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Carbohydrate Research 298 (1997) 117–121

CARBOHYDRATE RESEARCH

The correlation between adhesion of schizophyllan to yeast glucan and its effect on regeneration of yeast protoplast

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Received 30 July 1996; accepted 28 October 1996

Abstract

Schizophyllan, a water-soluble (1 → 3)-β-D-glucan with a triple-helical conformation, adheres to yeast glucan and curdlan gel. As the molecular weight of schizophyllan decreases, both its adhesion to the water-insoluble glucans and its ability to promote the regeneration of yeast protoplasts are reduced. Therefore, we hypothesize that schizophyllan can surround yeast protoplasts by adhering to a fragment of yeast glucan remaining or/and resynthesized on the cell surface and that this encapsulation allows regeneration of the protoplast cells to occur at very high frequency. © 1997 Elsevier Science Ltd. All rights reserved.

Keywords: Schizophyllan; Biological activity; Adhesion; Yeast glucan

1. Introduction

Schizophyllan is secreted by the fungus *Schizophyllum commune* and consists of a main chain of (1 → 3)-β-linked D-glucose residues with one (1 → 6)-β-linked D-glucosyl side chain for every three D-glucose residues of the main chain [1]. This water-soluble polysaccharide exists in a triple-helical conformation in water [2,3] and shows antitumor activity [4]. The antitumor activity of schizophyllan has been correlated to its ordered conformation [5]. Recently, we reported that schizophyllan is effective in promoting the regeneration of protoplast cells of *Saccharomyces cerevisiae* [6] and that this biological activity is also correlated to the ordered conformation [7].

The cell walls of yeast are composed of complex polymers including yeast glucan, yeast mannan, chitin, protein, and lipid [8]. It is reasonable to assume that yeast glucan might be the most structurally important cell-wall polymer because the protoplast can be prepared with (1 → 3)-β-D-glucanase, and schizophyllan has a remarkable effect on the regeneration of protoplasts. Although the major component of the yeast glucan was reported to be a (1 → 3)-β-D-glucan having branches at C-6 [9,10], its structure has not been fully elucidated because of its complexity and insolubility in water. Therefore, the interaction of schizophyllan with curdlan, a well-characterized β-glucan, was analyzed. Curdlan, which is produced by *Alcaligenes faecalis* var. *myxogenes* and some strains of *Agrobacterium*, is a water-insoluble and linear (1 → 3)-β-D-glucan [11,12] with antitumor activity

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PII S0008-6215(96)00299-6

[13] and some interesting physicochemical features, including the formation of a resilient gel [14,15].

In the present paper, the adhesive property of schizophyllan to yeast glucan was studied in order to investigate the mechanism of the biological activity of schizophyllan, that is, promotion of the regeneration of yeast protoplasts.

2. Materials and methods

Materials.—Curdlan, pullulan and dried yeast, zymolyase 20T, and arabinogalactan from larch wood were purchased from Wako Pure Chemical Industries (Osaka), Seikagaku Kogyo (Tokyo), and Sigma (MO, USA), respectively. Schizophyllan, xyloglucan from *Tamarindus indica*, and xanthan and locust bean gum were obtained by Taito (Kobe), Dainippon Pharmaceutical (Osaka), and San-EI Gen F.F.I. (Osaka), respectively. Pustulan was kindly provided by Dr. T. Nakajima of the Tohoku University. Cyclosophoran and succinoglycan were from our laboratory stocks.

Preparation of yeast glucan and yeast mannan.—Yeast glucan and yeast mannan were prepared from dried yeast according to the method described by Edwards [16].

Preparation of curdlan gel.—Curdlan (3 g) was suspended in 40 mL of distilled water (20 °C) and mixed with 60 mL of boiling water to prepare a soft gel and then heated at 120 °C for 2 h to prepare a hard curdlan gel. After homogenizing with a Nissei AM-9 Homogenizer (Nissei Co. Ltd., Tokyo) at 10,000 rpm for 2 min, the curdlan gel was heated again at 120 °C for 2 h and then rinsed with distilled water.

