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ORIGINAL RESEARCH PAPER

Enzymatic modification of schizophyllan

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Abstract An enzymatic method was developed for the progressive modification of the polysaccharide schizophyllan. Fungal strains *Hypocrea nigricans* NRRL 62555, *Penicillium crustosum* NRRL 62558, and *Penicillium simplicissimum* NRRL 62550 were previously identified as novel sources of β-endoglucanase with specificity towards schizophyllan. Concentrated enzyme preparations from these strains showed specific activities of 1.7–4.3 U β-glucanase/mg protein. Using dilutions of these enzymes in time course digestions, schizophyllan was progressively modified to reduced molecular weight species. Glucose and oligosaccharides were found only in the more complete digestions, and thus modified schizophyllan can be produced quantitatively, without loss, to small molecules. Permethylation analysis confirmed that modified schizophyllan retains the fundamental linkage structure of native schizophyllan. Modified schizophyllan species showed progressively reduced viscosity profiles, and all exhibited pseudoplasticity in response to shear thinning.

Keywords β-Glucanase · Modified schizophyllan · Schizophyllan · Viscosity

Introduction

Schizophyllan is a homoglucan with a β-1,3-linked backbone and single β-1,6-linked glucose side chains at every other residue, produced by the fungus *Schizophyllum commune* (Rau 1999). It acts as a biological response modifier and a non-specific stimulator of the immune system (Zhang et al. 2013). It is used in vaccines, anti-cancer therapies, and as a bioactive cosmetics ingredient (Rau 2002). It also is being tested for use in biomaterials applications, such as enhanced petroleum recovery (Anonymous 2012).

Native schizophyllan has a molecular weight range of 6–12 × 10^6 g/mol (Rau et al. 1990). Aqueous solutions exhibit pseudoplastic behavior, and viscosity thus depends on both concentration and shear rate (Rau 2002). Viscosity also depends on the molecular weight of the polymer, and for certain applications it would be useful to be able to modify the molecular weight in a controlled manner to alter the viscosity of schizophyllan. Kojima et al. (1984) state that the high viscosity of schizophyllan makes its preparation and

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administration difficult for clinical uses. Schizophyllan with a range of viscosities may be useful for cosmetic products of varying viscosities, such as lotions, creams, and ointments. Modified molecular weight also could be important to tailor schizophyllan for biomaterial applications.

Few studies have appeared concerning the modification of schizophyllan molecular weight. Tabata et al. (1981) reported the ultrasonic degradation of schizophyllan to a low-molecular weight polysaccharide. Similarly, Kojima et al. (1984) used hydrodynamic shear to break down schizophyllan. Enzymatic treatment of schizophyllan with endoglucanases could offer a useful alternative approach. However, little information has been available on the enzymatic degradation of schizophyllan. *S. commune* produces an endo-β-1,3-glucanase (Prokop et al. 1994), and Rau (2002) proposed that the organism can consume schizophyllan as a carbon source, contributing to a loss of polysaccharide molecular weight in late cultures. Fontaine et al. (1997) reported that schizophyllan was slightly hydrolyzed by one of two exo-β-1,3-glucanases associated with the cell walls of *Aspergillus fumigatus*. Sutivisedsak et al. (2013a) identified novel fungal sources of β-glucanase for the enzymatic degradation of schizophyllan. Enzymes from these fungi appear to be endoglucanases that have higher specific activities against schizophyllan than commercial sources of β-glucanase (Sutivisedsak et al. 2013a). In the current study, these enzymes are tested for the controlled, progressive modification of schizophyllan to lower molecular weight forms.

Materials and methods

Production of β-glucanase and enzymatic modification of schizophyllan

Fungal strains *Hypocrea nigricans* strain NRRL 62555, *Penicillium crustosum* strain NRRL 62558, and *Penicillium simplicissimum* strain NRRL 62550 were isolated as previously described (Sutivisedsak et al. 2013a) and maintained in the ARS Culture Collection, Peoria, IL. Strains were grown on potato/dextrose/agar at 28 °C for 7–10 days. Liquid preinocula cultures (10 ml in 50 ml flasks) contained 0.67 % (w/v) yeast nitrogen base, 0.2 % (w/v) asparagine, and 0.5 % (w/v) KH_2PO_4, amended with 1 % (w/v) cosmetic-grade schizophyllan (European Technologies Inc., Denver, Colorado). Pre-inocula were grown for 7 days at 28 °C, 200 rpm. Production cultures were 500 ml of the same medium in 1.8 Fernbach flasks, inoculated with 5 ml pre-inoculum and grown for up to 14 days under the same conditions. Cultures were centrifuged for 60 min at $10{,}900\times g$ to produce cell-free culture supernatants. Experiments were carried out in triplicate and standard errors are shown.

