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Original Contributions

Filamentous Marine Fungi as Producers of *O*-Glycosylhydrolases: β-1,3-Glucanase from *Chaetomium indicum*

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Abstract: Ninety fungal strains (42 species) isolated from marine habitats were studied for their ability to produce extracellular enzymes. Cultural filtrates of these strains were shown to contain a series of glycosidases (β-glucosidases, *N*-acetyl-β-glucosaminidases, β-galactosidases α-mannosidases) and glucanases (1,3-β-glucanases, amylases) which varied with habitat. The level of activity depended on the species of fungi. Several promising strains capable of producing both individual enzymes and a set of enzymes for splitting carbohydrate-containing compound have been isolated. Optimal conditions for growth of *Chaetomium indicum* and for biosynthesis of β-1,3-glucanase were determined. β-1,3-Glucanase was isolated using ion-exchange chromatography, ultrafiltration, and gel filtration. The presence of 2 enzyme forms was shown; both forms were exo-β-1,3-glucanases.

Key words: marine mycelial fungi, *Chaetomium indicum,* β-1,3-glucanase, amylase, cellulase, *N*-acetyl-β-glucosaminidase.

INTRODUCTION

Glycosylhydrolases that break down plant and microbial biomass participate in carbon turnover on the Earth. The enzymatic degradation of carbohydrates is the basis of many biotechnological processes, in which filamentous fungi are widely used as sources of enzymes. Studies of enzymatic systems of filamentous fungi are of great theoretical and practical interest.

Marine fungi are less studied than other ecologic groups. Obligatory marine fungi are still the main focus of attention (Kohlmeyer and Volkmann-Kohlmeyer, 1991). However, recently interest in studying marine facultative fungi has been growing since many new metabolites have been isolated from them which have not been found in terrestrial fungi (Kobayashi and Ishibashi, 1993; Liberra and Lindequist, 1995; Toske et al., 1998). Among the metabolites of marine fungi, enzymes degrading polysaccharides are of interest (Schaumann and Weide, 1995; Pointing et al., 1998). To increase the yield of desired products, glucanases are used for destroying the cellular walls of plants and microorganisms. Using these enzymes, the structure and biological role of glucans can be established. Highly purified β-1,3-glucanase has been successfully employed for the enzyme transformation of laminaran into a biologically active glucan, translam (Zvyagintseva et al., 1995).

The purpose of our work was to study the distribution of some glycosylhydrolases (glycosidases and glucanases) in

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filamentous fungi inhabiting marine environment, to isolate and characterize β-1,3-glucanases from the marine facultative fungus *Chaetomium indicum.*

Materials and Methods

Fungous Strains and the Growth of Fungi

The studied fungous strains belong to 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 (35 strains) and in Pociet Bay of the Sea of Japan (55 strains). These strains were cultivated on medium, 1 L of which contained: 1 g of nonpurified agar, 1 g of KH_2PO_4, 0.5 g of yeast extract, 0.5 g of $MgSO_4$ $7H_2O$, 0.02 g of $FeSO_4$, pH 7.0. The fungus *Chaetomium indicum,* inhabiting the brown alga *Fucus evanescens,* was used as a producer of β-1,3-glucanase. Glucose, glycerol, laminaran, a mixture of laminarioligosaccharides, and chitosan were applied to study the dependence of this enzyme biosynthesis on a source of carbon. Activity of the enzyme in a culture liquid was determined in a stationary phase of the growth of this fungus.

Reagents

Laminaran and fucoidan were isolated from the brown alga *Laminaria cichorioides* by a previously described method (Zvyagintseva et al., 1999). 1,3;1,6-β-D-Glucan (translam) was obtained by enzymatic transformation of laminaran from *L cichorioides* (Zvyagintseva et al., 1995). β-1,3-Glucanase from *Spisula sachalinensis* was isolated by the method of Sova et al. (1970). Aubasidan from *Aureobasidium pullulan* was kindly provided by Professor I.P. Elinov (St.-Peterburg, Russia). Pustulan, lichenan, zymosan, yeast glucan, pachyman, carboxymethyl-pachyman (CM-pachyman), galactan, agarosa, chitosan, a mixture of laminarioligosaccharides, methyl ether of glycine, and halistanol sulfate were kindly provided by our colleagues from PIBOC. Standard proteins (bovine serum albumin [BSA] and myoglobin), carboxymethyl-cellulose (CM-cellulose), amylopectin, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CME-carbodiimide), diethylpyrocarbonate, *p*-chloromercuribenzoate, EDTA, *N*-bromosuccinimide, sodium azide, acetylimidazol, and chromatographic sorbents were commercial preparations.

Principal Analytic Methods

Protein concentration in solution was determined by the method of Lowry et al. (1951) and by absorption at 280 nm. Reducing sugars were assayed by the method of Nelson (1944). Liquid chromatography of sugars was performed using a JEOL-JLC-6AH automatic liquid analyzer (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 to 9 ml/h. Carbohydrates were determined with the orcinol–sulfuric acid reagent. *p*-Nitrophenyl laminarioligosaccharides (the products of transglycosylation) were determined by high-performance liquid chromatography (HPLC) 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 (vol/vol). Oligosaccharides were detected at 300 nm.