Preparation of depolymerized schizophyllan (D.S.).—Schizophyllan (475 kDa) was partially hydrolyzed at 100 °C in 85% dimethyl sulfoxide containing 0.01 M H_2SO_4, and the D.S. samples obtained were further separated into size groups by gel-permeation chromatography, as described previously [7]. Molecular weights of separated D.S. samples were estimated by using a HPLC system with a low-angle laser-light-scattering detector, LS-8000 (Tosoh, Tokyo) [7].

Assay for adhesion of schizophyllan and D.S. samples to yeast glucan.—In a screw-capped vial, 50 mg of yeast glucan was mixed with 2 mL of a 0.05% sample. The vial was rotated at a speed of 15 rotations per min at 30 °C for 2 h. After centrifugation, the amount of carbohydrate in the supernatant was estimated by the phenol–H_2SO_4 method [17].

Adhesion of water-soluble polysaccharides and D.S. samples to curdlan gel.—Water-soluble polysaccharides and D.S. samples were applied to columns (2.0 × 15 cm) packed with curdlan gel and eluted with distilled water (100 mL) at a flow rate of about 0.5 mL/min. A solution (1 mL) containing 1 mg of sample was tested, and the amount of carbohydrate eluted from the column was analyzed colorimetrically [17].

X-ray diffraction pattern.—The X-ray diffraction patterns of yeast glucan, curdlan, curdlan gel, and schizophyllan in powdered form were recorded with a X-ray diffractometer Miniflex (Rigaku Denki, Tokyo) [15,18].

Frequency of regeneration (F.R.) of yeast protoplasts.—The capacities of schizophyllan and D.S. samples to promote the regeneration of protoplast cells of *Saccharomyces cerevisiae* were tested, as reported previously [6,7]. The cells (5×10^7/mL) of *S. cerevisiae* Kyokai No. 7 at mid-logarithmic phase were digested by shaking gently at 30 °C for 60 min in 0.1 M phosphate buffer (pH 7.5) containing 0.8 M KCl and 1.25 mg of Zymolyase 20T. The protoplast cells obtained were gently centrifuged and then suspended in 0.8 M KCl. The diluted solutions were transferred into soft-agar solutions consisting of 0.1% schizophyllan or D.S. sample, 0.8 M KCl, and 0.8% agar, and spread on agar plates consisting of PYD medium (2% polypepton, 1% yeast extract, and 2% glucose), 0.8 M KCl, and 2% agar. The plates were incubated at 30 °C for 4–5 days and colonies were counted. The F.R. value was calculated from the formula: [(number of colonies on plate)/(number of protoplast cells used)] × 100.

Formations of schizophyllan and D.S. complexes with Congo red.—Schizophyllan and D.S. samples were complexed with Congo red according to the method reported by Tabata et al. [19]. Each sample (7 mg) was dissolved in 2.4 mL of an alkaline solution, mixed with 0.1 mL of 0.04% Congo red, and the absorption spectrum was then recorded with spectrophotometer UV-300 (Shimadzu, Kyoto) from 600 nm to 400 nm. Alkaline solutions containing from 0.05 M to 0.3 M NaOH were tested.

3. Results and discussion

Effects of D.S. samples on regeneration of yeast protoplasts and their adsorption to yeast glucan.—We previously reported that the effect of schizophyllan on regeneration of yeast protoplast reduced as its

molecular weight decreased and finally disappeared [7]. It was found that schizophyllan can adhere specifically to yeast cells as described below, and the adhesion of D.S. samples to yeast glucan was assayed in test tubes. As shown in Table 1, the adhesion and the F.R. value of the 90-kDa sample were also almost at the same level as native schizophyllan, but F.R. values of other D.S. samples diminished along with the adhesion as their molecular weights decreased. This suggests that the effect of schizophyllan on regeneration of yeast protoplasts is related to its adhesion to yeast glucan.

Adhesion of water-soluble polysaccharides and D.S. samples to curdlan gel.—Of the water-soluble polysaccharides tested, only schizophyllan showed a considerable promotion of the regeneration of yeast protoplasts [6]. Therefore, it appeared necessary to confirm that schizophyllan adheres specifically to yeast glucan. However, preparation of large amounts of yeast glucans is difficult, and so a column packed with curdlan gel was used to test the adhesion of water-soluble polysaccharides and the D.S. samples.

