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Short communication

Triple helix conformation of botryosphaeran, a (1→3;1→6)-β-D-glucan produced by *Botryosphaeria rhodina* MAMB-05

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ABSTRACT

Botryosphaeran, a (1→3;1→6)-β-D-glucan produced by *Botryosphaeria rhodina* MAMB-05, was found to be present in a triple helix conformation from helix–coil transition studies using Congo Red. The triple helix conformation was disrupted at increasing alkali concentrations. Conformational changes were also observed using phenanthrene as a fluorescent probe, and the fluorescence intensity decreased 80% in the presence of dimethyl sulfoxide. The results confirmed the triple helix conformation of botryosphaeran, an important property manifesting biological response modifying activity.

1. Introduction

Botryosphaeran, a water-soluble exopolysaccharide (EPS) produced by *Botryosphaeria rhodina* MAMB-05 consists of a linear backbone chain comprising (1→3)-β-D-glucopyranosyl groups to which are attached branched chains of (1→6)-linked β-glucosyl and diglucosyl residues (Barbosa, Steluti, Dekker, Cardoso, & Corradi da Silva, 2003). Botryosphaeran can present more or less ramification points along the backbone chain according to the substrates used during fermentation (Corradi da Silva et al., 2005). This biomacromolecule was found to be non-mutagenic by the micronucleus test, and in animal studies botryosphaeran exhibited strong antimutagenic activity (Miranda et al., 2008). In the sulfonated form, this biopolymer presented anticoagulation activity (unpublished data).

The structure-functional relationships of biological response modifying activities are related to molecular conformation, the degree of branching, and molecular weight of the biopolymer (Bohn & BeMiller, 1995; Leung, Liu, Koon, & Fung, 2006). Three conformers of soluble β-1,3-glucans are known; single helix, triple helix, and random coil. Some related fungal (1→3;1→6)-β-D-glucans, such as scleroglucan and schizophyllan, adopt a triple helix conformation that strongly influences biological activity (Yoshitomi et al., 2005).

The objectives of the work reported here were to examine whether botryosphaeran, because of its biological activity, also exhibited conformational structure as a triple helix by helix–coil transition studies, and to determine the conformational properties of the triple helix as a function of the solvent nature, and the influence of salts on chain flexibility.

2. Materials and methods

2.1. Cultivation and production of botryosphaeran

Botryosphaeria rhodina MAMB-05 was maintained at 4 °C on potato dextrose agar. Inoculum was prepared and fungal cultures grown on media containing glucose or fructose (50 g l^{-1}) in submerged cultivation at 28 °C for 72 h as previously described (Steluti et al., 2004). Fungal cultures were harvested and the supernatant containing EPS (EPS_{GLC}, EPS_{FRU} for glucose- and fructose-grown cultures, respectively) recovered by centrifugation (3000*g*/15 min at 4 °C). Absolute ethanol (4 volumes) was added, the EPS allowed to precipitate overnight at 4 °C and recovered. It was then dissolved in water, exhaustively dialysed at 4 °C against several changes of water over 48 h, and the dialysate lyophilised.

2.2. Helix–coil transition analyses

Lyophilised EPS was dissolved in de-ionised water, or in aqueous salt solutions (Table 1) at the desired concentration, by prolonged stirring at 37 °C. For studies on the effect of alkali

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Table 1
The effect of urea and NaCl on the viscosity loss of botryosphaeran (EPS_{GLC}) in aqueous solution