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). Glycanases were assayed by accumulation of the reducing sugars after incubation with the corresponding polysaccharides at 37°C for 20 minutes. The measurement was carried out at 750 nm. Glycosidases were determined under the same conditions with *p*-nitrophenyl derivatives of the corresponding sugars as the substrates. The measurement was carried out at 410 nm. The amount of the enzyme catalyzing the formation of 1 μmol of a reaction product (glucose or *p*-nitrophenol) per 1 minute under these conditions was taken as 1 unit of activity. Specific activity was defined as 1 unit of enzyme per 1 mg of protein.

Isolation and Purification of β-1,3-Glucanase

A filtrate of the cultural liquid from the facultative fungus *C. indicum* was concentrated by ultrafiltration on a membrane, PM-30 (Amicon, U.K.), and dialyzed at 4°C for 24 hours against 0.05 M succinate buffer (pH 5.2). This filtrate was then applied to a CM-cellulose column (2.0 × 14 cm)

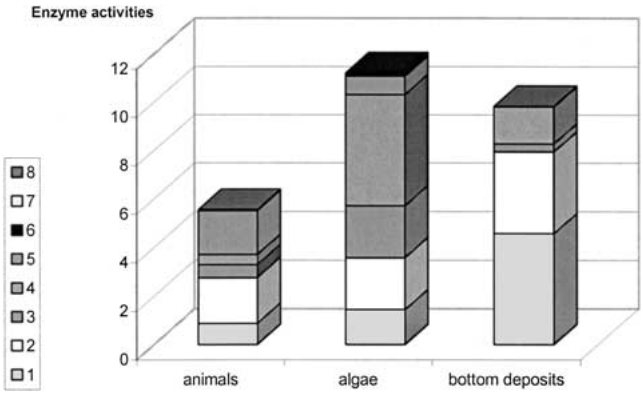

Figure 1. *O*-Glycosylhydrolases of marine fungi from various habitats: (1) β-1,3-giucanase; (2) amylase; (3) *N*-acetyl-β-glucosaminidase; (4) β-glucosidase; (5) β-galactosidase; (6) mannosidase; (7) pustulanase; (8) cellulase. Average values were taken.

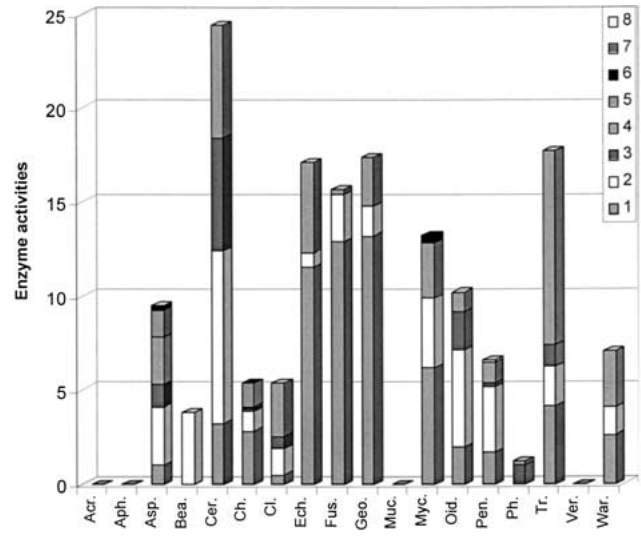

Figure 2. Distribution of *O*-glycosylhydrolases in marine fungi by genera: (1) β-1,3-glucanase; (2) amylase; (3) *N*-acetyl-β-glucosaminidase; (4) β-glucosidase; (5) β-galactosidase; (6) mannosidase; (7) pustulanase; (8) cellulase. Average values were taken.

equilibrated with 0.05 M succinate buffer (pH 5.2) and eluted with 0 to 0.5 M NaCl gradient (400 ml of each solution). The fractions showing β-1,3-glucanase activity were combined, dialyzed, and subjected to rechromatography on CM-cellulose with 0 to 0.3 M NaCl gradient. The fractions containing β-1,3-glucanase were concentrated to 3 ml by ultrafiltration and applied to a Biogel P-200 column (1.5 × 18 cm) equilibrated with 0.05 M succinate buffer, pH 5.2.

Estimation of Molecular Mass

Molecular mass of β-1,3-glucanase was estimated by gel filtration on Biogel P-200 column (1.5 × 18 cm). BSA (68 kDa), β-1,3-glucanase from *S. sachalinensis* (22 kDa) (Sova et al., 1992), and myoglobin (17.8 kDa) were used as standard proteins. Myoglobin and BSA were controlled by absorption at 280 nm; β-1,3-glucanases from *S. sachalinensis* and *C. indium* were controlled by enzymatic activities. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed in 9% to 20% gradient gel under denaturing conditions by the method of Laemmli (1970).

pH Optimum

Reaction mixture containing 20 μl of the enzyme solution, 100 μl of 0.2 M citrate-phosphate buffer (pH 3.4–7.4), and 400 μl of laminaran (1 mg/ml) was incubated at 37°C for 20 minutes. Reducing ability was determined by the method of Nelson (1944).

pH Stability

The enzyme solution (500 μl) in 0.1 M citrate-phosphate buffer at various values of pH was kept at room temperature. Aliquots (50 μl) were then taken in definite intervals (from 20 minutes to 24 hours) to determine residual activity.

