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Novel sources of β-glucanase for the enzymatic degradation of schizophyllan☆

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ABSTRACT

Schizophyllan is a homoglucan produced by the fungus *Schizophyllum commune*, with a β-1,3-linked backbone and β-1,6-linked side chains of single glucose units at every other residue. Schizophyllan is commercially produced for pharmaceutical and cosmetics uses. However, surprisingly little information is available on the biodegradation of schizophyllan. Enzymes that attack schizophyllan could be useful for controlled modifications of the polymer for novel applications. Enrichment cultures were used to isolate 20 novel fungal strains from soil samples, capable of growing on schizophyllan as a sole carbon source. Three additional strains were isolated as contaminants of stored schizophyllan solutions. Strains showing the highest levels of β-glucanase activity were identified as *Penicillium simplicissimum*, *Penicillium crustosum*, and *Hypocrea nigricans*. β-glucanases also showed activity against the similar β-glucans, laminarin and curdlan. By comparison, commercial β-glucanase from *Trichoderma longibrachiatum* and laminarinase from *Trichoderma* sp. showed lower specific activities toward schizophyllan than most of the novel isolates. β-glucanases from *P. simplicissimum* and *H. nigricans* exhibited temperature optima of 60 °C and 50 °C against schizophyllan, respectively, with broad pH optima around pH 5.0. Partial purifications of β-glucanase from *P. simplicissimum* and *P. crustosum* demonstrated the presence of multiple active endoglucanase species, including a 20–25 kD enzyme from *P. simplicissimum.*

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1. Introduction

Schizophyllan is a polysaccharide produced by *Schizophyllum commune*, a white-rot fungus and ubiquitous mushroom. It is a homoglucan with a β-1,3-linked backbone and single β-1,6-linked glucose side chains at every other residue [1,2]. Schizophyllan acts as a biological response modifier and a non-specific stimulator of the immune system. It is used in vaccines, anti-cancer therapies, and as a bioactive cosmetics ingredient. Schizophyllan can form oxygen-impermeable films for food preservation [3]. It also has been tested for use in enhanced petroleum recovery [2,4].

As a natural polysaccharide, it can be assumed that schizophyllan is biodegradable, and many of its applications rely on this assumption. However, surprisingly little information is available on the biodegradation of schizophyllan. *S. commune* has been reported to produce endo-β-1,3-glucanase [5], and Rau [2] proposed that the organism can consume schizophyllan as a carbon source, contributing to a loss of polysaccharide molecular weight in late cultures. Lo et al. [6] described β-glucosidases from *S. commune*. Fontaine et al. [7] reported that schizophyllan was slightly hydrolyzed by one of two exo-β-1,3-glucanases associated with the cell walls of *Aspergillus fumigatus*. On the other hand, Kanzawa et al. [8] found that exo-β-1,3-glucanase from *Bacillus circulans* rapidly hydrolyzed curdlan and laminarin, but did not attack schizophyllan. Tanji et al. [9] reported that schizophyllan was partially degraded at a very slow rate in rats, to lower molecular weight forms of <10,000 that were excreted in urine. It is potentially valuable to identify enzymes that attack schizophyllan, particularly for use in controlled modifications of the polymer for novel applications.

In the current study, 23 novel strains were isolated that were capable of growing on schizophyllan as a sole carbon source. Strains showing the highest activities against schizophyllan were identified, and β-glucanase activities were characterized. Results indicate that novel fungal isolates are promising sources of schizophyllan-degrading enzymes.

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Table 1
β-glucanase activity against schizophyllan produced by newly isolated strains[a]

Strain number	Isolation sites (near Peoria, Illinois)	β-glucanase (U activity/mL)[b]
1-1	Woodland	0.24 ± 0.02
1-2		0.16 ± 0.03
1-3		0.17 ± 0.03
2-1	Prairie grassland	0.005 ± <0.001
2-2		<0.001
2-3		0.003 ± <0.001
2-4		0.001 ± <0.001
3-1	Hayfield/woodland border	<0.001
3-2		<0.001
4-1	Pond shore	0.007 ± <0.001
4-2		0.001 ± <0.001
6-1	Clay soil	0.008 ± <0.001
6-2		0.002 ± <0.001
6-3		0.21 ± 0.05
6-4		0.19 ± 0.01
7-1	Marshland	<0.001
7-2		0.08 ± 0.02
8-1	Backyard/gardening area	0.007 ± <0.001
8-2		0.003 ± <0.001
8-3		0.003 ± <0.001
9-1	Laboratory contaminant	0.026 ± <0.001
9-2		0.032 ± <0.001
9-3		0.029 ± <0.001

