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EXPERIMENTAL ARTICLES

The Role of Chitinase in the Antifungal Activity of *Bacillus* sp. 739

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Abstract—Investigation of the crude extracellular chitinase of *Bacillus* sp. 739, an antagonist of phytopathogenic fungi, discerned a relationship between the chitinase and antifungal activities of this bacterium. Purified chitinase lost its ability to inhibit the growth of micromycetes. The antagonistic (antifungal) activity of crude chitinase was found to be located in a low-molecular-weight fraction of the enzyme, which does not possess chitinase activity. Both crude and purified chitinase were able to lyse the cell walls of intact mycelium. Accordingly, it may be inferred that the antagonistic activity of *Bacillus* sp. 739 against micromycetes is largely determined by low-molecular-weight nonenzymatic substances, whereas the role of chitinase is to utilize chitin, which is ubiquitously present in soil.

Key words: chitinase, antagonism, antifungal activity, mycolytic enzymes, bacilli.

In recent years, there has been increasing activity in the search for microbial antagonists of fungi that are causative agents of crop diseases [1, 2]. Of great interest in this regard are microorganisms that produce mycolytic enzymes, particularly chitinase (EC 3.2.1.14). This enzyme hydrolyzes chitin, one of the major components of fungal cell walls. Some bacterial strains exhibit a direct relationship between their abilities to suppress the growth of phytopathogenic fungi and to produce chitinases [3].

Some bacilli are very promising in the biocontrol of plant diseases [1, 4, 5]. The antagonism between bacilli and micromycetes is related to the synthesis of a broad range of substances that possess antibiotic activity [6, 7]. The role of chitinases in these phenomena are unknown.

The aim of the present work was to study the role of the chitinolytic complex of *Bacillus* sp. 739 in the antagonism between this bacterium and phytopathogenic micromycetes.

MATERIALS AND METHODS

Experiments were carried out with *Bacillus* sp. 739, an antagonist of some phytopathogenic fungi and the primary component of the biocontrol agent bacispecin BM [8].

The antifungal activity of this bacterium was assayed using four test phytopathogenic fungal strains isolated by N.F. Galimzyanova, i.e., *Fusarium solani* Mart. App. et Wr., *F. oxysporum* (Schlecht.) Snyd. et Hans., *Helminthosporium sativum* Pam., King et Bakke (= *Drechlera sorokiniana* (Sacc.) Subram. et Gain. or *Bipolaris sorokiniana*), and *Alternaria alternata* (Fr.) Keissl., as well as the collection strain *Fusarium culmorum* (W. G. Smith) Sacc. VIZR-227.

Bacillus sp. 739 was maintained on a medium containing (g/l) colloidal chitin, 5.0; peptone, 3.0; corn extract, 1.0; $(NH_4)_2HPO_4$, 1.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; and agar, 16.0. To isolate extracellular chitinase, the bacterium was grown in a liquid medium of the same composition except that agar was omitted. The cultivation was carried out at 37°C for 72 h in 250-ml flasks on a UBMT-12-250 shaker (Russia) at 160 rpm. To obtain the culture liquid (CL) of *Bacillus* sp. 739 which had trace chitinase activity, the bacterium was grown in a liquid medium containing (g/l) potato starch, 10.0; yeast extract, 3.0; peptone, 3.0; corn extract, 3.0; $(NH_4)_2HPO_4$, 2.0; and KH_2PO_4, 2.0.

Fungal strains were maintained and grown at 28°C using Czapek medium, either liquid or agar-solidified.

Crude chitinase was prepared from the CL supernatant by salting-out with ammonium sulfate (70% saturation) and dialyzing the resultant preparation against 50 mM phosphate–citrate buffer (pH 6.0). The complete desalting of the ammonium sulfate–precipitated fraction (ASPF) was achieved by passing it through a (2.5×40 cm) column with Sephadex G-10 (Pharmacia, Sweden). Chitinase was then further purified by batch affinity sorption on colloidal chitin. For this purpose, an enzyme solution was mixed with 0.5% (dry weight) colloidal chitin and the mixture was incubated at room temperature for 1 h under continuous stirring. The sorbent was separated from the liquid phase by centrifugation and washed twice with an equal volume of cold 25 mM phosphate–citrate buffer (pH 6.0) to remove non-

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Table 1. Purification of the chitinase of *Bacillus* sp. 739