Botryosphaeran[a] ($g\,l^{-1}$)	Urea		NaCl	
	Concentration (M)	Viscosity (Cp)[b]	Concentration (M)	Viscosity (Cp)[b]
1	0.2	57.6 ± 1.23	0.005	36.0 ± 1.37
	0.5	52.0 ± 0.89	0.05	22.8 ± 0.64
	1.0	48.6 ± 1.46	0.1	20.4 ± 0.89
2	0.2	84.8 ± 2.32	0.005	42.6 ± 1.28
	0.5	72.8 ± 2.16	0.05	38.4 ± 1.29
	1.0	67.2 ± 1.98	0.1	35.6 ± 2.01
3	0.2	345.0 ± 5.89	0.005	254.4 ± 3.54
	0.5	326.4 ± 4.65	0.05	148.8 ± 3.86
	1.0	312.8 ± 3.23	0.1	145.2 ± 3.73
4	0.2	441.6 ± 6.32	0.005	346.2 ± 3.96
	0.5	438.0 ± 4.59	0.05	253.4 ± 2.98
	1.0	380.4 ± 3.29	0.1	230.8 ± 3.86

[a] EPS_{GLC}.
[b] Mean ± SEM.

concentration and dimethyl sulfoxide (Me_2SO), solutions of EPS were adjusted with a few drops of 5 M NaOH solution, or Me_2SO, so as to maintain a constant EPS concentration.

Helix–coil transition experiments using Congo Red dye (CR, Sigma) were performed according to Ogawa, Watanabe, Tsurugi, and Ono (1972). An equal volume of solutions of EPS ($1\,g\,l^{-1}$) and CR (91 μM solution) was mixed in sodium hydroxide or Me_2SO, and the absorption maximum of the resulting solution immediately measured. The CR-complexes formed with dextran and laminarin (Sigma), and paramylon (Fluka) were also examined for comparative purposes.

Fluorescence spectral analyzes were performed as described by Zhang, Zhang, and Cheng (2000). EPS_{GLC} was first dissolved in water and a phenanthrene-MeOH (Sigma) stock solution added as a fluorescence probe. The EPS and fluorescence probe concentration was adjusted to $1\,g\,l^{-1}$ and 5 μM, respectively. Excitation and emission slits were set at 5.0 and 3.0 nm on a fluorimeter at wavelengths of 252 and 364 nm, respectively. Viscosity was determined with a Brookfield Viscometer (Model ED-II equipped with an S-18 spindle operated at 2 rpm).

The Tukey test was performed to confirm statistical significance of all data using Statistica Version 6.0. (www.statsoft.com; StatSoft, Inc. 2001).

3. Results and discussion

3.1. Helix–coil transition of botryosphaeran in NaOH solution

In the presence of alkali (NaOH), coil chains of EPS can affect the stability of inter- and intra-molecular hydrogen bonds and a conformational transition is observed (Young, Dong, & Jacobs, 2000). Helix or random coil conformational states have been detected by a simple method that consists of the formation of a complex between the biopolymer and the dye CR, which shows a red shift of the λ_{max} in the visible spectrum. Ogawa et al. (1972) proposed this finding to determine the conformation of (1→3)-β-D-glucans based on the ability of dye to interact with the carbohydrate possibly due to hydrogen bond formation with innate free hydroxyl linkages in a triple helix molecular form.

A complex of dye and botryosphaeran (CR-EPS_{GLC}) was formed in aqueous solution, demonstrating that the EPS from *B. rhodina* MAMB-05 has a native conformational state as a triple helix (Fig. 1). CR-EPS_{GLC} formation can be explained by enhancement of λ_{max} from 495 to 511 nm. A decrease in the maximum wavelength from 510 to 492 nm in the range of 0.2–0.25 M NaOH could be explained as resulting from disruption of the triple helix, and was in agreement with observations by Moresi, Bruno, Crognale, and Petruccioli (2003). This behavior has frequently been observed in (1→3;1→6)-β-D-glucans, and triple helix resistance to NaOH incrementally varies according to the kind of polysaccharide. For example, a β-D-glucan produced by *Pleurotus tuber-regium* presented a maximum absorption at λ_{530} that declined after addition of NaOH in the range from 0.1 to 0.16 M (Chenghua et al., 2000). In *Pleurotus florida*, however, the conformational transition of a similar β-D-glucan occurred at lower concentrations of alkali (0.05 M), and the dye-inclusion complex formed showed a λ_{max} at 511 nm (Rout, Mondal, Chakraborty, Pramanik, & Islam, 2005). Gradual addition of alkali instigates the helix–coil transition without contributing to hydrolysis of the β-linked glucosidic chains. The native conformation is easily restored through neutralization and dialysis (Young et al., 2000).

