

CHARACTERIZATION OF A CHITINASE WITH ANTIFUNGAL ACTIVITY FROM A NATIVE *SERRATIA MARCESCENS* B4A

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ABSTRACT

Chitinases have the ability of chitin digestion that constitutes a main compound of the cell wall in many of the phytopathogens such as fungi. In the following investigation, a novel chitinase with antifungal activity was characterized from a native *Serratia marcescens* B4A. Partially purified enzyme had an apparent molecular mass of 54 kDa. It indicated an optimum activity in pH 5 at 45°C. Enzyme was stable in 55°C for 20 min and at a pH range of 3-9 for 90 min at 25°C. When the temperature was raised to 60°C, it might affect the structure of enzymes lead to reduction of chitinase activity. Moreover, the K_m and V_{max} values for chitin were 8.3 mg/ml and 2.4 mmol/min, respectively. Additionally, the effect of some cations and chemical compounds were found to stimulate the chitinase activity. In addition, Iodoacetamide and Idoacetic acid did not inhibit enzyme activity, indicating that cysteine residues are not part of the catalytic site of chitinase. Finally, chitinase activity was further monitored by scanning electronic microscopy data in which progressive changes in chitin porosity appeared upon treatment with chitinase. This enzyme exhibited antifungal activity against *Rhizoctonia solani*, *Bipolaris* sp, *Alternaria raphani*, *Alternaria brassicicola*, revealing a potential application for the industry with potentially exploitable significance. Fungal chitin shows some special features, in particular with respect to chemical structure. Difference in chitinolytic ability must result from the subsite structure in the enzyme binding cleft. This implies that why the enzyme didn't have significant antifungal activity against other Fungi.

Key word: Antifungal; Characterization; Chitinase; *Serratia marcescens* B4A

INTRODUCTION

Plants, in contrast with vertebrates, have no immune system. So they affect pathogens easily leading to remarkable yield loss altogether (40, 32). One of the most important

pathogens in plants is fungi. Chemical fungicides are extensively adopted in current of plant diseases. Therefore, biological control tactics become an important approach to facilitate sustainable agriculture (51). farming practices to protect crops from diseases. However, recently their utilization

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has attracted increased scrutiny since chemical fungicides are highly toxic. They can cause environmental contamination and/or the presence of fungicide residues in food products induce pathogen resistance (6, 26). Because of these limitations of chemical fungicides, it seems necessary to search for an alternative control strategy. Biological control or the use of microorganisms or their secretions to prevent plant diseases offers an attractive harmless alternative or supplement for the control tactics become an important approach to facilitate sustainable agriculture (51).

Since chitin is the major component of most fungal cell walls, a principal role has been attributed to enzymes from the chitinolytic system (12). Enzymatic lysis of fungal cell walls through extracellular chitinases has been implicated as a mechanism of biocontrol by bacterial agents (24, 63, 64). Extensive studies over the past two decades on chitinases have been done by a large number of laboratories. This is mostly due to the antifungal property of chitinases (27, 11). Microbial production of chitinase has captured worldwide attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method (25).

Chitinases (EC 3.2.1.14) catalyze the hydrolysis of chitin, a linear homopolymer of β -1,4-linked N-acetyl-D-glucosamine (GlcNAc) residues. This polysaccharide is present in the cell walls of fungi and green algae and in the exoskeleton of many crustacean and insects (35). The carbohydrate active enzyme (CAZy) database (<http://www.cazy.org/>) classifies carbohydrate enzymes into functional families, which are further subdivided into structurally related families designated by number. Following this classification, chitinases are listed as GH family-18 and GH family-19. Family 19 is generally highly conserved and contains mainly plant chitinases. Family 18 includes a large number of diversely evolved chitinases from plants, animals, bacteria and fungi (62).

Bacterial chitinases extracted from several Actinobacteria and *Streptomyces* species (59, 56, 17), and the ones extracted

from plants (46, 19, 58) both have potential applications in the biocontrol of plant pathogenic fungi and insects (13). In addition to control of phytopathogens fungi, other different applications of chitinase such as target for biopesticides (8, 31), Estimation of fungal biomass (30), Mosquito control (28) and Morphogenesis (38) have been discovered. Biological control of plant pathogens provides an attractive alternative means for management of plant disease without the negative impact of chemical fungicides that are usually costly and can cause environmental pollution, and may induce pathogen resistance (5).

This research aims at characterizing native chitinase *Serratia marcescens* B4A and investigating its antifungal activity on pathogen fungi that attacks important economical plants as well as the comparison of its characteristics with those of previously described antifungal chitinase in order to ascertain whether it is a novel antifungal compound or not.

