM; 4.45 mM barbital sodium and 127 mM NaCl pH 7.4 (veronal buffered saline (VBS)) supplemented with 0.1% (w/v) gelatin, 2 mM MgCl₂, and 8 mM ethylene glycol bis(2-aminoethyl ether) tetraacetic acid (EGTA)), placed in a flat-bottomed 96-well microplate, and then incubated for 10 min at 37°C. In the resulting mixture, 50 μ l of sclerogen-M and -MB (82.3 μ g/ml final concentration as bovine serum albumin (BSA)) were added and further incubated from 60 min at 37°C. After incubation, the plates

were cooled on ice, and 50 μ l of rabbit red blood cells (RRBC) suspended in VEM (7.5×10^8 cells/ml) after washing with VBS-ethylenediaminetetraacetic acid (EDTA) (VBS containing 0.1% (w/v) gelatin and 10 mM EDTA) were added to each well. The turbidity of RRBC was measured during incubation at 25°C every 15 min for 60 min by using a microplate reader at 630 nm.

Hemagglutination Assay Packed sheep red blood cells (SRBC), four gourps (A, B, O, and AB) of human red blood cells (HRBC), or mouse red blood cells from C3H/HeJ (MRBC) were washed with phosphate buffered saline (PBS) three times and then suspended to 1.5% (v/v) with the same buffer. Twenty five μ l of sclerogen in saline (20.9 ng/ml to 42.8 μ g/ml final concentration as BSA) were placed in each well of a round bottomed 96-well microplate, and 0.2% of gelatin in PBS (75 μ l) and the prepared SRBC, HRBC, or MRBC (25 μ l) were added. The plate was shaken for 30 s at r.t. and incubated for 1.5 h at 37°C. Then, the plate was placed at r.t. overnight and assessed for hemagglutination ability. As a positive control, concanavalin A (Con A) was used in the range of 48.8 ng/ml to 100 μ g/ml as a final concentration.

Electrophoresis Gel electrophoresis in the absence of SDS was carried out in 12.5% polyacrylamide slab gels according to the method of Ornstein⁷⁾ and Davis,⁸⁾ and in the presence of SDS by the method of Laemmli.⁹⁾ The details were given in a previous paper.³⁾

Chemical Analyses Protein concentration was determined by the BCA protein assay reagent¹⁰⁾ (BSA as a standard). Amino acid analysis was performed in a Hitachi L-8500 amino acid analyzer after hydrolysis in 6 N HCl at 110°C for each time.

Results

Conditions of Phosphate Buffer Extraction from Sclerotia

We have demonstrated that the P.B. extracts (3S) of sclerotia of *S. sclerotiorum* IFO 9395 showed significant mitogenic activity after heat denaturation. To determine the appropriate conditions of phosphate buffer extraction from sclerotia, we compared the efficiency of extraction at four kinds of pH (5.0, 6.0, 7.0, and 8.0) and for various periods (3, 6, 12, 24, and 48 h). Consequently, no significant difference was observed in each 3S extracted by the four kinds of pH, and the 3S have already been extracted within 3 h (data not shown). Furthermore, the mitogenic activity of each 3S was similar to each other (data not shown). So, we determined to use the extraction condition for 3 h at 37°C in 10 mM P.B. pH 6.8 for further analyses. The components of 3S extracted by this condition were carbohydrate (29%), protein (69%), and phosphate (19%).

Fig. 1. Elution Profile of DEAE-Adsorbed 3S on TSK-gel HW-55F

DEAE-adsorbed 3S was applied to the column (1.7 $\times$ 50 cm) of TSK-gel HW-55F equilibrated with 10 mM P.B. pH 6.8. Fractions of 2.7 ml were collected at 7°C and protein was monitored by UV absorption measurement at 280 nm (—). Mitogenicity of aliquots of fraction ($\square$) were measured by using the splenocytes from C3H/HeJ (5×10^5 cells/culture) under FCS free conditions described in Materials and Methods. Arrows indicate the void volume. a, bovine serum albumin (66 kDa); b, papain (23.4 kDa); c, cytochrome c (12.4 kDa). V_t = fr. 75.

