J. Microbiol. Biotechnol. (2008), **18**(4), 759–766

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Enzymatic Deacetylation of Chitin by Extracellular Chitin Deacetylase from a Newly Screened *Mortierella* sp. DY-52

Kim, Young-Ju, Yong Zhao, Kyung-Taek Oh, Van-Nam Nguyen, and Ro-Dong Park*

Glucosamine Saccharide Materials-National Research Laboratory (GSM-NRL), Division of Applied Bioscience and Biotechnology, Institute of Agricultural Science and Technology, Chonnam National University, Gwangju 500-757, Korea

Received: August 17, 2007 / Accepted: October 16, 2007

Among more than a hundred colonies of fungi isolated from soil samples, DY-52 has been screened as an extracellular chitin deacetylase (CDA) producer. The isolate was further identified as *Mortierella* sp., based on the morphological properties and the nucleotide sequence of its 18S rRNA gene. The fungus exhibited maximal growth in yeast peptone glucose (YPD) liquid medium containing 2% of glucose at pH 5.0 and 28°C with 150 rpm. The CDA activity of DY-52 was maximal (20 U/mg) on the 3rd day of culture in the same medium. The CDA was inducible by addition of glucose and chitin. The enzyme contained two isoforms of molecular mass 50 kDa and 59 kDa. This enzyme showed a maximal activity at pH 5.5 and 60°C. In addition, it had a pH stability range of 4.5–8.0 and a temperature stability range of 4–40°C. The enzyme was enhanced in the presence of Co^{2+} and Ca^{2+}. Among various substrates tested, WSCT-50 (water-soluble chitin, degree of deacetylation 50%), glycol chitin, and crab chitosan (DD 71–88%) were deacetylated. Moreover, the CDA can handle *N*-acetylglucosamine oligomers $(GlcNAc)_{2-7}$.

Keywords: Chitin deacetylase, water-soluble chitin, *Mortierella* sp. DY-52, *N*-acetylchitooligomers

Next to cellulose, chitin is one of the most abundant, easily obtained, and renewable natural polymers in the world. It is commonly found in the exoskeletons or cuticles of many invertebrates [10] and in the cell walls of most fungi and some algae [19]. In several Zygomycetes species, it exists in its deacetylated form as chitosan.

Chitosan is a biopolymer with unique properties favorable for a broad variety of industrial applications [21]. Presently, chitosan is produced by the thermochemical alkaline deacetylation of chitin. However, the process is environmentally unsafe and not easily controlled, leading to a broad and heterogeneous range of products [6]. To develop an alternative, controlled, nondegradative, and well-defined process for chitosan production, studies of fungal chitin deacetylase have been initiated [1, 2, 5, 7, 13, 18].

Recently, chitin deacetylase (CDA; E.C. 3.5.1.41), the enzyme that catalyzes the conversion of chitin to chitosan by the deacetylation of *N*-acetylglucosamine residues, has attracted increasing interest, owing to its possible applicability for preparation of specific novel chitosan oligomers and polymers from chitin oligomers or pretreated chitin [14]. It was first identified and partially purified from extracts of the fungus *Mucor rouxii* [2]. Since then, the presence of this enzyme activity has been reported in several other fungi [1, 5, 7, 13, 18] and in some insect species [3] and bacteria strain [22].

To apply CDA for enzymatic conversion of chitin to chitosan industrially, intensive screening of strong CDA producers is still necessary because only limited information is available [28]. In the present study, we screened a new extracellular CDA-producing fungus, which was further identified as *Mortierella* sp. In addition, the properties of the newly screened CDA were investigated, including cultivation conditions for CDA production.

Materials and Methods

Isolation of Fungi from Soils

Dried soil samples of 1 g were diluted in 50 ml of distilled water, and pre-incubated at 30°C on a rotary shaker operating at 180 rpm for 12 h. Fungi were isolated by a dilution plate technique with peptone rose bengal agar medium. The medium composition was 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 g peptone, 10 g glucose, 0.03 g rose bengal, 0.05 g streptomycin, and 20 g agar, per liter.

Culture Conditions for Chitin Deacetylase Production

Fungal strains for CDA production were incubated for 5 to 7 days on PDA (potato dextrose agar) slants at 25°C. The spore suspension

*Corresponding author
Phone: 82-62-530-2133; Fax: 82-62-530-0876;
E-mail: rdpark@chonnam.ac.kr

(10^6 spores/ml) was allowed to germinate in a 100-ml Erlenmeyer flask containing 50 ml of YPD culture medium (20 g glucose, 3 g yeast extract, 10 g peptone, per liter). The pH was adjusted to 4.5 by HCl. Cultivation of the fungi was carried out at 28°C with shaking at 150 rpm for 5 days.

Intra- and Extracellular Chitin Deacetylase from Fungi

After cultivation of each of the fungi in YPD liquid medium for 5 days, mycelium and culture supernatant were separated by filtration through a suction funnel with Whatman No. 2 filter paper. The culture supernatant was assayed for extracellular CDA enzyme. The mycelium was washed with cold distilled water and then homogenized by grinding with liquid nitrogen in a mortar. The homogenate was then diluted in Tris-HCl buffer (50 mM, pH 7.2) with the ratio of 1:7 (w/v). The resulting supernatants obtained from centrifugation at 10,000 rpm for 30 min were assayed for intracellular CDA enzyme.