Cyclosophoran [20] and pustulan [21] were selected as (1 → 2)-β-D-glucan and (1 → 6)-β-D-glucan, respectively. Xanthan [22] and xyloglucan [23] were used because of their (1 → 4)-β-D-glucan backbone chains. Succinoglycan [24] was also tested because 75% of its backbone consists of (1 → 4)-β-D-glucosidic linkages. Locust bean gum [25] and arabinogalactan [25] with backbones of (1 → 4)-β-D-mannan and (1 → 3)-β-D-galactan, respectively, were also tested. Yeast mannan [26] and pullulan [26] were tested as α-glycans. As shown in Fig. 1, only schizophyllan showed a considerable adhesion to curdlan gel among the ten glycans tested.

Table 1
Effect of schizophyllan and D.S. samples on the regeneration of protoplast cells of *S. cerevisiae* and their adhesion to yeast glucan

	Adhered amount [a] (mg)	F.R. [b]
Schizophyllan (475 kDa)	0.63	1.2
90-kDa sample	0.58	1.4
66-kDa sample	0.41	7.1×10^{-1}
36-kDa sample	0.28	2.4×10^{-1}
3.5-kDa sample	0.14	1.5×10^{-3}

[a] The amount (mg) adhered to the yeast glucan (50 mg) was calculated from the difference between the amount applied and the amount not bound.
[b] Frequency of regeneration.

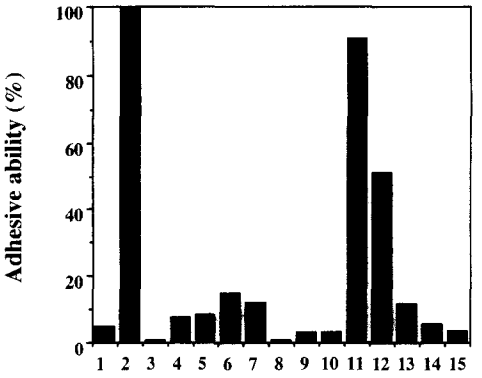

Fig. 1. Adhesive properties of water-soluble polysaccharides and D.S. samples to curdlan gel: 1, cyclosophoran; 2, schizophyllan; 3, xanthan; 4, xyloglucan; 5, pustulan; 6, succinoglycan; 7, locust bean gum; 8, arabinogalactan; 9, yeast mannan; 10, pullulan; 11, 90-kDa sample; 12, 66-kDa sample; 13, 36-kDa sample; 14, 3.5-kDa sample; 15, glucose.

In tests of the adhesion of D.S. samples to the column of curdlan gel, the 90-kDa sample almost entirely adhered, but most of the 36-kDa sample and 3.5-kDa sample were eluted without adhesion. In the case of the 66-kDa sample, about half of the sample adhered. We previously reported that D.S. samples more than 23 kDa consist of triple helical chains in water and D.S. samples smaller than 5.9 kDa are single coils [7], and it can be inferred that the 36-kDa, 66-kDa, and 90-kDa samples used in this study are triple strands. This suggests that the remarkable difference in their adhesion to curdlan gel might arise from a difference in their triple helical conformations.

Complex formation of D.S. samples with Congo red.—Conformationally ordered schizophyllan forms a complex with Congo red in dilute alkaline solution producing a shift of the adsorption maximum (λ_{max}) [19], thereby allowing the D.S. samples to be tested for complex formation with Congo red (Fig. 2). In 0.05 M NaOH, λ_{max}'s of the 36-kDa, 66-kDa, and 90-kDa samples were all significantly shifted, indicating that they adopt ordered conformations. However, in the case of the 36-kDa sample, the λ_{max} returned rapidly to the original value for uncomplexed Congo red as the alkaline concentration increased, suggesting that the conformation of the 36-kDa sample was unstable. The ordered conformation of the 36-kDa sample might be not as stable as that of the 90-kDa sample because the number of intermolecular hydrogen bonds between adjacent chains is decreased. Assuming that about half of the molecules in a 66-kDa sample have unstable conformations as in the 36-kDa sample, and that the other half have stable

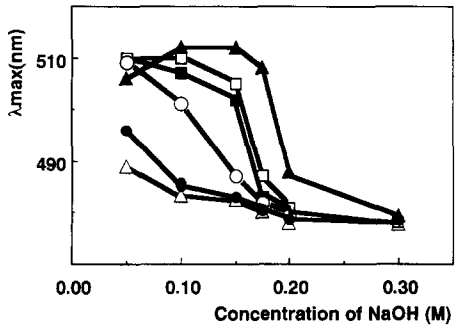

Fig. 2. Changes in the adsorption maximum (λ_{max}) of solutions mixed with Congo red and D.S. samples in various concentrations of sodium hydroxide: schizophyllan (▲); 90-kDa sample (□); 66-kDa sample (■); 36-kDa sample (○); 3.5-kDa sample (●); none (△).

conformations as in the 90-kDa sample, would explain the adhesion data for the 66-kDa sample shown in Fig. 1.