Isolation and Characterization of the Mitogen, Sclerogen

To isolate the mitogenic substance, 3S was applied to the column of DEAE-Sephadex A-25 equilibrated with 10 mM P.B. pH 6.8. After being washed with the same buffer, the column was eluted with 1 M NaCl containing 10 mM P.B. pH 6.8. The first peak of the adsorbed fraction was collected, and then the fraction was applied to the column to TSK-gel HW-55 equilibrated with the same buffer. As shown in Fig. 1, only one peak corresponding to about 30 kDa monitored by ultraviolet (UV) absorption measurement was detected. From the results of the mitogenic activity of each fraction after autoclaving, two peaks (frs. 1 and 2, respectively) were detected (Fig. 1). However, on SDS-PAGE analysis, although fr. 2 showed a clear single band at 32 kDa under a reducing condition which corresponded to the main band

Fig. 2. SDS-PAGE Analysis of Sclerogen Following Isolation by Ion-Exchange Chromatography and Gel Filtration

Each sample was subjected to 12.5% SDS-PAGE under reducing conditions. Lane 1, crude 3S; lane 2, DEAE-adsorbed 3S; lane 3, fr. 1 from gel filtration on TSK-gel HW-55F (Fig. 3); lane 4, fr. 2 (sclerogen) from the gel filtration.

TABLE I. Amino Acid Composition of Sclerogen

Amino acids	mol/mol
Asp (Asn)	34.3 $\pm$ 2.2 ^{a)}
Thr	20.1 $\pm$ 0.7
Ser	17.1 $\pm$ 3.4
Glu (Gln)	28.0 $\pm$ 1.2
Gly	25.2 $\pm$ 4.1
Ala	15.2 $\pm$ 1.6
Cys	n.d.
Val	14.4 $\pm$ 0.6
Met	4.5 $\pm$ 0.8
Ile	12.5 $\pm$ 1.0
Leu	17.9 $\pm$ 0.9
Tyr	7.5 $\pm$ 0.6
Phe	9.7 $\pm$ 0.5
Lys	19.2 $\pm$ 2.3
His	7.4 $\pm$ 1.5
Trp	n.d.
Arg	6.5 $\pm$ 0.4
Pro	12.4 $\pm$ 0.6

a) The values given are the mean $\pm$ S.D. of the three hydrolyses for 24, 48, and 72 h, expressed as each mol of amino acid per one mol of sclerogen. The values of Cys and Trp are not determined (expressed as n.d.).

Fig. 3. Elution Profiles of Sclerogen on Chromatofocusing

Native sclerogen (sclerogen-M) was applied to the column of PBE 94 (10 ml) equilibrated with 0.025 M imidazole-HCl buffer pH 7.4. The column was eluted with a step gradient by using Polybuffer 74 adjusted to pH 4.0. Fractions of 1.6 ml were collected and protein (—) and pH (---) were monitored by ultraviolet absorption measurement and pH meter, respectively.

Fig. 4. Effect of Heat Denaturation on the Expression of Mitogenic Activity by Sclerogen

Splenocytes from C3H/HeJ (5×10^5 cells/culture) were cultured with native sclerogen (sclerogen-M, ○) or heat denatured sclerogen (sclerogen-MB, ●) for 30 min in boiling water or in their absence (△) under FCS free conditions. The concentrations of sclerogen-M and -MB were expressed as BSA measured by the BCA protein assay reagent.¹⁰⁾

in 3S, no bands were detected in fr. 1 (Fig. 2). The reason for this observation will be discussed later. Thus, we determined fr. 2 as the isolated mitogenic substance from 3S, and designated it sclerogen. The yield of sclerogen was 1.4% from sclerotia, determined by the UV absorption measurement. By amino acid analysis, sclerogen was found to mainly contain Glu (Gln), Asp (Asn), and Gly more than any other amino acids (Table I). Interestingly, sclerogen contained a small proportion of aromatic amino acids such as Tyr and Phe. The pI of sclerogen determined by chromatofocusing was approximately 5.9, and the elution profile gave a single peak (Fig. 3), suggesting that sclerogen is slightly anionic molecule.

Sclerogen Requires Heat Denaturation to Express Mitogenicity To evaluate whether sclerogen also requires heat denaturation to express mitogenic activity as 3S, we compared the mitogenicity of sclerogen before and after the heat denaturation. Both sclerogen sterilized with only a $0.45 \mu\text{m}$ membrane filter (sclerogen-M) and sclerogen-M further treated in boiling water for 30 min (sclerogen-MB) were prepared as testing samples. As shown in Fig. 4, although sclerogen-M showed no mitogenicity, sclerogen-MB showed significant activity against spleen cells similar to the case of 3S-MB from the previous results.³⁾ On the other hand, little or no mitogenicity of sclerogen were

Fig. 5. Mitogenic Activity of Sclerogen to Thymocytes

Thymocytes (5×10^5 cells/culture) from C3H/He were cultured with sclerogen-M (○) or -MB (●) or double distilled-water (△) in the presence of 5% FCS for 72 h. As a positive control, Con A (▲) was added to the culture. The concentrations of sclerogen-M and -MB were expressed as BSA, and expressed as the lyophilized-weight in the case of Con A.

