Heat Induced Conformational Changes Generate Mitogenicity to Splenocytes by Sclerogen from *Sclerotinia sclerotiorum* IFO 9395

Hisami SHINOHARA, Naohito OHNO, and Toshiro YADOMAE*

Laboratory of Immunopharmacology of Microbial Products, Tokyo College of Pharmacy, 1432–1 Horinouchi, Hachioji, Tokyo 192–03, Japan.
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The fungal mitogen, sclerogen, obtained from sclerotia of *Sclerotinia sclerotiorum* IFO 9395 showed significant mitogenic activity to murine splenocytes after heat denaturation in relation to polymerization. To evaluate the conditions generating mitogenicity, we performed several chromatographic and spectral analyses. After heat denaturation of sclerogen, significant reduction of intrinsic fluorescence, significant changes on the ultraviolet absorption spectrum, significant changes on the circular dichroism spectrum, and an extreme change of the surface charge to anionic, were observed. These results strongly suggested that local as well as overall conformational changes of sclerogen associated with a high molecular mass and polyanionic charges are important in generating mitogenicity to murine splenocytes.

Keywords fungal mitogen; anionic charge; mitogenicity; sclerotia; *Sclerotinia sclerotiorum*; conformation; sclerogen

Introduction

We previously demonstrated the existence of immunomodulators in hot water extracts from sclerotia of *Sclerotinia sclerotiorum* IFO 9395. The extracts, termed TSHW, showed B cell mitogenicity, polyclonal B cell activation (PBA), reticuloendothelial system stimulation, and antitumor activities. The physicochemical characterization of active substances in TSHW has suggested that in addition to antitumor (1→3)-β-D-glucans, heterogenous strongly anionic polymer fraction showed immunomodulating activities. The latter fraction was found to strong contribute to the mitogenicity and PBA. Chemical modifications of the fraction have suggested that the activity was strongly related to the anionic groups, but purification of the active substances was unsuccessful because of the severe denaturation accompanied by chemical and physical cross-linking. In our recent studies a unique fungal mitogen, sclerogen, isolated from the phosphate buffer extracts (3S)[1] from sclerotia of *S. sclerotiorum* IFO 9395 showed significant mitogenicity to murine splenocytes after heat denaturation.[2] The native form of sclerogen did not show any mitogenicity at all. We also demonstrated that, after the heat-denaturation of sclerogen, the majority of sclerogen was retained on the top of the gel in normal polyacrylamide gel electrophoresis (PAGE) and also was retained using an ultrafiltration membrane of 200 kDa cut off. These data suggested that a certain specific conformation of sclerogen seen after heat denaturation is quite important for the generation of mitogenicity, and that sclerogen would be strongly related, at least a part, to the active substance in TSHW.

In this study, to assess the conformational changes occurring after heat denaturation of sclerogen and their role in the generation of mitogenicity, we analyzed some optical spectra (ultraviolet (UV), fluorescence and circular dichroism (CD)). We also measured the isoelectric point (pI) by chromatofocusing.

Materials and Methods

Microorganisms The mycelia of *S. sclerotiorum* IFO 9395 were obtained from the Institute for Fermentation, Osaka, Japan (IFO), and were cultured on potato-sucrose agar at 25 °C.[3] The sclerotia were collected after 3—4 weeks and lyophilized.

Preparation of Sclerogen from the Buffer Extracts of Sclerotia The preparation methods for the buffer extracts (3S) from sclerotia were as described previously.[1] Sclerogen was isolated from 3S using TSK-gel HW-55(F) gel filtration followed by diethylaminoethyl (DEAE)-Sephadex A-25 ion-exchange chromatography.[2a]

Analyses Protein concentrations were measured by BCA protein assay reagent.[4] The UV spectra were measured by Hitachi 557, the fluorescence spectra by Hitachi 650-60, and the CD spectra by a Jasco J-500C spectropolarimeter. Chromatofocusing was performed using PBE 94 (Pharmacia). The column was eluted with a linear gradient from pH 7.4 to 4.0 of Polybuffer 74 (Pharmacia), and then with 1 M NaCl.

