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ORIGINAL ARTICLE

Characterization of Antifungal Chitinase from Marine *Streptomyces* sp. DA11 Associated with South China Sea Sponge *Craniella Australiensis*

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Abstract The gene cloning, purification, properties, kinetics, and antifungal activity of chitinase from marine *Streptomyces* sp. DA11 associated with South China sponge *Craniella australiensis* were investigated. Alignment analysis of the amino acid sequence deduced from the cloned conserved 451 bp DNA sequence shows the chitinase belongs to ChiC type with 80% similarity to chitinase C precursor from *Streptomyces peucetius*. Through purification by 80% ammonium sulfate, affinity binding to chitin and diethylaminoethyl-cellulose anion-exchange chromatography, 6.15-fold total purification with a specific activity of 2.95 Umg^{-1} was achieved. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed a molecular weight of approximately 34 kDa and antifungal activities were observed against *Aspergillus niger* and *Candida albicans*. The optimal pH, temperature, and salinity for chitinase activity were 8.0, 50°C, and 45 g‰ psu, respectively, which may contribute to special application of this marine microbe-derived chitinase compared with terrestrial chitinases. The chitinase activity was increased by Mn^{2+}, Cu^{2+}, and Mg^{2+}, while strongly inhibited by Fe^{2+} and Ba^{2+}. Meanwhile, SDS, ethyleneglycoltetraacetic acid, urea, and ethylenediaminetetraacetic acid were found to have significantly inhibitory effect on chitinase activity. With colloidal chitin as substrates instead of powder chitin, higher V_{max} (0.82 mg product/min·mg protein) and lower K_m (0.019 mg/ml) values were achieved. The sponge's microbial symbiont with chitinase activity may contribute to chitin degradation and antifungal defense. To our knowledge, it was the first time to study sponge-associated microbial chitinase.

Keywords *Craniella australiensis* · *Streptomyces* sp. · Chitinase · Gene cloning · Purification · Property · Antifungal activity

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Introduction

Chitin, a linear β-1, 4-linked homopolymer of *N*-acetylglucosamine, is one of the three most abundant polysaccharides in nature besides cellulose and starch. The antifungal activity and highly biocompatible quality make the chitin and its derivatives particularly useful for biomedical applications, such as wound healings, cartilage tissue engineering, drug delivery, and nerve generation (Shi et al. 2006; Yan et al. 2006). Chitin's biodegradable and antifungal properties are also useful for environmental and agricultural uses and food technology and cosmetics (Lin and Lin 2005; Rabea et al. 2003; Goosen 1997).

Although more than 1,000 metric tonnes of chitin are produced annually in the aquatic biosphere alone, there is no substantial accumulation of chitin in ocean sediments (Keyhani and Roseman 1999). This is because a bioconversion process is naturally driven by chitinolytic marine bacteria (Hirono et al. 1998; Suginta et al. 2000). These bacteria can convert chitin into organic compounds that then can be used as nitrogen and carbon sources. For example, chitin is an excellent carbon and nitrogen source for many *Streptomyces* strains (Robbins et al. 1988).

Chitinases are glycosyl hydrolases, which can catalyze the degradation of chitin. Chitinases are present in a wide range of organisms such as bacteria, fungi, insects, plants, and animals. Henrissat and Bairoch (1993) classified chitinases into two families, namely families 18 and 19, based on amino acid sequence similarity. Family 18 includes chitinases found in bacteria, fungi, viruses, animals, and class III or V of plant chitinases. Family 19 includes class I, II, and IV chitinases of plant origin only, with the exception of chitinase C from *Streptomyces griseus* HUT 6037 (Ohno et al. 1996), and chitinases F and G from *S. coelicolor* (Saito et al. 1999). Chitinase genes from some marine bacteria have been cloned and characterized (Techkarnjanaruk and Goodman 1999; Tsujibo et al. 1993). Typically, chitinase enzymes are composed of at least three functional domains, namely catalytic domain, chitin-binding domain, and cadherin-like domain or fibronectin type III-like domain (Morimoto et al. 1997; Watanabe et al. 1990).

Chitinolytic enzymes have wide-ranging applications such as preparation of pharmaceutically important chitooligosaccharides and *N*-acetyl D-glucosamines, preparation of single cell protein, isolation of protoplasts from fungi and yeast, control of pathogenic fungi, treatment of chitinous waste, and control of malaria transmission (Dahiya et al. 2006). Chitinases also show biofunctional potential such as lytic activity (Patil et al. 2000). The production of inexpensive chitinolytic enzymes is important in the use of chitin-containing waste particularly in the seafood industry, which not only can solve environmental problems but also do with added value in certain cases (Wang et al. 1995; Suginta et al. 2000). Compared with chitinases derived from terrestrial organisms, marine chitinases with higher pH and salinity tolerance may contribute to special biotechnological application. Therefore, novel marine chitinases are of great importance.