MATERIALS AND METHODS

Chemicals

Chitin powder was obtained from shrimp shells of *Penaeus indicus* by the modified Method of Takiguchi (42). Shrimps were purchased from the markets in Abadan (Iran) and then the shells were isolated, cleaned, washed and dried. For elimination of mineral, dried shells were kept in HCl 10% for 24 h. After washing, NaOH (2M) was added for 24 h. Then the shells were dried again and powdered. 3, 5-dinitrosalicylic acid (DNS), N-acetyl D-glucosamine and Bovine serum albumin (BSA) were obtained from sigma (St. Louis, Mo. USA). All other chemicals were purchased from Merck (Darmstadt, Germany) and were reagent grade.

Microorganisms and Culture Condition

Serratia marcescens B4A showing high chitinase activity (61) was isolated in our laboratory from water, wastewater and soil of shrimp culture ponds in Abadan and Boushehr (south of

Iran) using agar plates containing nutrient agar 2% and colloidal chitin 0.1% at 30 °C and pH 7.5 for 3 days. For the production of chitinase in Liquid medium, the isolated strain was cultured in an Erlenmeyer flask (250 ml) containing 0.5% colloidal chitin, 0.05% MgSO₄.7H₂O, 0.03% KH₂PO₄, 0.07% K₂HPO₄, 0.03% yeast extract, 0.03% peptone, 0.1% NaCl, 0.1% (NH₄)₂SO₄, and 0.1% v/v trace elements solution (0.16% MnSO₄.2H₂O, 0.14% ZnSO₄.7H₂O, 0.5% FeSO₄.6H₂O and 0.2% CaCl₂) for 48 h at 30 °C on a shaker incubator (200 rpm).

Enzyme Assay

For the measurement of chitinase activity, colloidal chitin was selected as the substrate. The reaction mixture containing 0.5 ml of 1% w/v colloidal chitin and 0.5 ml enzyme solution was incubated at 45 °C for one hour. Then 3 ml 3, 5-dinitrosalicylic acid reagent was added for stopping the reaction followed by heating at 100 °C for 5 min. After centrifugation, determination of reducing sugar in supernatant was accomplished by the modified method of Miller (29). Absorbance was measured at 530 nm using UV spectrophotometer along with substrate and blanks. For determination of enzyme unit, serial dilutions of N-acetylglosamine (from 0 to 50 mM) were prepared. One unit (U) of the chitinase activity was defined as amount of enzyme required to release 1 mmol of N-acetyl D-glucosamine (as a standard) from chitin / min.

Partial Purification and Determination of Protein Concentration

Serial concentrations of ammonium sulfate from 25 to 85% was added slowly to the enzyme solution and left at 4 °C for at least 3 h with vigorous stirring. Then the solution was centrifuged at 10,000 g for 15 min. Next, sediment was dissolved in minimum phosphate buffer 20 mM in optimum pH. After that, the concentrated solution dialyzed in phosphate buffer twice and was checked for maximum chitinolytic activity. Finally the protein concentration was determined by

Bradford Method (4) using bovine serum albumin as a standard solution and the absorbance was measured at 595 nm.

Determination of Molecular Weight

The partially purified enzyme was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 12%) to determine its molecular weight and its purity by Laemmli method (18). Enzyme solutions obtained from culture medium in three conditions (a) without nitrogen resources, (b) without colloidal chitin but with glucose (both of them were partially purified) and (c) partially purified enzyme, and were used in SDS-PAGE electrophoresis for binding's competition. Then the proteins were separated from each other at constant voltage (100v). Next the electrophoresized gel bands were colored with coomassie brilliant blue R-250 and destained using acetic acid and methanol. Finally the relative molecular mass of the protein was estimated using standard molecular weight (prestained protein ladder; Fermentas).

Effect of Temperature and pH on Enzyme Activity and Stability

First enzyme (1U/ml) and substrate were kept at proper temperature separately to reach thermal equilibrium. Then chitinase activity was examined by incubating the enzyme in 1% w/v colloidal chitin as substrate at various temperatures (10-90 °C) for 15 min at pH 7.9 (optimum pH); after that the residual activity of the chitinase was measured at once. To determine the temperature stability, chitinase initially was pre-incubated at the different temperatures (10-90 °C). Every 10 min (up to 90 min) samples were placed in ice for 30 min and then colloidal chitin was added and enzyme assay was done at 25 °C.

To examine the optimum pH of the chitinase, 1% w/v colloidal chitin was prepared as the substrate at different pHs (2-10) by 50 mM phosphate- sodium acetate- glycine buffer and the chitinase was added to the substrate and then incubated in 45 °C for 15 min. Finally, the remaining activities were

determined. Monitoring the pH stability, chitinase was added to buffers with different pH for 1.5 h at 25°C. Followed by enzyme assay in optimum pH. Also pH stability of chitinase at 3, 8 and 10 at 25°C in 3 h (every 15 min) were determined.