Culture supernatants were concentrated ~50-fold using centrifugal ultrafiltration concentrators with 10 K membranes (Pall Corp., Port Washington, New York). Concentrated culture supernatants were stored at 4 °C with 0.02 % (w/v) sodium azide.

β-Glucanase assays were performed as previously described (Sutivisedsak et al. 2013a) by a modification of the dinitrosalicylic acid method with commercial schizophyllan as the substrate. One unit of enzyme activity is defined as the amount necessary to release 1 μ mole of glucose equivalents per min under the conditions tested. Protein was determined by the Bradford method.

For preparation of enzymatically modified schizophyllan, 1 % (w/v) solutions of commercial schizophyllan in 50 mM sodium acetate, pH 5.0, were incubated at 28 °C with 0.125, 0.625, or 1.25 U β-glucanase/ml for 0–6 h. Reactions were terminated by heating for 1 min at 100 °C.

Analysis of enzymatically-modified schizophyllan

Polysaccharide molecular weights were determined by size exclusion chromatography as previously described (Leathers et al. 2010). Schizophyllan solutions were filtered though a 0.45 μm filter (Pall Corp.), applied to a Shodex SB-806 M high performance size exclusion chromatography (HPSEC) column (Showa Denko, Tokyo, Japan) and eluted with 50 mM NaNOz at 0.5 ml/min. The column was calibrated with a set of eight pullulan molecular weight standards ranging from 5.8×10^3 Da to 1.66×10^6 Da (Showa Denko). Separations were monitored using a Shodex OR-1 optical rotation detector (Showa Denko).

Glucose and oligosaccharides were determined as previously described (Leathers and Manitchotpisit 2013). Samples were applied to an Aminex HPX-87H column (Bio-Rad, Hercules, CA) equilibrated with 0.02 % phosphoric acid and eluted at 0.5 ml/min at

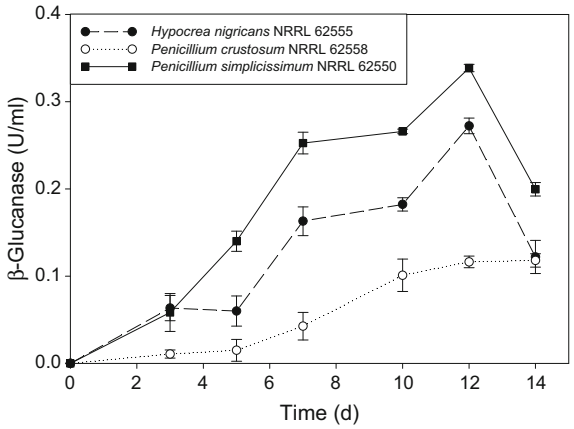

Fig. 1 Time course of β-glucanase production by *Hypocrea nigricans* strain NRRL 62555, *Penicillium crustosum* strain NRRL 62558, and *Penicillium simplicissimum* strain NRRL 62550. Triplicate 500 ml cultures were grown in basal medium containing schizophyllan as a carbon source. β-Glucanase assays were performed using schizophyllan as a substrate. *Error bars* represent the standard error

Table 1 Concentrated culture supernatants used for enzymatic modification of schizophyllan

Species	Strain number	β-glucanase activity[a] (U/ml)	Specific activity (U/mg protein)
Hypocrea nigricans	NRRL 62555	6.2 ± 0.5	1.9 ± 0.2
Penicillium crustosum	NRRL 62558	7.5 ± 1.2	1.7 ± 0.3
P. simplicissimum	NRRL 62550	18.7 ± 0.2	4.3 ± 0.1

[a] Determined on commercial schizophyllan. Standard errors are indicated

25 °C. Glucose and oligosaccharides were detected by refractive index.

Permethylation linkage analysis was performed as previously described (Price 2004) by adapting the method of Ciucanu and Kerek (1984). The methylated product was hydrolyzed, acetylated, and analyzed by GC/MS as previously described (Price 2004).

Schizophyllan solution viscosities were measured as previously described (Sutivisedsak et al. 2013b) using a TA Instruments (New Castle, Delaware) ARES LS-1 controlled strain rheometer with a 25 mm titanium parallel plate. All tests were performed at 25 °C using a peltier plate.

