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Purification and characterization of chitin deacetylase from *Scopulariopsis brevicaulis*

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Abstract

An extracellular chitin deacetylase from *Scopulariopsis brevicaulis* has been produced using chitin as sole carbon resource of culture medium. The enzyme activity was 10–11 units ml^{-1} culture supernatant after the strain was shaken at 200 rpm and 29 °C for 96 h. The enzyme was purified 74-fold at 38% yield through ammonium sulfate precipitation, and Sephadex G-25, and G-100 column chromatography. The apparent molecular weight of 55 kDa, as determined by SDS-PAGE and gel filtration chromatography, suggested that the enzyme exists as a single component. The enzyme was active on chitooligosaccharides with at least two *N*-acetyl-glucosamine residues, but the activity increased with the number of *N*-acetyl-glucosamine residues. When hexa-*N*-acetylchitohexaose was used as substrate, the optimum pH for enzyme activity was determined to be 7.5, and the optimum temperature was 55 °C. Under these conditions, the activity of enzyme was studied on water-soluble chitosan, chitin from *Aspergillus niger* and shrimp crystalline chitin. The structures of products were characterized by FT-IR, XRD and potentiometric titration. The results indicated that degree of substrate crystallinity had an important effect on enzyme activity. The enzyme had high deacetylating activity on amorphous chitin from *A. niger* mycelium (37% deacylation) and water-soluble chitosan (33%) but low. activity on shrimp crystalline chitin (3.7%).

Keywords: *Scopulariopsis brevicaulis*; Chitin deacetylase; Purification; Characterization

1. Introduction

Chitin, a linear polysaccharide composed of 1,4-linked 2-acetamido-2-deoxy-β-D-glucose units, is one of the most abundant, easily obtained, and renewable natural polymers. It is widely distributed in crustaceans, insects, and in certain fungi (Bartnicki-Garcia, 1968). Up to now, no important industrial applications have been found due to its insolubility in aqueous and organic solvents. Chitosan, the *N*-deacetylated derivative of chitin, is soluble in acid solutions, and has a wide range of uses; such as a natural insecticide (Thome & Van Daele, 1986), an antimicrobial agent (Liu, Du, Wang, Hu, & Kennedy, 2004; Liu, Du, Yang, & Zhu, 2004), a biopolymer for binding metals (Wan, Petrisor, Lai, Kim, & Yen, 2004), or a base for cosmetics (Hirano, 1989). At present, deacetylation of chitin to chitosan is usually achieved by thermo chemical treatment of chitin to remove the acetyl groups. This process uses strong alkali at high temperatures for extended periods of time, so it is environmentally unsuitable and not easily controlled, leading to a broad and heterogeneous range of products (Chang, Tsai, Lee, & Fu, 1997).

Deacetylation can also be achieved enzymically using chitin deacetylase (EC3.5.1.41) under mild conditions (Trudel & Asselain, 1990) which overcome most of disadvantages in the alkali treatment method. Chitin deacetylase had been isolated partially from extracts of many fungi (Araki & Ito, 1975; Kauss, Jeblick, & Yong, 1983; Siegrist & Kauss, 1990) and occurs in some insect species (Arachami, Gowri, & Sundara-Rajulu, 1986). The purification of chitin deacetylase from *Mucor rouxii* (Kafetzopoulos, Martrinou, & Bouriotis, 1993), *Absidia coerulea* (Gao, Katsumoto, & Onodera, 1995) and *Aspergillus nidulans* (Alfonso, Nuero, Santamaria, & Reyes, 1995) have been reported.