Although many studies concerned with chitinase gene cloning and expression have been reported (Aunpad and Panbangred 2003; Liu et al. 2005; Morimoto et al. 1997; Robbins et al. 1998) and chitinases were also found in marine bacteria such as *Alteromonas sp.* strain O-7 (Tsujibo et al. 1993), *Vibrio anguillarum* and *V. parahaemolyticus* (Hirono et al. 1998), *Salinivibrio costicola* (Aunpad and Panbangred 2003), and *Microbulbifer degradans* (Howard et al. 2003), few reports on chitinase from microorganisms associated with marine sponge were found. In this study, for the chitinase of *Streptomyces* sp. DA11 isolated from South China sponge *Craniella australiensis*, the gene cloning, purification, properties, kinetics, and antifungal activity were investigated.

Materials and Methods

Sponge Sample, Microorganism, and Fermentation Conditions Marine sponge *C. australiensis* (Porifera, Class Demospongiae, Order Choristida, Family Craniellidae) was collected by SCUBA diving at a depth of about 20 m in the South China Sea around Sanya Island and enclosed in axenic bags immediately. Before bacterial isolation, sponge samples were stored at 4°C. The collected sponge was identified by Professor Jin-He Li in the Institute of Oceanology, Chinese Academy of Sciences (Li and Liu 2006).

Strain DA11 was isolated from sponge *C. austrialiensis* and was identified as *Streptomyces* sp. by 16S rDNA sequencing (GenBank accession no. DQ180128). The methods for isolation and screening of the chitinase-producing strain *Streptomyces* sp. DA11 were described as before (Li and Liu 2006).

The medium for chitinase production by *Streptomyces* sp. DA11 consisted of galactose 5.00 g/l, peptone 12.5 g/l, colloid chitin 2.62 g/l, $MgSO_4{\cdot}7H_2O$ 0.10 g/l, KH_2PO_4 0.45 g/l, K_2HPO_4 1.05 g/l, $MgSO_4{\cdot}7H_2O$ 0.5 g/l, $FeSO_4{\cdot}7H_2O$ 0.03 g/l, $ZnSO_4$ 0.03 g/l, and powder chitin 12.5 g/l. Each flask (250 ml) containing 100 ml of the fermentation medium was inoculated with 5% (*v*/*v*) seed culture, the cell concentration of which was 2.6 g/l cell dry weight, then cultured at 28°C on the rotary shaker (180 rpm).

DNA Extraction, Chitinase Gene PCR, and Alignment Analysis of the Deduced Amino Acid Genomic DNA of *Streptomyces* sp. DA11 was extracted using the method of Mehling et al. (1995). Two oligonucleotides primers chi1, 5′ attgtcgacacctgggaccagccgct3′, and chi2, 5′ttagcatgcg ccgaa gaagtcgtacg3′ (Liu et al. 2005), were used for chitinase gene polymerase chain reaction (PCR). Twenty-five microliters of the PCR mixture consisted of 2.5 μl of 10× buffer, 3.5 μl of $MgCl_2$ (25 mM), 2 μl of dNTP (2.5 mM), 0.5 μl of P1 (20 pmol/μl), 0.5 μl of P2 (20 pmol/μl), Taq polymerase (5 U/μl), and 20 ng template DNA. PCR amplification was carried out as follows: initial denaturation step for 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 2 min, with a further 10-min extension at 72°C. The PCR product was purified with 3S Spin Agarose Gel DNA Purification Kit (Shenergy Biocolor Bioscience & Technology Company, China) and was ligated into the pUCmT vector. Then the vector was transformed into $CaCl_2$-competent *Escherichia coli* DH5a and the positive recombinants were screened on X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside)-isopropyl-β-D-thiogalactopyranoside–ampicillin–tetracycline indicator plates by color-based recombinant selection. Positive clones were identified by PCR amplification with pUCmT vector primer pairs T7 (5′-TAATACGACTCACTA TA GGG-3′) and M13 (5′-CAGGAAACAGCTATGACC-3′) using the same PCR program as described above.

The PCR product was sequenced using ABI 3730 DNA Sequencer by Bioasia Biotechnology Company, China and was submitted to GenBank with accession No. EU369114.

The deduced amino acid sequence was searched for homology by BLASTp in NCBI protein databases. Finally, the deduced amino acid sequence was compared with the sequences of the chitinase in GenBank (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST).

