Heat Induced Generation of the Mitogenic Substance(s) Responding to Murine Splenocytes Obtained from Sclerotia of *Sclerotinia sclerotiorum* IFO 9395

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We have demonstrated that hot water extracts of sclerotia of *Sclerotinia sclerotiorum* IFO 9395 (TSHW) show various immunomodulating activities and mitogenic substance(s) were recovered from the β-1,3-glucanase resistant-fraction (EDP) (Shinohara *et al. Chem. Pharm. Bull.*, 37, 2174 (1989)). In this paper, we examined whether or not the mitogenic substance(s) were also obtained from the other methods, phosphate buffer extraction. Although the native extracts (3S-M) sterilized with a membrane filter showed a slight mitogenicity to murine splenocytes, 3S-M denatured in boiling water (3S-MB) showed significant activity. Treatment of 3S-M for only 1 min in boiling water or 10 min at 70 °C was sufficient to show significant mitogenic activity. After heat treatment of 3S-M in boiling water for 30 s, the main band corresponding to that of 3S-M was not clearly observed. Instead, new bands appeared at the top of the gel in normal-polyacrylamide gel electrophoresis (normal-PAGE), suggesting that many physicochemical changes occurred during the heat treatment. These findings suggest that heat denaturation of the substance(s) from sclerotia was one of the triggering mechanisms expressing mitogenic activity to murine splenocytes.

Keywords *Sclerotinia sclerotiorum*; sclerotia; mitogenic activity; heat denaturation; buffer extract; post-translational modification; polymerization

Mitogens which trigger proliferative responses of lymphocytes without any antigenic specificities are known to be obtained from bacteria such as lipopolysaccharides (LPS),[1] *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP)[2] and from plant lectins (*e.g.* concanavalin A (Con A),[3] phytohemagglutinin (PHA)[4]). The biochemical mechanisms of these mitogens that act on the lymphocyte activation have been extensively investigated[5] but are not clarified in detail. One of the reasons for this complexity is due to the multiple action of cytokines that are released from several cells by the action of these mitogens.

A series of our studies have demonstrated that hot water extracts from the fruit bodies of fungi also contain mitogens.[6] Especially the hot water extracts (TSHW) of sclerotia of *Sclerotinia sclerotiorum* IFO 9395 belonging to Ascomycotina, Discomycetes, Helotiales showed various immunomodulating activities (B cell mitogenicity, polyclonal B cell activating, reticuloendothelial system stimulating, and antitumor activities).[7] We have also demonstrated that the mitogenic activity of TSHW was concentrated into the β-1,3-glucanase resistant-fraction (EDP), suggesting that the mitogenic substance was not a carbohydrate part, but was probably a protein part.[8] However, some previous works in our laboratory using hot water extracts of fruit bodies from *Peziza vesiculosa*[9] and *Angelica acutiloba* KITAGAWA[10] suggested that these kinds of mitogenic substances showed complicated physicochemical properties. Furthermore, these substances, including TSHW, were not detected by polyacrylamide gel electrophoresis (PAGE) and reversed-phase high performance liquid chromatography (RP-HPLC) (unpublished data). Since hot water extraction was done under very drastic conditions, it was hard to extract the active material(s) from sclerotia without any post-translational modifications and it was difficult to analyze its original structure. The reason for having charge and molecular weight heterogeneities is probably due to some post-translational changes during extraction.

The purpose of this paper is to obtain direct evidence to support the contribution of post-translational modifications to show mitogenicity. In this study, we tried to obtain the immunomodulator(s) from sclerotia without any modifications by using mild buffer extraction. The data suggest that the mitogenic activity of the extracts was apparently observed after heat denaturation.

Materials and Methods

Mice Six- to 12-week-old male ICR, C3H/HeN, and BALB/c nu/nu mice were obtained from SLC, Japan, and C3H/HeJ mice were obtained from CLEA, Japan, Inc., Tokyo. They were housed under specific pathogen-free conditions for the duration of the experiments.

Materials and Microorganisms The mycelia of *Sclerotinia sclerotiorum* IFO 9395 were obtained from the Institute for Fermentation, Osaka, Japan (IFO), and were cultured on potato-sucrose agar media at 25 °C in our laboratory.[11] After 3—4 weeks, the yielded sclerotia were picked up and lyophilized. The prestained SDS molecular weight markers were purchased from Sigma Chem. Co., Ltd. Silver stain kit Wako was purchased from Wako Pure Chemical Industries, Ltd.

