Carbohydrate Research, 254 (1994) 213–219
Elsevier Science B.V., Amsterdam

Preparation and properties of metabolically ^{3}H- or ^{13}C-labeled (1 → 3)-β-D-glucan, SSG, from *Sclerotinia sclerotiorum* IFO 9395

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(Received January 25th, 1993; accepted August 13th, 1993)

ABSTRACT

Metabolically labeled (1 → 3)-β-D-glucan (SSG) obtained from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395, with the radio (^{3}H) or the stable isotope (^{13}C) was prepared. The specific radioactivity of ^{3}H-SSG was increased in accordance with the amount of D-[^{3}H]glucose added, and was 15–20 kBq/mg SSG when 222 kBq/mL of D-[^{3}H]glucose was added. Physicochemical analyses of ^{3}H-SSG suggested a structural similarity between SSG and ^{3}H-SSG. ^{13}C NMR spectra of ^{13}C-labeled SSG (^{13}C-SSG) revealed that a strong signal of the ^{13}C-nucleus was observed at the C-1 or C-2 position when D-[1-^{13}C]glucose or D-[2-^{13}C]glucose was used, respectively, suggesting that most of the glucose residues in SSG were directly taken up from the medium. In addition, part of the ^{13}C-nucleus was incorporated into the SSG molecule at all carbon atoms after metabolic degradation and reconstruction of the glucose molecule. Analyses of the culture filtrate and the mycelium of the fungus suggested that part of the glucose was also metabolized to trehalose and mannitol.

INTRODUCTION

Several kinds of polysaccharides are known to possess immunomodulating effects and exhibit significant antitumor activities[1]. In Japan, two kinds of glucans, lentinan (LTN; from *Lentinus edodes*)[2] and schizophyllan (SPG; from *Schizophyllum commune*)[3], have been applied clinically in cancer patients. The antitumor and immunomodulating activities of (1 → 3)-β-D-glucans have been extensively studied by many research groups. However, the mechanisms of action of these glucans have not been fully examined at the molecular level and are far from being clarified. One of the reasons for the difficulties is the complexity of the immune systems and the other is the gel properties of these glucans.

SSG is a (1 → 3)-β-D-glucan obtained from the liquid-cultured broth of a fungus, *Sclerotinia sclerotiorum* IFO 9395[4,5], belonging to the *Ascomycotina*, and

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SSDI 0008-6215(93)E0306-L

possesses immunomodulating and antitumor activities[6–9]. The number of branching points in SSG is greater than in LTN and SPG (the ratios of branching points to main-chain glucosyl residues in SSG, LTN, and SPG are 1:2, 2:5, and 1:3, respectively). The available evidence from our laboratory suggested that the most characteristic biological activities of SSG are the significant antitumor and immunomodulating activities even with oral administration[10,11]. While many interesting immunopharmacological activities of SSG have been demonstrated, there are many points to be examined, including pharmacokinetics, tissue distribution, mechanism of action, and metabolic degradation. All of these studies are necessary to understand fully the immunopharmacological activities at the molecular level.

We have previously investigated the distribution in vivo of the antitumor glucan, grifolan (GRN; from *Grifola frondosa*), using a chemically modified derivative (labeled by periodate oxidation and borotritide reduction; ^{3}H-I/B-GRN) and suggested that ^{3}H-I/B-GRN was concentrated in the reticuloendothelial system[12], and that products with lower molecular weights are formed by oxidative degradation. However, some difficulties appeared during these studies, such as whether the chemically modified glucan was metabolized in the same way as the parent glucan. Therefore, it is better to use the metabolically labeled glucan to clarify these questions. Here we report the preparation and properties of metabolically labeled SSG.

EXPERIMENTAL

Materials.—D-[1-^{3}H]Glucose (555 MBq/nmol) was purchased from American Radiolabeled Chemicals Inc., St. Louis, MO. D-[1-^{13}C]Glucose and D-[2-^{13}C]glucose were purchased from Cambridge Isotope Laboratories, Woburn, MA, USA.

Preparation of ^{3}H-SSG.—*S. sclerotiorum* IFO 9395 was grown in medium (25 mL each) containing D-glucose (2%), yeast extract (0.3%), and polypepton (1%) with reciprocal shaking at 25°C for 4 days. At 24 h after inoculation, 5550 kBq of D-[1-^{3}H]glucose was added. The mycelia and broth were then separated by centrifugation, the broth was mixed with 1 vol of EtOH, and the fibrous products were collected. These products were washed with aq 50% EtOH and dissolved in 8 M urea. The resulting solution was applied to columns (5 mL) of DEAE Sephadex A-25 (Cl^- form) and SP Sephadex C-25 (Na^+ form). The eluate was dialyzed against tap and distilled water, and precipitated by EtOH.

