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Conformation of carboxylated schizophyllan in aqueous solution

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ABSTRACT

Carboxylated schizophyllan (sclerox) samples of different degrees of oxidation were molecularly characterized by size exclusion chromatography equipped with a multi-angle light scattering detector (SEC-MALS) in 0.10 M aqueous NaCl solution. The molar mass distribution obtained by SEC-MALS shows that sclerox of low degree of oxidation is dissolved mainly as the trimer, whereas the trimer and single chain coexist in solution of sclerox of high degree of oxidation. The trimer of sclerox is much more flexible than the fully ordered triple helix of the parent schizophyllan and easily dissociates into single chains upon heating.

1. Introduction

Schizophyllan, an extracellular polysaccharide produced by the fungus *Schizophyllum commune* (Kikumoto, Miyajima, Yoshizumi, Fujimoto, & Kimura, 1970; Kikumoto, Miyajima, Kimura, Okubo, & Komatsu, 1971), is a linear β-1,3-glucan, consisting of the repeating unit of three β-1,3-glucose with one side chain of β-1,6-glucose as illustrated in Fig. 1A. It is known that schizophyllan stimulates the immune system and has an antitumor activity. Norisuye et al. discovered that schizophyllan exists as a rigid triple helix in water (Kashiwagi, Norisuye, & Fujita, 1981; Norisuye, Yanaki, & Fujita, 1980; Sato, Norisuye, & Fujita, 1981; Yanaki, Norisuye, & Fujita, 1980) and its triple helical conformation is related to the antitumor activity (Norisuye, 1985; Okamura et al., 1986). In the triple helix, three schizophyllan chains assemble by intermolecular hydrogen bonds, and the side chain glucoses are located outward to the helical core (Takahashi, Kobatake, & Suzuki, 1984).

The schizophyllan triple helix is stable in water, whereas it dissociates to single chains in dimethylsulfoxide (DMSO) and water-DMSO mixtures (Norisuye et al., 1980; Sato, Norisuye, & Fujita, 1983; Sato, Sakurai, Norisuye, & Fujita, 1983), as well as in water at high pH and/or high temperature (Kashiwagi et al., 1981). Such a denaturation process is irreversible and the dilution of the DMSO solution with water does not produce the original triple helix but more or less random aggregations at finite concentrations (Sato, Sakurai, et al., 1983). Stokke et al. have shown that the change of the solvent conditions produces trimer, circular and more complex aggregated species as a result of the renaturation from schizophyllan single chains in very dilute solutions (Falch & Stokke, 2001; Stokke, Elgsaeter, Brant, Kuge, & Kitamura, 1993).

Since carboxylation of polysaccharides modifies their physicochemical properties and biological functions, various neutral polysaccharides have been oxidized to introduce carboxylate groups (Casu et al., 1984; Hofreiter, Wolff, & Mehltretter, 1957). Crescenzi, Gamini, Paradossi, and Torri (1983) synthesized a new ionic polysaccharide ('sclerox') from scleroglucan by two successive oxidations with sodium periodate and sodium chlorite, respectively. Here, scleroglucan is a polysaccharide produced by a fungus different from *Schizophyllum commune*, having the same chemical structure and triple helical conformation as schizophyllan. Periodate oxidation and subsequent chlorite oxidation are specific reactions including the ring-opening of the side chain, so that the degree of substitution of polysaccharide can be controlled by the amounts of periodate in the reaction (Christensen, Aasprong, & Stokke, 2001; Stokke, Elgsaeter, Smidrød, & Christensen, 1995). Carboxylated scleroglucan (Fig. 1B) shows polyelectrolyte character unlike the parent scleroglucan, such as pH and salt dependences of solution properties (Coviello, Dentini, & Crescenzi, 1995; Coviello, Dentini, Crescenzi, & Vincenti, 1995). Coviello et al. (1998) performed small angle X-ray scattering measurements on sclerox

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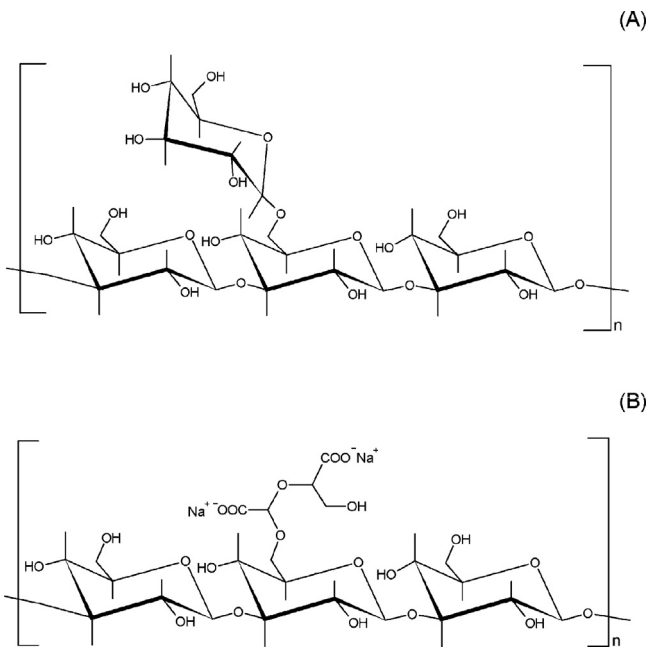

Fig. 1. Chemical structure of the repeating unit of schizophyllan (A) and carboxylated schizophyllan (B).

in aqueous solution, and concluded that sclerox exists as a mixture of the triple helix and single chains. However, because scleroglucan is produced as a gel-like component adhered with mycelium, its isolation from the fungus is made under a severe condition, so that partial irreversible denaturation and aggregation of the triple helices may take place (see above) in isolated scleroglucan samples, which makes the parent scleroglucan sample be poorly soluble in water (Yanaki & Norisuye, 1983). These partial denaturation and aggregation make difficult to study the detailed molecular conformation for both scleroglucan and sclerox prepared from scleroglucan.