Assay of Chitinase Activity Chitinase activity was determined by a dinitrosalicylic acid (DNS) method (Miller 1959). This method works on the concentration of *N*-acetyl glucosamine, which is released as a result of enzymic action (Massimiliano et al. 1998; Ulhoa and Peberdy 1991). The reaction mixture contained 0.5 ml of 1.5% colloidal chitin in 10 mM phosphate buffer (pH 7.5) and 0.5 ml of the enzyme sample. *Streptomyces* sp. DA11 was cultured for 96 h on the rotary shaker (180 rpm) at 28°C. After centrifugation (9,000 ×*g*, 30 min), the cell-free broth supernatant was collected as crude enzyme. The purified enzyme after ammonium sulfate precipitation, chitin affinity binding, and diethylaminoethyl (DEAE)-cellulose column chromatography were prepared according to the procedure in the following enzyme purification part. The well-vortexed mixture was incubated at 50°C for 1 h. The reaction was terminated by placing the tubes in boiling water bath for 10 min. After cooling to the room temperature, the reaction mixture was centrifuged at 1,600 g for 5 min. Then, 0.5 ml of the supernatant and 0.5 ml of DNS reagent were mixed together and incubated in boiling water bath for 10 min. After cooling to the room temperature, the absorption of the test sample was measured at 540 nm using UV spectrophotometer (V-2102PCS, Shanghai, China) along with substrate and enzyme blanks. All measurements of enzymatic activity and measurements of protein content were performed in triplicates for each sample.

One unit of the chitinase activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min at 50°C. The protein concentration was determined according to Bradford (1976) with bovine serum albumin as a standard.

Enzyme Purification and Determination of Molecular Weight In the case of chitinase purification, precipitation by ammonium sulfate followed by affinity binding to chitin and DEAE-cellulose anion-exchange chromatography were carried out according to Mukherjee and Sen (2006). Cell-free culture broth was precipitated with ammonium sulfate (80%). The pellets obtained were dissolved in phosphate buffer (10 mM, pH 7.5) and dialyzed overnight. A 10-ml volume of colloidal chitin (20 mg/ml) and 15 ml of 1 M sodium chloride were added and increased to 35 ml using the same buffer. The mixture was left on ice for 1 h and centrifuged to remove unbound protein. The pellet was washed extensively with the same phosphate buffer and suspended in 15 ml of the same buffer. The suspension was incubated for 6 h at 35°C under gentle shaking (90 rpm) to digest colloidal chitin with the absorbed chitinase. The resulting clear supernatant was again precipitated with 80% ammonium sulfate, centrifuged, and dialyzed. Ion-exchange chromatography was done using a DEAE-cellulose column (2.5×7 cm). The column was packed with overnight-swollen DEAE-cellulose in 10 mM phosphate buffer (pH 7.5) and eluted stepwise with 0.1 to 0.6 M NaCl. The pooled protein fractions were dialyzed, stored at –20°C, and used as the purified enzyme.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the Laemmli (1970). After SDS-PAGE, gel was stained with 0.05% coomassie brilliant blue R-250. The chitinase purified by DEAE-cellulose column chromatography was used for the following investigation of antifungal activity, properties, and kinetics.

Bioassay of Antifungal Activity Antifungal activity was detected by hyphal extension inhibition (Roberts and Selitrennikoff 1986). *Aspergillus niger* (AS 3.5487) and *Candida albicans* (AS 2.2086), from the Chinese Biodiversity Information Center in the Institute of Microbiology, Chinese Academy of Sciences, were used as indicator strains. The fungal cultures were inoculated on beef extract peptone plates. Discs containing the finally purified enzyme (20 μl) were placed on the plates and incubated at 28°C for 5 days; the positive and negative controls were nystatin and beef extract peptone culture medium, respectively.

Effects of Temperature, pH, Salinity, and Metal Ions on Enzyme Activity Optimum temperature for the purified chitinase activity was determined from 30°C to 60°C; temperature stability was tested by the pre-incubation of enzyme at temperatures from 30°C to 60°C for 1 h. The effect of pH was determined by incubating the purified chitinase at pH 6.0–12.0. The pH stability was tested by pre-incubation of the purified chitinase in buffers with different pH from 6.0 to pH 12.0 at 50°C for 1 h. The remaining enzyme activity was tested after the treatment.

The enzyme activity at various salinity was measured with g‰ psu; the enzyme was treated at the optimized temperature, pH, and in a saltwater solution with psu range from 5 g‰ to 50 g‰. The effects of metal ions were assayed after the addition of metal ions Ca^{2+}, Fe^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Mg^{2+} to the reaction mixtures at a final concentration of 5 mM.