A cDNA of the *M. rouxii* encoding chitin deacetylase has been isolated, sequenced and further characterized (Kafezopoulos, Thireos, Vournakis, & Bouriotis, 1995). The chitin

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deacetylase from the *M. rouxii* and *A. coerulea* exhibited similar molecular weights (75 kDa), amino-terminal sequences, optima pH's (4.5) and optima temperatures (50 °C), and substrate specificities (four to six sequential *N*-acetylglucosamine residues). The CAD from *A. nidulans* exhibited different molecular weight (27 kDa), optimum pH (7.0), and substrate specificity (glycol chitin and oligomers of *N*-acetylglucosamine). Recently Martinou, Koutsiouli, and Bouriotis (2003) succeeded in cloning the chitin deacetylase gene from *Saccharomyces cerevisiae* and expressing it in *Escherichia coli.* The resultant chitin deacetylase apparent molecular weight, optimum temperature and pH were 43 kDa 50 °C and 8.0, respectively (Martinou, Koutsioulis, & Bouriotis, 2002). However, until now, name of these enzymes can be applied to the production of chitosan from chitin in the industrial field yet, due to very limited extent of deacetylation on such macromolecular substrates. Thus, finding a chitin deacetylase with high activity against the polysaccharide still is a very interesting quest.

In this paper, an extracellular chitin deacetylase from *Scopulariopsis brevicaulis* was produced using chitin as sole carbon resource in the culture medium, and isolated and purified by a very simple method. Its properties such as optimum temperature, thermal stability, pH, molecular weight and substrates were investigated, and its effect of deacetylation was evaluated with shrimp crystalline chitin, chitin from *Aspergillus niger* and water-soluble chitosan.

2. Materials and methods

2.1. Materials

S. brevicaulis was isolated from the soil around the chitin factory in Jinan of china. The soil around the chitin factory has plenty of chitin, and so those microorganisms producing chitin deacetylase have greater scope for growth in this soils. *N*-acetylchitooligosaccharides (dimer, trimer, tetramer, pentamer and hexamer) and 3-methyl-2-benzothiazolinonehydrazone were purchased from Sigma (St Louis, USA). Sephadex G-25, G-75 and Sephadex G-100, were purchased from Pharmacia (Uppsala, Sweden). *N*-acetyl-D-glucosamine and shrimp crystalline chitin were purchased from Liao Nin Xin Die Biotechnology Co. Ltd (Dalian, China). D-Glucosamine·HCl was purchased from Zhejiang Aoxing Biotechnology Co. Ltd (Taizhou, China). Molecular weight markers were purchased from Bio-Rad (Hercules, USA). Water-soluble chitosan (54% degree of deacetylation with an average molecular weight of 280,000) and chitin from *A. niger* (21% degree of deacetylation with an average molecular weight of 120,000) were prepared in our laboratory based on the method in the literature (Synowiecki & Al-Khateeb, 1997). All other chemicals used were commercial products of analytical grade.

2.2. Organism and culture conditions

S. brevicaulis was cultivated on agar slants [sucrose (3%), $NaNO_3$ (0.2%), K_2HPO_4 (0.1%), KCl (0.05%), $MgSO_4$, (0.05%)] for 72 h at 28 °C. The cells were harvested into sterile water. This suspension (6 ml, containing 5.0×10^8 cells ml^{-1}), was inoculated into a 500 ml Erlenmeyer flask containing liquid medium (80 ml, $NaNO_3$, 0.16 g; K_2HPO_4, 0.08 g; KCl, 0.04 g; $MgSO_4$, 0.04 g; peptone, 0.08 g; chitin, particle size less than 100 mesh, 0.4 g). The flask was shaken at 200 rpm and 29 °C for 96 h.

2.3. Preparation of chitin deacetylase

After incubation, and working throughout at 4 °C, the culture (120 ml) was centrifuged at $10{,}000\times g$ for 30 min. The supernatant was collected and mycelium (4 g) was homogenized in a cold mortar with sterilized silica powder (60 mesh, 6 g) to break the cells; the supernatant of the culture was added to the homogenate to form a suspension. The suspension was centrifuged at $10{,}000\times g$ for 30 min, and the supernatant was used as the crude extract. Ammonium sulfate was added to the crude extracts to 35% saturation. After 120 min, the solution was centrifuged at $18{,}000\times g$ for 30 min. Ammonium sulfate was added to the supernatant to 80% saturation again. After 18 h, the precipitated proteins were collected by centrifugation at $20{,}000\times g$ for 30 min.