Effects of Metal Ions and Chemical Compounds

The enzyme was added to a final concentration of 1 mM in the substrate of different metal ions such as (chloridized Li^+ , Al^{3+} , Ba^{2+} , Co^{2+} , Fe^{3+} , K^+ , Mg^{2+} , Mn^{2+}), (sulfated Cu^{2+} , Ni^{2+} , Zn^{2+}), (Ag^+ (AgNO_3), Ca^{2+} (CaCO_3), Cr^{3+} ($\text{K}_2\text{Cr}_2\text{O}_7$)) and chemical compounds (Citric acid, EDTA, SDS, PMSF, Iodoacetamide, Idoacetic acid, Tween 20 and Tween 80). Then they were incubated at 45°C for 1 h. The relative activity of the treated enzyme to that of the untreated one was explained by the percentage ratio.

Michaelis and Rate Constant Determination

The Michaelis constant (K_m) and the maximum velocity (V_{max}) of the enzyme (1U/ml) were measured by the substrate concentration ranged from 0.0 mg/ml to 0.9 mg/ml. The K_m and V_{max} were accounted from a double reciprocal plot by the Lineweaver-Burk method (23).

Scanning Electron Microscopy (SEM)

In order to observe the effect of chitinase on the morphological changes of chitin, chitin in the absence and presence of enzyme were provided and fixed in a mixture of 2.5% glutaraldehyde v/v and 2.5% paraformaldehyde v/v in 0.1 M phosphate buffer (prepared freshly) for 2 h. After being washed with distilled water, fixed samples were dehydrated through a graded ethanol (a series of 25-50-75-95 and 100% 5 min), and then freeze dried. The dried samples were sputtered coated with gold. All the samples were seen and photographed in a SEM (LEO, 1455 VP, Germany).

Antifungal Activity of Chitinase

For the detection of chitinase antifungal activity, the zone

of inhibition assay (57) was carried out. The mycelium of seven phytopathogenic fungi including: *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Bipolaris* sp, *Fusarium graminearum*, *Trichoderma reesei*, *Alternaria raphani*, *Alternaria brassicicola* were inseeded in the center of the Petri plates containing potato dextrose agar (PDA). When the diameter of the colony was almost 2 cm, four sterile blank paper disks were located around with equal distance from the center of the plates.

Equal aliquots (50 μl) of phosphate buffer 0.02 M, cell-free media, partially purified chitinase, and 5-min-boiled partially purified chitinase were presented onto disks 1 to 4 respectively and then incubated at 25°C until the colony started growing. What we observed was the control disk didn't indicate the inhibition of fungal growth but the others produced a crescent of inhibition around the disks due to with antifungal chitinase.

RESULTS AND DISCUSSION

Molecular Weight and Protein Concentration

An enzyme solution partially purified by ammonium sulfate 65 % with maximum activity was examined for purity and molecular weight determination by SDS-PAGE. Molecular weight of the enzyme was estimated to be almost 54 kDa (Fig.1). Molecular weights of chitinases obtained from different strains of *Serratia* were 47-60 kDa (50). The molecular weight of chitinases from *Serratia marcescens* QM13 1466 (34) and *Serratia plymuthica* HRO_C 48 (14) were 58 kDa and 60.5 kDa respectively. The molecular weight of chitinases obtained from *Enterobacter* sp. was about 60 kDa. Molecular weight of chitinase from *Enterobacter* sp. NRG4 (9), *Enterobacter* sp. G-1 (33), *Enterobacter agglomerans* (7) and *Enterobacter aerogenes* (47) were 60 kDa, 60 kDa, 61 kDa and 42.5 kDa respectively that were almost similar to our results.