Temperature Optimum

A standard mixture of the enzyme and laminaran was incubated at temperatures from 20° to 70°C for 20 minutes, and the concentration of reducing sugars was then measured.

Thermal Stability

Aliquots of the enzyme solution (50 μl) were kept at various temperatures for 10 minutes. Laminaran solution (450 μl, 1 mg/ml) was then added to these probes and incubated at 37°C for 20 minutes to determine residual activity.

Effect of Inhibitors

The reaction mixture containing 50 μl of the enzyme solution in 0.05 succinate buffer (pH 5.2) and 50 μl of an inhibitor solution was incubated at room temperature for

Table 1. Distribution of *O*-glycosylhydrolases in Filamentous Fungi

No.	Fungus	No. of samples	Enzyme activity, U/mg protein[a]							
			β-1,3-Glucanase	Amylase	Pustulanase	Cellulase	*N*-Acetyl-β-glucosaminidase	β-Glucosidase	β-Galactosidase	α-Mannosidase
1	*Acremonium harticola*	1	0	0	0	0	0	0	0	0
2	*Aphanocladium album*	1	0	0	0	0	0	0	0	0
3	*Aspergillus flavipes*	2	0–4.7	0	0	0	0.4–7.5	4.0–15.5	2.8–4.7	0
4	*Aspergillus flavus*	4	0–7.0	0–1.4	0	0	0–1.4	0–19.7	0–4.2	0–0.6
5	*Aspergillus fumigatus*	1	2.0	2.7	0.3	0	6.7	4.0	0	0
6	*Aspergillus fumigatus var. ellipticus*	1	0	0	0	0	0	0	0	0
7	*Aspergillus hallophilicus*	2	0	3.0–4.5	0	0	0–0.4	0	0	0
8	*Aspergillus niger*	2	0	0	0	0	0.1–0.2	0.7	0–0.7	0.3–0.7
9	*Aspergillus sydowii*	4	0–9.8	0–21.4	0	0	0–1.8	0–2.1	0	0
10	*Aspergillus varians*	1	0	0	0	0	5.3	8.0	5.0	0
11	*Aspergillus versicolor*	1	0	0–2.8	0	0	0–1.1	0–1.7	0	0.7
12	*Aspergillus wentii*	1	0.7	8.4	0	0	0	0.3	0	0
13	*Beauveria albae*	1	0	3.8	0	0	0	0	0	0
14	*Ceriosporiopsis cicumvestita*	1	3.2	9.2	0	0	6.0	6.0	0	0
15	*Chaetomium globossum*	3	0	0	0	0	0	0	0–7.2	0
16	*Chaetomium indicum*	1	4.1	0.7	0	0	1.0	0.5	0.5	0
17	*Chaetomium olivaceum*	2	2.8–9.9	0–5.5	0	0	0	0	0	0
18	*Cladosporium algarum*	2	0	0–6.8	0	0	0.4–2.9	1.1–20.5	0	0
19	*Cladosporium atroseptum*	1	0	0	0	0	0	0.4	0	0
20	*Cladosporium brevi-compactum*	5	0	0–7.6	0–0.06	0	0–1.5	0–10.7	0	0
21	*Cladosporium oxysporum*	1	0	0	0	0	0	0	0	0
22	*Cladosporium sphaerospermum*	6	0–7.8	0–3.1	0	0	0–3.1	0–6.1	0	0
23	*Echinobotrium atrum*	1	11.5	0.8	0	0	0	4.8	0	0
24	*Fusarium oxysporum* var. *orthoceras*	2	4.1–26.7	0	0	0	0	0–0.8	0	0
25	*Fusarium* sp.	1	7.8	7.6	0	0	0	0	0	0
26	*Geomyces pannorus*	4	6.7–19.6	0–6.3	0	0	0	0–1.1	0	0
27	*Mucor* sp.	1	0	0	0	0	0	0	0	0
28	*Myceliophthora lutea*	3	0–11.1	0–6.2	0	0	0	0–6.2	0	0–1.2
29	*Oidiodendron truncatum*	2	0–3.9	0–10.3	0	0	1.5–2.6	0.8–1.3	0	0
30	*Penicillium chrisogenum*	4	0–1.8	0–17.5	0	0–0.7	0–2.5	0–8.7	0	0
31	*Penicillium citrinum*	1	0	6.0	0	*0*	0	2.1	0	0
32	*Penicillium commune*	2	0	0	0	0	0	0	0	0
33	*Penicillium cordubense*	3	0–17.5	0	0	0	0	0–4.0	0	0

