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Purification and Characterization of Antifungal Chitinase from *Pseudomonas* sp. YHS-A2

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A strain producing a high amount of chitinase was isolated from soil, identified as *Pseudomonas* sp., and tentatively named *Pseudomonas* sp. YHS-A2. An extracellular chitinase of *Pseudomonas* sp. YHS-A2 was purified according to the procedure of ammonium sulfate saturation, affinity adsorption, Sephadex G-100 gel filtration and Phenyl-sepharose CL-4B hydrophobic interaction column chromatography. The molecular weight of the purified enzyme was estimated to be 55 kDa on SDS-PAGE was confirmed by active staining. Optimal pH and temperature of the enzyme are pH 7.0 and 50°C, respectively, and the enzyme is stable between pH 5.0 and 8.0 and below 50°C. The main products of colloidal chitin by the chitinase were N-acetyl-D-glucosamine and N,N'-diacetylchitobiose both of which were detected by HPLC analysis. The enzyme is supposed to be a random-type endochitinase which can degrade any position of β-1,4-linkages of chitin and chitooligosaccharides. The chitinase inhibited the growth of some phytopathogenic fungi, *Fusarium oxysporum*, *Botrytis cineria*, and *Mucor rouxii* and these antifungal effects were thought to be due to the characteristics of endochitinase.

Chitin is the second most abundant biomass in nature and the polymer of N-acetyl-D-glucosamine. Recently, chitin and its derivatives have been used in many fields, for example, in food, pharmaceutical, agricultural, and the environmental industry. However, if chitin is treated by concentrated acid and alkali, the byproducts lead to hazards and environmental pollution.

Chitinase (E.C.3.2.1.14), which hydolyzes the β-1,4-linkages of N-acetyl-D-glucosamine polymers of chitin, is being studied because of its potential use in various biotechnological fields, and many chitinases and their genes have been reported from bacteria (10, 25, 31), fungi (3, 33), plants (5, 12, 22, 32), and even from human (11, 24). Especially plant chitinases, which are induced by phytopathogenic fungi with chitin in their cell wall, are supposed to be antifungal agents. Many plants excrete chitinases even if plants do not have chitin in their cell wall, and some, but not all, inhibit the growth of fungi (14, 15, 23). Since chitin is one of the major components of fungal cell wall, it is believed that plant chitinases play an important role in the self defense mechanism of the plants (1).

There are some differences in chitinases produced by microorganisms. Yeast and fungal chitinases play a role in cell division (17, 18). On the other hand, bacterial chitinases are used for the uptake of nutrients because chitin is a good carbon and nitrogen source (8). Like plant chitinases, some chitinases produced by microorganisms, have antifungal activity but in many cases, microorganism-produced chitinases inhibit fungal growth much less than plant chitinases (6, 27). In addition, most of the bacterial chitinases do not have antifungal characteristics because their chitinases are mainly exo-type chitinases (26). Nevertheless, since bacterial chitinases and their genes are easier to manipulate and regulate, they are potential antifungal agents. So, as described in this paper, we isolated and identified a novel bacterium which produces extracellular chitinase with high antifungal activity, and we purified its chitinase and investigated some characteristics of the enzyme.

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Key words: chitinase, antifungal, *Pseudomonas*, endochitinase, phytopathogenic fungi

MATERIALS AND METHODS

Materials and Medium

Chitin powder extracted from crab shell, N-acetyl-D-glucosamine (GlcNAc), chitobiose (N,N'-diacetylchitobiose), chitotriose (N,N',N''-triacetylchitotriose), chitotetraose (N,N',N'',N'''-tetraacetylchitotetraose), *p*-nitrophenyl-N-acetylglucosaminide, and *p*-nitrophenyl-N,N'-diacetylchitobiose were purchased from Sigma Chemical Co. and colloidal chitin

and glycol chitin were prepared by the modifications of the methods of Lee (20) and Molano (21), respectively. All the other chemicals were of special grades. Two media were used in our experiments; isolating medium for screening of chitinase producing bacteria and activation medium for seed culture. The composition of the isolating medium and activation medium was 6 g/l colloidal chitin, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.3 g/l KH_2PO_4, 0.7 g/l K_2HPO_4, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 0.001 g/l $ZnSO_4$, 0.001 g/l $MnCl_2$, and 10 g/l nutrient broth (Difco), 5 g/l glucose, respectively. For fatty acids analysis, the selected strain was cultivated on Tryptic Soy broth agar (TSBA, Difco) medium.