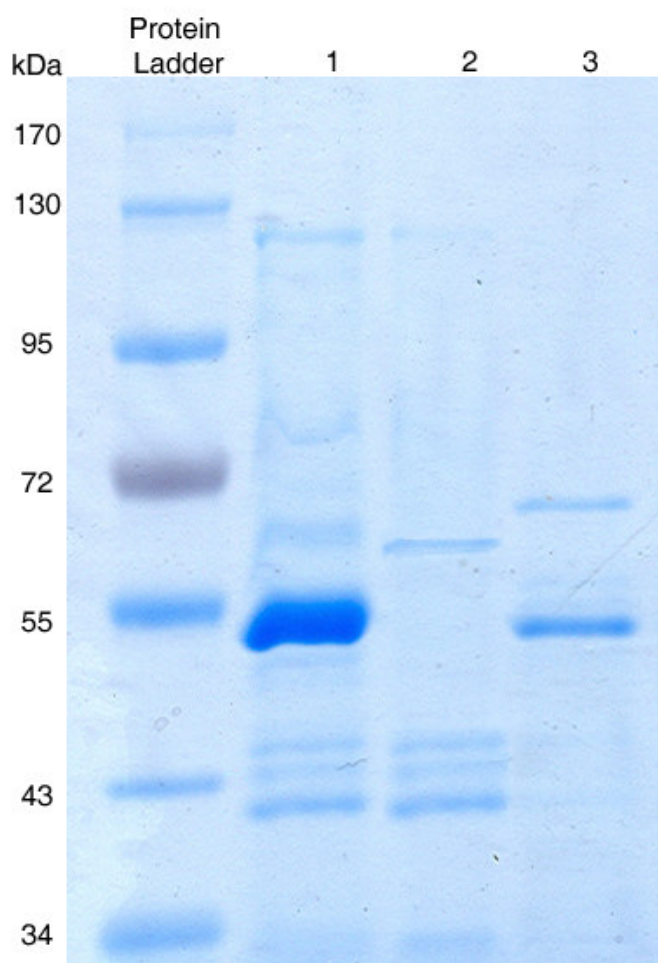
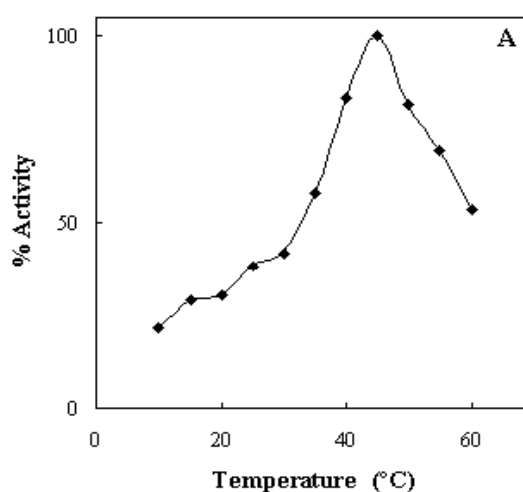


Figure 1. SDS-PAGE analysis of chitinase; a direct comparison between *Serratia marcescens* B4A cultured with different media. Lane 1, partial purified chitinase by ammonium sulfate 65% which obtained from culture media with colloidal chitin as sole carbon source lane 2, glucose as sole carbon source, chitinase band wasn't found; and lane 3, without nitrogen resource which produced a small amount of chitinase compared with lane 1.

Effect of Temperature on the Enzyme

The enzyme was active in the temperature range of 20-60°C with optimal activity at 45°C (Fig. 2A), however chitinase thermostability was continuously decreased either when the time of incubation or the temperature increased (Fig. 2B, C). Chitinase was quite stable under 50°C for 20 min (Fig. 2B) and

the result of enzyme stability at 50 to 60°C in the absence of substrate showed that it can maintain more than 50% of its original activity (Fig. 2C). To our surprise, we found out that a 5°C increase in temperature, would result in a 40 minute decrease in the stability suggesting a remarkable conformational changes in the enzyme integrity which in turn affects its activity (half-life of enzyme is less than 20 minutes at 55°C and 60°C compare to 50°C). Thus, when the temperature was raised to 60°C, it might affect the structure of enzymes and lead to reduced chitinase activity. The optimum temperature of chitinases obtained from other *Serratia* strains like *S. marcescens* BJL 200, *S. marcescens* 2170, *S. marcescens* NK1(50), *S. plymuthica* HRO_C 48 (14) and *S. marcescens* QM13 1466 (34) were 50-60 °C, 60 °C, 47 °C, 55 °C and 30 °C respectively. Other *Enterobacter* strains that produced chitinase like *Enterobacter* sp. G-1 (33), *E. aerogenes* (43) and *E. agglomerans* (7) their optimum temperature were 40°C, 55°C and 40°C respectively. *Enterobacter* sp. NRG4 (9) showed a maximum activity at 45°C and it was stable at 45°C for 1h which is similar to our results. Different temperature profile of chitinase produced by variety of bacterial sources has been reported. For example the optimum temperature activity of chitinase produced by *Bacillus* sp. NCTU2 (53) and *Alcaligenes xylosoxidans* (45) were 60°C and 50°C respectively.