Preparation of the Buffer Extracts from Sclerotia The lyophilized sclerotia were powdered and suspended in 10 mM phosphate buffer (P.B.) pH 6.8, and then incubated for 24 h at 37 °C with stirring. After centrifugation at 8000 rpm for 20 min, the supernatant was passed through a 0.45 μm millipore filter to remove the microsegment of sclerotia. The filtrated supernatant was then subjected to ultrafiltration cutting off material less than 10 kilodaltons (kDa). The retained fraction, designated 3S, was used in this experiment. 3S was composed of protein 40—60%, carbohydrate 20—30%, and phosphate 10—20%.

Mitogenic Activity ICR mice were killed by cervical dislocation and the cell suspension from the spleen was prepared by teasing the organ in ice cold RPMI 1640 medium supplemented with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), penicillin and streptomycin. After centrifugation (1200 rpm for 5 min at 4 °C), the packed cells were treated with a 0.83% (w/v) ammonium chloride solution to lyse red cells. The treated cells were washed with fresh medium 3 times, resuspended in the above medium, and viability was assessed by the trypan blue dye exclusion test. The cell suspension was adjusted to 5×10^6 viable cells per ml. Both 3S sterilized only with a 0.45 μm millipore filter (3S-M) and further treatment of 3S-M in boiling water (3S-MB) were prepared as testing samples, and 25 μl of each diluted sample was placed in a flat bottomed 96-well tissue culture plate and 2-fold enriched medium (25 μl) was added for osmotic stabilization. Then 100 μl of the cell suspension prepared above were added to each well. The plates were incubated for 48 h at 37 °C in a 5% CO_2 incubator. Twenty hours before harvesting, 0.5 μCi of tritiated thymidine (^{3}H-TdR), specific activity 38 Ci/mmol (ICN Biomedicals Inc., California, U.S.A.), was added to the culture in a volume of 20 μl. At the end of the culture, the cells were harvested and radioactivity was measured in a liquid scintillation counter. Results were expressed as the arithmetic mean ± S.D. of triplicate cultures. Concentrations of 3S

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were expressed as protein (bovine serum albumin) contents measured by Lowry-Folin method.

Polyacrylamide Gel Electrophoresis (PAGE) In normal-PAGE analysis, 3S was solubilized with 70% glycerol in 0.5 M Tris, pH 6.8, and electrophoresed as described by Ornstein[12a)] and Davis.[12b)] In sodium dodecyl sulfate (SDS)-PAGE analysis, 3S was solubilized with the above buffer supplemented with 10% SDS and 10% β-mercaptoethanol, and performed under the reducing condition, as described by Laemmli.[13)] Each page was carried out on slab gel (2.5% stacking gel and 10% separating gel, 90 (W) × 75 (H) × 1 (D) mm) and done at a constant current (15 mA) using a PS-520D power supply instrument (Advantec Toyo Kaisha, Ltd.) and an AE-6050 mini slab gel electrophoresis instrument (ATTO Corporation). The gels were stained with silver stain kit Wako. The molecular weight of 3S was determined to compare with that of prestained SDS molecular weight markers given in kilodaltons.

Results

Condition of the Expression of the Mitogenicity of 3S Previously, we obtained the mitogenic substances from hot water extracts of sclerotia of *S. sclerotiorum* IFO 9395.[7)] To examine whether 10 mM phosphate buffer extracts of sclerotia (3S) show mitogenicity, powdered sclerotia was incubated at 37 °C in the buffer for 24 h with stirring. After ultrafiltration cutting off less than 10 kDa, the extracts were sterilized by passing through a 0.45 μm millipore filter (3S-M). To induce the denaturation, a portion of 3S-M was boiled in water (3S-MB). Both of these materials were added to murine splenic culture and incubated for 48 h. The mitogenic activity was evaluated with the incorporation of ^{3}H-TdR into the nucleus of splenocytes as described in Materials and Methods. As shown in Fig. 1A, 3S-M showed slight mitogenic activity in comparison with that of the control, but 3S-MB showed significant mitogenic activity to ICR spleen cells in a dose dependent manner. Similar results were obtained when the splenocytes from other strains of mice (BALB/c Nu/Nu and C3H/HeJ) were used in this assay (Fig. 1B, C). Interestingly, 3S-M suppressed ^{3}H-TdR incorporation at the highest dosage in which