34	*Penicillium estinogenum*	1	0	0	0	0	0	0	0		0
35	*Penicillium simplicissimum*	1	0	0	0	0	0	0	0		0
36	*Penicillium verrucosum* var. *cyclopium*	2	0–3.6	0–5.5	0	0	0	0	0		0
37	*Penicillium verrucosum* var. *verrucosum*	6	0–8.0	0–23	0	0–0.8	0–1.3	0–5.2	0		0
38	*Phoma sp. (Epicoccum st.)*	1	0	0	0	0	1.0	0.2	0		0
39	*Trichoderma viride*	1	4.1	2.1	0	0	1.2	10.3	0		0
41	*Verticillium tenerum*	1	0	0	0	0	0	0	0		0
42	*Wardomyces inflatus*	2	0–5.2	0–2.9	0	0	0	0–6.0	0		0

[a]An interval of values of specific activity. For screening investigation: U = n mole/mg protein.

15 minutes. To determine activity by a standard method, laminaran solution (400 μl) was then added to the mixture.

Activity Toward Various Substrates

The enzyme solution (20 μl) was added to a substrate solution (500 μl, 1 mg/ml). This enzyme was incubated with laminaran for 20 minutes; the incubation with other substrates was continued for 3 to 24 hours. Relative hydrolysis rate was given as a percentage from the hydrolysis rate of laminaran.

Characterization of Laminaran Hydrolysis Products

The enzyme solution (0.2 ml) was added to laminaran (11.8 ml, 15 mg) and incubated at 37°C. Aliquots of 1 ml were then taken in definite intervals, and the products of the reaction were analyzed on a Jeol-JLC-6AH liquid chromatograph. The increase of concentration of reducing sugars was also controlled. The reaction was stopped by boiling.

Transglycosylation

The enzyme solution in buffer (40 μl) was added to the solution (4 ml) containing 10 mg of laminaran and 10 mg of *p*-nitrophenyl-β-D-glucopyranoside. The reaction mixture was incubated at 37°C. Aliquots of 50 μl were then taken in definite intervals and added to acetonitrile (200 μl). The probes were analysed by HPLC.

Results and Discussion

Contents of some *O*-glycosylhydrolases (glycosidases and polysaccharide hydrolases) were determined in cultural filtrates from 90 facultative marine fungous strains that belong to 42 species. It has been established that glycosidases are widely distributed in cultural filtrates of these strains: β-glucosidases (in 40 samples), *N*-acetyl-β-glucosaminidases (in 28 samples), β-galactosidase (in 8 samples), α-mannosidases (in 5 samples). Among enzymes degrading polysaccharides, amylases (in 34 samples) and β-1,3-glucanases (in 29 samples) are the most widespread, whereas the enzymes splitting pustulan (in 2 samples) and CM-cellulose (in 3 samples) are rare. The enzymes hydrolyzing agar and fucoidan were not found under the conditions described.

It has been shown that the habitat affects the enzyme composition of facultative fungi. Amylases and β-1,3-glucanases are more often found in fungal strains from bottom deposits; *N*-acetyl-β-glucosaminidases and β-glucosidases are frequently produced by fungi associated with the alga *Fucus evanescens;* β-galactosidases are common in fungi associated with marine animals (sponges, holothurians) and brown algae (Figure 1).

The distribution of *O*-glycosylhydrolases in fungi of various genera is presented in Figure 2. β-1,3-Glucanases are more frequent in fungi of the genera *Fusarium, Geomyces*, and *Echinobotrium. N*-Acetyl-β-glucosaminidases are usual in fungi from the genera *Trichoderma.* It should be noted that the genera *Acremonium*, *Aphanocladium, Mucor*, and *Verticillium*, which had no enzymes, were represented by only one strain of each (Table 1). If more strains were tested, conceivably producers of *O*-glycosylhydrolases could be found. Three β-1,3-glucanases and β-1,6-glucanase in cultural filtrate from *Acremonium persicinum* have already been isolated and investigated (Pitson et al., 1995, 1997).

Different species of the same genera also differed in their enzyme composition (Table 1). For example, various representatives of the genera *Penicillium* produced the following enzymes: β-1,3-glucanase and β-glucosidase from *P. cordubense;* amylase and β-glucosidase from *P. citrinum;* β-1,3-glucanase and amylase from *P. verrucosum* var. *cyclopium;* β-1,3-glucanase, amylase, β-glucosidase, *N*-acetyl-β-glucosaminidases, and cellulase from *P. verrucosum* var. *verrucosum. O*-Glycosylhydrolases were not found in cultural filtrates of the examined strains of *P. commune*, *P. estinogenum,* and *P. simplicissimum.* Thus our studies allowed us to reveal a series of strains promising as producers both of individual enzymes and of a set of enzymes splitting carbohydrate-containing compounds.