The precipitated proteins were dissolved in distilled water (10 ml). The insoluble material was removed by centrifugation at $20{,}000\times g$ for 30 min and the supernatant was then applied to a Sephadex G-25 column (16 mm diam × 80 cm). The column was eluted with 50 mM Tris–HCl, pH 7.5. The fractions were monitored by UV assay (at 280 nm) and for chitin deacetylase and those enzymically active were combined and applied to a Sephadex G-100 column (16 mm diam × 60 cm). The column was eluted with 50 mM Tris–HCl, pH 7.5, monitoring the fractions (5 ml) at 280 nm. The fractions containing chitin deacetlyase activity were combined and dialyzed overnight against distilled water (5 l). After dialysis, the enzyme was lyophilized with and suspended in distilled water again (5 ml).

2.4. Enzyme activity assay

A chitin deacltylase enzyme activity assay based on the literature method (Kauss & Bauch, 1988; Martinou, Kafetzopoulos, & Bouriotis, 1995; Ride & Drysdale, 1972) was performed in glass tubes using 50 mM Tris–HCl buffer pH 7.5 (50 μl), hexa-*N*-acetylchitohexaose (100 μg) in water (100 μl), and enzyme preparation (50 μl). Incubation time was 15 min at 55 °C, and the reaction was terminated by the addition of 5% (w/v) $KHSO_4$ (250 μl).

For color formation 5% w/v $NaNO_2$ (250 μl) was added, and the tubes capped immediately and allowed to stand with occasional shaking for 15 min, and 12.5% w/v aqueous 3-methy1-2-benzothiazolinonehydrazone hydrochloride (250 μl, freshly prepared each day) added and the mixture heated at 100 °C for 3 min. After cooling to room temperature, 0.5% w/v $FeCl_3$ (250 μl) was added and the developing color was read after 30 min at 650 nm. Standard curves were prepared with D-glucosamine·HCl standard (0–6 μg).

Units of enzyme activity were estimated by using hexa-*N*-acetylchitohexaose (166 nmol) as substrate in 50 mM Tris–HCl buffer pH 7.5 (50 μl). Incubation time was 15 min at 55 °C (that it is the optimum temperature of this chitin deacetylase), and the reaction was terminated by the addition of 5% w/v $KHSO_4$ (250 μl).

One unit of chitin deacetylase activity is defined as the amount of the enzyme required to produce 1 μmol of acetate/min. when incubated with hexa-*N*-acetylchitohexaose as described above.

2.5. Characterization of the enzyme

The molecular weight of chitin deacetylase was estimated by gel filtration and SDS-PAGE. Crude enzyme was applied to a Sephadex G-75 column (16 mm × 60 cm), eluted with 50 mM Tris–HCl buffer pH 7.5 at a flow rate of 0.3 ml/min. The apparent molecular weight was calculated by comparison of its elution volume with those of molecular weight markers including phosphorylase b (97,000), serum albumin (66,200), ovalbumin (43,000), carbonic anhydrase (28,700) and tobacco mosaic virus coat protein (17,500).

Polyacrylamide gel electrophoresis under denaturing conditions was performed in 12% w/v polyacrylamide gels. Samples were boiled for 5 min with 15% w/v sucrose and 2.5% w/v SDS in 125 mM Tris–HCl buffer pH 6.7 without 2-mercaptoethanol or dithiothreitol. Pre-stained molecular weight markers were tobacco mosaic virus coat protein (17,500), carbonic anhydrase (28,700), ovalbumin (43,000), serum albumin (66,200), phosphorylase B (97,000).

Protein content was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) and Peterson (1977) using bovine serum albumin as standard.