Fig. 6. Effect of the Heat Treatment Time on the Pattern of Sclerogen in Normal-PAGE

Each of sclerogen-M ($8.75 \mu\text{g}$) was treated in boiling water for the indicated time and subjected to 12.5% normal-PAGE.

TABLE II. Evaluation of the Polymerization of Sclerogen after Heat Denaturation^{a)} by 200 kDa Cut Off Ultrafiltration Membrane

Form	Protein content ^{b)} (μg)	
	Untreated	Pass-through fr.
Native	216.0	173.8 (80.5%) ^{c)}
Boiled	204.2	14.4 (7.1%)

a) Native sclerogen were treated in boiling water for 30 min, and then applied to the ultrafiltration membrane UK-200 which cut off 200 kDa. b) Protein concentrations of each fraction were measured by using BCA protein assay. c) Parentheses mean the percentage of the recovery of protein content in the pass-through fraction after ultrafiltrating.

observed against thymus cells in comparison with that of Con A (Fig. 5), suggesting that sclerogen did not act on thymocytes.

When sclerogen was treated in boiling water for 0—60 min, new bands and the top of the gel in normal-PAGE were observed accompanied with the heat denaturation (Fig. 6). These results indicated that significant physicochemical changes of sclerogen proceeded to express mitogenicity against murine splenocytes. To evaluate whether these physicochemical changes were the result of the polymerization of sclerogen, we demonstrated the polymerization by using an ultrafiltration membrane which

TABLE III. Responses of Splenocytes to Allogeneic Splenocytes in Primary MLR^{a)}

Sample	Dose ($\mu\text{g}/\text{culture}$)	Responder only	Responder plus syngeneic stimulator	Responder plus allogeneic stimulator
Sclerogen-M	0.35	30402 $\pm$ 552	31900 $\pm$ 3787	87853 $\pm$ 22243
	1.75	25584 $\pm$ 2500	22319 $\pm$ 1539	59975 $\pm$ 13062
	8.75	5761 $\pm$ 451	2715 $\pm$ 224	7254 $\pm$ 790
Sclerogen-MB	0.35	35682 $\pm$ 2761	36913 $\pm$ 2108	76120 $\pm$ 24800
	1.75	36535 $\pm$ 4569	46304 $\pm$ 1398	80561 $\pm$ 23070
	8.75	46624 $\pm$ 3492	42632 $\pm$ 6117	138279 $\pm$ 38098
Control	—	30003 $\pm$ 4578	36288 $\pm$ 4380	37312 $\pm$ 11424

a) Splenocytes from C3H/He were cultured with mitomycin C-treated splenocytes from C3H/He or C57BL/6 (as syngeneic and allogeneic stimulator, respectively) in the absence or presence of sclerogen-M or -MB at the presented dose. The numbers of stimulator and responder cells were described in Materials and Methods. Results are presented as the mean $\pm$ S.D. of the incorporation of ^3H -TdR. Allogeneic stimulator only = 1024 $\pm$ 425 cpm.

Fig. 7. Effect of Sclerogen to Alternative Pathway of Complement

Human serum was incubated with 10.7 $\mu\text{g}/50\ \mu\text{l}$ of sclerogen-M (Δ) or -MB ($\blacktriangle$) or in their absence ($\bullet$) for 60 min and the ability of the lysis of RRBC was measured, as described in Materials and Methods. To assess the spontaneous lysis of RRBC, no serum ($\circ$) was added to the well.

cut off 200 kDa. As shown in Table II, although the same protein concentration in the passed- and retained-fractions after ultrafiltration were observed in native sclerogen, apparent differences were observed in heat denatured sclerogen. These results suggested that sclerogen was clearly polymerized more than 200 kDa after heat denaturation.

Biological Activities of Sclerogen To evaluate the other biological effects of sclerogen *in vitro*, we examined whether sclerogen-M and/or -MB augment the primary MLR. Consequently, sclerogen-M as well as -MB augmented the primary MLR (Table III). Sclerogen-MB more effectively augmented the MLR response than sclerogen-M.