Protein Inhibitor of the Chitinase The effects of chitinase inhibitors, ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), SDS, urea, and theophylline at a concentration of 1.0 mM, on chitinase activity

were measured with no protein inhibitor addition as control. The residual activity was determined in triplicates.

Enzyme Kinetics The effect of substrates on chitinase activity was studied using powder chitin and colloidal chitin, respectively. The enzyme was pre-incubated in 0.2 M phosphate buffer (pH 8.0) at 50°C for 20 min. The kinetic constants K_m and V_{max} were determined from the respective Lineweaver–Burk plots. These assays were set up in triplicates. If a plot of $1/V$ and 1/[Substrate] is linear, the y-intercept, x-intercept, and slope is equal to $1/V_{max}$, $1/K_m$, and K_m/V_{max}, respectively (Imoto and Yagshita 1981).

Results and Discussion

Alignment Analysis of the Chitinase Gene from Streptomyces sp. DA11 One DNA fragment of 451 bp (No. EU369114) was obtained by PCR using chi1 and chi2 primers. Figure 1 shows the alignment analysis of amino acid sequences of this chitinase (EU369114) with some reported chitinases from GenBank including ChiC of *S. lividans* (BAA02168) and *S. coelicolor* A3 (2) (BAA75644), Chi63 of *S. plicatus* (AAA26720), *S. peucetius* (AAF43629), and *Saccharophagus defradans* 2–40 (DAA01336). Chi63 and ChiC listed here belong to the chitinases from *Streptomyces*, and they are representative chitinases of the group A of family 18. BLAST search showed that chitinase C precursor (AAF43629) from *S. peucetius* had the highest degree of amino acid similarity 80% to the chitinase from *Streptomyces* sp. DA11. The alignment result suggested that the chitinase from *Streptomyces* sp. DA11 also belong to ChiC.

Enzyme Purification and SDS-PAGE Traditionally, ammonium sulfate precipitation is preferred for the purification of chitinase (Wiwat et al. 1999; Blaak et al. 1993). Recently, the combined strategy of ammonium sulfate precipitation and chromatography has been successfully used for the purification of chitinase from microorganisms such as *S. marcescens* NK1 (Nawani and Kapadnis 2001) and *Bacillus cereus* YQ 308 (Chang et al. 2003). In this study, based on Table 1, the specific activity of the purified enzyme by 80% ammonium