The enzyme activity of chitin deacetylase was estimated on shrimp crystalline chitin, chitin from *A. niger*, water-soluble chitosan (54% degree of deacetylation with an average molecular weight of 280,000), and six different *N*-acetylchitooligosaccharides. The substrates (50 μg) were added to 50 mM, Tris–HCl buffer pH 7.5, then chitin deacetylase (12 units) was added. Enzymatic reactions were performed in a total volume of 1 ml, incubation for 120 min at 55 °C, and the reaction was terminated through heating at 100 °C. Acetate released was determined using a literature method: the acetate was translated to citric acid by catalysis with acetyl-CoA synthetase, citrate synthase and malate dehydrogenase following the addition of ATP and CoA, then determining the content of citric acid (Martinou et al., 2002).

2.6. Analysis of the structure

IR spectra of water-soluble chitosan and the product of deacetylation were recorded as KBr pellets on a NEXUS (Madison, USA) spectrophotometer.

X-ray diffraction (XRD) patterns of the sample were measured with a Shimadzu Labx-XRD-6000 diffractometer (Kyoto, Japan) and using a Cu Kα target at 40 kV and 50 mA. The diffraction angle ranged from 10 to 40°.

2.7. Determination of the degree of deacetylation (DD)

Chitosan (0.1 g) was dissolved in a known excess of 0.1 M HCl (10 ml). From the titration of this solution with 0.1 M NaOH; a pH curve with two inflexion points was obtained. The amount of acid consumed between these two points was considered to correspond to the amount of the free amino groups in the chitosan (Tolaimate et al., 2000).

3. Results and discussion

3.1. Purification and molecular weight determination of chitin deacetylase

In general, chitin deacetylase has been isolated and purified from mycelium extracts of fungi, such as *M. rouxii* (Kafetzopoulos et al., 1993; Martinou, Kafetzopoulos, & Bouriotis, 1993), *A. coerulea* (Gao et al., 1995) and *A. nidulans* (Alfonso et al., 1995). Recently, chitin deacetylase was isolated from the culture filtrate of the fungus *Colletotrichum lindemuthianum*. In the current work, a strain of *S. brevicaulis* was selected to produce chitin deacetylase. When chitin was used as sole carbon resource of the culture medium, *S. brevicaulis* produced extracellular chitin deacetylase (10–11 units ml^{-1} culture supernatant).

In the purification of chitin deacetylase from *S. brevicaulis* the column chromatography on Sephadex G-25 removed proteins with low molecular weight (data not shown). Subsequent chromatography on Sephadex G-100 (Fig. 1) showed that the solution contained five fractions. Based on the deacetylating activity of eluate against hexa-*N*-acetylchitohexaose, the main fraction with maximum activity was identified and collected. Subsequent purification (Table 1) resulted in 74-fold purification and an overall yield of 38%. This method of enzyme purification was very simple, and the yield was higher than the yields of chitin deacetylase obtained from other fungi (Gao et al., 1995; Kafetzopoulos et al., 1993; Martinou et al., 1993, 2002; Tsigos & Bouriotis, 1995).

The purified protein gave a single band on SDS-PAGE indicting its purity and its apparent molecular weight of 55 kDa. As the apparent molecular weight of the chitin deacetylase was different from that in other reports (Gao et al., 1995; Kafetzopoulos et al., 1993; Martinou et al., 1993, 2002; Tsigos & Bouriotis, 1995). It is suggested that this particular chitin deacetylase should be a novel enzyme. The effect of deacetylation (Table 2) also proved that the purified protein was chitin deacetylase. Moreover, gel filtration chromatography on Sephadex G-75 confirmed the apparent molecular weight of the chitin deacetylase as 55 kDa, the symmetrical shapes of the peak further indicating that the purity of the product was very high (Fig. 2).