Isolation and Identification of Chitinase-producing Bacterium

The soil samples obtained from the west coast of Korea were incubated on the solid isolation medium containing 1.8% (w/v) of agar powder and we selected bacterium showing a clear zone. The supernatant of the selected strains was used for chitinase assay using the dinitrosalicilic acid (DNS) method (20). The strain producing chitinase activity was identified by Gram staining diagnostic kit (Sigma) and Vitek® gram negative index (GNI) kit analysis. Major fatty acid composition was analysed by gas liquid chromatography (GLC) with a fused silicultivated capiliiary column (25 m × 0.2 mm). The bacteria were cultivated on TSBA at 30°C for 24 h and then 50 mg of cells were harvested. The cells were treated by NaOH and HCl in aqueous methanol, and extracted with hexane/ether. The analysis was carried out under 170-300°C temperature conditions with nitrogen as a carrrier gas. The result was analyzed according to a microbial identification system (MIS, Hewlett Packard 5890A).

Purification of Chitinase Produced by *Pseudomonas* YHS-A2

Unless otherwise stated, the following steps were done at 4°C.

Step 1. Preparation of crude enzyme solution. The selected strain was cultured and centrifuged at 12,000× g for 15 min. In the supernatant of 3.0 liters, finely ground solid ammonium sulfate was slowly added to give 60% saturation, and incubated with continuous stirring. After incubation for 12 h, the precipitate was collected by centrifugation (20,000× g, 25 min), dissolved in 50 ml of 20 mM potassium phosphate buffer (pH 7.0) and then dialysis was carried out.

Step 2. Affinity adsorption. To the crude enzyme solution, the same volume of 1% (w/v) colloidal chitin solution was added and incubated for 1 h in ice. This mixture was centrifuged (12,000× g, 10 min) and supernatant was discarded. After adding an equal volume of the same buffer to the precipitates and suspending it completely, the mixture was incubated at 37°C for 3 h. It was centrifuged (12,000× g, 10 min) and supernatant was obtained and freeze-dried.

Step 3. Gel filtration on Sephadex G-100. The concentrated sample (3 ml) was applied to a Sephadex G-100 column (1.8× 90 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0. The active fractions were obtained and concentrated by ultrafiltration (Amicon Co.).

Step 4. Chromatography on Phenyl-sepharose CL-4B. For further purification, the concentrated active fraction was applied onto a Phenyl-sepharose CL-4B column (2.6× 26 cm) equilibrated with 10 mM sodium phosphate buffer containing 25% ammonium sulfate, and eluted gradiently to 0% ammonium sulfate. The active fraction was collected and ultrafiltrated to concentrate.

SDS-PAGE and Active Staining Using Glycol Chitin

Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) was carried out with a Modular Mini-Protein II Electrophoresis System (BIO-RAD, U.S.A.) and active staining was done as described in Asselin (29). 12% (w/v) polyacrylamide gel containing 0.01% (v/v) glycol chitin and 0.1% (w/v) SDS was used for electrophoresis. After electrophoresis, gel was incubated at 37°C for 24 h in 0.1 M sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 to remove SDS, washed with distilled water sufficiently, and incubated for a further 12 h at 37°C to react chitinase with the glycol chitin. Then gel was stained with 0.01% (w/v) Calcoflour white M2R (Sigma, U.S.A.) in 0.5 M Tris-HCl (pH 8.9) buffer for 2 h and destained with distilled water for 2 h at room temperature. The active band appeared to be dark under the UV transilluminator.