Step	Volume, ml	Chitinase		Protein, mg	Purification factor	Yield, %
		units	units/mg protein			
CL	500	51.000	0.02	2970	1	100
Ammonium sulfate (70% saturation)	10	33.86	0.05	645.6	3	66
Sephadex G-100;	42	22.18	0.12	185.1	7	44
Sorption on chitin (preparation I)	7	12.24	0.54	22.67	32	24
Biogel P-100 (preparation II)	37	8.18	0.96	8.51	56	16

Table 2. The antagonistic activity of various chitinase preparations and their fractions isolated from the culture liquid of *Bacillus* sp. 739*

Preparation	Chitinase activity, units	Growth inhibition zone diameter, mm				
		F. solani	*F. oxysporum*	*F. culmorum*	*H. sativum*	*A. alternata*
ASPF I (growth medium with chitin)	~0.17	7	8	8	6	6
ASPF I (100°C)	0	0	0	0	0	0
ASPF II (growth medium with starch)	<0.01	3	2	0	2	1
Preparation I	~0.09	22	17	14	24	14
ASPF I fraction nonadsorbed on chitin	~0.01	0	0	0	0	0
Preparation II	~0.14	0	0	0	0	0
Low-molecular-weight fraction of preparation I (see Fig. 1)	0	20	14	14	21	10

* The diameters of growth inhibition zones were measured on the 3rd day of the test fungi incubation on agar media at 28°C.

adsorbed proteins. Chitin was resuspended in the same buffer in a volume comprising 20% that of the original enzyme solution. The suspension was incubated at 37°C for 24 h. The nonhydrolyzed fraction of this suspension was removed by centrifugation, and the supernatant (preparation I) was purified by gel filtration on Biogel P-100 (Bio-Rad, United States). The resultant chitinase (preparation II), which was free of most low-molecular-weight impurities, was lyophilized in an Inei-26 freeze drier. The results of the chitinase isolation are shown in Table 1.

The total chitinase activity was determined as follows: One milliliter of the enzyme solution in 50 mM phosphate–citrate buffer (pH 6.0) was mixed with 0.5 ml of a 1% suspension of colloidal chitin in the same buffer. The mixture was incubated at 50°C for 1 h, and the concentration of reducing sugars in the supernatant was determined by the ferricyanide method [9]. One unit of chitinase activity was defined as the amount of enzyme which catalyzes the formation of 1 μmol *N*-acetyl-*D*-glucosamine in 1 ml of the reaction mixture per minute.

The antagonistic activity of the enzyme preparations was assayed at 28°C by the agar wells method. The diameter of the growth inhibition zones in a lawn of the test fungi was measured on the 3rd and 7th day of incubation. Enzyme solutions for this series of experiments were sterilized by filtration through 0.45-μm-pore-size filters.

The mycolytic activity of chitinase was assayed in the following way: The washed mycelium of the test fungi was suspended in a mixture (1 : 1) of 0.9% NaCl and 0.05 M Na phosphate buffer (pH 6.0). One milliliter of this suspension was mixed with 0.05 ml of the enzyme preparations and incubated at 28°C for 24 h. Structural alterations in the mycelium were examined microscopically under a magnification of 400×. Alternatively, the mycolytic activity of chitinase was measured by the formation of reducing sugars. To this end, an enzyme solution containing ca. 0.5 units/ml chitinase in 50 mM phosphate–citrate buffer (pH 6.0) was supplemented with 1% (w/v) air-dry mycelium and incubated at 20°C for 8 h or at 37°C for 3 h, depending on the aim of the experiment. The concentration of the reducing sugars produced was determined by the ferricyanide method [9]. The remaining mycelium was washed, dried at 105°C to complete desiccation, and weighed to determine the mycelium loss as a percent of the dry weight of mycelium in the control sample.

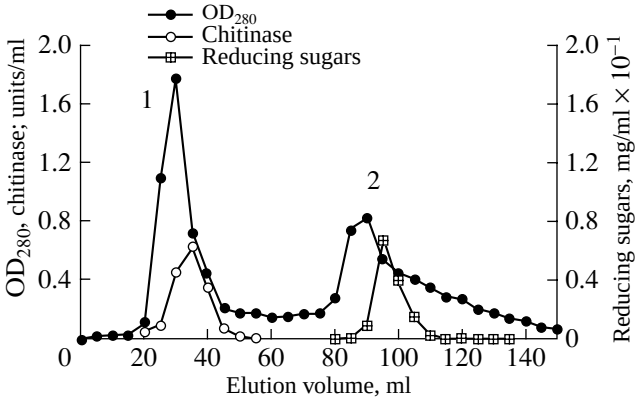

Fig. 1. Gel filtration of preparation I (fraction with affinity for chitin) on a (2.5 × 30 cm) column with Biogel P-100. Elution with 25 mM Tris–HCl (pH 7.1) at a flow rate of 0.5 ml/(cm^2 min). The fraction volume is 5 ml. Peak 1 corresponds to chitinase and other high-molecular-weight substances. Peak 2 corresponds to the products of chitin hydrolysis and other low-molecular-weight substances.