3.2. Characteristics of chitin deacetylase

Through purification, the activity of chitin deacetylase dropped to 6 units ml^{-1} mainly because the solution became diluted in the purifying process. Optimum pH (7.5) and

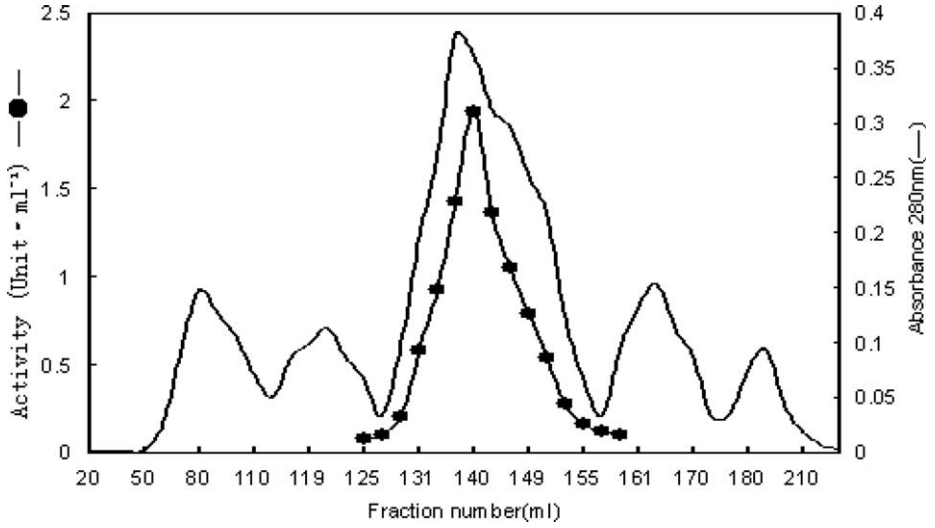

Fig. 1. Purification of chitin deacetylase on Sephadex G-100 chromatography. (–•–) chitin deacetylase activity; (–) absorbance at 280 nm.

temperature (55 °C) of the enzyme were deduced using hexa-*N*-acetylchitohexaose as substrate. The pH optimum was different from that of previous reports (Gao et al., 1995; Kafetzopoulos et al., 1993) and the enzyme showed high activity in the pH range 7.0–9.0 (Fig. 3). The high optimal temperature of the enzyme (Fig. 4) was 55 °C. This temperature is higher than that of the chitin deacetylase from *A. coerulea* (50 °C) (Gao et al., 1995) and *A. nidulans* (50 °C) (Alfonso et al., 1995). In Fig. 4, the curve B is the residual activity of chitin deacetylase after incubation for 1 h at different temperatures. The residual activity was assayed at the high optimal temperature of the enzyme (55 °C). The results showed that the enzyme possessed high thermal stability; full activity was retained after the enzyme had been pre-incubated in the range of 30–55 °C for 1 h (Fig. 4).

3.3. Deacetytion of *N*-acetylchitooligosaccharides substrates

In order to test the deacetylaton specificity of the enzyme, six *N*-acetyl-chitooligosaccharides with degrees of polymerization 2–6 were used as substrates (Table 2). The results showed that the enzyme had the deacetylating activity for the *N*-acetyl-chitooligosaccharides with DP of 2–6, but not for 2-acetamido-2-deoxy-glucose (GlcNAc). This was accord with the data for chitin deacetylase from *C. lindemuthianum* (Tsigos & Bouriotis, 1995), but different from those of *M. rouxii* and *A. coerulea*. The enzyme from *M. rouxi* required at least four contiguous *N*-acetylglucosamine residues for catalysis (Kafezopoulos et al., 1995), and the enzyme from *A. coerulea* is active on chitooligosaccharides with more than two *N*-acetylglucosamine residues (Gao et al., 1995). Furthermore, the activity of the enzyme increased with increases in degree of polymerization of the *N*-acetylchitooligosaccharides. In the range of substrates studied and under the conditions studied, the enzyme had the maximum activity for the hexa-*N*-acetylchitohexaose and the extent of deacetylation was 56%.