Effects of pH and Temperature on Activity and Stability of the Purified Chitinase

For investigation of optimum pH for enzyme reaction and pH stability, the purified enzyme was treated with buffers with various pH at 40°C for 30 min. The buffers were adjusted from pH 3 to 10 using citrate buffer (from pH 3 to 6), potassium phosphate buffer (from pH 6 to 8), and boric-NaOH (from pH 8 to 10). The effects of reaction temperatures and heat stability between 0 and 80°C were investigated.

Analysis of Products Reacted with Chitinase

Prepared colloidal chitin was reacted with purified chitinase for 3 h at 45°C, centrifugated (12,000× g, 10 min) to remove unreacted chitin, and filtrated to remove enzymes. HPLC was performed with a carbohydrate HPLC column (3.9× 300 mm, Waters) using 80% acetonitrile, and the products were detected at 210 nm.

Antifungal Activity of Chitinase

Some phytopathogenic fungi, *Mucor rouxii*, *Phycomyces nitens*, *Fusarium oxysporum*, and *Botrytis cineria*, were used to assess the antifungal effect of the purified chitinase. On the potato dextrose agar medium, soft agar containing fungal spores were poured and the purified chitinase loaded paper discs were put on the surface of the medium. After

incubation at 24°C for 3 days, the diameter of clear zone resulting from cell wall lysis was measured.

RESULTS AND DISCUSSION

Isolation and Identification of Chitinase-producing Bacteria

Hundreds of bacteria producing chitinase were isolated and a bacterium which had the highest chitinase activity among them was selected. This strain was a gram negative, rod type bacteria and was shown to have 93% homology with *Pseudomonas aeruginosa* by Vitek® Gram Negative Index (GNI) kit anaysis (Table 1). However, the result of GLC analysis was different. The major fatty acids were 16 : 1 (47.8%), 16 : 0 (36.1%), 18 : 1 (9.6%), and 12 : 0 (4.8%) as shown in Fig. 1. According to IMS, this strain was similar to the human- and animal-pathogenic *Pseudomonas* RNA and showed similarity with groupIII (7) and *Janthinobacterium lividum*, a well-known purple pigmented bacterium. However, this strain did not produce purple pigment or esculin hydrolysis. Therefore, the strain used in this experiments was named tentatively as *Pseudomonas* sp. YHS-A2.

Table 1. Morphological and biochemical characteristics of *Pseudomonas* sp. YHS-A2.

Characteristics	Result
Mortality	+
Gram stain	-
Shape	rod
Colony shape	round and smooth
Colony color	white fluorescent
Lysine decarboxylase	-
Ornithine decarboxylase	-
Arginine decarboxylase	+
Malonate	+
Tryptophan deaminase	-
DP 300	-
p-Coumaric	-
Acetamide	+
Esculin hydrolysis	-
Glucose oxidation	+
Lactose oxidation	-
Maltose oxidation	+
Mannitol oxidation	+
Polymyxin B	+
β-Galactosidase (ONPG)	-
H_2S	-
Raffinose fermentation	-
Sorbitol fermentation	-
Inositol fermentation	-
Adonitol fermentation	-
L-Arabinose fermentation	-
Glucose fermentation	-
Urease	-
Citrate	+

Purification of Chitinase and Its Molecular Weight

An extracellular chitinase produced by *Pseudomonas* sp. YHS-A2 was purified by ammonium sulfate saturation, affinity adsorption, Sephadex G-100 gel filtration (Fig. 2A) and Phenyl-sepharose CL-4B hydrophobic interaction column chromatography (Fig. 2B) and the results of the purification are summarized in Table 2. The enzyme was purified to 11.0 fold compared to culture broth and overall recovery yield was 21.9%. The analysis of SDS-PAGE and active staining showed a single band which was estimated to be about 55 kDa (Fig. 3).