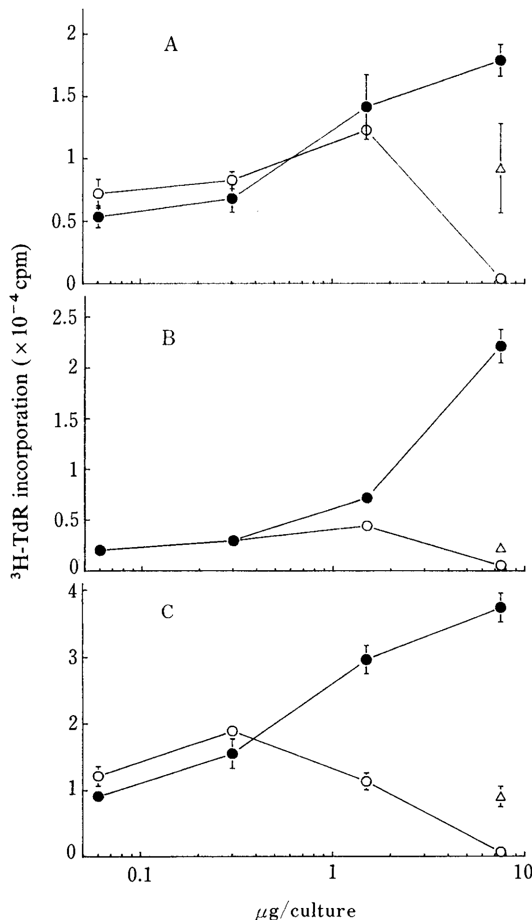

Fig. 1. Mitogenic Activity of 3S on Some Strains of Mice Spleen Cells

Spleen cells (5 × 10^{5} cells/culture) from ICR (A), C3H/HeJ (B), and BALB/c nu/nu (C) mice were cultured with 3S-M (○) or 3S-MB (●) or in their absence (△) under the conditions described as Materials and Methods, and the mitogenicity was assessed.

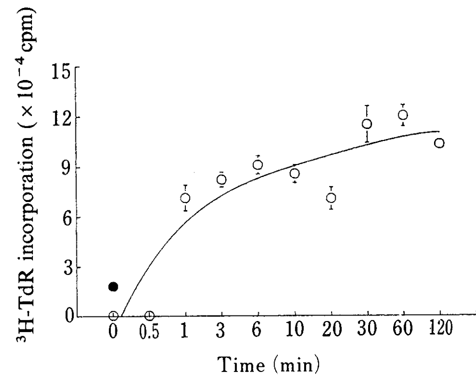

Fig. 2. Effect of the Heat Treatment Time on the Expression of Mitogenicity by 3S

Each 3S-M (6.7 μg/25 μl as protein) was treated for the indicated time at 100 °C (○), and the mitogenicity was evaluated against ICR splenocytes. As control, double distilled water (●) was added to the culture.

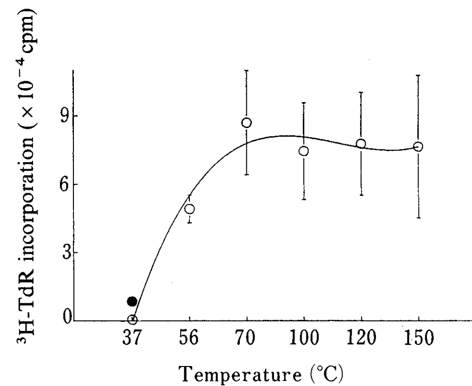

Fig. 3. Effect of the Heat Treatment Temperature on the Expression of Mitogenicity by 3S

Each 3S-M (8.1 μg/25 μl as protein) was treated at the indicated temperature for 10 min, and the spleen cells from ICR mice were cultured in the presence (○) or the absence (●) of each fraction.

Table I. Mitogenic Activity of Heat-Denatured Protein and Glucose Mixture[a)]

Added proteins	Heat treatment	^{3}H-TdR incorporation	
		Mean cpm ± S.D.	S.I.[b)]
Papain	−	12902 ± 1376	1.41
	+	13311 ± 2373	1.45
Pepsin	−	5713 ± 1257	0.62
	+	6023 ± 279	0.66
Lysozyme	−	8069 ± 136	0.88
	+	1859 ± 240	0.20
Control	−	9166 ± 715	1.00

a) Spleen cells (5 × 10^{5}/culture) from ICR mice were cultured with 100 μM of the presented proteins and glucose (100 μM) with or without the heat treatment. *b)* Stimulation index (S.I.) = mean cpm in each experimental group/mean cpm in control group.