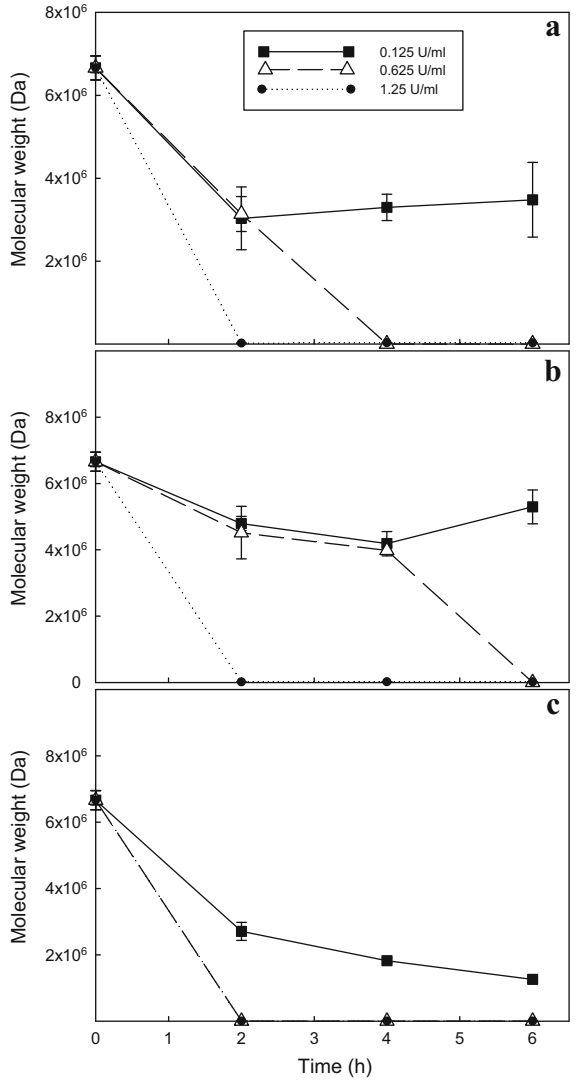

Fig. 2 Time course of enzymatic modification of schizophyllan by β-glucanase from **a** *Hypocrea nigricans* strain NRRL 62555, **b** *Penicillium crustosum* strain NRRL 62558, and **c** *Penicillium simplicissimum* strain NRRL 62550. *Error bars* represent the standard error

Results and discussion

Production of β-glucanase for enzymatic modification of schizophyllan

Sutivisedsak et al. (2013a) described novel sources of β-glucanase for the enzymatic degradation of schizophyllan. In particular, *H. nigricans* strain NRRL 62555, *P. crustosum* strain NRRL 62558, and *P. simplicissimum* strain NRRL 62550 were considered

Table 2 Generation of oligosaccharides and glucose during enzymatic modification of schizophyllan

β-glucanase source	β-glucanase (U/ml)	Time (h)	Oligosaccharides[a]	Glucose (mg/ml)
Control (native schizophyllan)			0	<0.1
Hypocrea nigricans	0.125	2	0	<0.1
Strain NRRL 62555	0.125	4	0	<0.1
	0.125	6	0	<0.1
	1.25	2	4	6.7
Penicillium crustosum	0.125	2	0	<0.1
Strain NRRL 62558	0.125	4	0	<0.1
	0.125	6	0	<0.1
	0.625	6	3	4.8
	1.25	2	2	12
P. simplicissimum	0.125	2	0	<0.1
Strain NRRL 62550	0.125	4	0	<0.1
	0.125	6	4	6.0
	0.625	2	4	11