Identification of Fungus DY-52

Identification of the fungus was carried out on the basis of morphological characteristics and the nucleotide sequence of the 18S rRNA. To determine the 18S rRNA sequence, the genomic DNA was isolated by the G-spin Genomic DNA Extraction Kit (Intron Inc., Suwon, Korea). Universal primers (ITS1-F:5'-CTTG-GTCATTTAGAGGAAGT-3', and ITS4-R:5'-TCCTCCGCTTATTG-ATATGC-3') were synthesized and used for the PCR reactions. The polymerase chain reaction (PCR) was performed to amplify the partial 18S rRNA fragment of the fungus using a GeneAmp Thermal Cycler (Model 2400; Perkin-Elmer, Norwalk, CT, U.S.A.). The amplified PCR product was purified from the agarose gels using a gel elution kit (Bioneer Inc., Daejeon, Korea) and cloned into a pGEM-T vector (Promega Co., Madison, WI, U.S.A.). The nucleotide sequence of the 18S rRNA gene of DY-52 was determined using an Applied Biosystem (ABI373) DNA automated sequencer (PE Applied Biosystem, Foster City, CA, U.S.A.) and then aligned with reference sequences obtained from the GenBank databases (NCBI, Bethesda, MO, U.S.A.). The fungus, DY-52, was identified as a *Mortierella* sp. The DNA manipulations for the cloning, transformation, plasmid isolation, and ligation were done according to the method described by White *et al.* [29].

Enzyme Assays

Chitin deacetylase (CDA) activity was estimated using a substrate, WSCT-50. Enzyme activity assay was performed under standard conditions at 50°C in a test tube containing 2.5 mg of WSCT-50 dissolved in 0.5 ml of 50 mM sodium glutamate buffer (pH 4.5) and 0.1 ml of crude enzyme. After incubation for 1 h, the reaction was terminated by heating at 100°C for 15 min. The acetic acid released in the enzymatic deacetylation was analyzed by a gas-liquid chromatography (GLC) method [27]. One unit of chitin deacetylase activity was defined as the amount of enzyme that released 1 μmol acetic acid per min under the standard conditions described above. WSCT-50 as the substrate was prepared according to the method of Sannan *et al.* [20]. The degree of deacetylation was measured by a colloidal titration method [24].

Effect of pH, Temperature, Acetate, and Metal Ions on Enzymatic Deacetylation

To determine the optimum temperature, enzyme reaction was controlled at various temperatures (20–70°C) for 1 h. For the temperature stability, enzyme was pre-incubated at various temperatures (20–70°C) for 1 h without substrate. To determine the optimum pH, the enzyme reaction was carried out in 50 mM sodium glutamate buffer (pH 3.5–5.5), 50 mM phosphate buffer (pH 5.0–8.5), 50 mM Tris-HCl buffer (pH 7.0–9.5), and 50 mM Na_2CO_3 buffer (pH 9.0–11.5) for 1 h. For the pH stability, the enzyme was pre-incubated in the various pH buffers at 4°C overnight. Various concentrations of acetate and metal ions were added to the enzyme reaction mixture to study their effect on the enzyme activity.

Deacetylation of *N*-Acetylchitooligosaccharides $(GlcNAc)_{2-7}$ and Other Chitinous Substrates

In order to test the substrate specificity of this enzyme, *N*-acetylchitooligomers [$(GlcNAc)_{2-7}$] and other chitinous substrates were incubated with the enzyme under standard enzyme assay conditions. WSCT-50 was used as the reference substrate.

Detection of Chitin Deacetylase Activity After SDS-PAGE

Polyacrylamide gel electrophoresis under denaturing condition was performed as described by Laemmli [15] in 12.5% polyacrylamide gels containing 0.1% (w/v) glycol chitin. After SDS-PAGE, gels were incubated at 37°C for 2 h with gentle shaking in 50 mM sodium glutamate buffer (pH 4.5) containing 1% purified Triton X-100 and 1% skim milk for nature of CDA enzymes. Then, the gels were incubated overnight at 37°C with gentle shaking in 50 mM sodium glutamate buffer (pH 4.5) containing 1% purified Triton X-100. After incubation, gels were transferred in a fresh solution of 0.01% Calcofluor white M2R in 0.5 M Tris-HCl (pH 8.6) buffer for 7 min. The gels were washed by distilled water for at least 2 h. Proteins exhibiting enzyme activity were visualized under UV light as a light band [26].

Other Methods

To determine the cell growth of the fungus, the separated mycelium was dried at 80°C for 24 h and the dry weight was evaluated. Reducing sugar was measured by Schales' method [9]. The protein concentration was determined by the Bradford method [4], using bovine serum albumin as the standard.