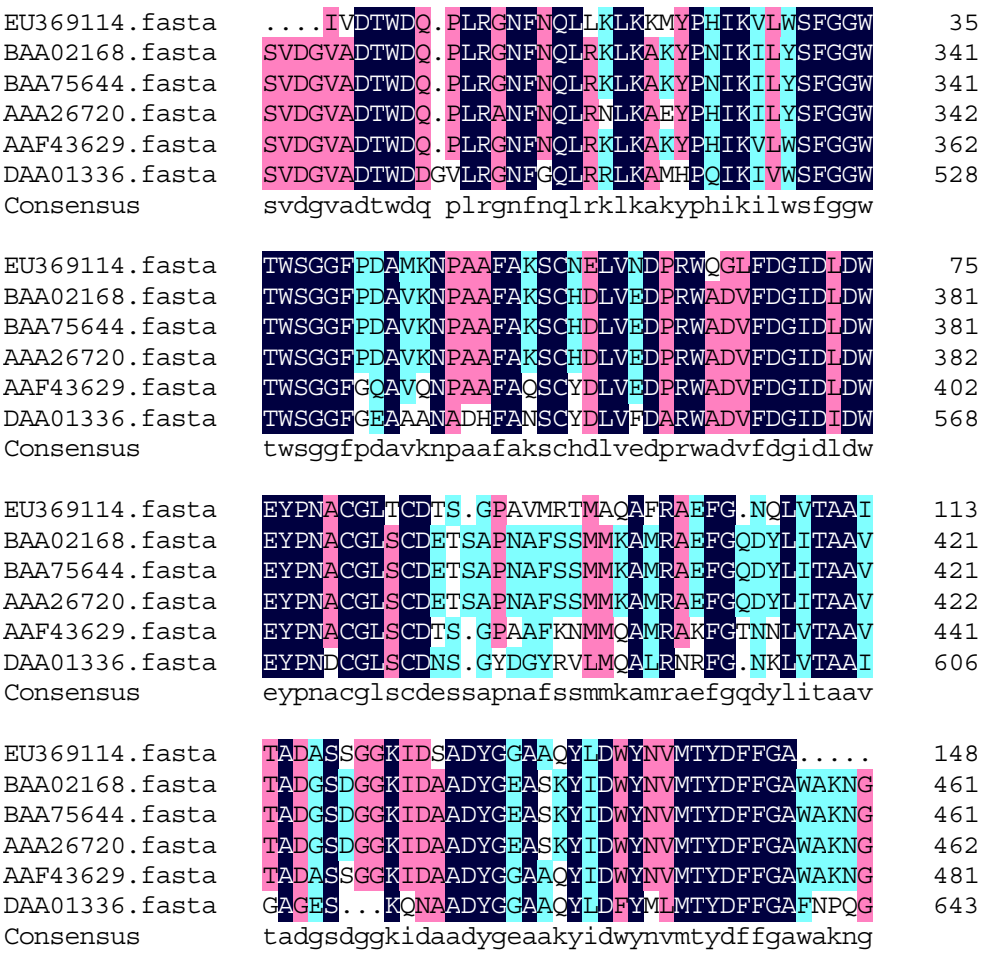

Fig. 1 Multiple alignments of chitinase amino acid sequences from *Streptomyces* sp. DA11 (EU369114), *S. lividans* (BAA02168), *S. coelicolor* A3 (2) (BAA75644), *S. plicatus* (AAA26720), *S. peucetius* (AAF43629), and *S. defradans* 2–40 (DAA01336)

sulfate precipitation was 1.18°Umg^{-1}, which was 2.46 times higher than that of the crude enzyme (0.48°Umg^{-1}). The sequential treatment of chitinase with chitin affinity binding and DEAE-cellulose anion-exchange chromatography resulted in 2.95°Umg^{-1} of specific activity, which was 6.15 times higher than that of crude enzyme, suggesting that the combined strategy of ammonium sulfate precipitation and chitin affinity binding and anion-exchange chromatography is effective for the purification of chitinase from *Streptomyces* sp. DA11.

Microbial chitinases weigh from 20 to 120°kDa. The molecular weight of chitinase from *Streptomyces* sp. is between 30 and 68°kDa (Tsujibo et al. 2000; Vetrivel et al. 2001). In Fig. 2, the *Streptomyces* sp. DA11 chitinase purified by affinity binding to chitin and DEAE-cellulose column chromatography shows a single band with an estimated molecular weight of 34°kDa.

Antifungal Activity After 5°days of incubation, a circularity of growth inhibition appeared around the perimeter of the discs containing the purified chitinase obtained in the study (Fig. 3). The average diameter of inhibition zone on the tested fungal plates was 10.98±0.49°mm for *A. niger* and 10.48±0.45 mm for *C. albicans*, respectively. Obviously, both of the tested fungi were suppressed by the purified chitinase (20 μl), indicating that the obtained chitinase has the potential to be an antifungal agent. Potential use of chitinases as biocontrol agents has been reported (Freeman et al. 2004). For example, the obvious antifungal activity of chitinase from the novel *Streptomyces* strain was proved (Hoster et al. 2005). Chitinases from *S. griseus* also showed activity against fungi such as *Aspergillus* sp., *Phycomyces blakesleeanus*, and *Trichoderma reesei* (Williams et al. 1983). Hoster et al. (2005) reported chitinase activity against *A. nidulans* and phytopathogens such as *Botrytis cinerea*, *Fusarium culmorum*, *Gulgnardia bidwellii*, and *Sclerotia sclerotiorum*.

Effects of Temperatures, pH, Salinity, and Metal Ions on Chitinase Activity The maximum activity was found at 50°C according to Fig. 4, which was in consistent with that of the reported chitinases derived from mesophilic *Streptomyces* or *Bacillus* species (Watanabe et al. 1999; Wiwat et al. 1999). The enzyme maintained activity over 85% from 30°C to 45°C. These results were similar to those of the chitinase from a novel *Streptomyces* strain isolated from marine sediment, which was reported to be active at temperatures from 20°C to 65°C, and the highest activity was recorded between 45°C and 50°C (Hoster et al. 2005). However, according to Carrillo and Gomez (1998) and Gomes et al. (2001), the optimum temperature for chitinase of *Streptomyces* was found to be 40°C. These observations indicated that chitinases from *Streptomyces* from different environment showed the different temperature sensibility.

At the same time, Fig. 4 shows the enzyme has the maximum activity at pH 8.0 and was stable at pH 6–9, which was higher than that of chitinase from *Thermococcus chitonophagus* (Andronopoulou and Vorgias 2003), *Streptomyces* strain (Hoster et al. 2005), and *Bacillus* sp. DAU101 (Lee et al. 2007), indicating the characteristic of marine microorganisms derived enzyme. As for other species of *Streptomyces*, the optimum pH for chitinase activity was found to be from 3.3 to 7.5 (Carrillo and Gomez 1998; Gomes et al. 2001). It is known that pH influences the proton-donating or proton-accepting groups (ionization) in the catalytic site. The higher pH tolerance of this chitinase makes it possible be used as a catalyst under basic condition.