[a] Number of oligosaccharide species observed

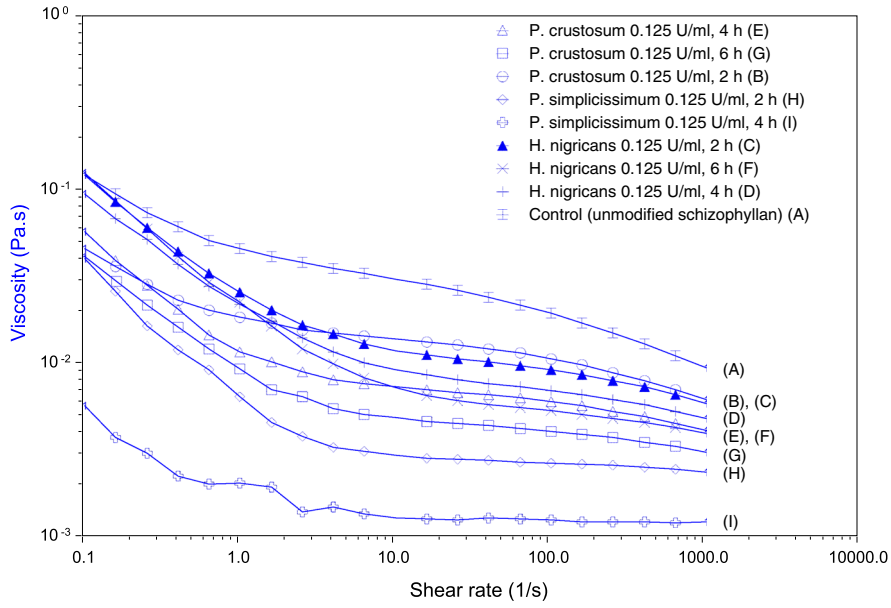

Fig. 3 Solution viscosity properties of native and modified 1 % (w/v) solutions of schizophyllan

promising for their activity and specificity against schizophyllan (Sutivisedsak et al. 2013a). In the current study, enzyme production was carried out in 500 ml shake flask cultures over a 14 day time course (Fig. 1). Under these conditions, enzyme production was optimal for all thee strains at 12 days.

Culture supernatants were concentrated ~50-fold by ultrafiltration (Table 1). Concentrated culture supernatants showed specific activities of 1.7–4.3 U β-glucanase/mg protein. By comparison, commercial sources of β-glucanase (from *Trichoderma longibrachiatum* and *Aspergillus niger*) showed less than 0.1 U/mg (Sutivisedsak et al. 2013a).

Production and characterization of enzymatically modified schizophyllan

Aqueous solutions of commercial schizophyllan (cosmetic grade) were partially degraded over 6 h using

concentrated culture supernatants containing β-glucanase at 0.125, 0.625, and 1.25 U/ml (Fig. 2). Native schizophyllan exhibited an apparent molecular weight of 6.7×10^6. Digestions producing molecular weights of approx. 10^3 or less were considered complete, representing glucose or oligosaccharides.

Partial digestions produced modified schizophyllan species from 1.7×10^4 to 5.3×10^6 Da (Fig. 2). This represents a broad range of 0.2–80 % of native schizophyllan molecular weights. β-glucanases from different species appeared to act at different rates and to different degrees of completion, even at equivalent enzyme activities. For example, enzyme from *P. simplicissimum* strain NRRL 62550 (Fig. 2c) was most active on schizophyllan, effecting complete digestion in 2 h at 0.625 U/ml. Enzyme from *P. crustosum* strain NRRL 62558 (Fig. 2b) was least active, requiring 6 h to produce complete digestion at 0.625 U/ml. Using all thee enzyme sources over a 6 h time course, it was possible to progressively modify schizophyllan to reduced molecular weight species.

Partial digestions of schizophyllan were tested for the presence of free glucose and oligosaccharides (Table 2). Glucose and oligosaccharides were found only in the more complete digestions. This confirms that the enzymes were endoglucanases rather than exoglucanases or debranching enzymes. More importantly, this means that reduced molecular weight schizophyllan species can be produced quantitatively, without loss to small molecules.

Modified schizophyllan species shown in Table 2 were also characterized by permethylation linkage analysis (data not shown). Although native schizophyllan was recalcitrant to quantitative permethylation linkage analysis, mass fragmentations showed a major peak corresponding to 2,4,6-methylglucose (representing the $1 \rightarrow 3$ linked backbone linkages), and two less abundant peaks corresponding to 2, 4-methylglucose (representing the regular $3 \rightarrow 6$ linked branches) and 2,3,4,6-methylglucose (representing the terminal branched glucose residues). These fragmentations reflect the characteristic structure of native schizophyllan. All modified schizophyllan species showed the same relative proportions of these mass fragmentations. This indicates that modified schizophyllan retains the same fundamental linkage structure of native schizophyllan, and further confirms that the enzymes used here are endoglucanases rather than debranching enzymes.

Modified schizophyllan species, listed in Table 2 that did not include free glucose or oligosaccharides, were further characterized for their solution viscosity properties (Fig. 3). Native schizophyllan exhibited characteristic pseudoplastic behavior in response to shear thining. Modified schizophyllan species showed progressively reduced viscosity profiles, all exhibiting pseudoplasticity (Fig. 3).

In conclusion, we report here for the first time progressive modification of native schizophyllan to lower molecular weight forms by partial degradation using recently described fungal β-glucanases. Modified schizophyllan was produced in a broad range of molecular weights, and could be produced without loss of material to free glucose or oligosaccharides. Modified schizophyllan appeared to retain the fundamental structure of native schizophyllan, and reduced molecular weight species exhibited progressively reduced solution viscosity profiles. Using this method, it should be possible to tailor the viscosity properties of schizophyllan for specific commercial applications.

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