All experiments were performed in triplicate. The results were processed in terms of Student's *t*-test statistics at a 5% level of significance using Origin 5.0 software.

RESULTS AND DISCUSSION

A comparison of the antifungal activities of the ammonium sulfate–precipitated fractions of the culture liquid of *Bacillus* sp. 739 grown in the cultivation medium containing either colloidal chitin or starch showed that they were considerably different (Table 2). The enzyme preparation that had a high chitinolytic activity efficiently inhibited the growth of the test micromycetes. The heating of this preparation at 100°C for 5 min completely destroyed its antifungal activity.

It should be noted that this observation does not prove that the antifungal activity of crude chitinase is related to this enzyme, since it is known that many nonprotein antibiotics are inactivated at temperatures exceeding 60°C [6, 7]. Indeed, further studies showed that after the gel filtration of crude chitinase on Biogel P-100, the chitinase fraction (preparation II) lost the ability to inhibit the growth of the test fungi, whereas the fraction containing low-molecular-weight substances, including the products of the enzymatic hydrolysis of colloidal chitin (Fig. 1), possessed antifungal activity. Some oligosaccharides produced from chitin are known to have fungicidal activity [10]. Therefore, their presence in the crude chitinase may be responsible for the antagonistic activity. Alternatively, the antifungal activity of the low-molecular-weight fraction may be due to some antibiotic substances capable of reversible adsorption on colloidal chitin. This suggestion is confirmed by the absence of antifungal activity in the fraction that does not adsorb on chitin (Table 2). Visual examination of the germination of *H. sativum* spores in the growth inhibition zones of Czapek agar suggested that the mechanisms of germination impairment by chitinase preparation I and its low-molecular-weight fraction are similar (Fig. 2). Therefore, the absence of antifungal activity in pure chitinase (preparation II) may indicate that the antifungal activity of *Bacillus* sp. 739 is largely due to nonenzymatic low-molecular-weight substances.

Table 3. Changes in the dry weight of the mycelia of the test phytopathogenic fungi incubated with the crude chitinase of *Bacillus* sp. 739

Test fungi	Dry weight of mycelium, %			
	control		experiment	
	mg	%	mg	%
Fusarium oxysporum	125	100	92	74
F. culmorum VIZR-227	125	100	90	74
Helminthosporium sativum	125	100	79	63

In spite of the fact that the chitinase preparation, which lacks the low-molecular-weight substances, does not possess antifungal activity, such chitinase preparation exhibited notable mycolytic activity, especially toward the *Fusarium* and *Helminthosporium* fungi. The micrographs presented in Fig. 3 show that both crude chitinase (preparation I) and pure chitinase (preparation II) caused the swelling of fungal mycelium and the formation of vacuoles. This was sometimes accompanied by the degradation of hyphal cell walls and the release of intracellular components into the medium.

The involvement of the chitinolytic complex of *Bacillus* sp. 739 in the observed alterations in the structure of fungal mycelia was confirmed by experimental data on the accumulation of reducing sugars in the reaction mixture (Fig. 4). When the incubation temperature was increased from 20 to 37°C, the accumulation rate of reducing sugars also increased, indicating the enzymatic origin of the process of cell wall destruction (Fig. 4b). These data agree well with the data on mycelium weight loss after treating the mycelium with chitinase (Table 3).