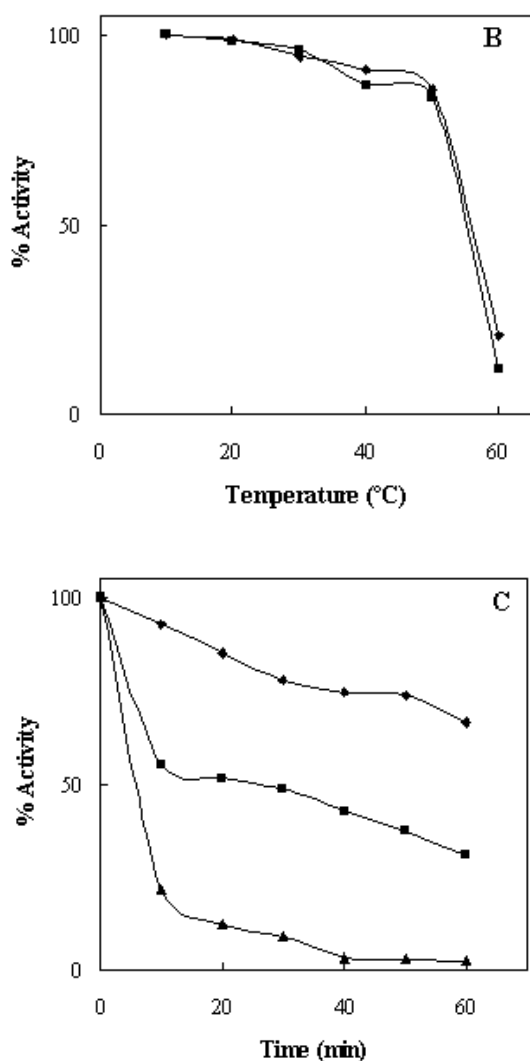
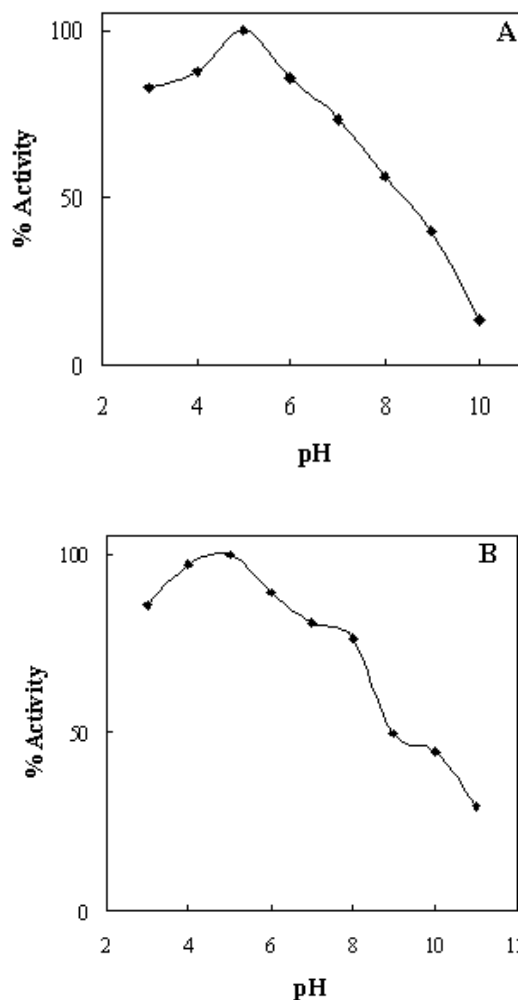


Figure 2. (A) Temperature profile of enzyme in 20 mM phosphate buffer, pH 5.0. (B) Temperature Stability of enzyme at 10 (◆), and 20 (■) minute from 10 to 60°C. (C) Thermal inactivation of chitinase activity at 50-60 °C. The assay were incubated at various temperatures ([◆] 50°C; [■] 55°C; [▲] 60°C) prior to the addition of substrate, followed by cooling in an ice bath. The activity at zero time was taken as 100 activity.

Effect of pH on the Enzyme

Chitinase showed optimum activity at pH 5 as justified by its pH profile (Fig. 3A) at 45 °C. Chitinase was stable at pH range of 3-9 for 90 min at 25 °C (Fig. 3B). Remaining activity of enzyme in pH 3, 8 and 10 at 25 °C for 180 min was more

than half of the original activity (Fig. 3C). Other *Serratia* strains that produced chitinase like *S. marcescens* BJL 200, *S. marcescens* NK1 (50) and *S. plymuthica* HRO_C 48 (14) showed a maximum activity at pH 6, 6.2 and 6.6 respectively, while chitinase produced by other strains of *Enterobacter*, such as *Enterobacter* sp. G-1 (33) and *E. aerogenes* (43) have been reported to have a pH optima 7 and 6 respectively. Also another chitinase produced by *Enterobacter* sp. NRG4 (9) with optimum pH 5.5 showed pH stability from pH 4.5 to 8.0, but in our test result the enzyme is active at a wider pH range and more stable. The optimal pH for the other bacterial chitinases such as *Aeromonas* sp. No.10S-24 (44) and *Bacillus* sp. NCTU2 (53) was reported 4 and 6.3, but they were stable between pH range 4 to 9 and 4 to 8.5 respectively.