TLC of acid hydrolysate of ^{3}H-SSG.—^{3}H-SSG (2000 μg/mL) was mixed with 1/3 vol of 4 M CF_3CO_2H and heated at 100°C for 5 h. After concentration to dryness, the residue was applied to a cellulose sheet (200 × 200 mm, Merck), which was developed for 160 mm with 5:5:1:3 EtOAc–pyridine–AcOH–H_2O. The R_f values of the monosaccharides (D-glucose, D-galactose, and D-mannose) were determined using an alkaline $AgNO_3$ reagent. The R_f values of acid hydrolysis products were determined by counting the radioactivity of the strips on the TLC sheet.

Preparation of ^{13}C-SSG and NMR studies.—*S. sclerotiorum* was grown in 25 mL of the above-mentioned medium containing 100 mg of D-[^{13}C]glucose. ^{13}C-SSG was purified in basically the same way as ^{3}H-SSG. ^{13}C NMR spectra (400 MHz) of SSG were recorded at 60°C for solutions in Me_2SO-d_6 with a Bruker AM-400 spectrometer. The spectra were obtained in the pulsed FT mode with complete proton decoupling. The pulse angle was ca. 70°. All spectra were obtained from 1000 to 2000 (^{13}C-SSG) or 40000 to 50000 (unlabeled SSG) scans with a 655-ms pulse interval. Chemical shifts were recorded relative to that of Me_2SO-d_6 (39.5 ppm).

RESULTS AND DISCUSSION

Preparation of ^{3}H-SSG.—In order to prepare metabolically labeled SSG, we chose D-glucose as the ^{3}H source. Since a general procedure for metabolic labeling had not been established, the growth curve of *S. sclerotiorum* was studied in order to determine the optimum conditions to prepare ^{3}H-SSG with a high specific activity. As shown in Fig. 1, the pH of the culture medium and wet weight of the mycelia changed from early on in the culture period. Residual glucose in the culture medium was reduced gradually, but 25% of the glucose remained in the medium even after 96 h. The quantity of SSG rapidly increased after 48 h of culture. Considering these parameters, we decided to add D-[^{3}H]glucose to 25 mL of culture medium 24 h after inoculation and to harvest after 96 h. The specific activity of SSG increased as the amount of D-[^{3}H]glucose increased, and reached the highest level when 5550 kBq (222 kBq/mL) of D-[^{3}H]glucose was added (15 ~ 20 kBq/mg SSG) (Fig. 2). The specific activity was equivalent to 3% of the total radioactivity of the D-[^{3}H]glucose added.

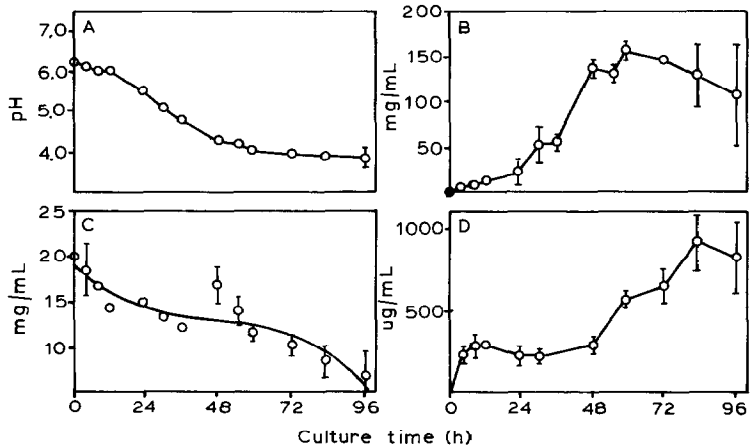

Fig. 1. Growth curve of *S. sclerotiorum* IFO 9395 in YPG medium at 25°C. Pre-cultured mycelium (1 mL) was inoculated into 25 mL of YPG medium at 0 h, and shaken reciprocally at 25°C. Residual glucose was assayed by the Glucose B-Test (Wako), and the quantity of SSG was assayed by the orcinol–sulfuric acid method. A, pH; B, mycelium weight; C, residual glucose; D, quantity of SSG.