β-1,3-Glucanase from the facultative fungus *C. indicum,* one of the most efficient producers of this enzyme, was studied. Optimal conditions for the growth of this fungus and for biosynthesis of β-1,3-glucanase were found. In this connection, the dependence of this enzyme's biosynthesis on the carbon source used in the nutrition medium was investigated. When cultivating this fungus, the formation of β-1,3-glucanase was observed in any variation of the cultural medium (Table 2) that allowed the assignment of this enzyme as constitutive. As seen from Table 2, when laminaran at a concentration of 2 g/L was used as a carbon source, β-1,3-glucanase had maximal activity. We suggest that β-1,3-glucanase biosynthesis is subjected to catabolite repression. In these experiments, glycerol and chitosan were repressive agents.

Combining methods of ion-exchanging chromatography on CM-cellulose, ultrafiltration, and gel filtration on Biogel P-200 (Figures 3–5), a preparation of β-1,3-glucanase with specific activity of 13.5 U/1 mg of protein and yield of 1.2% was isolated without impurities of other enzymes (Table 3). Some principal properties of the enzyme were studied. Molecular mass of this β-1,3-glucanase determined by gel filtration on Biogel P-200 is about 54 kDa. PAGE showed the preparation to contain 2 proteins. Major and minor bands corresponded to molecular masses 63 and 70 kDa, respectively (Figure 6). Lower molecular masses obtained by gel filtration are characteristics of many β-1,3-glucanases (Sova et al., 1992). The β-1,3-glucanase obtained had rather high thermostability (Figure 7) and pH stability; temperature optimum was 60°C (Figure 8). This enzyme did not change its activity at room temperature for 24 hours (pH 4.5–7.5), but it was inactivated for 3 hours at pH 3–4.

A curve of the dependence of enzymatic activity on pH had 2 maxima: 4.4 and 5.6 (Figure 9). These data indicate the presence of 2 forms of β-1,3-glucanase, which have almost the same molecular masses and isoelectric points. Existence of several forms of the same enzyme is well known. For instance, 3 forms of exo-β-1,3-glucanase were isolated from cultural filtrates of a fungus, *Acremonium persicinum* (Pitson et al., 1995).

A method of inhibitory analysis was applied to clear up the role of some functional groups in the catalytic activity of β-1,3-glucanase (Table 4). *N*-Bromosuccinimide, specifically oxidizing tryptophan residues in weakly acidic medium, completely inactivated this enzyme. This fact suggests an important role of these tryptophan residues in the catalytic activity of the enzyme isolated; it is rather typical for most β-1,3-glucanases of various functional types (Svetasheva et al., 1984). Acetylimidazol, interacting with tyrosine and histidine residues, inhibited 50% of its activity. Likely, acetylimidazol specifically modified tyrosine residues (important for enzyme activity), since diethylpyrocarbonate (a specific reagent for histidine) did not decrease enzyme activity.

Using a series of glucans and glycosides with different types of bonds, this β-1,3-glucanase was established to be specific to β-1,3-bonds in glucans. It hydrolyzed relatively low molecular mass glucans (laminaran and translam) at a rather high rate; hydrolysis of a series of slightly soluble glucans with mixed type of bonds (yeast glucan, lichenan,

Table 2. Effect of Different Compounds on Biosynthesis of β-1,3-Glucanase from *C. indicum*

Compound	Concentration, g/L	Specific activity of β-1,3-glucanase
Laminaran	0.5	0.19
Laminaran	1	0.58
Laminaran	2	0.76
Laminarioligosaccharides	2	0.44
Agar	2	0.60
Chitosan	2	0.06
Glucose	2	0.41
Glycerol	2	0.03

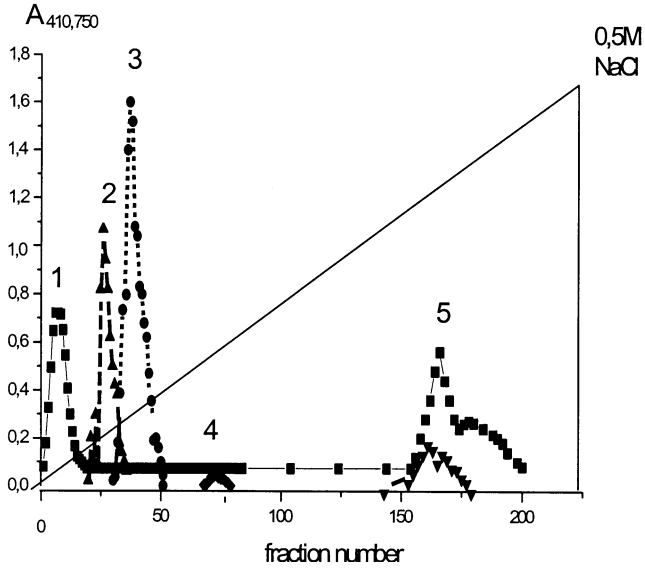

Figure 3. Ion-exchange chromatography of culture filtrate from *C. indicum* on a CM-cellulose: (1) protein; (2) amylase; (3) β-1,3-glucanase; (4) β-glucosidase; (5) CM-cellulase.