Characteristics of the Enzyme

The chitinase activity was measured at various pH's ranging from 3.0 to 10.0. The optimum pH was found to be 7.0 as shown in Fig. 4A. pH stability was also determined after 30 min of incubation. The chitinase was stable between pH 5.0 and 8.0. When enzyme activity was measured between 0 and 80°C for 1 h, the highest activity was recorded at 50°C (Fig. 4B) Heat stability at 20, 30, 40, 50°C was detected until 6hr and the results showed that the enzyme became unstable at 50°C (Fig. 4C).

Product Analysis of the Chitinase

It is very important to clarify what the main product of the chitinase reaction is because there is a significant difference between the two different types of chitinases;

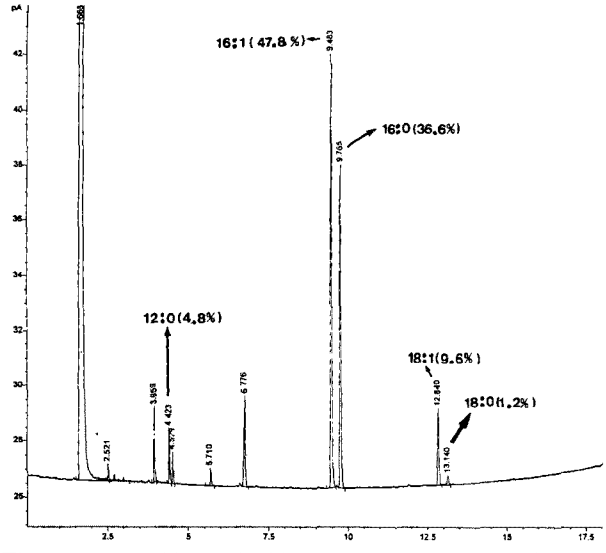

Fig. 1. Fatty acid gas-liquid chromatogram of *Pesudomonas* sp. YHS-A2.

The strain was cultivated on TSBA at 30°C for 24 h and then 50 mg cells were harvested, treated by NaOH in aqueous methanol 100°C for 20 min, and extracted with hexane/ether. The GLC analysis was carried out in below conditions; a fused silicultivated capiliiary column (25 cm ×0.2 mm), column temperature (170-270°C), injector temperature (250°C), detector temperature (300°C), and carrier gas was nitrogen. The result was analyzed according to a microbial identification system (MIS; Hewlett Packard 5890A). The strain cultivated on TSBA at 30°C.

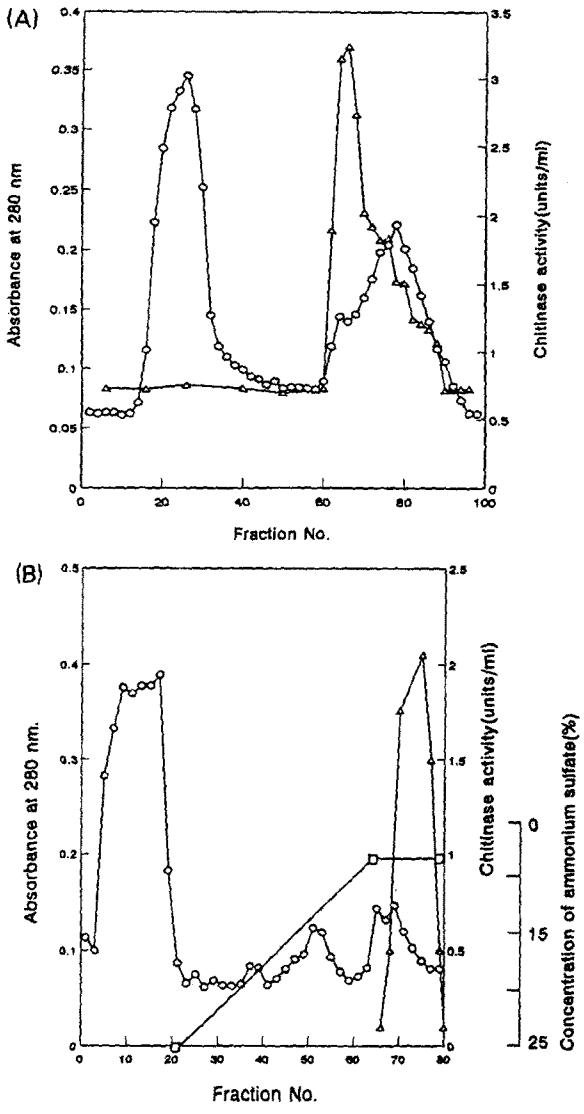

Fig. 2. Column chromatography of chitinase on Sephadex G-100 (A) and Phenyl-sepharose CL-4B (B).