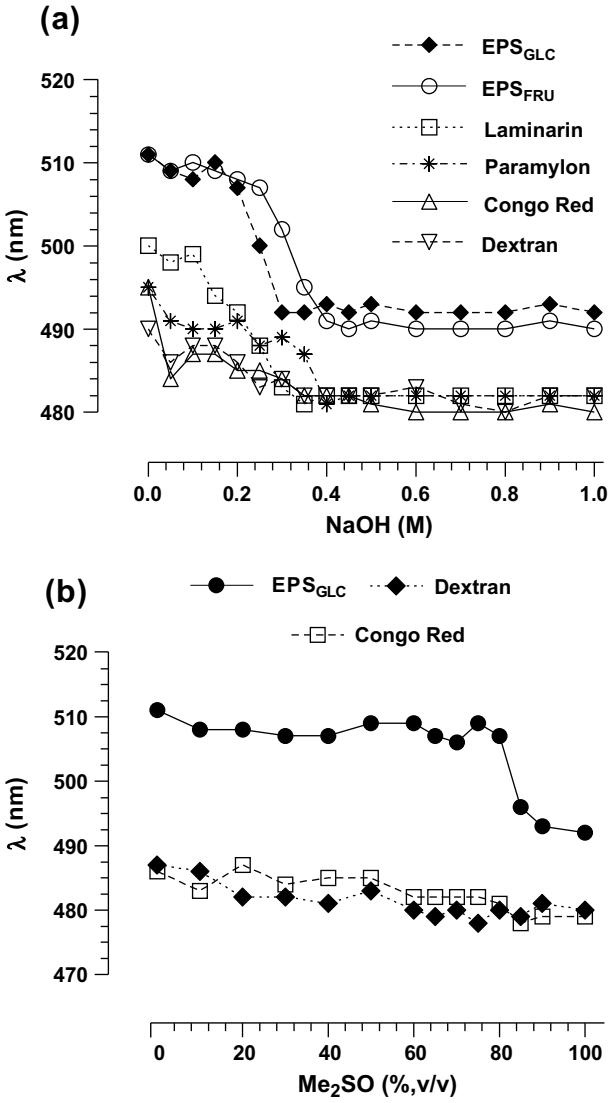

Fig. 1. Helix–coil transition analysis of (a) botryosphaerans (EPS_{FRU} and EPS_{GLC}), laminarin, and paramylon in the presence of different concentrations of NaOH, and (b) botryosphaeran (EPS_{GLC}) in the presence of different concentrations of Me_2SO. Dextran was used as random coil model.

Conformational changes in EPS_{GLC} solutions were also observed in fluorescence analyses with phenanthrene as an indicator of conformational state, as it specifically associates with a partially opened triple helix, a simple helix or stretched/random coils (Young et al., 2003). Interaction of phenanthrene with EPS_{GLC}

entailed a significant ($p < .05$) decrease of fluorescence intensity in the presence of NaOH concentrations higher than 0.2 M (Fig. 2).

3.2. Helix–coil transition of different botryosphaerans in NaOH solution

Botryosphaeria rhodina MAMB-05 produces a family of extracellular botryosphaerans when grown on different substrates (Steluti et al., 2004) that possess different degrees of ramification at C-6; EPS_{FRU} was more ramified (31%) than EPS_{GLC} (22%) (Corradi da Silva et al., 2005). Laminarin (*Laminaria digitata*) are lesser-branched (1→3;1→6)-β-D-glucans having 5–10% ramification (Young et al., 2000), while paramylon (*Euglena gracilis*) is a linear (1→3)-β-D-glucan with no branching at C-6 (Barsanti, Vismara, Passarelli, & Gualtieri, 2001).