Table 1
Purification of chitin deacetylase

Method of purification	Total protein (μg)	Enzyme activity (units)	Specific activity (units mg^{-1})	Activity (%) yield	Purification (fold)
Crude extract	2326	82	0.035	100	1
Ammonium sulfate	207	56	0.27	68.29	8
Sephadex G-25	105	48	0.46	58.54	13
Sephadex G-100	12	31	2.58	37.80	74

One unit of enzyme liberates 1 μmol of acetate/min.

3.4. Deacetylation of chitin substrates

The structures of products from use of shrimp crystalline chitin, chitin from *A. niger* and water-soluble chitosan as substrates were investigated. In the FT-IR spectrum of the water-soluble chitosan (Fig. 5B), the absorption bands at 1655,

Table 2
Deacetylation of *N*-acetylchitooligosaccharides and chitin substrates

Substrate	ASAR (nmol)	Acetate released (nmol)	Degree of deacetylation of substrate (%)	Degree of deacetylation of product (%)	Deacetylation (%)
GlcNAc	224	0	0	0	0
$(GlcNAc)_2$	234	28	0	12	12.0
$(GlcNAc)_3$	237	67	0	28	28.3
$(GlcNAc)_4$	240	88	0	37	36.7
$(GlcNAc)_5$	240	102	0	43	42.5
$(GlcNAc)_6$	240	135	0	56	56.3
Shrimp crystalline Chitin	1043	39	13	16	3.7
Chitin from *Aspergillus niger* water-soluble chitosan	763	254	21	47	33.3
	655	221	54	70	33.7

ASAR, amount of substrate as acetyl residues.

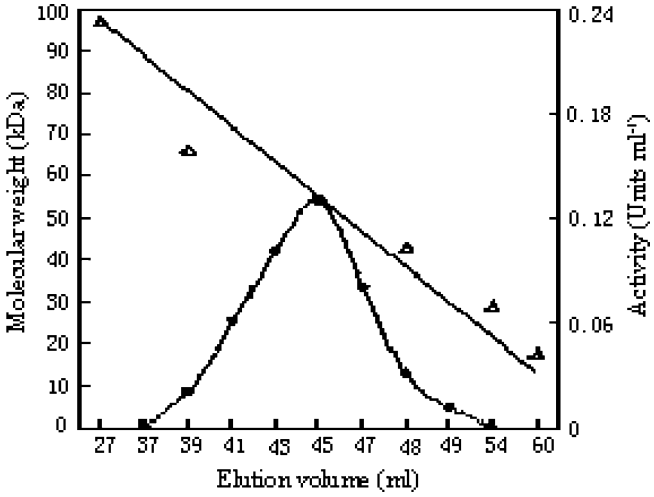

Fig. 2. Estimation of molecular weight of chitin deacetylase by gel filtration chromatography. –•– chitin deacetylase activity; –△– molecular weight standards.

1550 and 1325 cm^{-1} are characteristic peaks of acetamido groups and had been reported to be the amide I, II, III bands, respectively (Van de Velde & Kiekens, 2004). The absorption band at 1600 cm^{-1} was the amino characteristic peak; the band at 1377 cm^{-1} was due to the methyl symmetrical deformation mode (Brugnerotto, Goycoolea, Arguelles-Monal, Desbrieres, & Rinaudo, 2001). After enzyme deacetylation (Fig. 5A), the absorbance of the band at 1655 cm^{-1} was reduced, the bands at 1550, 1377 and 1325 cm^{-1} disappeared, and the absorbance of the band at 1600 cm^{-1} increased, which indicated that the acetyl groups were partially removed. The degree of deacetylation of enzyme-treated products was quantitatively determined using potentiometric titration (Tolaimate et al., 2000). The results (Table 2) showed that the degrees of deacetylations caused by enzyme-treatment of water-soluble chitosan and chitin from *A. niger* were 33.7 and 33.0%, respectively. This further indicated that the deacetylation of the enzyme was significant. However, the deacetylation rate of shrimp crystalline chitin was only 3.7%, although this was