Table 2. Summary of purification procedures of the chitinase.

Purification step	Total protein (mg)	Total activity (units)	Sp. activity (unit/mg)	Yield (%)	Fold
Culture broth	1,004.0	9,173.0	9.0	100.0	–
Ammonium sulfate precipitation	328.3	8,012.1	14.4	87.3	2.7
Affinity adsorption	98.3	5,835.2	59.4	63.6	6.6
Sephadex G-100	50.2	4,015.1	80.0	43.8	8.9
Phenyl-sepharose CL-4B	20.2	2,007.0	99.4	21.9	11.0

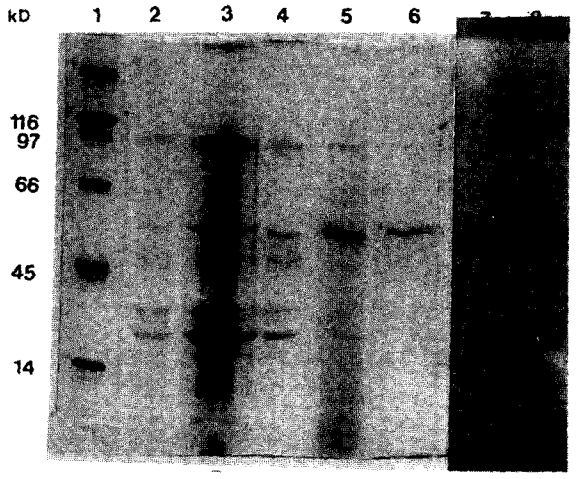

Fig. 3. SDS-PAGE (lanes 1-6) and active staining (lanes 7, 8) of chitinase in each purification steps.

Lane 1, molecular weight marker; lane 2, culture broth; lane 3, after 60% ammonium sulfate precipitation; lane 4, after affinity adsorption; lane 5, after Sephadex G-100 chromatography; lane 6, after Phenyl-sepharose CL-4B; lane 7, after 60% ammonium sulfate precipitation (crude enzyme); lane 8, after Phenyl-sepharose CL-4B (purified enzyme).

exochitinase and endochitinase, but until now, it remains as a very confused concept because no one method of determining whether a chitinase is endo- or exo-type has achieved broad acceptance (9, 30). According to the nomenclature of Tronsmo and Harman (28), there are two exochitinases. When whole products of chitinase are N-acetyl-D-glucosamines, the enzyme is N-acetyl-D-glucosaminidase (exochitinase) and another exochitinase is 1,4-β-chitobiosidase (chitobiosidase) that excises chitin polymer to produce only N,N'-diacetylchitobiose (chitobiose). However, exochitinases have been reported to have much less antifungal activity (13) and many plant chitinases which inhibit fungal growth were endochitinases (19). Since most of the bacterial chitinases are used for the uptake of nutrients, they have different functions compared with plant chitinases, and bacterial endochitinases are relatively rare. It may be advantageous for bacteria to take up smaller saccharides (mono- or disaccharides) than larger ones because most of the chitinase-producing bacteria use chitin as carbon and nitrogen sources. So, bacteria is thought to produce exochitinase more than endochitinase. *Pseudomonas* sp. YHS-A2 also used colloidal chitin as sole carbon and nitrogen sources (data not shown). In addition, when colloidal chitin was used as unique carbon and nitrogen sources, *Pseudomonas* sp. YHS-A2 grew best (data not shown). However, some exochitinases are known to help in the inhibition of the