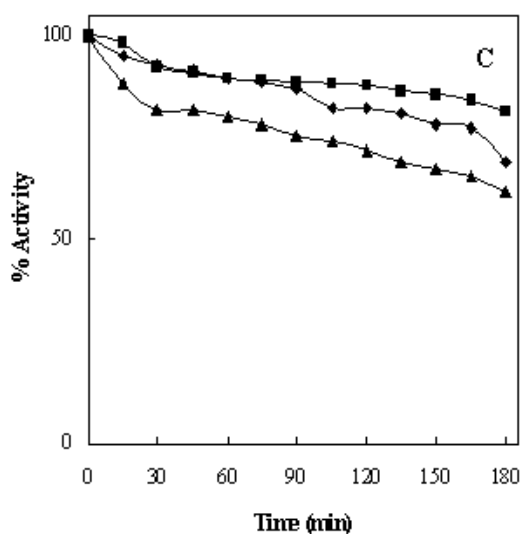


Figure 3. (A) Effect of pH on enzyme activity at 25 °C. A mixture of glycine, acetate, and phosphate buffer at a concentration of 50 mM was used. (B) pH stability of enzyme at pH 3.0-11.0 for 90 min at 25 °C. (C) Remaining activity of enzyme at pH 3.0 (♦), 8.0 (■), and 10.0 (▲) at 25°C. Before addition of substrate, followed by cooling in an ice bath. The activity at zero time was take at 100% activity.

Effects of Metal Ions and Chemicals Compounds on Chitinase Activity

Results of chitinase activity at pH 5 in the presence of different chemical compounds and metal ions with final concentration 1 mM were shown at table 1. Iodoacetamide and Idoacetic acid, a chemical modifier of cysteine residues almost didn't change the enzyme activity indicating that cysteine residues are not involved in catalytic site of chitinase (2). In addition, it's notable that all the surface-active detergents like SDS and Tweens (20 and 80) were stimulator of chitinase activity by 2%, 23% and 20% respectively. Perhaps these results are based on the fact that surface-active reagents might increase the turnover number of chitinase by increasing the contact frequency between the enzyme active site and the substrate which are accomplished through lowering the surface tension of the aqueous medium (15). Interestingly PMSF at

1mM concentration didn't inhibit the chitinase activity significantly; therefore it could be used to control the serine proteases activity of crude extract for preventing exhaustive degradation of proteins during downstream processes.

To investigate the effect of presence or absence of cations, on the activity, we used Li^+ , Ag^+ , Al^{3+} , Ba^{2+} , Ca^{2+} , Cr^{3+} , Cu^{2+} , Fe^{3+} , K^+ , Ni^{2+} , Mg^{2+} , Zn^{2+} , and EDTA. In 1mM concentration, all of them offered partial inhibition (Table 1). These results are in line with those reported for other bacterial chitinase (14, 33, 45). Nevertheless Co^{2+} and Mn^{2+} were found as stimulator of chitinase activity 13% and 36% respectively (Table 1). Contrary to our results, Co^{2+} (14, 43) and Mn^{2+} (60, 48) inhibited bacterial chitinases activity but Mn^{2+} stimulated the fungal chitinases activity (20).

Table 1. Effect of metal ions, metal chelators and chemical compounds on Chitinase activity

Metal ion/Chemical compound	Residual activity of Chitinase at 1mM
Control	100
Li^+	94
Ag^+	92
Al^{3+}	76
Ba^{2+}	94
Ca^{2+}	97
Co^{2+}	113
Cr^{3+}	91
Cu^{2+}	91
Fe^{3+}	99
K^+	89
Ni^{2+}	80
Mg^{2+}	88
Mn^{2+}	136
Zn^{2+}	91
Citric acid	101
EDTA	69
SDS	102
PMSF	96
Iodoacetamide	101
Iodoacetic acid	102
Tween 20 (at 0.1 v/v)	123
Tween 80 (at 0.1 v/v)	120

Michaelis Constant and Maximal Velocity

The substrate concentration effect on the hydrolysis rate of chitin degradation was studied. The K_m (for Chitin) and V_{max} values of this enzyme were calculated by Lineweaver-Burk (Fig. 4). The values of K_m and V_{max} were 8.3 mg/ml and 2.4 mmol/min, respectively. The K_m values of chitinase from different organisms were 1.41 mg/ml for *Enterobacter* sp. NPG4 (9), 2.88 mg/ml for *E. aerogenes* (43), 3 mg/ml for *A. xylosoxidans* (45) and *Bacillus* sp. WY22 (54).