RESULTS

Screening and Identification of the CDA-producing Fungus DY-52

Among the more than 100 isolates screened, strain DY-52 was isolated for an extracellular CDA producer. It showed a very high specific enzyme activity of 3.75 U/mg, which was almost 10-fold higher than that of *M. rouxii* (0.39 U/mg), which is an intracellular CDA producer (Table 1). Total CDA activity (30.8 U) from a 50 ml culture of DY-52 was 6 times higher than that of *M. rouxii* (5.2 U).

To identify the isolate DY-52, the growth shape of the fungal colony, spore shape, and 18S rRNA nucleotide sequence were analyzed. It grew very slowly, akin to the way of clouds spreading. It produced a small number of spores per ml and then most existed as spawns. The spores

Table 1. Comparison of CDA activity between DY-52 and *Mucor rouxii*.

Strain	Extracellular			Intracellular		
	U/ml	U/mg	Total activity (U)	U/ml	U/mg	Total activity (U)
DY-52	0.60	3.75	30.0	0.09	0.04	0.78
M. rouxii (ATCC 24905)	0.02	0.07	1.0	0.40	0.39	4.2

After cultivation of *Mortierella* sp. DY-52 and *Mucor rouxii* in 50 ml of YPD broth medium for 5 days and 3 days, respectively, extra- and intracellular enzymes were prepared and the activities were measured as described in Materials and Methods.

resembled a circle of 21.6 μm diameter. In a comparison of the partial nucleotide sequence of the 18S rRNA with that of *Mortierella* sp., 606 out of 628 nucleotides were identical, indicating 96% similarity. Based on these results, DY-52 was identified to be a *Mortierella* sp. (Fig. 1).

Optimization of Culture Condition for CDA Production

To determine the effect of carbon source for CDA production, various carbon sources (2% each) were examined in the liquid medium. The highest CDA activity was observed at 3 days of cultivation, where glucose was added. Powder chitin and swollen chitin also induced the extracellular CDA but in a lesser extent than glucose. Little CDA activity was detected in MeOH addition (Fig. 2A). The mixtures of glucose plus maltose and glucose plus swollen chitin (1% each) also induced CDA, but the addition of glucose plus swollen chitin delayed the maximum induction of CDA to the 5th day (Fig. 2B). Therefore, only glucose was added as a carbon source for production of CDA in subsequent experiments. It was found that CDA production was maximal at initial pH 4.5–5.0 and decreased dramatically below pH 4.0 (Fig. 3). About 60% of the maximum CDA production can be achieved at pH 6.0. The optimum temperature was 25–28°C, and less than 60% of the maximum activity was observed at 20°C and 30°C (Fig. 3B). In addition, the optimum agitation speed for CDA production was found to be 150 rpm (Fig. 3C).

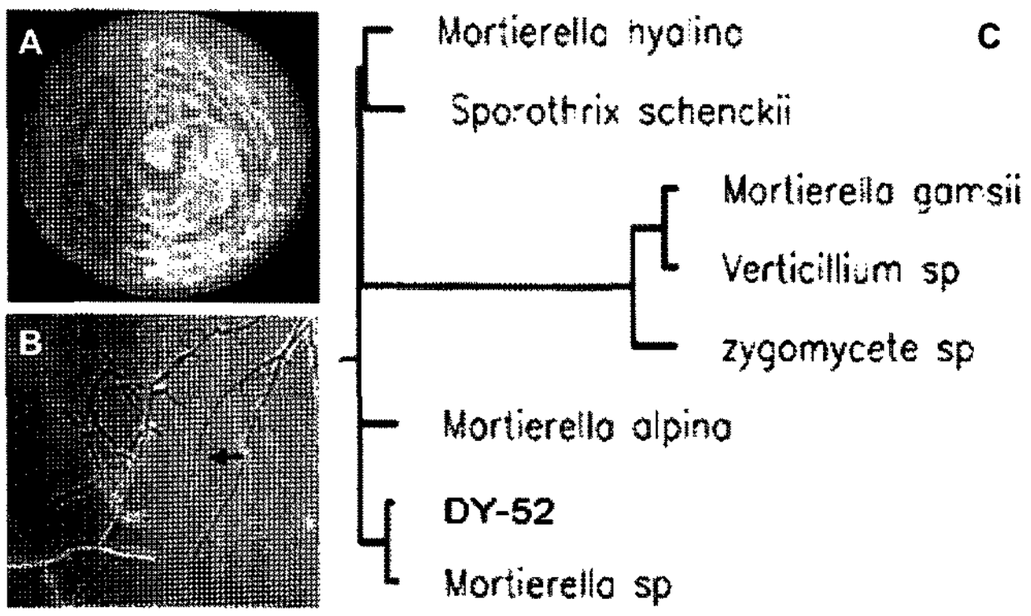

Fig. 1. Photographs of colony (**A**) and sporangia (100×) by microscopy (**B**), and a phylogenic tree of the 18S rRNA gene (**C**) of *Mortierella* sp. DY-52.

