Journal of Applied Phycology (2006) 18: 375–380
DOI: 10.1007/s10811-006-9043-9

Comparative characterization of laminarinases from the filamentous marine fungi *Chaetomium indicum* Corda and *Trichoderma aureviride* Rifai

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Key words: marine mycelial fungi, *Chaetomium indicum, Trichoderma aureviride,* laminarinase, O-glycosylhydrolases

Abstract

Marine filamentous fungi (103 strains) isolated from various marine habitats were studied for their ability to produce extracellular O-glycosylhydrolases. Cultural filtrates of these strains were shown to contain a series of glycanases (laminarinases, amylases, cellulases, pustulanases) and glycosidases (β-glucosidases, N-acetyl-β-glucosaminidases, β-galactosidases, α-mannosidases). Two species of marine fungi from different habitats were chosen for isolation of laminarinases and detailed study on enzyme properties. The fungus *Chaetomium indicum* associated with the alga *Fucus evanescens* C. Agardh was collected near the Kuril Islands, and *Trichoderma aureviride* was sampled from bottom deposits of South China Sea. Properties of extracellular laminarinases were similar: temperature optimums (40–45 °C), molecular masses (54–56 kDa), K_m (0.1–0.3 mg mL^{-1}). Temperature stability of laminarinase of *C. indicum* was significantly higher than those from *Trichoderma aureveride*. It is shown that these enzymes are specific to β-1,3-bonds in glucans, release predominantly glucose from laminaran and do not catalyze reaction of transglycosylation. Accoding to these data enzymes are exo-1,3-β-D-glucan-glucanohydrolases (EC 3.2.1.58). Inhibitor analysis demonstrated the significant role of tryptophan and tyrosine residues in the catalytic activity of enzymes. Molecules of *T. aureviride* laminarinase contained the functionally important thiol group.

Introduction

Laminaran, 1,3-β-linked glucan, is an important storage polysaccharide of many brown seaweeds (Phaeophyta) and is produced by these algae in large quantities. Seaweeds, including the Phaeophyta, are well known to be colonized by marine fungi. These microorganisms are capable of degrading laminaran and other carbohydrates, and may therefore have a role in the breakdown of seaweeds.

Systematic analysis of the composition and level of O-glycosylhydrolase activity of marine filamentous fungi have not previously been conducted. However, effective producers of carbohydrate metabolism enzymes are found among these microorganisms (Grant & Rhodes, 1992; Pointing et al., 1999; Lee, 2000; Burtseva et al., 2003).

The aim of our work was to study the distribution of some O-glycosylhydrolases (glycosidases and glycanases) in filamentous fungi inhabiting the marine environment, and to isolate and to characterize the properties, specificity and type of action of laminarinases from the marine facultative fungi *Chaetomium indicum* and *T. aureviride.*

Materials and methods

Fungal strains and the growth of fungi

The studied fungal strains were obtained from the Collection of Marine Microorganisms (KMM) of the Pacific Institute of Bioorganic Chemistry (PIBOC), Far

East Branch of the Russian Academy of Sciences. All these fungi were collected during marine expeditions aboard the research vessel "Akademik Oparin" near the Kuril Islands and Pociet Bay of the Sea of Japan, as well as the South China Sea.

Fungi were grown on modified Tubaki's medium, which contained (g L^{-1} sea water): non-purified sheet agar (3.0), peptone (1.0), KH_2PO_4 (1.0), yeast extract (0.5), $MgSO_4 \cdot 7H_2O$ (0.5), $FeSO_4$ (0.02), pH 7.0. The fungus *Chaetomium indicum,* inhabiting the brown alga *Fucus evanescens*, and the fungus *T. aureviride* from the bottom sediments, were used as producers of laminarinases.

Determination of activity

A standard reaction mixture contained the enzyme solution (20 μL) in succinate buffer (pH 5.2) and the substrate solution (500 μL, 1 mg mL^{-1}). Glycanases were assayed by accumulation of the reducing sugars after incubation with the corresponding polysaccharides at 37 °C for 20 min. Glycosidases were determined under the same conditions with *p*-nitrophenyl derivatives of the corresponding sugars as the substrates. The amount of the enzyme catalyzing the formation of 1 μmole of a reaction product (glucose or *p*-nitrophenol) per one minute under these conditions was taken as one unit of activity. Specific activity was defined as one unit of enzyme per one mg of protein. Activity of the enzymes in a cultural liquid was determined in a stationary phase of the growth of these fungi.

Principle analytical method

Reducing sugars were assayed by the method of Nelson (1944). Protein concentration in solution was determined by the method of Lowry et al. (1951). Liquid chromatography of sugars was performed using a JEOL-JLC-6AH automatic liquid analyser (Japan) on a Biogel P-2 column (0.9 × 90 cm) in 0.05 M sodium acetate buffer (pH 5.2) containing 0.2 M NaCl at a flow rate of 7–9 mL h^{-1}. Carbohydrates were determined with the orcinol-sulfuric acid reagent. Products of transglycosylation were determined by HPLC method using a Du Pont 8800 chromatograph with an Ultrasil-NH_2 column (10 × 25 mm). The column was eluted with acetonitrile:H_2O, 80:20 (v/v). Oligosaccharides were detected at 300 nm.

Isolation and purification of laminarinases

The following steps were used for purification of laminarinases: ultrafiltration on a membrane PM-10, PM-30, gel filtration on Biogel P-200, Sepharose CL-6B, Superdex 75 HR 10/30 columns, cation exchange chromatography on CM-cellulose, 15 *Q* PE, 15 *S* PE columns, hydrophobic chromatography on Phenyl-Sepharose 6.

Estimation of molecular mass

Molecular mass of laminarinases was estimated by gel filtration on Biogel P-200 (*C. indium*) and Sepharose CL-6B columns (*T. aureviride*).

Michaelis constants

Michaelis constants were calculated according to the Lineweaver-Burk method (Dixon & Webb, 1958).

Results and discussion

One hundred and three strains of marine fungi isolated from various marine habitats were studied for their ability to produce extracellular enzymes. Distribution of O-glycosylhydrolases in fungi of various genera is presented in Figure 1. It has been established that glycosidases are widely distributed in cultural filtrates of these fungal strains: β-glucosidases (in 47 samples), N-acetyl-β-glucosaminidases (in 36 samples), β-galactosidase (in 9 samples), α-mannosidases (in 5 samples). Among enzymes degrading polysaccharides, amylases (in 38 samples) and laminarinases (in 33 samples) are most widespread, whereas the enzymes splitting pustulan (in 6 samples) and CM-cellulose (in 4 samples) are rare. The enzymes hydrolysing agar and fucoidan were not found under the conditions described.

Two species of marine fungi from different habitats were chosen for isolation of laminarinases and detailed study of enzyme properties. *C. indicum* associated with the alga *Fucus evanescens* was collected near the Kuril Islands and *T. aureviride* was sampled from bottom deposits of the South China Sea. Composition of O-glycosylhydrolases produced by these two species was nearly identical: laminarinase, amylase, N-acetyl-β-D-glucosaminidase, β-D-gluco- and galactosidase. Cellulase in addition to these enzymes was found in cultural liquid of *C. indicum*, but pustulanase was found in cultural liquid of *T. aureviride*.