The weak, if any, antifungal activity of the *Bacillus* sp. 739 chitinase confirms the earlier observations of Roberts and Selitrennikoff that the antifungal activity of bacterial chitinases is significantly lower than that of plant chitinases [11]. These authors relate this phenomenon to the fact that bacterial chitinases are predominantly of the exo-type, which have from one to two orders of magnitude lower antifungal activity than endo-chitinases. Along with this, the antifungal activity of chitinolytic complexes may depend on other compo-

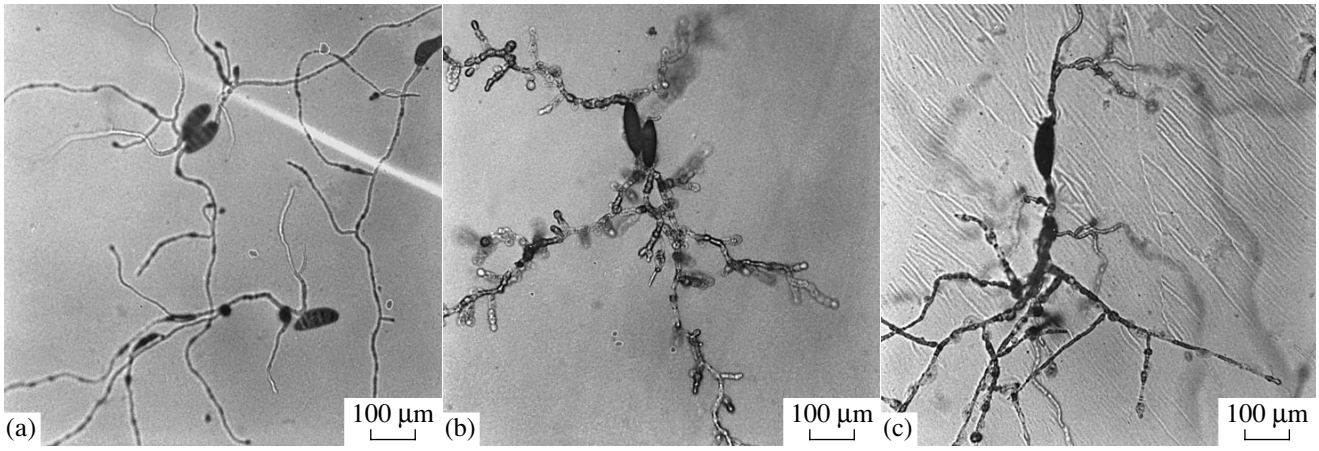

Fig. 2. Germination of *Helminthosporium sativum* spores on Czapek agar at 28°C in the diffusion zones of chitinase and of the low-molecular-weight fraction. Light microscopy at a magnification of 100×. (a) Control; (b) preparation I (for explanation, see the text); and (c) the low-molecular-weight fraction of preparation II (peak 2 from Biogel P-100).