Profile of CDA Production at the Optimum Condition

Fig. 4A shows the cell growth and CDA enzyme activity during fermentation for *Mortierella* sp. DY-52 in YPD medium with 2.0% concentration of glucose at a temperature of 28°C, with shaking at a speed of 150 rpm. *Mortierella* sp. DY-52 grew linearly up to the 4th day followed by a stationary phase for more than 3 days. CDA was secreted dramatically for the first 2 days, and then maintained a constant level (0.6 U/ml and 20 U/mg on the 3rd day), and subsequently declined sharply from the sixth day. The pH of the culture fluid, residual sugar concentration, and protein concentration were also measured (Fig. 4B). The pH maintained almost constant acidic level between 4 and 6, except reaching pH 7 at the second day. The level of residual sugar decreased constantly from 110 mM to 20 mM at day 7. The extracellular protein contents maintained at a

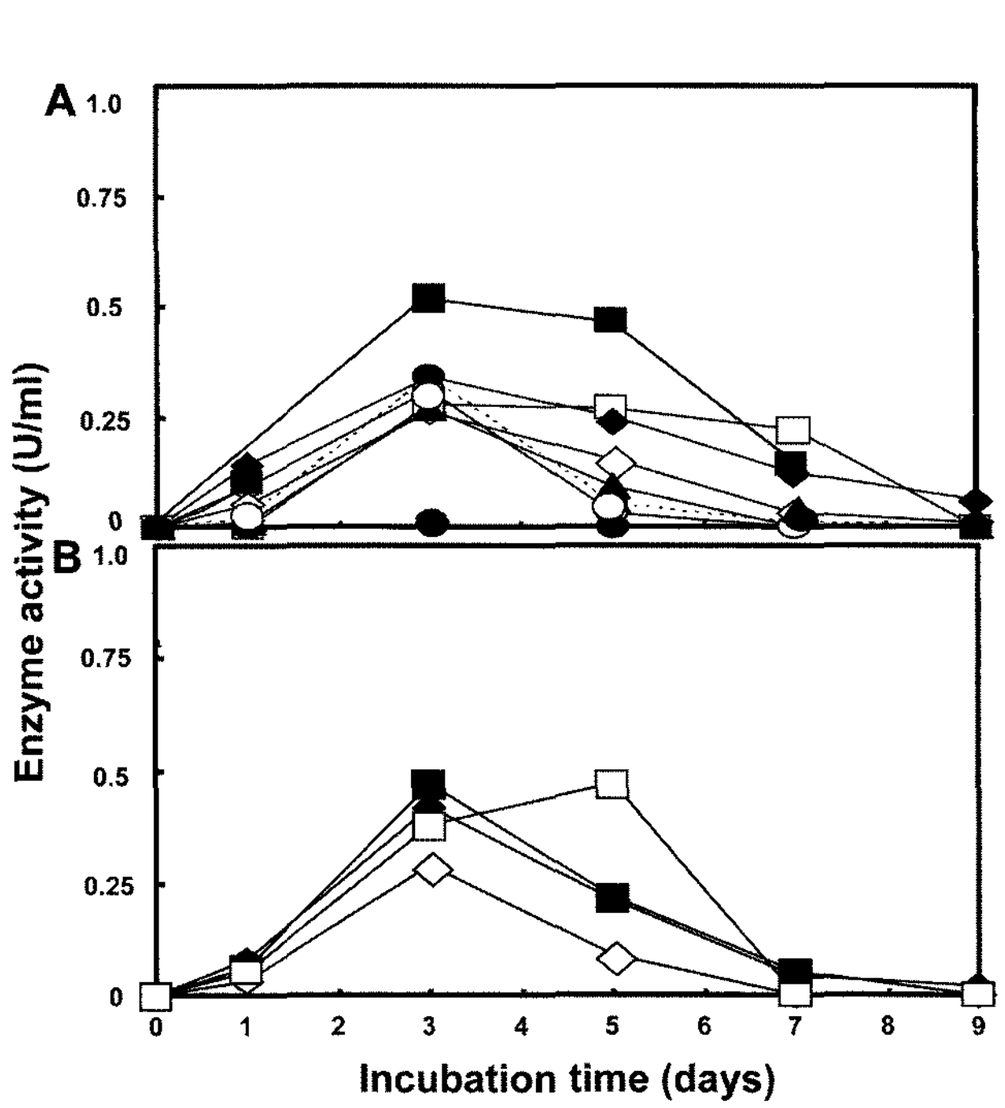

Fig. 2. Effect of carbon sources on CDA production of *Mortierella* sp. DY-52.

A. Carbon sources (-○- None; ··●·· Sucrose; -■- Glucose; -□- Fructose; -▲- Chitin powder; -▲- Swollen chitin; -◆- Glycerol; -●- MeOH) at 2% was added to the YPD growth medium for *Mortierella* sp. DY-52. **B**. Mixtures of 1% glucose and 1% other carbon sources (-◆- Fructose; -■- Maltose; -△- Sucrose; -□- Swollen chitin) were added.