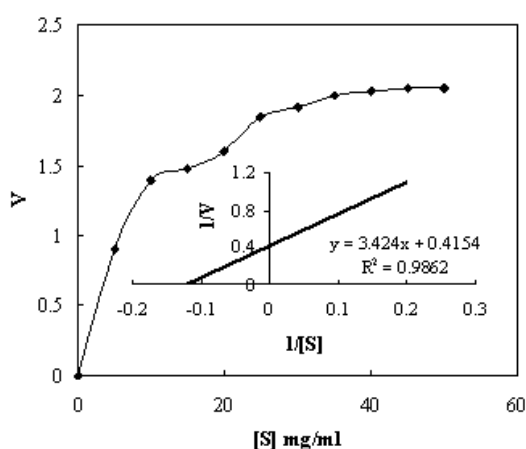
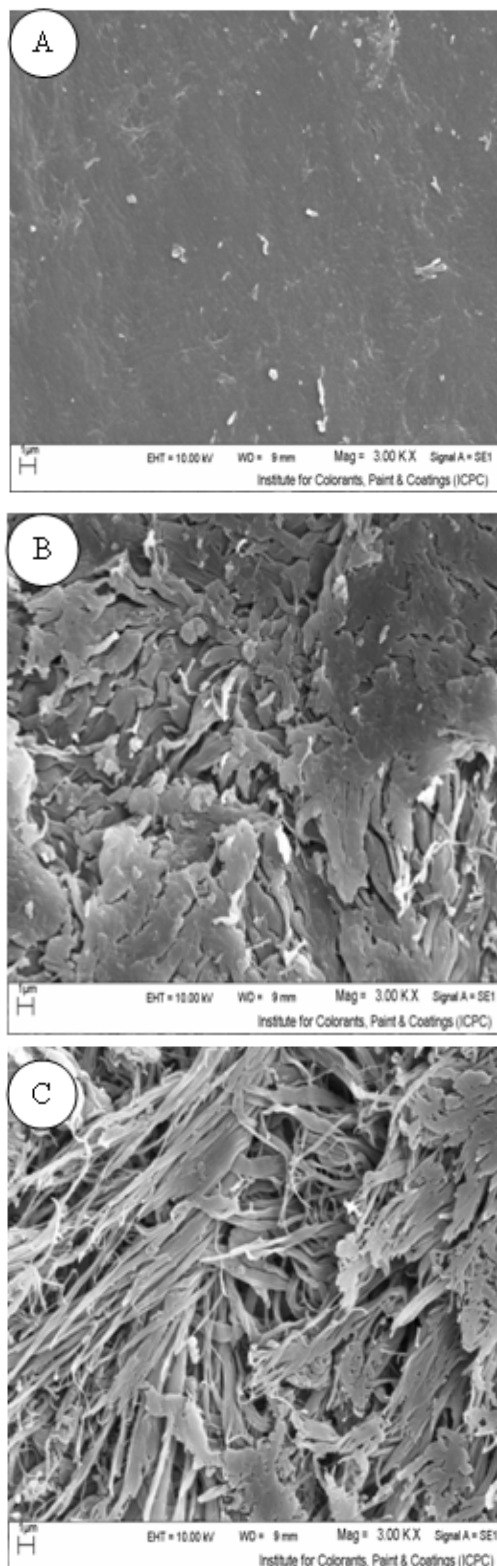


Figure 4. Michaelis-Menten curve of chitinase activity. (Inset) Lineweaver-Burk plot relating *Serratia marcescens* B4A chitinase reaction velocity to chitinase concentration.

Pattern of Degradation on Chitin

Observing the morphological changes of chitin powder treated by chitinase, we performed scanning electronic microscopy experiments. In the absence of chitinase, chitin powder showed almost a smooth surface (Fig. 5-A). However, when chitin was treated by chitinase (Fig. 5B, C and D), it showed cracks and pores in chitin surface and the number of pores increased significantly by increasing the time of exposure. Such an observation has been found for chitinase isolated from *penicillium* sp. LYG0704 by Lee et al. in 2009 (20) showed the same morphological changes. Our results implied that isolated chitinase is quite effective on chitin as the

direct evidence because of evidence from scanning electron microscopy data.



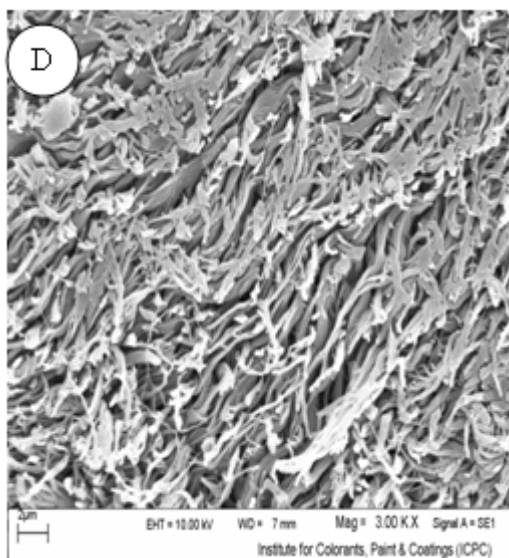


Figure 5. SEM of chitin powder treated with chitinase; (A) chitin without chitinase; (B) chitin treated with chitinase after 1h; (C) 2h; (D) 4h.