In the case of the effect of salinity on the chitinase activity, the maximum chitinase activity was found at 45 g‰ psu (Fig. 4). One of the characteristics of marine microorganisms is salt tolerance (Chandrasekaran 1996; Moriguchi et al. 1994). Salt-tolerant enzymes may play significant roles in the industrial processes that require high-salt environments (Moriguchi et al. 1994). In conclusion, based on Fig. 4, the chitinase from *Streptomyces* sp. DA11 appears to be with thermal stability, alkaline tolerance, and salinity tolerance, which will lead this enzyme to some special application in biotechnology.

Table 2 shows that the enzyme activity can be increased by Mn^{2+}, Mg^{2+}, and Cu^{2+}. The activiting effect of Co^{2+} and Zn^{2+} were not obvious. While metal ions Fe^{2+} and Ba^{2+} strongly inhibit the chitinase activity. Similarly, Lee et al. (2007) observed the chitinase activity of *Bacillus* sp. DAU101 was increased by Mg^{2+} and Co^{2+}, and Park et al.

Table 1 Purification of chitinase from *Streptomyces* sp. DA11

Enzyme	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	1,075.2±90.06	2,206.72±115.84	0.48±0.04	100	1
The purified enzyme[a]	236.21±13.11	200.18±15.01	1.18±0.06	9.07	2.46
The purified enzyme[b]	6.4±1.06	2.17±0.05	2.95±0.06	1.09	6.15

Results were presented as means±standard deviation, $n=3$

[a] Ammonium sulfate precipitation

[b] Chitin affinity binding and DEAE-cellulose column chromatography

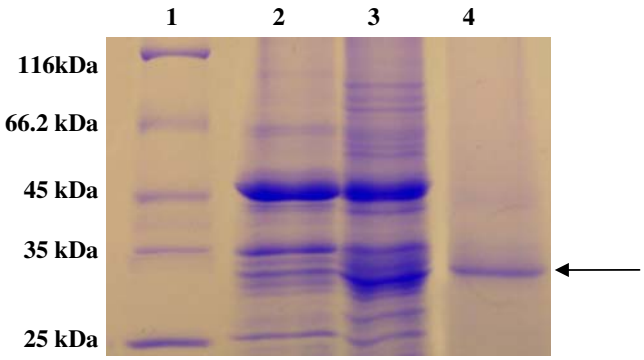

Fig. 2 SDS-PAGE of chitinase from *Streptomyces* sp. DA11. *Lane 1*—marker proteins; *lane 2*—crude enzyme in the fermentation culture supernatant; *lane 3*—enzyme after 80% ammonium sulfate precipitation; *lane 4*—enzyme purified by affinity binding to chitin and DEAE-cellulose column chromatography. *Arrow* indicates band of the purified chitinase

(2000) found the chitinase activity of *Vibrio* sp. 98CJ11027 was inhibited by Fe^{2+}.

Effects of Protein Inhibitors on Chitinase Activity Table 3 shows SDS, EGTA, urea, and EDTA have significantly inhibitory effect on the activity of chitinase; the activity were decreased to 28%, 36%, 37%, and 44% of the control, respectively. While theophylline shows moderate inhibition on chitinase activity, the residual activity was about 83%.

In the presence of denaturing agents, urea and SDS, the chitinase activity will be depressed significantly; the similar result was observed by Groleau et al. (2003). Chelating agents EGTA and EDTA can inhibit the enzyme activity, which was consistent with the report of Chisb (Lee et al. 2007), alpha-amylase (Ezeji and Bahl 2006), and protease (Tsuji et al. 1999), demonstrating that the purified enzyme should be a kind of metalloenzyme (Ramirez-Zavala et al. 2004). Theophylline was found to be a competitive inhibitor against a fungal family 18 chitinase by Rao et al. (2005), while only little effect could be found on marine chitinase from *Streptomyces* sp. DA11, indicating the different structure of this chitinase from that of the fungal family 18 chitinase.