X-ray diffraction patterns.—As the adhesion of schizophyllan to curdlan was obviously different from its adhesion to curdlan gel, as measured in a preliminary examination in test tubes, the crystallinities of yeast glucan, curdlan gel, curdlan, and schizophyllan were investigated by X-ray diffraction (Fig. 3). The diffraction patterns of yeast glucan, curdlan gel, and schizophyllan showed an intense peak near 6.4°, but that of curdlan showed no significant peak near 6.4°. It has been known that a heating treatment at 120 °C transfers curdlan from a single helical strand to another form (curdlan gel) with a triple helical conformation [27,28]. The occurrence of a triple helical

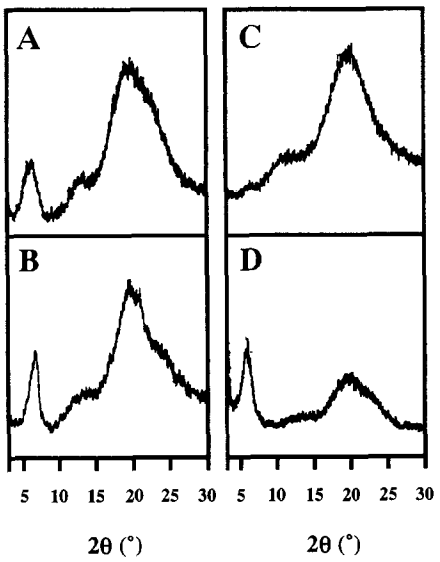

Fig. 3. X-ray diffraction patterns of yeast glucan (A), curdlan gel (B), curdlan (C), and schizophyllan (D).

structure in yeast glucan was suggested by studies of the molecular weights of both phosphorylated yeast glucan in water and native yeast glucan dissolved in dimethyl sulfoxide [29]. Schizophyllan exists as a triple-helical structure in water [3], and the ordered conformation was also detected in powder (see Fig. 3D). Thus, it is likely that the peak near 6.4° in the diffraction pattern of yeast glucan indicates ordered domains corresponding to the triple helical conformation.

4. Conclusions

The specific adhesion of schizophyllan to yeast glucan and curdlan gel requires high-molecular-weight molecules with a triple helical conformation. It is well known that xyloglucan can adhere strongly to cellulose [30,31]. Hayashi et al. reported that xyloglucan fragments containing eight or more sugar residues bind to cellulose [32]. Thus, it is likely that the mechanisms of adhesion are completely different for schizophyllan and xyloglucan.

The X-ray diffraction patterns of schizophyllan, yeast glucan, and curdlan gel have peaks corresponding to ordered conformations. On the other hand, when schizophyllan was converted to single coils by partial depolymerization, its adhesion and capacity to promote regeneration of yeast protoplast were reduced. These results show that the conformation of schizophyllan is correlated to both its biological activity and its adhesive properties.

Furthermore, this data allows us to propose a mechanism for the biological activity of schizophyllan. That is, yeast protoplasts encapsulated by schizophyllan via its adhesion to fragments of yeast glucans remaining on or/and resynthesized and exported to the cell surface become considerably more resistant to deleterious physical and physiological factors. The resulting protected environment allows the regeneration of yeast protoplasts to occur at very high frequency.

As the yeast glucan did not show any effect on the regeneration of yeast protoplast, it is likely that the water solubility of the polysaccharide is an important factor for its biological activity.

Acknowledgements

The authors thank Dr. P. Albersheim and Dr. W.S. York (Complex Carbohydrate Research Center, the

University of Georgia, Athens, GA, USA) for checking the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 05806043) from the Ministry of Education, Science, and Culture of Japan, and by Okasan-Kato foundation (96-3-4).

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