[a] Strains were isolated from enrichment cultures containing schizophyllan as a sole carbon source.
[b] Pure cultures were grown for 7 days in basal medium containing 1.0% schizophyllan as a sole carbon source.

2. Methods

2.1. Isolation of novel schizophyllan-degrading strains

Soil samples from the area of Peoria, Illinois were serially diluted according to Leathers et al. [10]. Specifically, 2.0 g of soil was diluted into 198 mL of sterile 0.2% agar in distilled water (water agar). This was shaken vigorously, and then 10 mL was transferred to 90 mL of water agar and mixed well. One milliliter of this suspension was then transferred into 9 mL of water containing 0.01% Tritron X-100. Aliquots (0.1 mL) of these final dilutions were used to inoculate 10 mL enrichment cultures containing 1.0% (w/v) schizophyllan (cosmetic grade, European Technologies, Inc., Denver, CO) as a sole carbon source in basal medium composed of 0.67% (w/v) yeast nitrogen base, 0.5% (w/v) KH_2PO_4, and 0.2% (w/v) bacto-asparagine (Difco Laboratories, Detroit). Enrichment cultures were grown for 7 days at 28 °C and 200 rpm. Serial dilutions were made onto solid medium containing potato dextrose agar (PDA, Difco), and isolates were single-colony purified at least three times. In addition, three strains were isolated as laboratory contaminants of stored schizophyllan solutions (Table 1).

2.2. Sequence identification of strains

Conidia were floated free of the mycelium with 2–3 mL of 70% ethanol and concentrated by brief centrifugation in a micro-centrifuge. Conidial pellets were suspended in 400 μL of CTAB buffer [11] in a 1.5 mL microfuge tube containing about 400 mg of 0.5 mm diameter glass beads. Cell walls were broken by vortex mixing of the glass beads and conidia. Chloroform (0.4 mL) was added to extract proteins and the aqueous and organic phases were separated by centrifugation. The aqueous phase was transferred to a clean microfuge tube, precipitated by addition of an equal volume of isopropanol and collected by centrifugation. The resulting pellet was rehydrated in 100 μL buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0). The DNA preparation was diluted 10 to 100 times in sterile distilled water for use in PCR amplifications. Beta tubulin was amplified using the primers and procedures of Glass and Donaldson [12]. Alternatively, DNA isolation was carried out using a ZR Fungal/Baterial DNA kit following the procedure according to Nawrot et al. [13], and strains were identified by the sequence of their 28s rRNA genes. Primers were NL-1 (59-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (59-GGTCCGTGTTTCAAGACGG) as described by O'Donnell [11].

2.3. Culture conditions for β-glucanase production on schizophyllan

Strains were grown on PDA slants at 28 °C for 7–10 days. An approximately 7 mm × 7 mm square of mycelium was used to inoculate 10 mL of CB basal medium containing 1.0% (w/v) commercial schizophyllan in a 50 mL flask with three 10 mm glass beads. Cultures were incubated at 200 rpm for 7 days at 28 °C, then centrifuged at 3220 × *g* to produce cell-free culture supernatants. Mycelial pellets were dried at 60 °C for 48 h. All experiments were carried out in triplicate and standard deviations are shown.