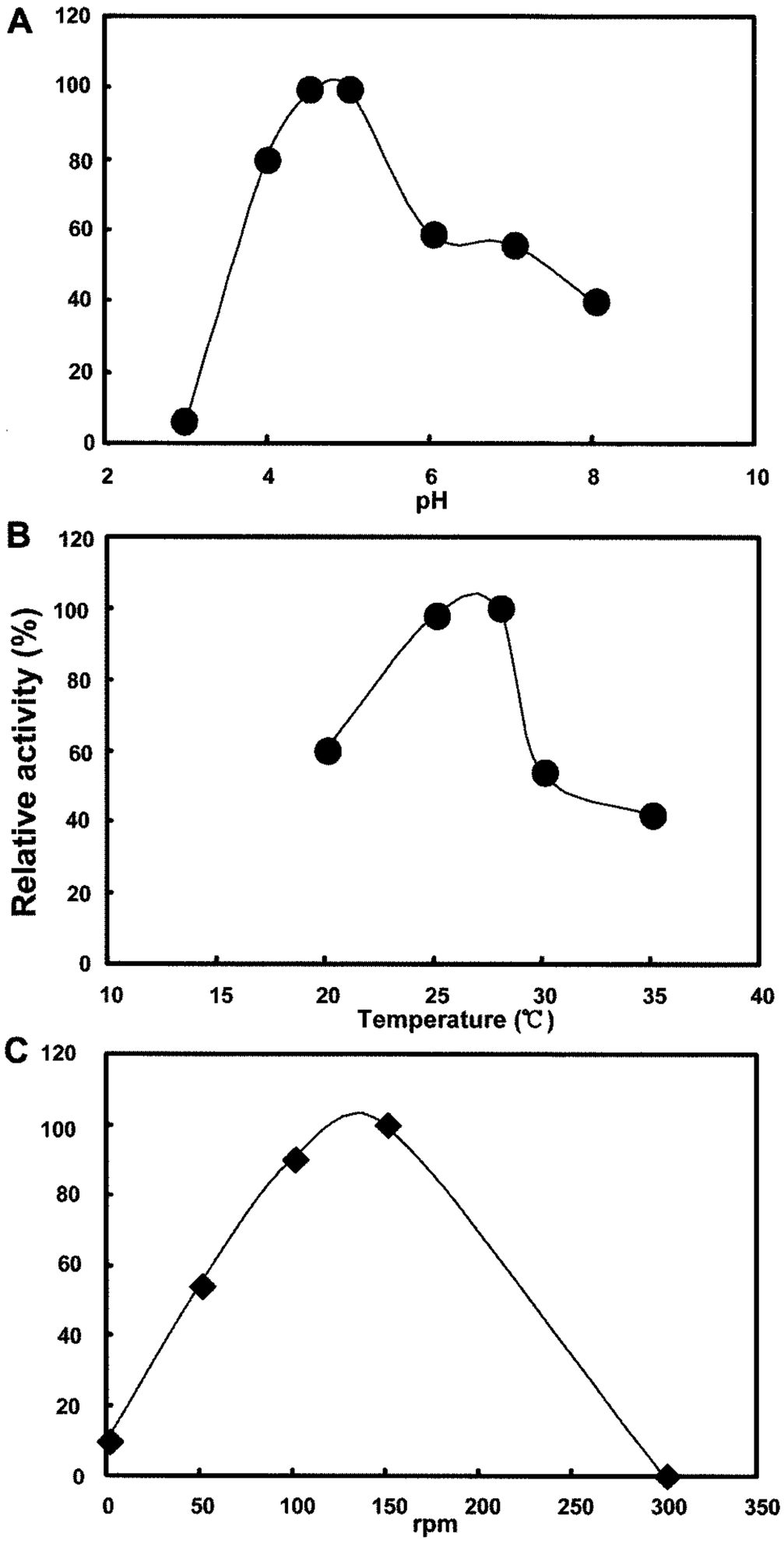

Fig. 3. Effects of initial pH (**A**), temperature (**B**), and agitation speed (**C**) on CDA production by *Mortierella* sp. DY-52.

low level for the first 4 days but increased strongly on the fifth day.

Enzyme Activity Characterization

To investigate the enzymatic deacetylation of water-soluble chitin (WSCT-50, degree of deacetylation 50%), 1–5 mg of WSCT-50 was dissolved in 1 ml of 50 mM sodium glutamate buffer (pH 4.5) and incubated with 0.2 ml of crude enzyme at 50°C for various times. The acetic acid released was proportional to the substrate amount (Fig. 5A) and the enzyme amount (Fig. 5B). The enzymatic conversion was very fast during the first 30 min and then slowed down