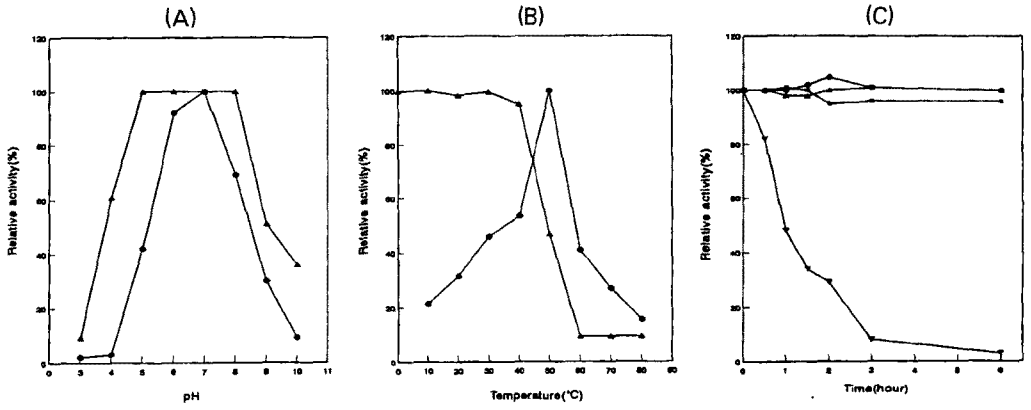

Fig. 4. Effects of pH and temperature on chitinase activities and stability of the extracellular chitinase.
(A): Effects of pH on the activitiy and stability at 40°C for 30 min. The optimal on activity (●) of purified chitinase and the pH stability (▲) on chitinase. (B): Effect of temperature on activity (●) and stability (▲) of chitinase at various temperature. (C) Effect of temperature on stability of chitinase during various time. (●), 20°C; (▲), 30°C; (■), 40°C; (▼), 50°C.

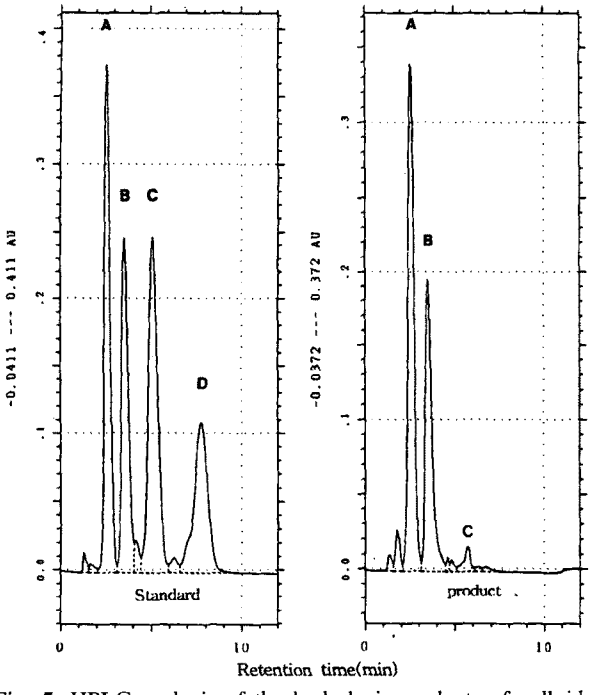

Fig. 5. HPLC analysis of the hydrolysis products of colloidal chitin by the chitinase.
Standard carbohydrate (left) and hydrolysis products (right). Peak A, $(GlcNAc)$; B, $(GlcNAc)_2$; C, $(GlcNAc)_3$; D, $(GlcNAc)_4$.