Antifungal Activity of Chitinase

Figure 6A-D presents antifungal activity of chitinase against a wide range of phytopathogenic fungi such as *Rhizoctonia solani* (Fig 6A), *Bipolaris* sp. (Fig 6B), *Aphanomyces raphani* (Fig 6C), and *Alternaria brassicicola* (Fig 6D). However, it had no antifungal effects on *S. sclerotiorum*, *F. graminearum*, and *T. reesei* (data not shown). Dahiya *et al.* (9) identified antifungal chitinase against *R. solani* from *Enterobacter* sp. NRG4 with molecular weight 64 kDa. Antifungal chitinase also isolated from *Bacillus subtilis* CH426 (55), *Bacillus cereus* J1-1 (49), *Bacillus* sp. DAU101 (21) *Cellulosimicrobium cellulans* 191 (13) with molecular weight 64 kDa, 65 kDa, 62 kDa and 61 kDa respectively. Obstruction with spore germination or germ tube elongation (39, 52), inflation and distortions of hyphal (1, 22, 10), release of chitin oligosaccharides from cell wall and cytoplasm leakage (47) were observed in fungi affected by chitinases.

The chitin has an intrinsic variability due to its natural origin and it exists in several forms with their specific properties each (3, 16). This polymer of fungi possesses

principally the same structure as the chitin occurring in other organisms. However, a major difference results from the fact that fungal chitin is associated with other polysaccharides which do not occur in the exoskeleton of arthropods (41). Difference in chitinolytic ability must result from the subsite structure in the binding cleft (36). This implies that why the enzyme didn't show significant antifungal activity against other Fungi.

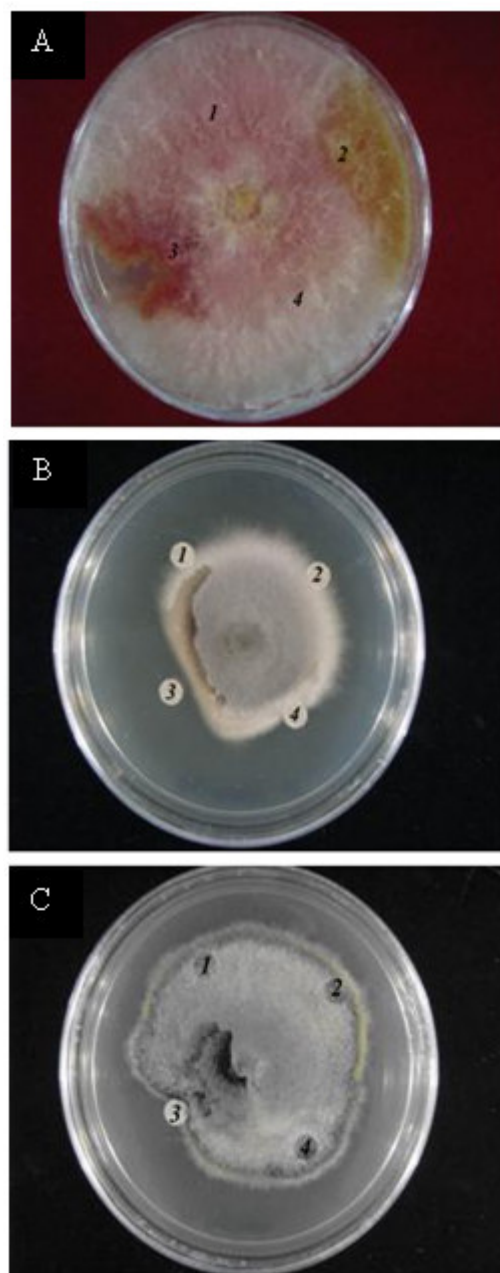




Figure 6. Antifungal activity of chitinase. In vitro growth inhibitory activity of chitinase against (A) *Rhizoctonia solani*, (B) *Bipolaris sp.*, (C) *Alternaria raphani*, (D) *Alternaria brassicicola*. (1) Control, phosphate buffer 0.02 M; (2) cell-free media; (3) partial purified chitinase; (4) partial purified chitinase that boiled for 5 min.

CONCLUSIONS

Protection of plants from disease produced by phytopathogenic fungi is one of the most important challenges in agriculture. The total losses as results of plant diseases reach almost 50% of the crop in developing countries. One-third of this is a consequence of fungal infections (40). Therefore finding biological products that could be used for biological fighting is very important in agriculture. Recent studies demonstrated that chitinase from plants (37, 40, 58) and microorganism (33, 7, 9, 43) are able to inhibit the fungal growth.

In this study, a native chitinase with antifungal activity against a wide range of phytopathogens was isolated from *Serratia marcescens* B4A, which is important since not all chitinases have antifungal activity. Fortunately this isolated microorganism is a native strain of Iran that its chitinase was found to be somewhat different from other reported chitinases in terms of its resistance to acidic medium. Furthermore, isolated chitinase in this study may have important implications

on agriculture such as the biological control of insects which are plants pests.

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