This paper reports the molecular conformation of carboxylated schizophyllan (also named sclerox) in solution. It is important for expanding the applicative fields of the polysaccharide to understand the physical properties in solution on the basis of the molecular conformation. The carboxylation, which occurs only in the side chains, largely alters physical properties of sclerox from parent schizophyllan by first introducing the carbonyl groups with periodate oxidation, and subsequent chlorite oxidation. We characterized the molecular conformation of sclerox with different degrees of carboxylation examined mainly by size-exclusion chromatography equipped with a multi-angle light scattering detector (SEC-MALS) to investigate the carboxylation effects on the triple helical conformation of schizophyllan. Since native schizophyllan is well soluble in both water and aqueous salt solutions, we can compare the molecular conformations after the carboxylation on their molar mass dependence. Furthermore, SEC-MALS can analyze both triple helix and single chain existing in the solution of sclerox separately, so that we can characterize more precise molecular conformation of sclerox with different degrees of carboxylation.

2. Experimental

2.1. Chemical modification of schizophyllan

According to Norisuye et al. (1980), a stock schizophyllan sample (SPG) supplied from Taito Co., Ltd. (now Mitsui Sugar Co., Ltd., Tokyo, Japan) was dissolved in water and separated into three fractions by fractional precipitation with deionized water as the solvent and acetone (Wako Pure Chemical Industries Ltd., Osaka, Japan) as the precipitant. The sample was freeze-dried from aqueous solution after the filtration with a DISMIC-25AS filter (pore size 0.45 μm, Toyo Roshi Co., Ltd., Tokyo, Japan). The fractional precipitation was repeated twice on the middle fraction. The fractionated samples were reprecipitated again in ethanol and freeze-dried from aqueous solution. The sample was dried in vacuo before use. The experimental details have been described in previous papers on schizophyllan for the sample preparation (Kikumoto et al., 1970) and degradation (Norisuye et al., 1980; Yanaki, Nishi, Tabata, & Kojima, 1983).

A middle fraction prepared above was oxidized by the following two-step oxidation using $NaIO_4$ and $NaClO_2$. In the first oxidation with $NaIO_4$ (Wako Pure Chemical Industries Ltd., Osaka, Japan), 120 mg of the fractionated schizophyllan sample was dissolved in 120 cm^3 of deionized water containing 13.3 cm^3 of 1-propanol (Wako Pure Chemical Industries Ltd., Osaka, Japan). An appropriate amount of aqueous 30 mM $NaIO_4$ solution was added to adjust the concentration equivalent to the repeating unit of schizophyllan. The reaction was performed by stirring the solution at room temperature in the dark for 24 h to obtain the corresponding dialdehydes. The second oxidation reaction was carried out in 0.5 M aqueous solution of $NaClO_2$ (Kanto Chemical Co. Inc., Tokyo, Japan) in the presence of 0.5 M acetic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan). Nitrogen gas was bubbled through the solution in the oxidation and the reaction was performed for 24 h in the dark at room temperature, and the pH condition was below pH 4 until the reaction was finished. The solution after the oxidations was dialyzed repeatedly with deionized water. The sodium salt of carboxylated sample was obtained by freeze-drying after neutralization with 0.01 M NaOH (Wako Pure Chemical Industries, Osaka, Japan).

Infrared spectra of the samples before and after carboxylation were obtained at room temperature on a JASCO FT/IR-4200 spectrometer (JASCO Co., Ltd., Tokyo, Japan). The KBr disk was prepared by mixing with each freeze-dried sample.

The degree of substitution (DS) was determined by potentiometric titration with 0.01 M NaOH in aqueous 0.10 M NaCl (Wako Chemical Industries Ltd., Osaka, Japan) at room temperature. The sample was transformed to the acid form before the measurement by mixing 20 cm^3 of the aqueous solution of 40 mg of the sample with 1 g of Amberlite IR-120B (Organo Co., Ltd., Tokyo, Japan) at room temperature for one day. The neutralization point was determined from the titration curve to calculate the DS of sclerox according to Fig. 1(B). In what follows, sclerox samples are coded as SC-*X*, where *X* represents the molar ratio of $NaIO_4$ to the side-chain glucose residue of schizophyllan in the solution at performing the oxidation reaction.

2.2. Size-exclusion chromatography equipped with a multi angle light scattering detector (SEC-MALS)

SEC-MALS measurements were made on a Shodex GPC-101 size exclusion chromatography apparatus (Showa Denko K.K., Kanagawa, Japan) connected with a DAWN HELEOS II MALS detector (Wyatt Technology Co., Santa Barbara, USA). A guard column, OHpak SB-G (Showa Denko K.K., Kanagawa, Japan) and two SEC columns, OHpak SB-806M-HQ (Showa Denko K.K., Kanagawa, Japan) were serially connected and warmed at 40 °C in a thermostated oven. The MALS detector and differential refractometer were connected to the columns in series to detect the scattering intensity and the concentration profiles. The flow rate was fixed to 1.0 $cm^3\,min^{-1}$. The MALS detector was calibrated by a standard poly(ethyleneoxide) sample of 2.7×10^4 g mol^{-1} (Tosoh Co. Ltd., Tokyo, Japan) and toluene. The refractive index increment for schizophyllan and sclerox solutions at the constant solvent chemical potential, $(\partial n/\partial c)_\mu = 0.141\ cm^{-3}\,g^{-1}$, was used in the