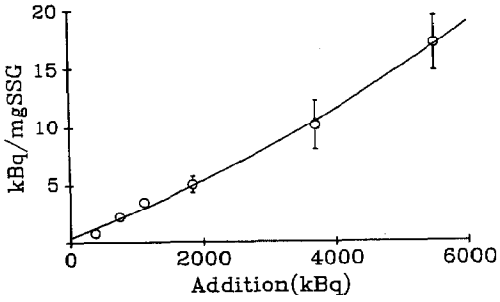

Fig. 2. Effect of D-[^{3}H]glucose concentration on the specific activity of ^{3}H-SSG. D-[^{3}H]Glucose was added to the culture 24 h after inoculation. After 96 h of culture, ^{3}H-SSG was prepared, and the specific activity measured.

The radioactivity in the supernatant solution after ethanol precipitation was almost equal to that in the culture medium. About 86% of the radioactivity in the supernatant solution disappeared during evaporation. To examine the residue, TLC was used. The R_f value of the residue corresponded to that of glucose (data not shown). These results suggested that > 90% of the D-[^{3}H]glucose added was metabolized by the mycelium but that only a small percentage of the D-[^{3}H]glucose was incorporated into the SSG molecule.

Structural analysis of ^{3}H-SSG.—SSG is a neutral β-D-glucan with a high molecular weight. To clarify whether the ^{3}H-SSG prepared has the same physicochemical properties as SSG, the chemical composition, electric charge, and molecular weight of ^{3}H-SSG were examined. In order to determine the carbohydrate composition of ^{3}H-SSG, it was completely hydrolyzed by 1 M CF_3CO_2H and TLC was performed. The R_f value of the main component in the hydrolysate corresponded to that of glucose (data not shown). The results also suggested that ^{3}H-SSG did not contain other carbohydrate components.

The molecular weight of ^{3}H-SSG was examined using a column of TSK GEL HW-60F (10 × 450 mm) with 0.3 M sodium hydroxide as solvent. As shown in Fig. 3, the elution pattern of ^{3}H-SSG measured by LSC corresponded to that of SSG. Ion-exchange chromatography using a column of DEAE Sephadex A-25 (Cl^- form, 15 × 30 mm), shown in Fig. 4, suggested that the majority of both the radioactivity and carbohydrate were present in the fraction eluted with 8 M urea. These data strongly suggested that ^{3}H-SSG possesses physicochemical properties similar to those of SSG.

Preparation and properties of ^{13}C-SSG.—It is known that ^{13}C NMR spectroscopy is very useful for primary- and ultra-structural analyses of (1 → 3)-β-D-glucan[13–15]. However, natural abundance spectra are time-consuming and we therefore prepared ^{13}C-enriched SSG (^{13}C-SSG) with the following objectives: (*i*) to study the biosynthetic pathway of SSG, and (*ii*) to study the metabolic pathways of SSG in animals. In order to prepare ^{13}C-enriched SSG, 20% of the total glucose in the medium was substituted with D-[^{13}C]glucose (D-[1-^{13}C]- or D-[2-^{13}C]-glucose), and

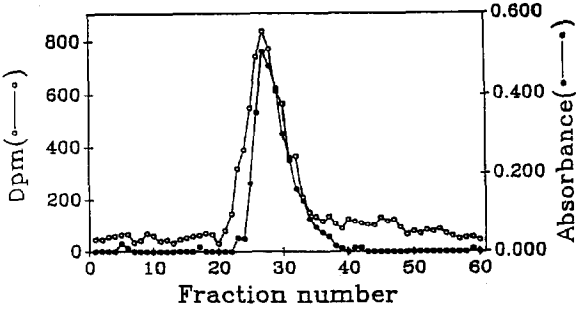

Fig. 3. Gel filtration chromatography of ^{3}H-SSG. A column (10×450 mm) of TSK-Gel HW 60F was equilibrated with 0.3 M NaOH, and labeled glucan and cold SSG were applied. Polysaccharide contents were measured by the phenol–sulfuric acid method. V_0, Fraction 23; V_t, Fraction 46.

^{13}C-SSG was separated in a similar way from ^{3}H-SSG and SSG. The resulting ^{13}C-SSGs showed high molecular weights similar to those of unlabeled SSG (data not shown).

Fig. 5A shows the ^{13}C NMR spectrum of ^{13}C-SSG when D-[1-^{13}C]glucose was used as the ^{13}C source. Since spectra with sufficient signal intensities could be measured within 30 min, incorporation of ^{13}C into the SSG molecule had occurred. We previously assigned the signals of SSG under conditions of natural abundance[16] using the chemical shifts of scleroglucan[17]. In the spectra in Fig. 5A, C-1s (main chain and branched glucose) showed quite a strong signal at 103 ppm. The weak signals at 60–61 ppm corresponded to C-6. Fig. 5B is the spectrum when D-[2-^{13}C]glucose was used as ^{13}C source. In this spectrum, C-2 (branched glucose) showed a rather strong signal at 73.5 ppm, and C-2s (main chain glucose with or without branching) showed a strong signal at 72.5 ppm. Furthermore, weak signals at 103 ppm (C-1) and 74–77 ppm (C-3 of branched glucose and C-5s) were observed. The ratio of ^{13}C-signals for all carbon atoms suggested that a small part