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3S-MB showed the maximum activity.

Conditions Required to Exhibit Mitogenicity by 3S To evaluate the conditions (time and temperature) to express the mitogenic activity of 3S, each 3S-M was treated at various periods in boiling water, and the mitogenic activities of those fractions were measured. As shown in Fig. 2, all 3S fractions treated more than 1 min showed mitogenicity. The data indicates that only a little heat denaturation was required to express mitogenicity by 3S.

Next, each sterilized-3S was treated for 10 min at various temperatures as shown in Fig. 3. The results indicated that mitogenicity was increased at as low as 56 °C treatment and reached the maximum at a temperature more than 70 °C. Based on the data that 3S incubated for 8 or 18 d at 37 °C become mitogenic (data not shown), the activity would result from certain physicochemical changes of 3S.

Generally, heat treatment of protein causes dramatic changes on conformation and function.[14] Furthermore, it is well known that proteins react with reducing sugar to make Maillard reactants.[15] To evaluate the possibility that heat denaturation of the protein induced mitogenicity under the same conditions as 3S, we examined the mitogenicity of three other heat denatured representative proteins (Table I). However, in all cases no activities were observed, suggesting that the expression of mitogenicity by 3S after heat denaturation seems to be the specific phenomenon.

Change of Electrophoretic Pattern of 3S during Heat Denaturation As mentioned above, 3S showed significant mitogenic activity after heat denaturation, and the change sufficiently occurred for 1 min at 100 °C and/or for 10 min at 70 °C. To evaluate what physicochemical changes occurred during heat denaturation, we examined the electrophoretic pattern of heat denatured-3S on normal- and/or SDS-PAGE. Consequently, 3S-M showed two major bands in normal-PAGE (Fig. 4A lane 1) and showed a major band corresponding to about 30 kDa in SDS-PAGE (Fig. 4B lane 3). After heat treatment of 3S-M for 30 min at 100 °C, a new band was detected at the top of the gel of normal-PAGE (Fig. 4A, lane 2). However, as shown in Fig. 4B (lane 4), significant changes after heat denaturation were not observed in SDS-PAGE.

To clarify the critical point at which the physicochemical change of 3S is induced during heat treatment, we compared the electrophoretic pattern of 3S at various heating times and/or temperatures. First, accompanied with the period of heat treatment, two major bands were gradually dispersed and disappeared and a new band appeared at the top of the gel of normal-PAGE within 30 s at which time mitogenicity was observed a little (Fig. 5A). In contrast, in SDS-PAGE, the major band could be detected clearly up to 120 min (Fig. 5B). Next, accompanied with an increasing reaction temperature, the main band of treated-3S at 70 °C was decreased compared with that of native 3S and a new band was detected at the top of the gel of normal-PAGE (Fig. 6A). In SDS-PAGE, although some minor bands which may be caused by heat degradation were detected, the main band corresponding to that of native 3S was apparently observed up to 150 °C (Fig. 6B). These results suggested that some physicochemical changes were induced during heat denaturation and that these changes may be mild aggregation (and/or polymerization) or reversible

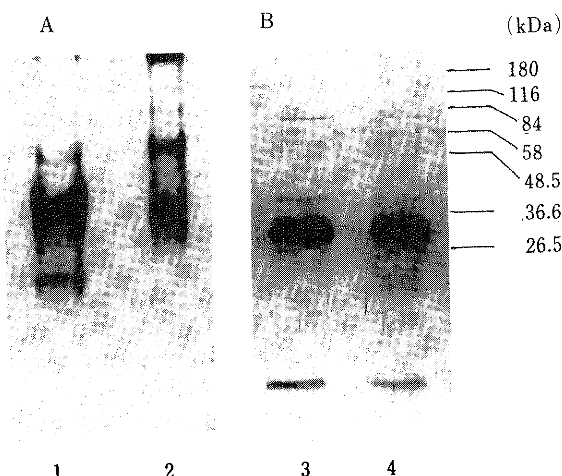

Fig. 4. Normal- and SDS-PAGE Pattern of 3S before and after Heat Treatment

Physical differences between native 3S (3S-M, lanes 1 and 3) and heat denatured 3S (3S-MB) treated for 30 min in boiling water (lanes 2 and 4) were assessed by (A) normal- and (B) SDS-PAGE analyses.