Results and Discussion

We described here our attempts to demonstrate the physicochemical changes of sclerogen following heat denaturation. In the first experiment to confirm the heat induced generation of mitogenicity, we examined the mitogenicity of sclerogen before and after heat treatment (100 °C, 30 min). As expected, sclerogen showed significant mitogenic activity after heat denaturation (Fig. 1). To analyze these changes precisely, we measured UV, fluorescence and CD spectra of the native- (non-mitogenic) and denatured- (mitogenic) forms of sclerogen (sclerogen-M and -MB, respectively). As shown below, significant changes were observed in all three spectra.

In UV spectra, the absorption maximum at around

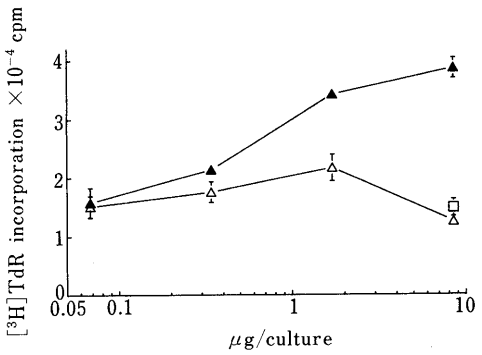

Fig. 1. Mitogenic Activity of Sclerogen before and after Heat Denaturation

Splenocytes (5×10^5 cells/culture) from C3H/He mice were cultured in the presence of sclerogen-M (△) or sclerogen-MB (▲), or in their absence (□) for 48 h without fetal calf serum. Twenty hours before harvesting, 0.5 μCi of tritiated thymidine ([3H]TdR) was added to the culture. Data are expressed as mean cpm ± S.D. of triplicate cultures.

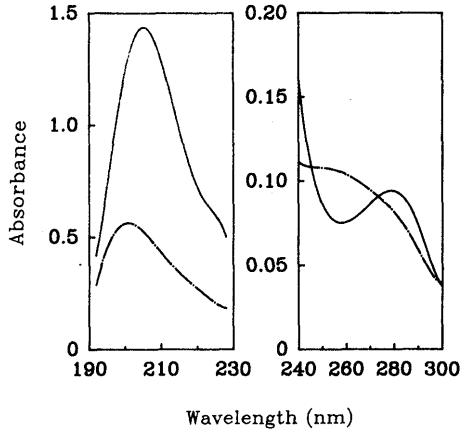

Fig. 2. UV Absorption of Sclerogen

UV absorption of sclerogen-M (——) or -MB (—·—) was measured by Hitachi 557. The sample concentration was 48.8 μg/ml.

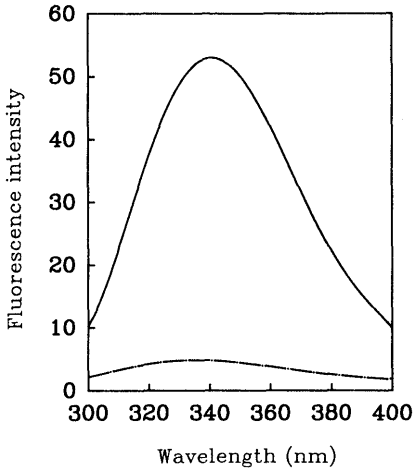

Fig. 3. Fluorescence Intensity of Sclerogen

Fluorescence intensity of sclerogen-M (——) or -MB (—·—) was measured by Hitachi 650-60. The sample concentration was 48.8 μg/ml and the excitation wavelength 280 nm.

280 nm in sclerogen-M disappeared in sclerogen-MB (Fig. 2). The absorption maximum corresponding to amide bonds (at about 200 nm) also decreased after heat denaturation (Fig. 2). The absorption around 280 and 200 nm would represent information of different parts of the polypeptides, such as aromatic side chain and amide bonds. Heat denaturation of sclerogen induced critical differences of the spectra of both regions, suggesting changes of the local as well as the overall conformation of sclerogen. Similar results were obtained for the fluorescence spectra (Fig. 3); a fluorescence maximum around 340 nm excited at 280 nm for sclerogen-M was significantly reduced in the case of sclerogen-MB. The spectral change would represent the reduction of surface exposed residues of Trp. Furthermore, the positive peak around 230 nm in the CD spectrum of sclerogen-M also disappeared after heat denaturation (Fig. 4). Compared with the spectra of poly-L-lysine, sclerogen-MB showed a significantly higher ratio of β-sheet conformation than sclerogen-M. These results suggested that these changes in the spectra are due to burying and/or modification of the chromophores and/or residue(s) during denaturation.