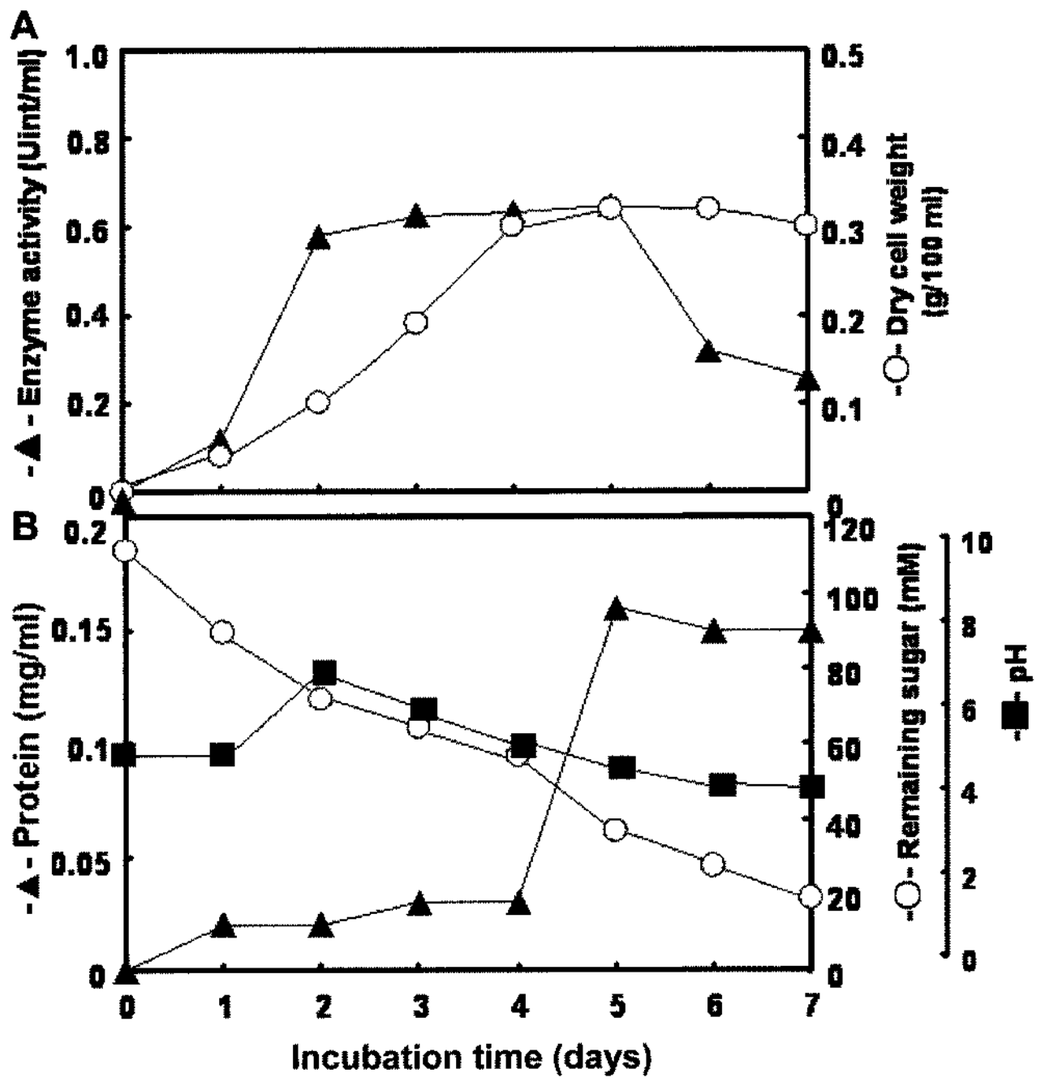

Fig. 4. Time course of CDA production during the culture of *Mortierella* sp. DY-52 at the optimum culture condition for 7 days.

A. CDA activity (-▲-) and dry cell weight (-○-). **B**. Protein content (-▲-), pH (-■-), and remaining sugar concentration (-○-).

with 5 mg WSCT-50 as substrate and 0.2 ml (1.2 U) enzyme, showing biphasic kinetics (Figs. 5A and 5B).

Optimum pH and Temperature

As shown in Fig. 6A, the optimum pH of the enzyme was estimated to be 5.5 using WSCT-50 as the substrate. At pH 4.5, the enzyme lost about 60% of the maximum activity. The enzyme was stable between pH 5 and 7.5 (Fig. 6B). Meanwhile, the optimum temperature was found to be 60°C with the same substrate (Fig. 7A). The enzyme was stable below 40°C, but the stability clearly disappeared at above 60°C (Fig. 7B).

Substrate Specificity

Under standard enzyme assay conditions, various chitinous substrates were tested as the substrate of the CDA. As shown in Table 2, the enzyme worked on WSCT-50, glycol chitin, and crab chitosan (DD 71–88%) for deacetylation with a wide range of affinities (7–100%). Among them, WSCT-50 was the most accessible substrate for the enzyme. Other chitins with different DD values and particle sizes were little deacetylated by the crude enzyme.

Results of deacetylation of $(GlcNAc)_{2-7}$ by DY-52 CDA is shown in Table 3. It was revealed that the enzyme could not only deacetylate $(GlcNAc)_{4-7}$ but also $(GlcNAc)_{2-3}$. The highest enzyme activity was achieved when chitin heptamer was used as a substrate. The longer the length of