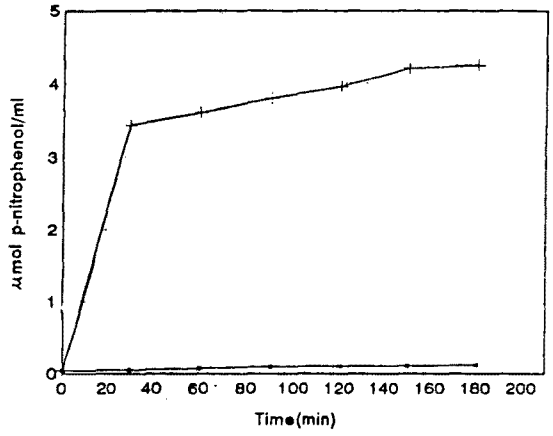

Fig. 6. Chitinase activity as measured by release of nitrophenol from *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (■) or *p*-nitrophenyl-β-D-N,N'-diacetyl-chitobiose (+).

growth of fungi even though they are exochitinases (16). To identify the products of the chitinase from *Pseudomonas* sp. YHS-A2 and the type of chitinase, the composition of the colloidal chitin suspension solution used as substrate in this work was determined by HPLC analysis but there were no small saccharides ranging from monomer to tetramer (data not shown). When the colloidal chitin suspension was reacted with purified chitinase from *Pseudomonas* sp. YHS-A2 for 3 h and compared with HPLC data for standard saccharides from monomer to tetramer (Fig. 5A), the main products were N-acetyl-D-glucoseamine and chitobiose and both chitotriose and chitotetraose were detected in much smaller quantities (Fig. 5B). Moreover, the purified chitinase cannot hydrolyze pNp-GlcNAc (Fig. 6), thus it is assumed that the chitinase is neither exochitinase (N-acetyl-D-glucosaminidase or chitobiosidase) nor chitobiase. Some endochitinases that cannot hydrolyze chitotriose (4) have been identified, and the chitinase from *Pseudomonas* sp. YHS-A2 can degrade chitotriose (data not shown) and a turbidity test showed that this enzyme is endo-type chitinase (data not shown). So, the chitinase was identified as a random-type endochitinase which can degrade any position of β-1,4-linkages of chitin and chitooligosaccharides.

Antifungal Activity of the Chitinase

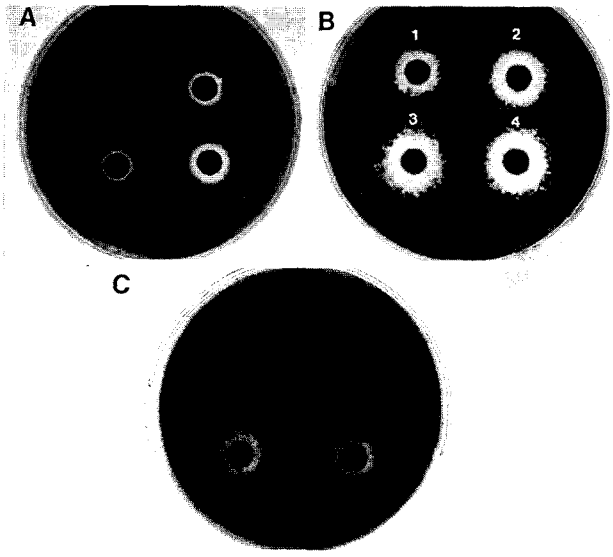

Fig. 7. Antifungal activity of the purified chitinase *Fusarium oxysporum* (A), *Botrytis cineria* (B), *Mucor Rouxii* (C).
Number denote the unit of the purified chitinase. 1, 100 μg; 2, 200 μg; 3, 300 μg; 4, 400 μg.

Four representatives of phytopathogenic organisms, *Fusarium oxysporum*, *Botrytis cineria*, *Mucor rouxii* and *Phycomycetes nitens* were used as test organisms. The purified chitinase inhibited the growth of three phytopathogenic fungi, *Fusarium oxysporum*, *Botrytis cineria* and *Mucor rouxii* (Fig. 7). When observed over time from day 1 to day 3, *Mucor rouxii* was less sensitive than *Fusarium oxysporum* and *Botrytis cineria*, which were inhibited completely. Most of the bacterial chitinases cannot inhibit the growth of fungi completely, but this may be due to the fact that they are exochitinases, N-acetyl-D-glucoaminidase or chitobiosidase (2, 25). By contrast, the purified chitinase produced by *Pseudomonas* sp. YHS-A2 inhibited fungal growth more effectively, which led to us characterising it as an endochitinase.

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