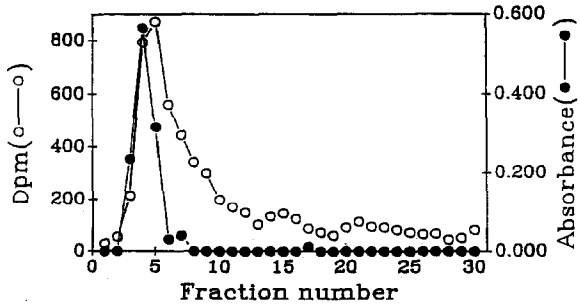

Fig. 4. DEAE Sephadex A-25 ion-exchange chromatography of ^{3}H-SSG. A column (15×30 mm) of DEAE Sephadex A-25 (Cl^-) was equilibrated with 8 M urea. Fractions 1 to 15 were eluted with 8 M urea, and fractions 16 to 30 were eluted with 2 M NaCl–8 M urea. Polysaccharide contents were measured by the phenol–sulfuric acid method.

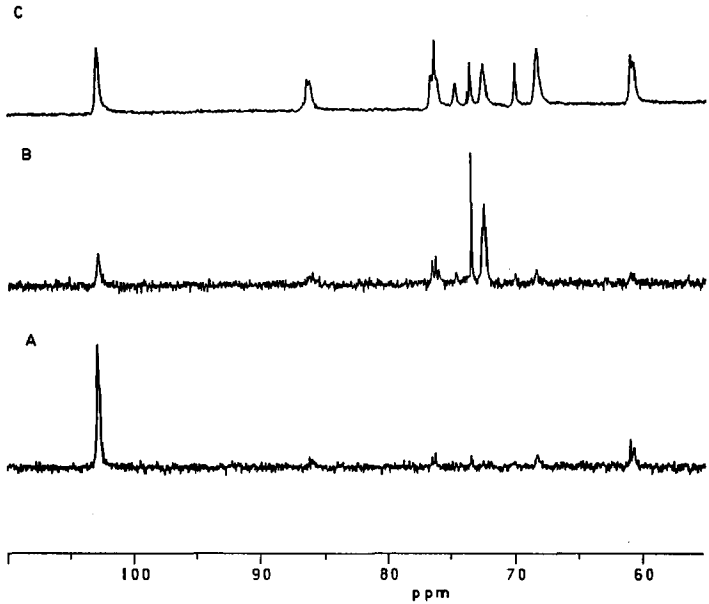

Fig. 5. ^{13}C NMR Spectra of ^{13}C-SSG in Me_2SO-d_6 at 60°C. ^{13}C-SSG (20 mg) was dissolved in Me_2SO-d_6 (1 mL), and ^{13}C NMR spectra were measured at 60°C. The solvent signal appeared at 39.5 ppm as a multiplet. A, ^{13}C-SSG obtained from medium containing D-[1-^{13}C]glucose; B, ^{13}C-SSG obtained from medium containing D-[2-^{13}C]glucose; C, unlabeled SSG.

of the glucose in the medium was incorporated into the SSG molecule after metabolic degradation and reconstruction.

Understanding the movement of the label is quite interesting and important in determining the biosynthetic pathway of SSG and glucose metabolism of the mycelia. To study this point, ^{13}C NMR spectra of the culture supernatant solution and the mycelium (both 96 h after inoculation) were measured. For the supernatant solution, in addition to the signals of C-1 or C-2, other carbon signals were also detected. For the mycelium, signals of trehalose and mannitol, as well as glucose, were detected. These findings strongly suggested the metabolic degradation and reconstruction of glucose during culture.

CONCLUSIONS

The biological significance of (1 → 3)-β-D-glucans has been established in various ways. Now, it is necessary to determine the mechanisms of action of these glucans at the molecular level. However, since (1 → 3)-β-D-glucans possess high molecular weights and complicated conformations, it is difficult to prepare synthetic glucan. Therefore, we prepared metabolically labeled (1 → 3)-β-D-glucans with radio and stable isotopes in this study. These included ^{3}H-SSG of 15-20 kBq/mg, and ^{13}C-SSG which showed sufficient ^{13}C NMR signals within 30 min.