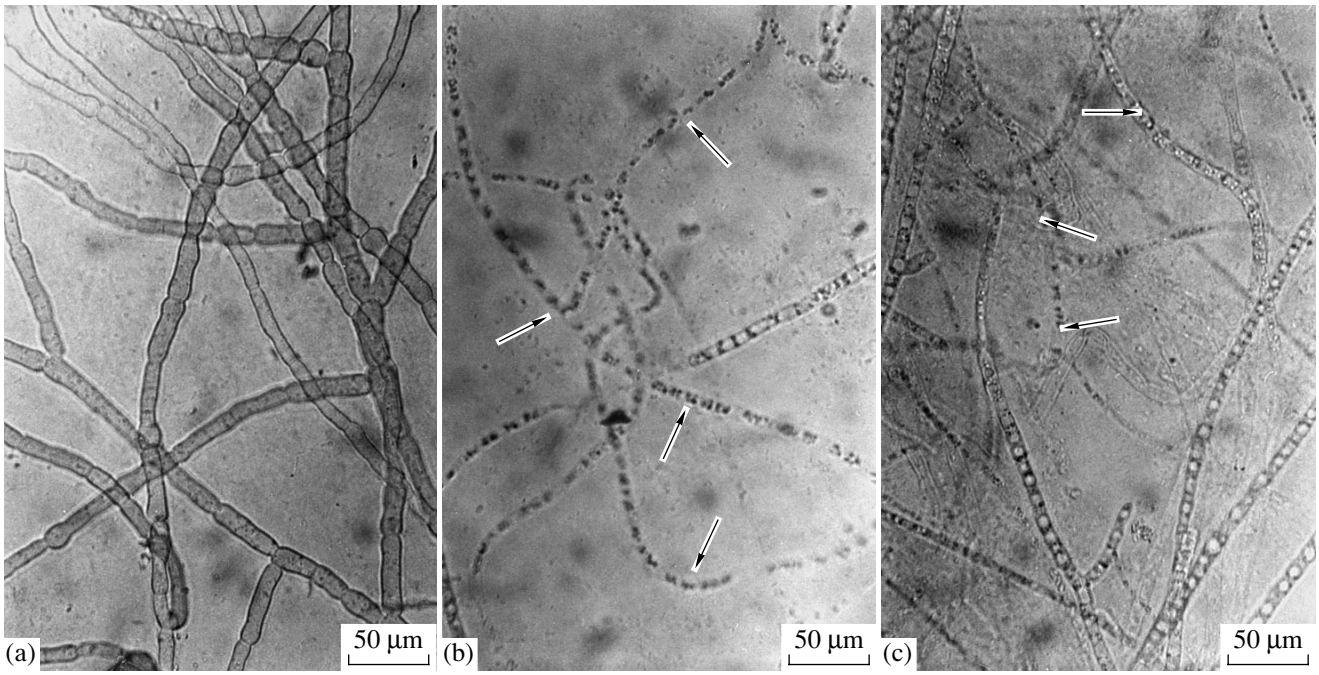

Fig. 3. The lytic action of *Bacillus* sp. 739 chitinase on the mycelium of *Helminthosporium sativum.* Light microscopy at a magnification of 400×. (a) Control; (b) preparation I; and (c) preparation II. The arrows point to the regions of mycelium destruction and vacuolization.

nents than their chitinases [12]. For instance, Lorito *et al.* [13] described the synergistic action of the endochitinase and chitobiosidase of *Trichoderma harzianum* P1. It should be noted in this regard that many bacillar chitinolytic complexes are dominated by chitobiosidases [14–16]. When considering bacillar chitinases as biocontrol agents against phytopathogenic fungi, some researchers do not clearly understand to what extent the antifungal activity of an enzyme preparation is due to its own activity [15].

To conclude, the antagonism between *Bacillus* sp. 739 and phytopathogenic fungi is associated with nonenzymatic low-molecular-weight substances. Unlike that of *Serratia marcescens* [12], the chitinase complex of *Bacillus* sp. 739 exhibits a high lytic activity towards mature micromycetous mycelia but is almost inactive towards young, actively growing mycelia. It is possible that the ecological significance of the chitinolytic complexes of most soil bacilli differs from that of mycoparasitic and entomopathogenic organisms and higher plants. Their role may lie in providing soil bacilli with

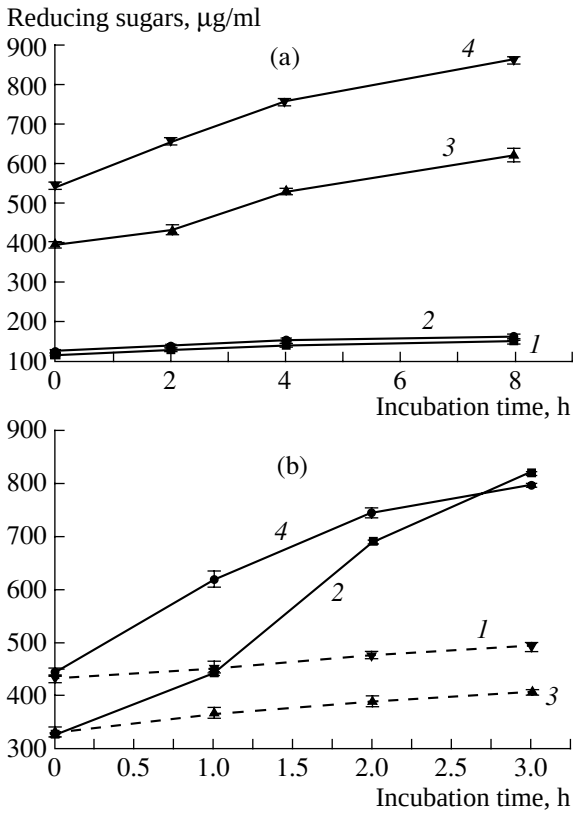

Fig. 4. The formation of reducing sugars from fungal mycelia under the action of the crude chitinase of *Bacillus* sp. 739. (a) Incubation temperature 20°C: *1* and *2*, *Fusarium culmorum* VIZR-227 (control and experiment, respectively); *3*, and *4*, *Helminthosporium sativum* (control and experiment). (b) Incubation temperature 37°C: *1* and *2*, *Fusarium solani* (control and experiment); *3* and *4*, *Alternaria alternata* (control and experiment).

easily metabolizable sources of carbon and nitrogen resulting from the enzymatic hydrolysis of chitin. This suggestion is confirmed by the ability of the chitinase of *Bacillus* sp. 739 to degrade only mature or dead fungal mycelium, which is always present in soils.

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