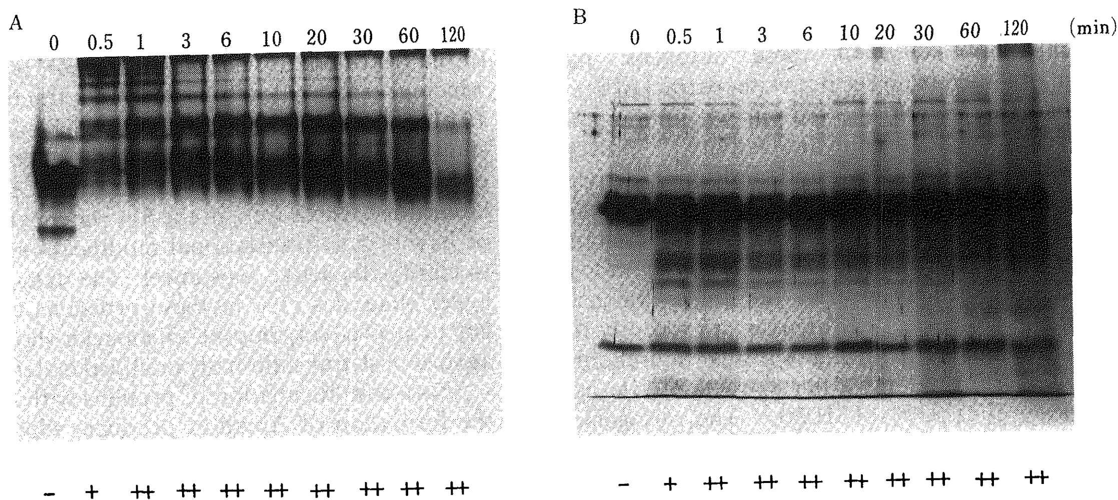

Fig. 5. Effect of the Heat Treatment Time on the Pattern in PAGE

Each of 3S-M was treated in boiling water for the indicated time and applied to each lane, then (A) normal-, and (B) SDS-PAGE analyses were performed as described in Materials and Methods.

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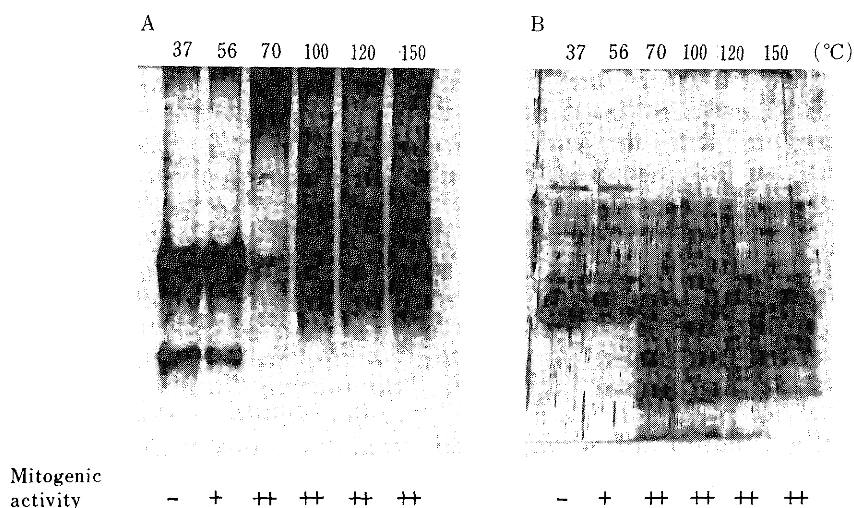

Fig. 6. Effect of the Heat Treatment Temperature on the Pattern in PAGE

Each 3S-M was treated for 10 min at the indicated temperature and applied to each lane of (A) normal-, and (B) SDS-PAGE.

modifications. Further studies are required on this point.