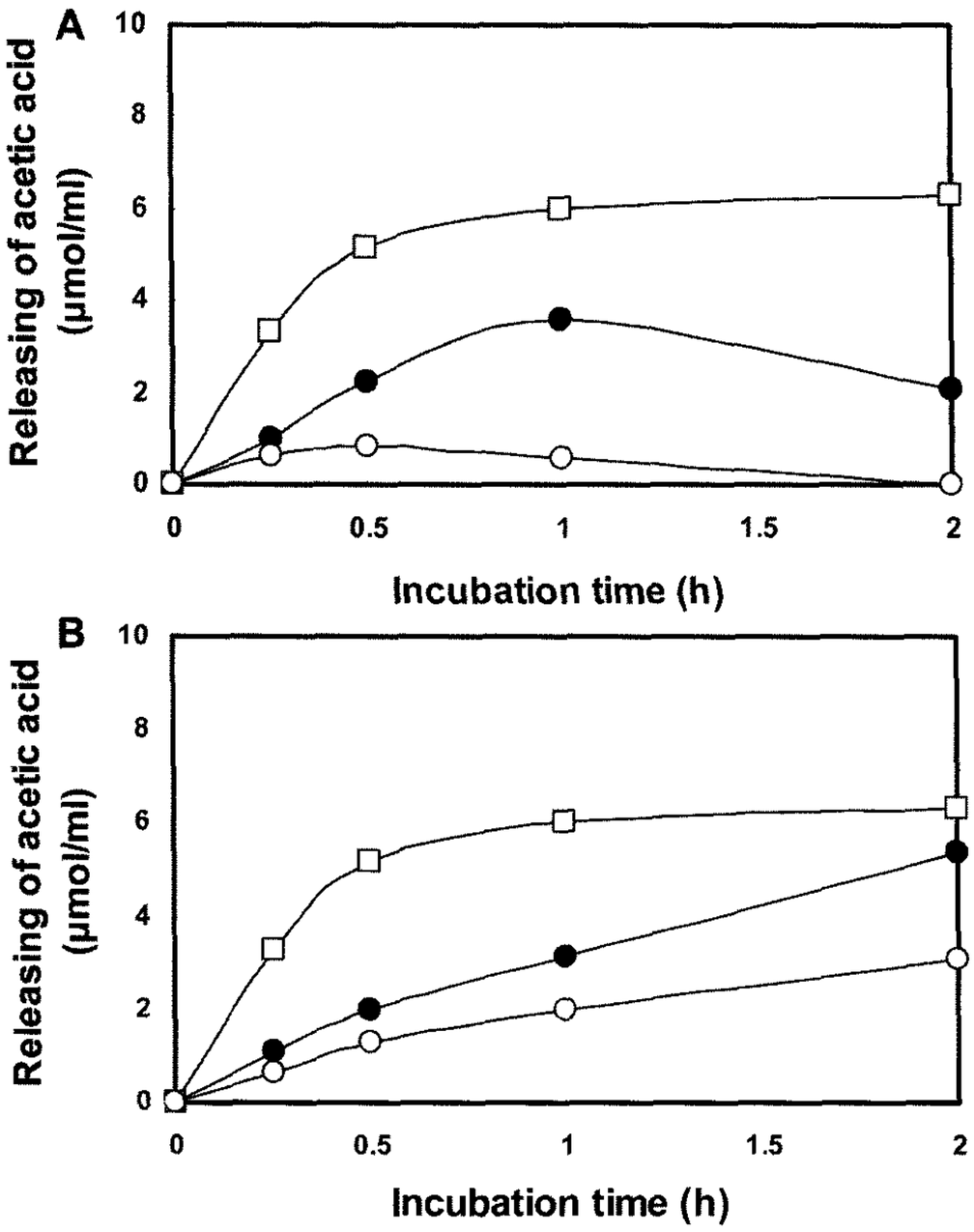

Fig. 5. Effects of the amount of substrate (**A**) and enzyme (**B**) on releasing of acetic acid by the CDA.

A. For enzyme assay, different amounts of substrates (-○- 1 mg/ml; -●- 3 mg/ml; -□- 5 mg/ml) were incubated with 0.2 ml of crude enzyme (0.6 U/ml) in a total volume of 1.2 ml for 2 h. **B**. Different amount of crude enzyme preparation (-○- 0.03 units; -●- 0.06 units; -□- 0.12 units) were incubated with 5 mg of WSCT-50 for 3 h in a total volume of 1.2 ml.

N-acetylchitooligosaccharide was, the higher was the rate of deacetylation.

Effect of Metal Ions on CDA Activity

As shown in Table 4, the enzyme activity was affected in the presence of various metal ions. Hg^{2+}, Zn^{2+}, EDTA, Ag^{+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, and Sn^{2+} were inhibitory at concentrations of 1 and 10 mM, whereas Ca^{2+} (10 mM), and Co^{2+} (0.01–10 mM) could act as activators. Specifucally, the activation by Co^{2+} was dramatic even at the very low concentration of 0.01 mM (Table 5). The effect of acetic acid, a product of the enzyme action, was also investigated. The activity was not inhibited in the presence of 0–1 mM acetic acid, sodium acetate, and magnesium acetate. The inhibition occurred from 2 mM of acetate and the enzyme activity could be inhibited to the level of 50–90% at 10 mM (data not shown).