EPS_{FRU} presented more resistance than EPS_{GLC} to conformational transitions in alkali medium as verified by a decrease in maximum wavelength (λ_{507} to λ_{495}) between 0.25 and 0.35 M NaOH. By comparison, the structural feature of less-ramified laminarin could be responsible for changes in its triple helix conformation at lower NaOH concentration (0.15 M; Fig. 1a), in agreement with the finding by Ogawa et al. (1972). In the case of paramylon, a transition form was observed between 0.2 and 0.4 M NaOH. A similar behavior was also described for a linear (1→3)-β-D-glucan extracted from the cell wall of an *Aspergillus* sp. (Ishibashi et al., 2004). The presence of ramification at C-6 appears to contribute to the enhancement of water solubility as the ramified chains are outside the triple helix thus avoiding the formation of insoluble aggregates (Bot, Smorenburg, Vreeker, Pâques, & Clark, 2001).

3.3. Effect of organic and inorganic salts on botryosphaeran conformation and viscosity

Denaturation of the triple helix can also occur through electrostatic interactions by the addition of salts that destabilize intramolecular interactions between the carbohydrate chains (Fariña, Siñeriz, Molina, & Perotti, 2001). An enhancement of ionic forces can also act on hydrogen bonds and disrupt the triple helix, as hydrogen bonds play a role in the stabilization of conformation.

Fig. 1b shows the effect of Me_2SO increment on EPS_{GLC} conformational state in aqueous solution. Me_2SO concentrations greater than 85% (v/v) instigated a dissociation of CR-EPS formed at a lower solvent content. Yanaki, Kojima, and Norisuye (1981) observed that the triple helix structure of scleroglucan was disrupted in 87% (v/v) Me_2SO as confirmed through intrinsic viscosity observations.

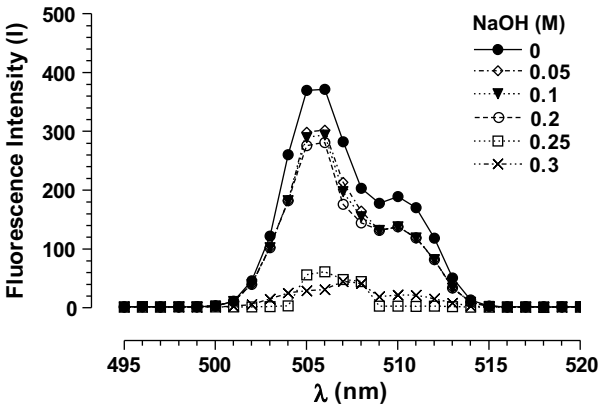

Fig. 2. Fluorescence analysis of botryosphaeran (EPS_{GLC}) in the presence of different concentrations of NaOH. Phenanthrene was used as a probe.

The effect of urea and NaCl additions on the relative viscosity of different aqueous solutions of EPS_{GLC} was also evaluated. The viscosity depends upon the conformational structure, degree of polymerization and molecular mass of the polysaccharide. Usually addition of a simple electrolyte protects the intermolecular electrostatic interactions and affects water mobility, decreasing the viscosity and the biopolymer assumes a more flexible configuration (Funami & Nishinari, 2007).

The viscosity decrement of EPS_{GLC} solutions in the presence of salts (urea and NaCl) was observed (Table 1), and similar results have been described for a diverse range of polysaccharides (Fariña et al., 2001). The presence of alkali (NaOH) at different concentrations, also promoted a loss of viscosity of a solution of EPS_{GLC}, and this effect was more pronounced at higher concentrations of EPS (data not shown). Botryosphaeran showed non-Newtonian pseudoplastic behavior in aqueous solutions, and viscosity decreased with increasing shear rates (unpublished data). A triple helical structure of EPS_{GLC} in solution appears be responsible for the pseudoplasticity. A small decrease of viscosity was observed in the presence of salts, and we conclude from this that the conformational state of EPS_{GLC} was not changed under these conditions. Sodium hydroxide as reported by Fariña et al. (2001), induces a structural denaturation of β-D-glucan generating changes in the viscoelastic behavior of these biopolymer solutions.

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