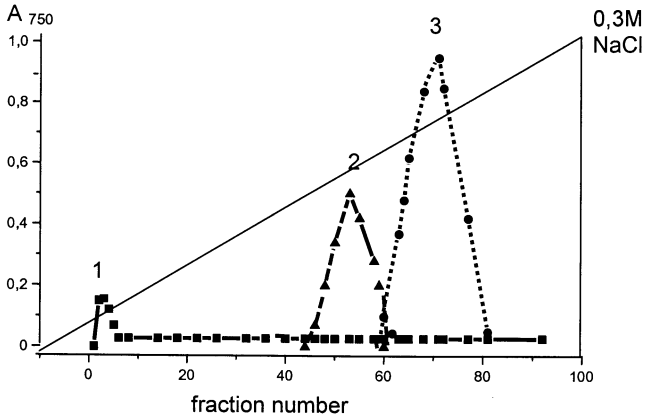

Figure 4. Rechromatography of β-1,3-glucanase from *C. indicum* on a CM-cellulose: (1) protein; (2) amylase; (3) β-1,3-glucanase.

zymosan, and some others) was significantly slower (Table 5). Glycosides were not effectively hydrolyzed.

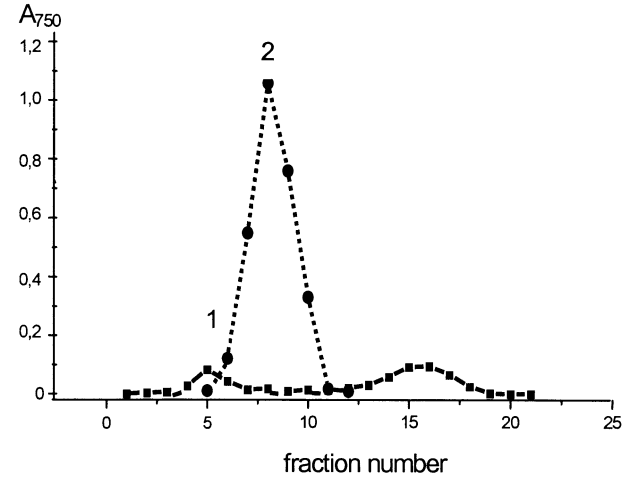

Figure 5. Gel filtration β-1,3-glucanase from *C. indicum* on a Bio-Gel P-200: (1) protein; (2) β-1,3-glucanase.

Some differences in the rates of hydrolysis of laminaran and translam depend on peculiarities of their structures (Figure 10). These glucans differ in the number and location of β-1,6-bonds, as well as in molecular mass. β-1,6-Bonded residues of glucose (10% of residues have β-1,6-bonds) are present in laminaran as branches of the main chain and distributed evenly along the chain of the glucan. Whereas about one third of β-1,6-bonded glucose residues in translam (residues have 25% of β-1,6-bonds) are included in the main chain, the rest of β-1,6-bonded glucose residues are located mainly in a nonreducing end of the molecule. Exo-type enzymes, which cleave monosaccharides or oligosaccharides from the nonreducing end of the polymer, are sensitive to the substituting groups located at this site (Sova et al., 1997).

The kinetics of hydrolysis of laminaran with β-1,3-glucanase at pH 4.4 and 5.6 was studied by the accumulation of the reaction products. The results obtained were characteristic for exoenzymes. Liquid chromatography

Table 3. Purification of β-1,3-Glucanase from *C. indicum*

Purification stage	Total amount of protein, mg	Total activity, U	Yield, %	Specific activity, U/mg protein	Degree of purification
Culture filtrate	3360	229.6	100	0.07	1
Ion-exchange chromatography on CM-cellulose	32	40.1	17.5	1.25	17.8
Rechromatography on CM-cellulose	4	27.9	15	6.97	102.5
Gel filtration on Bio-Gel P-200	0.2	2.7	1.2	13.5	159

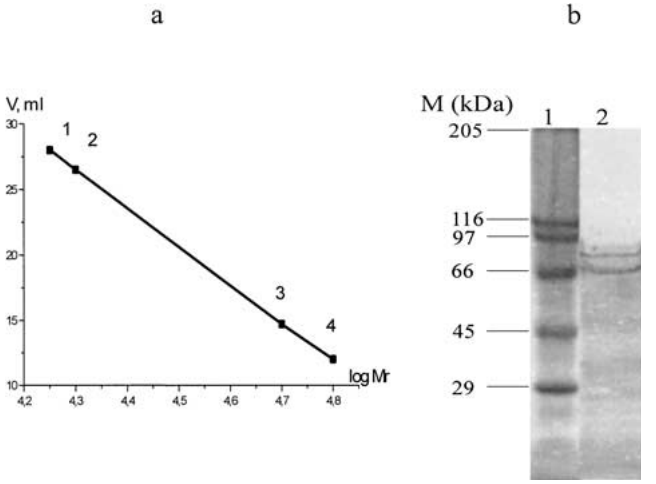

Figure 6. Determination of the molecular mass of β-1,3-glucanase from *C. indicum*. **a**: By gel filtration on a Bio-Gel P-200. (1) myoglobin, 17 kDa; (2) β-1,3-glucanase from *S. sachalinensis*, 22 kDa; (3) β-1,3-glucanase from *C. indicum;* (4) BSA, 68 kDa. **b**: By SDS/PAGE. Lane 1, molecular mass (M) markers; lane 2, β-1,3-glucanase.