Many studies of structure–activity relationships of these glucans have been undertaken. Under physiological conditions, however, the ^{13}C signals of (1 → 3)-β-D-glucans disappeared[18,19]. The structural analyses of (1 → 3)-β-D-glucans by NMR have therefore been performed using Me_2SO solutions in which glucans form a sol conformer. When glucans are oxidized and/or degraded, they lose the ability to form a gel and become readily soluble in water. Their ^{13}C-spectra can then be observed in neutral, aqueous, and physiological conditions[20–23]. Furthermore, many interesting points, such as pharmacokinetics and recognition in vivo and in vitro, are still not clarified, even though two (1 → 3)-β-D-glucans, schizophyllan and lentinan, have been applied clinically in Japan. Therefore, we thought that isotope-labeled (1 → 3)-β-D-glucans, ^{3}H- and ^{13}C-SSG, should be very helpful in investigating these points.

ACKNOWLEDGMENTS

The authors thank Miss N. Kato, Mr. H. Kuwasawa, Miss N. Asada, Mr. O. Niwano, Miss T. Miyajima, and Mr. M. Nishino for their technical assistance.

REFERENCES

1 N.R. Di Luzio, *Springer Semin. Immunopathol.*, 8 (1985) 387–400.
2 T. Taguchi, Y. Kaneko, and G. Chihara, *Biotherapy*, 2 (1988) 509–521 (in Japanese).
3 S. Fujimoto, K. Orita, T. Kondoh, T. Taguchi, K. Yoshida, K. Kimura, N. Ogawa, and H. Furue, *Biotherapy*, 2 (1988) 500–508 (in Japanese)
4 N. Ohno, I. Suzuki, and T. Yadomae, *Chem. Pharm. Bull.*, 34 (1986) 1362–1365.
5 N. Ohno and T. Yadomae, *Carbohydr. Res.*, 159 (1987) 293–302.
6 N. Ohno, K. Kurachi, and T. Yadomae, *J. Pharmacobio-Dyn.*, 10 (1987) 478–486.
7 I. Suzuki, K. Hashimoto, and T. Yadomae, *J. Pharmacobio-Dyn.*, 11 (1988) 527–532.
8 K. Hashimoto, I. Suzuki, M. Ohsawa, S. Oikawa, and T. Yadomae, *J. Pharmacobio-Dyn.*, 13 (1990) 512–517.
9 T. Sakurai, I. Suzuki, A. Kinoshita, S. Oikawa, A. Masuda, and T. Yadomae, *Chem. Pharm. Bull.*, 39 (1991) 214–217.
10 I. Suzuki, K. Hashimoto, N. Ohno, H. Tanaka, and T. Yadomae, *Int. J. Immunopharmacol.*, 11 (1989) 761–769.
11 T. Sakurai, K. Hashimoto, I. Suzuki, N. Ohno, S. Oikawa, A. Masuda, and T. Yadomae, *Int. J. Immunopharmacol.*, 14 (1992) 821–830.
12 T. Takeyama, I. Suzuki, N. Ohno, S. Oikawa, K. Sato, M. Ohsawa, and T. Yadomae, *J. Pharmacobio-Dyn.*, 11 (1988) 293–302.
13 H. Saito and M. Yokoi, *Bull. Chem. Soc. Jpn.*, 62 (1989) 392–398.
14 H. Saito, M. Yokoi, and Y. Yoshioka, *Macromolecules*, 22 (1989) 3892–3898.
15 H. Saito, Y. Yoshioka, M. Yokoi, and J. Yamada, *Biopolymers*, 29 (1990) 1689–1698.
16 N. Ohno, I. Suzuki, and T. Yadomae, *Chem. Pharm. Bull.*, 34 (1986) 1362–1365.
17 M. Rinaudo and M. Vincendon, *Carbohydr. Polym.*, 2 (1982) 135–144
18 N. Ohno, Y. Adachi, M. Ohsawa, K. Sato, S. Oikawa, and T. Yadomae, *Chem. Pharm. Bull.*, 35 (1987) 2108–2113.
19 Y. Adachi, N. Ohno, and T. Yadomae, *Carbohydr. Res.*, 198 (1990) 111–122.
20 Y. Adachi, N. Ohno, and T. Yadomae, *Carbohydr. Res.*, 177 (1988) 91–100.
21 N. Ohno, K. Kurachi, and T. Yadomae, *Chem. Pharm. Bull.*, 36 (1988) 1016–1025.
22 Y. Adachi, N. Ohno, M. Ohsawa, K. Sato, S. Oikawa, and T. Yadomae, *Chem. Pharm. Bull.*, 37 (1989) 1838–1843.
23 K. Kurachi, N. Ohno, and T. Yadomae, *Chem. Pharm. Bull.*, 38 (1990) 2527–2531.