Furthermore, we examined the ability of the activation of an alternative pathway of complement by assessing the lysis of RRBC by human serum after incubation with sclerogen. As shown in Fig. 7, consumption of the complement components was not observed in either sclerogen-M and -MB, suggesting that sclerogen is not the activator of the alternative pathway of complement.

To assess whether sclerogen is a mitogenic lectin as Con A, hemagglutination activity of SRBC, HRBC, and MRBC were performed. Con A used as a positive control showed hemagglutination activity more than 195.3 ng/ml (in the case of SRBC), more than 4 $\mu\text{g}/\text{ml}$ (in the case of all four groups of HRBC), and more than 160 ng/ml (in the case of MRBC). However, no agglutination of these RBC were observed by the addition of sclerogen-M and -MB within

the concentration range of 20.9 ng/ml to 42.8 $\mu\text{g}/\text{ml}$. Considering the results that sclerogen did not show agglutination of splenic lymphocytes under microscopic observation (data not shown), the mitogenicity of sclerogen was not due to the lectin-like property.

Discussion

We could isolate the fungal mitogen from the buffer extracts (3S) of sclerotia of *S. sclerotiorum* IFO 9395. The isolated mitogen, sclerogen, has a molecular mass of 32 kDa and showed unique characteristics as pro-mitogen which showed significant mitogenic activity after heat denaturation. Considering the physicochemical changes during heat denaturation, the condition to express mitogenicity by sclerogen may be due to the results from the homopolymerization. The reason why native 3S and sclerogen (3S-M and sclerogen-M, respectively) did not show mitogenicity at the highest dosage is probably due to the inhibition mechanism at a high dosage similar to the case of mitogenic lectin, such as Con A.

Although fr. 2 showed a clear single band at 32 kDa under the reducing condition which corresponded to the main band in 3S, no bands were detected in fr. 1 on SDS-PAGE (Fig. 2). The reason for this is due to the inability to permeate to the gel of SDS-PAGE, and may be from the results of polymerization. Previously, we have demonstrated the existence of mitogen in hot water extracts from the fruit bodies and mycelia of fungi.¹⁻³⁾ Mitogen was also found in hot water extracts from sclerotia of *S. sclerotiorum* IFO 9395.⁴⁻⁵⁾ Since hot water extraction would subject these kinds of mitogenic substances to show the complicated physicochemical properties during extraction from a fungal source, these substances could not be detected by SDS-PAGE and reversed-phase high performance liquid chromatography (unpublished data). The purpose of our series of studies about fungal mitogenic substance is to get the mitogenic protein which is detectable on these analyses. So, we used fr. 2 as the isolated mitogen (named sclerogen) from the buffer extracts (3S) of sclerotia of *S. sclerotiorum* IFO 9395. Furthermore, the passed-through fraction of 3S on ion-exchange chromatography (DEAE-Sephadex A-25) also showed mitogenic activity as well as the adsorbed fraction (data not shown). Interestingly, the passed-through fraction showed significant mitogenicity without the heat treatment (data not shown). However, this fraction would be composed of nucleic acid from the results of UV spectrum (data not shown). Accordingly, we did not recover all the substances to show mitogenicity in 3S but isolated the mitogenic protein which could be analyzed by ordinary methods.

Recently, Kino *et al.* reported a novel immunomodulatory protein from a fungus.¹¹⁾ The protein, named Ling Zhi-8 (LZ-8), was extracted from the homogenized mycelia of an oriental medicinal fungus, Ling Zhi (*Ganoderma lucidum*), and showed blast-formation stimulatory activity resembling lectins. LZ-8 also agglutinated SRBC *in vitro* like as lectins. However, they concluded that LZ-8 is not a lectin since mono- or dimeric haptenic sugars they examined did not inhibit the activity. Whether the heat-denatured LZ-8 show mitogenicity and agglutinate SRBC, HRBC, or MRBC are not certain, sclerogen did not agglutinate these RBC, suggesting that sclerogen is not a lectin and the action

pattern would be different from LZ-8.

What attracted us to sclerogen was the significant expression of mitogenic activity after heat denaturation. Only a few minutes of heat denaturation make sclerogen a potent mitogen, and drastic physicochemical changes occur in sclerogen during heat denaturation. To elucidate the changes generated during heat denaturation may be the important key to dissolving the problems as to what changes are needed to express mitogenic activity by sclerogen and why these post-translationally modified-substances should be recognized by murine splenocytes.

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