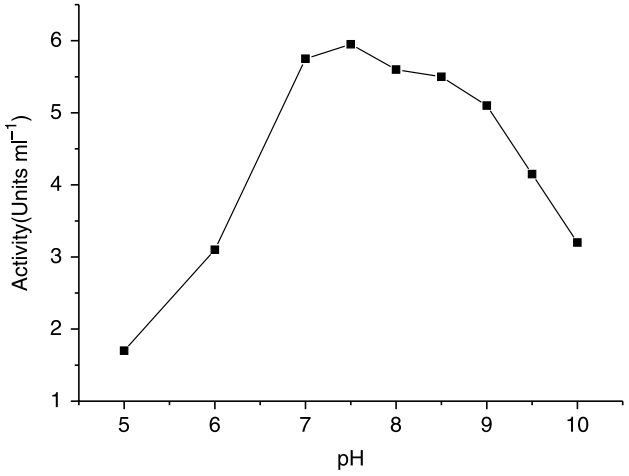

Fig. 3. Effect of pH on chitin deacetylase activity.

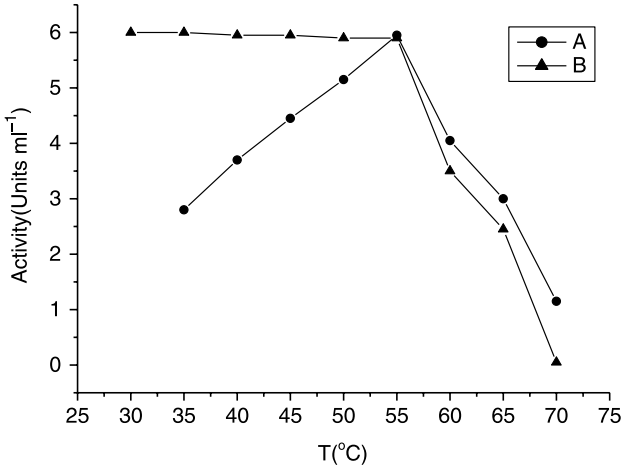

Fig. 4. Effect of temperature on chitin deacetylase activity and stability—(A) initial activity; (B) residual activity after incubation for 1 h.

higher than those of other researches (Gao et al., 1995; Tsigos & Bouriotis, 1995). Compared with water-soluble chitosan and chitin from *A. niger*, such low deacetylation rate of shrimp crystalline chitin could be related to its crystalline state solid.

The X-ray diffraction patterns of shrimp crystalline chitin, water-soluble chitosan and chitin from *A. niger* (Fig. 6), showed diffraction angles ranging from 10 to 40°. The diffraction pattern of shrimp crystalline chitin showed the characteristic peaks around 12.6, 19.5, 23.8, 26.3, 34.9 and 38.9°, the same as those previously reported, and are assigned to the corresponding diffraction planes (020), (110), and (110) of the shrimp type of α-form orthorhombic crystal structure (Zhang, Haga, Sekiguchi, & Hirano, 2000). But water-soluble chitosan and chitin from *A. niger* only had a broad diffraction peak around 20°, and the intensity of this peak was far less than that of the peak of shrimp crystalline chitin around 19.5°. Therefore, water-soluble chitosan and chitin from *A. niger* were amorphous, but the crystallinity of shrimp crystalline