Discussion

This paper demonstrated the generation of mitogenic substance(s) in the phosphate buffer extracts of sclerotia of *S. sclerotiorum* IFO 9395. The extracts, 3S, showed significant mitogenicity after heat denaturation. The expression of mitogenicity sufficiently occurred only for 1 min at 100 °C and/or for 10 min at 70 °C treatment. Accompanied with heat denaturation, the physicochemical changes (may be polymerization) were detected on normal-PAGE. To explain these results, we would consider the following possibilities: 1) the active substance(s) were generated from the result of its own conformational changes during heat denaturation, 2) inactivation of the material(s) which suppress ^{3}H-TdR incorporation and block the effect of the other mitogenic substance(s) in 3S-M, and 3) the new compounds made by heat denaturation from the materials showed no or little mitogenic activity. To clarify these problems, it would be necessary to purify the substance(s) from 3S which showed mitogenicity after heat denaturation.

Previously, we obtained mitogenic substances from hot water extracts of many fungal fruit bodies and liquid-cultured mycelia.[6] One of these mitogens, named vesiculogen, obtained from the hot water extract of the fruit body of *Peziza vesiculosa*, showed various immunomodulating activities, such as adjuvant activity,[16] polyclonal B-cell activation activity,[16,17] and antitumor activity.[18] The mitogenic substance(s) obtained from *P. vesiculosa* is a heat stable anionic polymer which was resistant to several hydrolytic enzyme digestions and the mitogenic activity did not strongly restrict the three dimensional structure of the molecule.[19] Moreover, we recently found the immunomodulator showing various immunomodulating activities from the hot water extracts of sclerotia of *S. sclerotiorum* IFO 9395 (TSHW).[7] The mitogenic activity of TSHW was due to the β-1,3-glucanase-resistant fraction, EDP, the polypeptides having molecular weight and charge heterogeneities.[8] These mitogens could not be analyzed by PAGE and/or RP-HPLC, so the precise chemical characteristics have not yet been clarified. The data shown in this paper (Fig. 5B) strongly supported the previous results that these mitogens could not be detected on PAGE and/or RP-HPLC. These observations suggest that heat denaturation causes the post-translational modifications, such as polymerization. Futhermore, based on the data shown in this paper, this phenomenon would be the reason that vesiculogen and TSHW showed mitogenic activity and had a molecular weight and charge heterogeneities. Since these mitogens were extracted from the fruit body and sclerotia under drastic conditions, the native form might show little or no mitogenic activity, and would be subjected to post-translational modifications during hot water extraction.

Many mitogens have been found from various species such as bacteria, plants, and fungi. Especially, bacterial lipopolysaccharide (LPS) and *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) have been precisely characterized and the active sites were confirmed by using various synthetic analogues.[20,21] The structure of the plant lectins acting as mitogens (*e.g.* Con A) have also been defined.[22] However, fungal mitogens have not yet been precisely characterized. For example, Krestin (PS-K),[23] the representative fungal immunomodulator obtained from the hot water extraction of the fruit body of *Coriolus versicolor*, is the glycoprotein which also showed mitogenicity in a fraction of more than 10 kDa,[24] but it is not yet known which part of the molecule showed mitogenic activity. Similar results were observed in oriental crude drugs and other fungi examined in our laboratory. These were in the hot water extracts so, because of having post-translational modifications, it was difficult to clarify the active substances. The data presented in this paper suggest a clue to these problems and could be the key to answer why murine splenocytes showed mitogenicity against post-translationally modified materials from fungi.

Short-term incubation of proteins with glucose results in the formation of Amadori products which are reversible, and further incubation forms a number of advanced glycosylation end-products (AGE) which are yellow-brown fluorescent chromophores that can cross-link proteins.[25] In contrast to the Amadori products, these adducts, once formed, are irreversible. Recently, Vlassara *et al.* reported

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AGE-proteins (AGE-bovine serum albumin (BSA) and AGE-myelin) were recognized and uptaken by macrophages,[25,26] so that there is a possibility of modulating the immune systems. To evaluate whether mitogenicity of 3S is due to a kind of AGE-protein, we treated some representative proteins under the same condition which showed mitogenicity by 3S. Consequently, no activities were detected in any cases, suggesting that mitogenicity after heat denaturation of 3S is not due to the production of AGE-proteins in 3S.

Since the mitogens from the hot water extracts of sclerotia of *S. sclerotiorum* could not be detected in PAGE analyses, it was difficult to analyze the primary structure of the mitogen. Although we have not answered clearly what changes occurred during heat denaturation in 3S, the data presented in this paper indicated the possibility that we were able to determine the structure of mitogenic substance(s) in fungi. The purification and structural characterization of 3S are in progress.

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