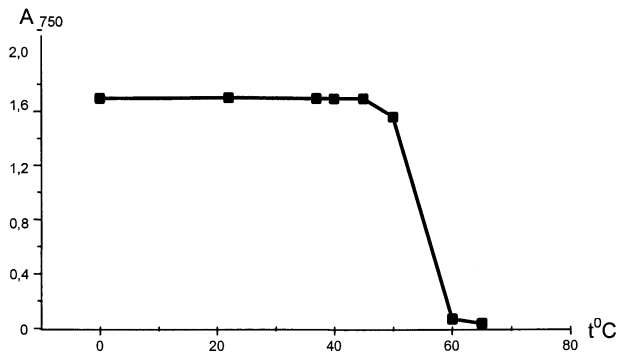

Figure 7. Thermal stability of β-1,3-glucanase from *C. indicum.*

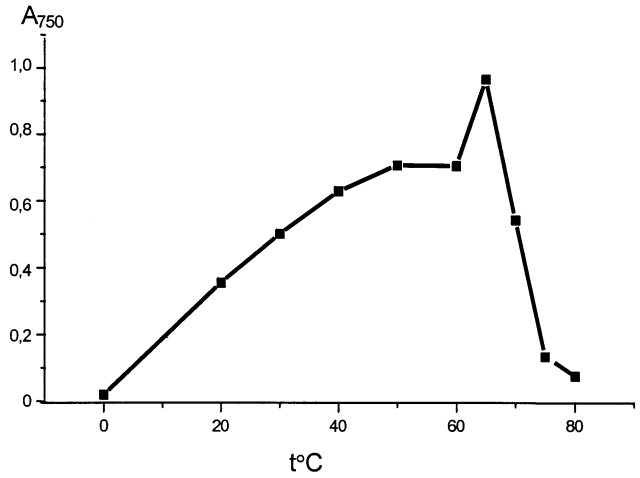

Figure 8. Temperature optimum of β-1,3-glucanase from *C. indicum*.

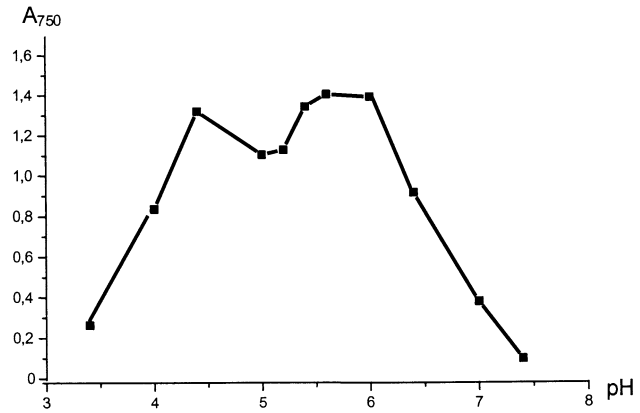

Figure 9. The pH optimum of β-1,3-glucanase from *C. indicum.*

showed glucose to be a main hydrolysis product in both cases (Figure 11). It should be noted that in the presence of exoenzyme, endoglycanase cannot be revealed by the methods described above, since in this case glucose is also the main hydrolysis product. To confirm the type of action, we attempted to determine the transglycosylation activity of β-1,3-glucanase.

Endoglycanases, cleaving internal bonds in polymer molecules, are known to catalyze both hydrolysis and transglycosylation, whereas exoglycanases catalyse hydrolysis only. To determine the transglycosylation activity of β-1,3-glucanases from *C. indicum,* a mixture of 2 substrates

Table 4. Effect of Chemical Reagents on β-1,3-Glucanase Activity

Reagent	Modified groups	Reagent concentration, M	Residual activity, %
p-Chloromercuribenzoate	-SH	5×10^{-3}	100
N-Bromosuccinimide	Trp	10^{-2}	0
EDTA	Me^{2+}	5×10^{-3}	100
Sodium azide	Me^{2+}	10^{-2}	100
Acetylimidazol	Tyr, Hys	10^{-2}	50
CME-carbodiimide	-COOH	10^{-2}	100
CME-carbodiimide and glycine methyl ester	-COOH	10^{-2}	100
Diethylpyrocarbonate	Hys	10^{-2}	94
Halistanol sulfate	—	10^{-2}	100