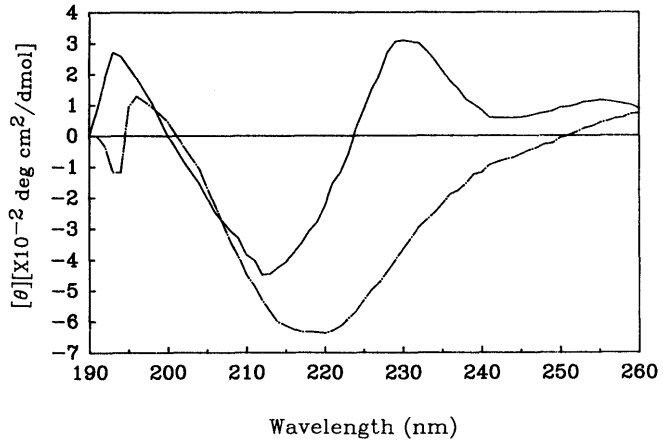

Fig. 4. CD Spectra of Sclerogen

CD spectra of sclerogen-M (——) or -MB (—·—) were measured by Jasco J-500C. The sample concentration was 48.8 μg/ml. Measurement conditions: wavelength expansion, 10 nm/cm; time constant, 16 s, sensitivity, 10^{-3} deg/cm; accumulation, 8 times.

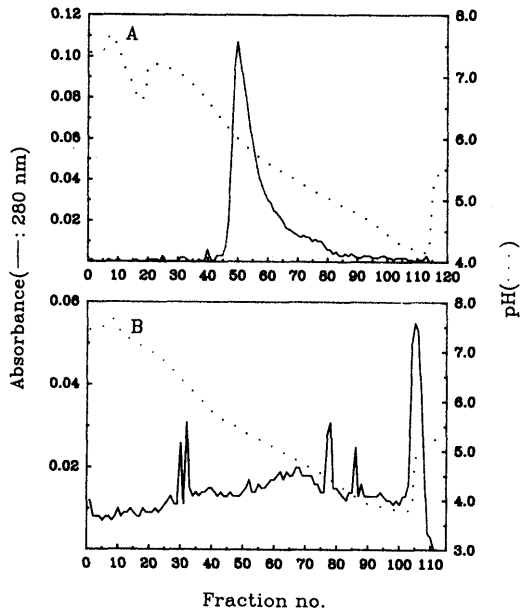

Fig. 5. Detection of the Conformational Changes of Sclerogen before and after Heat Denaturation by Chromatofocusing

Both forms of sclerogen (sclerogen-M, A; sclerogen-MB, B) were applied to a column of PBE 94 (10 ml) equilibrated with 25 mM imidazole–HCl buffer, pH 7.4. The column was initially eluted with a stepwise gradient using Polybuffer 74 adjusted to pH 4.0 (up to fr. 110 (A), or to fr. 100 (B)), and then with 1 M NaCl. Fractions of 1.6 ml were collected. Protein (280 nm; ——) and pH (------) were monitored by UV absorption measurement and a pH meter, respectively.

We showed previously that the mitogenic substance in the hot water extract of this fungus, EDP, was an anionic polymer.[3b] Sclerogen was polymerized after heat denaturation as assessed using a membrane filter.[2a] All spectral data shown in this paper suggested changes in the overall conformation of sclerogen by heat treatment. Considering these findings, it is suggested that the pI value of sclerogen would also be changed after heat denaturation. As shown in Fig. 5, the major part of sclerogen had an anionic pI below 4.0 after heat denaturation according to the results of chromatofocusing. In addition, recovery of the denatured sclerogen showed a pI lower than native sclerogen (pI 5.9) was about 70%. These results suggested

that the heat denaturation of sclerogen induced conformational changes associated with polymerization and anionization, and that these changes were important for the generation of mitogenicity of sclerogen.

There are several reports indicating the importance of the anionic pI for mitogenicity.[5,6] We also observed the importance of the polymeric and anionic structure in the generation of mitogenicity of vesiculogen from the fruit body of *Peziza vesiculosa*[7] and the activity of a mitogen obtained from an oriental crude drug, Tohki, *Angelica actiloba* KITAGAWA.[8] These results suggested that a high molecular mass and polyanionic charge may be necessary for the generation of mitogenicity by fungal mitogens.

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