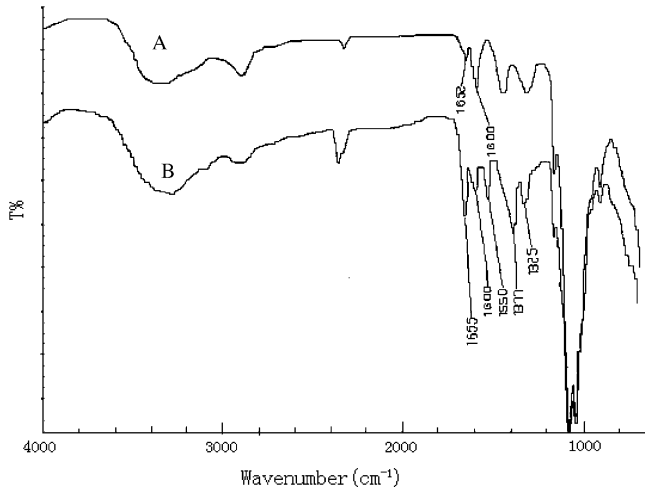

Fig. 5. FT-IR spectra of chitin deacetylase deacetylated water-soluble chitosan and water-soluble chitosan. (A) Product of deacetylated water-soluble chitosan with chitin deacetylase; (B) water-soluble chitosan.

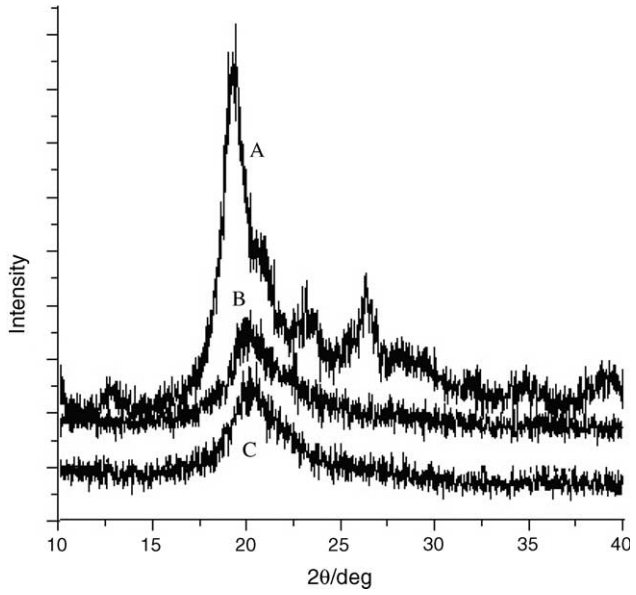

Fig. 6. X-ray diffraction patterns of (A) shrimp crystalline chitin; (B) chitin from *Aspergillus niger* and (C) water-soluble chitosan.

chitin was very high. This high crystallinity made the interaction between the molecules of shrimp crystalline chitin strong so that it was difficult for the enzyme to access the acetyl groups. Therefore for shrimp crystalline chitin, a pre-treatment for destroying chitin crystalline structure prior to enzyme addition is necessary in order to improve deacetylation rate. (The pre-treatment process is, briefly, as follows: cold sulfuric acid (15 ml, 98% w/w) was added slowly to a chitin (10 g) and water (2 ml) mix at 1 °C to form a micelle. Then the solution was neutralized with a NaOH aqueous solution. The precipitate was washed with distilled water and dried yielding an amorphous chitin.) The results were similar to those for chitin deacetylase from *C. lindemuthianum* (Tsigos & Bouriotis, 1995) and *M. rouxii* (Kafezopoulos et al., 1995).

4. Conclusions

In this study, a novel extracellular chitin deacetylase has been isolated from the culture medium of *S. brevicaulis* through ammonium sulfate precipitation, of Sephadex G-25 Sephadex G-100 column chromatographies. Compared with all other corresponding enzymes reported, the enzyme exhibits different properties, as thermostability, different pH optimum and enzyme activity. The enzyme is only active on chitooligosaccharides with at least two *N*-acetyl-glucosamine residues and has high deacetylating activity on amorphous chitin and chitosan. Thus, it has potential application in the effective deacetylation of chitinous and chitosanous substrates. However, the substrate recognition and the mechanism of enzyme action on chitooligosaccharides, chitin and chitosan were not completely clear. The structure and the active center of enzyme need to be studied further. At present, detailed works are now in progress.

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