Table 5. Substrate Specificity of β-1,3-Glucanase from *C. indicum*

Substrate	Type of bond, ratio	Molecular mass, kDa	Solubility[a]	Relative hydrolysis rate, %
Laminaran	β-1,3; β-1,6			
	90:10	5–6	sol.	100
Translam	β-1,3; β-1,6			
	75:25	8–10	sol.	80
Yeast glucan	β-1,3; β-1,6			
	90:10	>200	s. sol	3
Pachyman	β-1,3; β-1,6			0.02
	98:2	50–120	s. sol	
CM-pachyman	β-1,3; β-1,6			0.13
	98:2	>200	v.s.	
Zymosan	β-1,3; β-1,6	>200	s. sol	0.4
Aubasidan	β-1,3; β-1,6			
	50:50	500–550	s. sol	0.08
Lichenan	β-1,3; β-1,4			1
	70:30	10–74	s. sol	
Pustulan	β = 1,6	30	s. sol	0
Amilopectin	α = 1,4	2000	sol.	0
Xylan	β = 1,4	10–20	sol.	0
CM-cellulose	β = 1,4	>2000	v.s.	0
p-Nitrophenyl-acetyl-β-D-glucosaminide	β	0.342	sol.	0.05
p-Nitrophenyl-β-D-glucopyranoside	β	0.301	sol.	0.05
p-Nitrophenyl-β-D-galactopyranoside	β	0.301	sol.	0
p-Nitrophenyl-α-D-mannopyranoside	α	0.301	sol.	0

[a]Sol. indicates soluble; s. sol., slight soluble; v.s., viscid solution.

containing a donor (laminaran) and an acceptor (*p*-nitrophenylglucoside) was used. HPLC revealed no products of transglycosylation in the reaction mixture. This result suggests both the forms of β-1,3-glucanases to be exoenzymes. Using a natural inhibitor, we also received one indirect confirmation of this suggestion: halistanol sulfate (sulfated polyoxisteroid), which usually effectively inhibits endo-β-1,3-glucanases from marine mollusks (Zvyagintseva et al., 1986), did not inhibit β-1,3-glucanases from *C. indicum*.

Thus it appeared that β-1,3-glucanase from the filamentous marine fungus *C. indicum* hydrolysed β-1,3-bonds

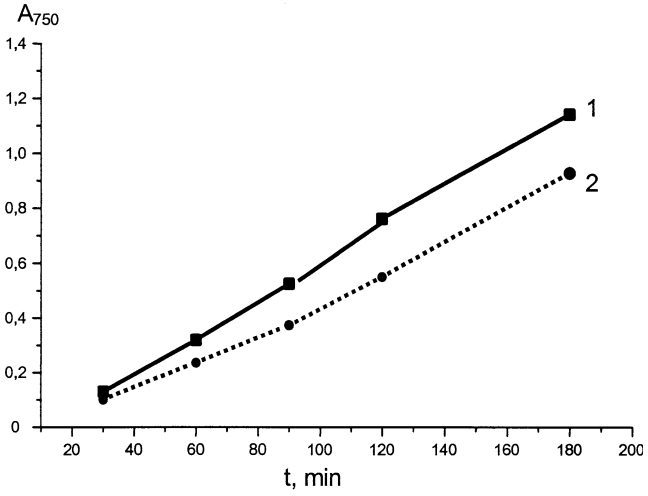

Figure 10. Hydrolysis of β-1,3;1,6-D-glucans with β-1,3-glucanase from *C. indicum*: (1) laminaran; (2) translam.

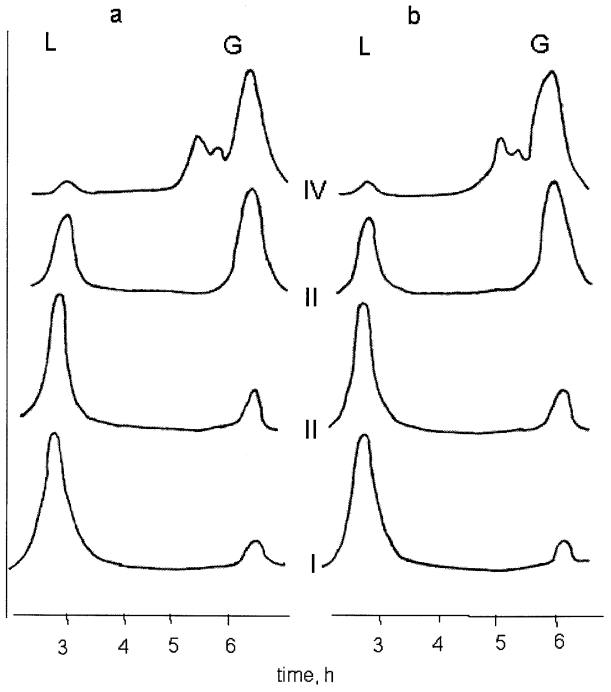

Figure 11. Gel filtration of the products of laminaran hydrolysis with β-1,3-glucanase from *C. indicum* at pH 4.4. **a**: pH 5.6. **b**: On Bio-Gel P-2 (Jeol-JLC-6AH, liquid chromatograph). L indicates laminaran; G, glucose; I–IV, incubation for 15, 30, 80 minutes and 28 hours, respectively.

in glucans and acted by an exohydrolytic mechanism. β-1,3-Glucanase from *C. indicum* is similar in its enzymatic properties to exo-β-1,3-glucanases from the filamentous fungus *A. persicinum* (Pitson et al., 1995).

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