2.4. Enzyme and protein assays

Quantitative β-glucanase assays were performed by the dinitrosalicylic acid (DNS) method [14] as modified by Leathers et al. [15]. Samples (5–20 μL) were incubated in a total volume of 205 μL containing 0.5% (w/v) substrate (schizophyllan or another β-glucan) in 50 mM sodium acetate buffer, pH 5.0, at 28 °C. Sample dilutions and incubation times were adjusted to ensure results were within the linear range of the assays. One unit of enzyme activity is defined as the amount of enzyme necessary to release 1 μmole of glucose equivalents per min under the conditions tested. Schizophyllan (cosmetic grade) was purchased from European Technologies, Inc., Denver, CO. Other β-glucan substrates (laminarin from *Laminaria digitata*, paramylon from *Euglena gracilis*, curdlan from *Agrobacterium* sp. (*Alcaligenes faecalis*), and barley β-glucan) were from Sigma-Aldrich, St. Louis. Commercial cellulases from *Aspergillus niger* and *T. viride* (Cellulysin) were from Calbiochem (La Jolla, CA). Commercial β-glucanases from *A. niger*, *T. longibrachiatum*, and *Bacillus subtilis*, as well as laminarinase from *Trichoderma* sp., were from Sigma–Aldrich. Temperature and pH optima were performed using the same assay. For studies of pH optima, substrate buffer was titrated to the desired test pH with acetic acid or sodium hydroxide before digestion, then returned to pH 5 before assays were developed, since the DNS assay is pH sensitive. Enzyme values are the mean of triplicate cultures and are characteristic of repeated experiments.

Rapid, semi-quantitative β-glucanase assays were performed using a solid medium plate assay. Samples (4 μL) were spotted directly onto the surface of freshly prepared plates containing 0.7% (w/v) Phytagel (Sigma–Aldrich Co., St. Louis) and 0.05% (w/v) commercial schizophyllan in 50 mM sodium acetate buffer, pH 5.0. Plates were incubated overnight and stained with an aqueous solution of 1 mg Congo red/mL for 45 min, then destained with 1.0 M NaCl for 30 min. Enzyme activity was observed as a clear zone. Laminarinase from *Trichoderma* sp. was used as a positive control.

Extracellular protein was estimated by the Bradford method [16], with bovine serum albumin as the standard.

2.5. Enzyme purification by fast protein liquid chromatography (FPLC)

Enzyme samples were purified using a fast protein liquid chromatography system (Biologic Duoflow, Bio-Rad, Hercules, CA). Cell-free culture supernatants were applied to a 5.0 mL High Q anion exchange column (Bio-Rad) equilibrated in 50 mM sodium acetate buffer, pH 5.0. Bound protein was eluted with an increasing gradient of 0.0 to 1.0 M NaCl in the same buffer. Alternatively, samples were adjusted to 1.0 M $(NH_4)_2SO_4$ by direct addition of solid $(NH_4)_2SO_4$ and then applied to a Phenyl Sepharose column (1.5 × 10 cm, GE Healthcare, Piscataway, NJ) equilibrated with 1.0 M $(NH_4)_2SO_4$ in 50 mM sodium acetate buffer, pH 5.0. Bound protein was eluted with a decreasing gradient from 1.0 M to 0.0 M $(NH_4)_2SO_4$ in the same buffer.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Active enzyme fractions from liquid chromatography were desalted using protein desalting spin columns (Pierce, Rockford, IL). Samples were denatured by heating for 4 min at 95 °C in 2× SDS sample buffer (4.0% (w/v) SDS, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 25% (w/v) 0.5 M Tris–HCl pH 6.8 and 5.0% (v/v) β-mercaptoethanol) and applied to an SDS-PAGE gel (3.0% stacking, 10% resolving). After electrophoresis at 100 V for approximately 1 h, the SDS PAGE gel was stained with SYPRO Ruby protein gel stain (Invitrogen, Grand Island, NY) for 16 h and rinsed with deionized water. Stained gels were visualized by UV transillumination.

2.7. Zymogram analysis

Samples from cell-free culture supernatants and liquid chromatography fractions were denatured as described above and applied to a 10% SDS-PAGE gel containing 0.04% (w/v) commercial schizophyllan within the gel matrix. Following electrophoresis, the gel was incubated in 50 mM sodium acetate buffer, pH 5.0 at 28 °C for 20 h, stained with an aqueous solution of 1.0 mg Congo Red/mL for 30 min, and destained with 1.0 M aqueous NaCl for 30 min. After rinsing with deionized water, enzyme activity was visualized on the gel as clear bands against a red background of stained schizophyllan. The clear band resulted from the degradation of schizophyllan.

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