Kinetic of Chitinase According to Fig. 5, with colloidal chitin as substrates, the K_m and V_{max} values were 0.019 mg/ml and 0.82 mg product/min·mg·protein, respectively. While using powder chitin, the K_m and V_{max} values were 0.078 mg/ml and 0.47 mg product/min·mg·protein, respectively. The higher V_{max} and lower K_m values indicated that colloidal chitin was a

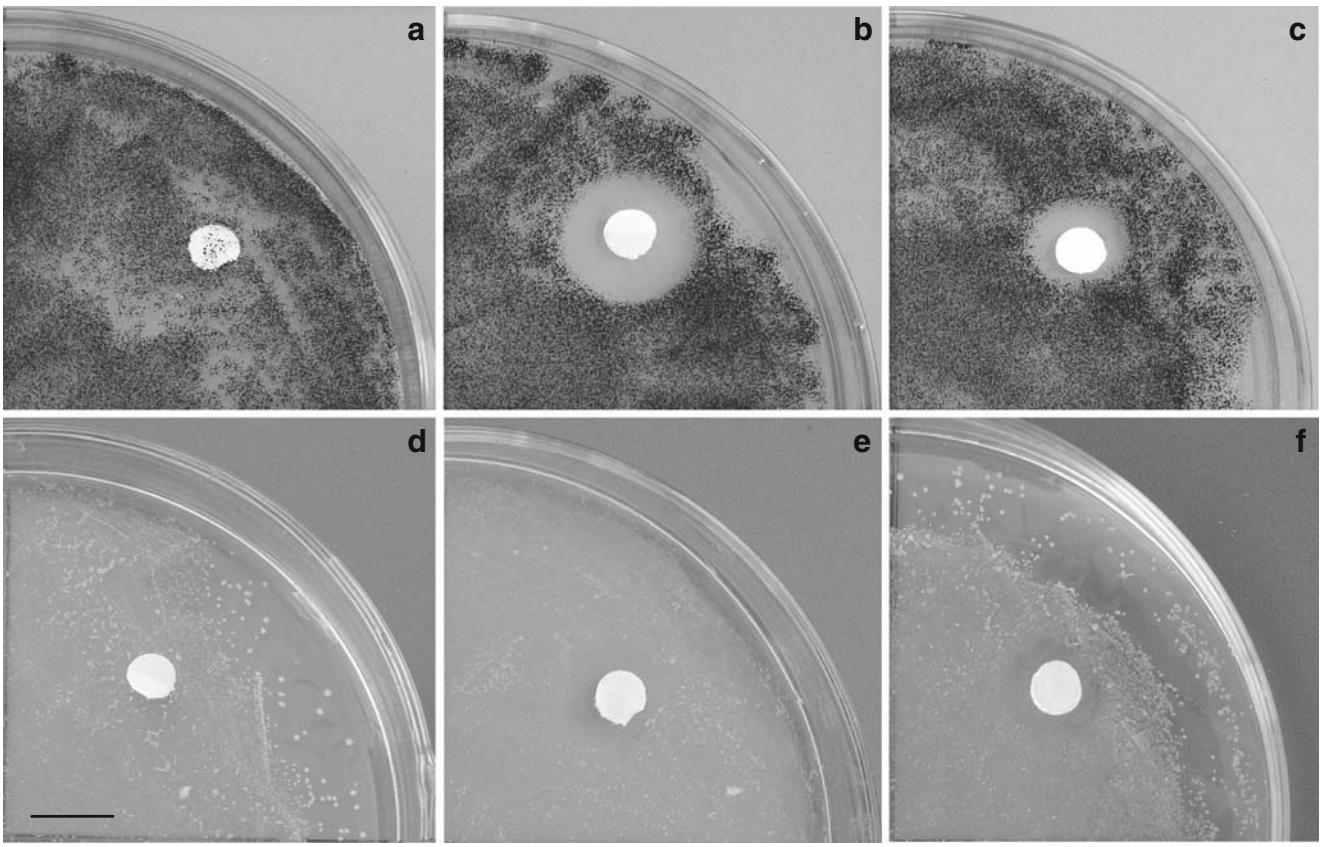

Fig. 3 Inhibition of chitinase against *A. niger* (**a** negative control, **b** positive control—nystatin, **c** chitinase) and *C. albicans* (**d** negative control, **e** positive control—nystatin, **f** chitinase). *Bar* means 10.0 mm

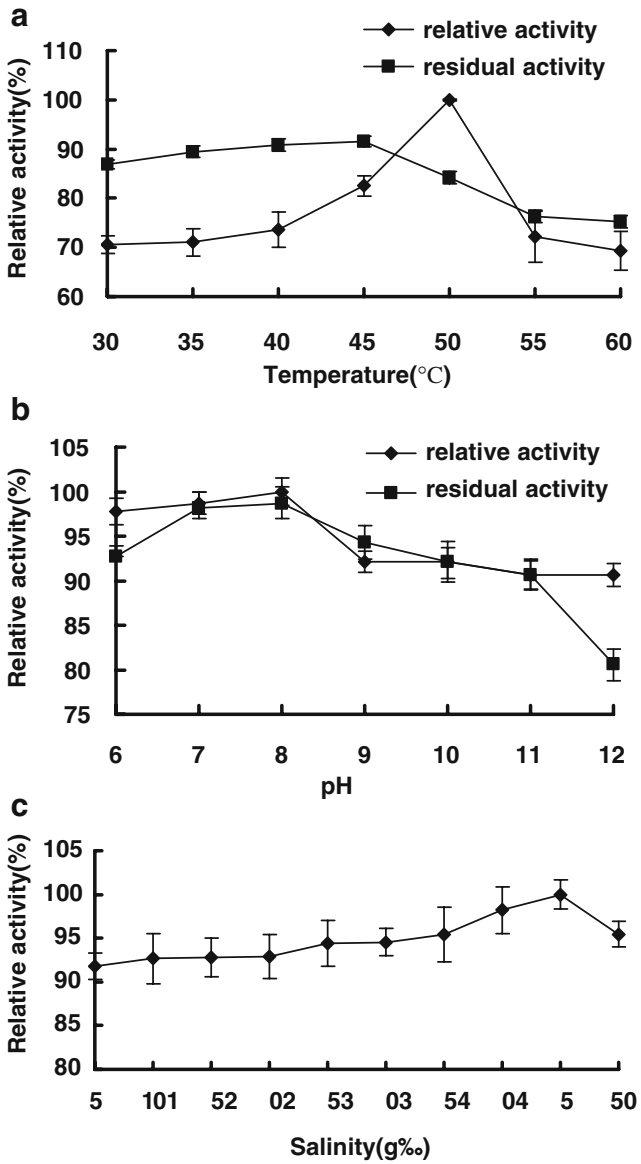

Fig. 4 Effects of temperature (**a**), pH (**b**), and salinity (**c**) on the activity of chitinase. Optimal temperature and pH (*unfilled square*), temperature and pH stability (*filled square*). Results were presented as means±standard deviation, $n=3$

Table 2 Effects of metal ions on chitinase activity

Metal ion	Relative activity (%)
Control	100
Mn^{2+}	137.7±8.2
Ca^{2+}	97.31±5.3
Co^{2+}	105.17±1.7
Fe^{2+}	76.92±0.9
Ba^{2+}	40.91±0.4
Cu^{2+}	113.02±7.9
Zn^{2+}	104.31±1.5
Mg^{2+}	114.68±1.9

Results were presented as means±standard deviation, $n=3$

Table 3 Effects of protein inhibitors on chitinase activity

Inhibitor	Relative activity (%)
Control	100
EDTA	43.65±0.901
EGTA	36.46±1.04
SDS	27.91±0.89
Urea	37.1±2.92
Theophylline	83.38±1.79

Data are given as means±standard deviation, n=3. The chitinase activity in the absence of the protein inhibitors was regarded as 100%

better substrate for the enzyme. The kinetic parameters of K_m and V_{max} were lower and higher, respectively, than those of a chitinase from *T. chitonophagus* when colloidal chitin was used as the substrate (Andronopoulou and Vorgias 2003), suggesting that the affinity for the substrate of the enzyme obtained in this study was different from that of chitinase from other microorganisms.

To our knowledge, this was the first report on chitinase from marine sponge-associated microorganisms. This study will lead to understand the relationship between sponge host and its microbial symbionts and find novel marine enzyme for biotechnological application. Based on this study, the sponge's microbial symbiont with chitinase activity may contribute to antifungal defense and the degradation of chitin into nutrients such as low molecular weight carbohydrates for sponge or other sponge-associated microorganisms.

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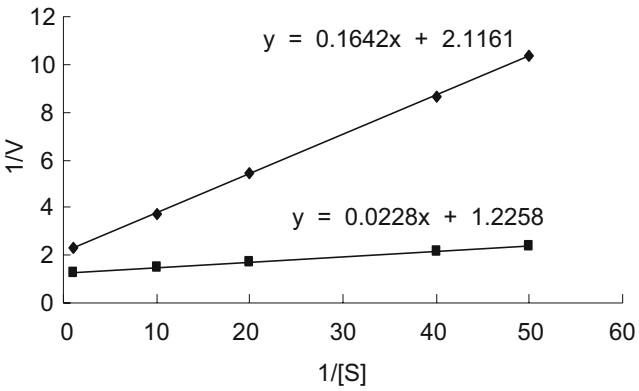

Fig. 5 Lineweaver–Burk plot for K_m and V_{max} values of the chitinase with powder chitin (*filled diamond*) and colloidal chitin (*filled square*) as substrates, respectively

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