Detection of Chitin Deacetylase Activity After SDS-PAGE

By both protein and activity stainings on SDS-PAGE by the method of Trudel and Asselin [26], it was revealed that

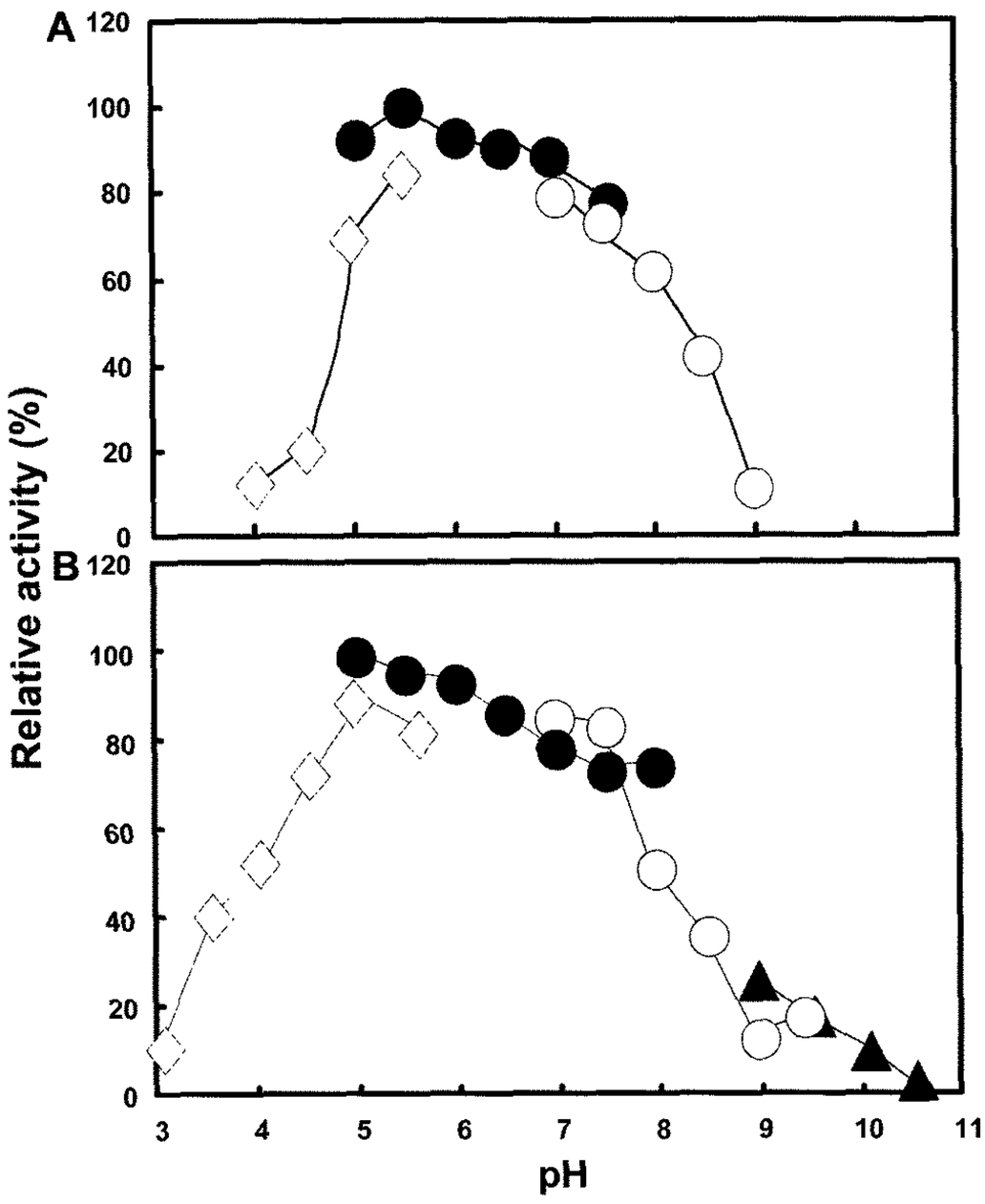

Fig. 6. Effects of pH on the activity (**A**) and stability (**B**) of CDA from *Mortierella* sp. DY-52.

The buffers used are -◇- 50 mM sodium glutamate buffer (pH 3.0–5.5); -●- 50 mM phosphate buffer (pH 5.0–8.5); -○- 50 mM Tris-HCl buffer (pH 7.0–9.5); and -▲- 50 mM Na_2CO_3 buffer (pH 9–11.5).

the crude enzyme preparation contained two isoforms of molecular mass 50 kDa and 59 kDa, respectively (Fig. 8).

DISCUSSION

Chitin deacetylase (CDA) is an enzyme that has been separated mostly either from fungal mycelium or from culture medium. However, extracellular CDA has more potential and is more meaningful to apply for bioconversion of chitin to chitosan in industry, since the enzyme preparation is much easier to obtain.

In this work, an extracellular CDA-producing fungus, DY-52, was screened from environmental soil samples and further identified as a *Mortierella* sp. by the morphological characteristics and the 18S rRNA gene. To our knowledge, this is the first report on CDA from *Mortierella* species. Furthermore, the CDA enzyme activity was detected by activity staining after SDS-PAGE (Fig. 8). The two white bands on activity staining (Fig. 8C) corresponding to two dark bands on SDS-PAGE (Fig. 8B) suggested the presence of two isoforms for the enzyme. The isoforms may represent the products of different *cda* genes or the posttranslational modification products of one gene. Purification of the

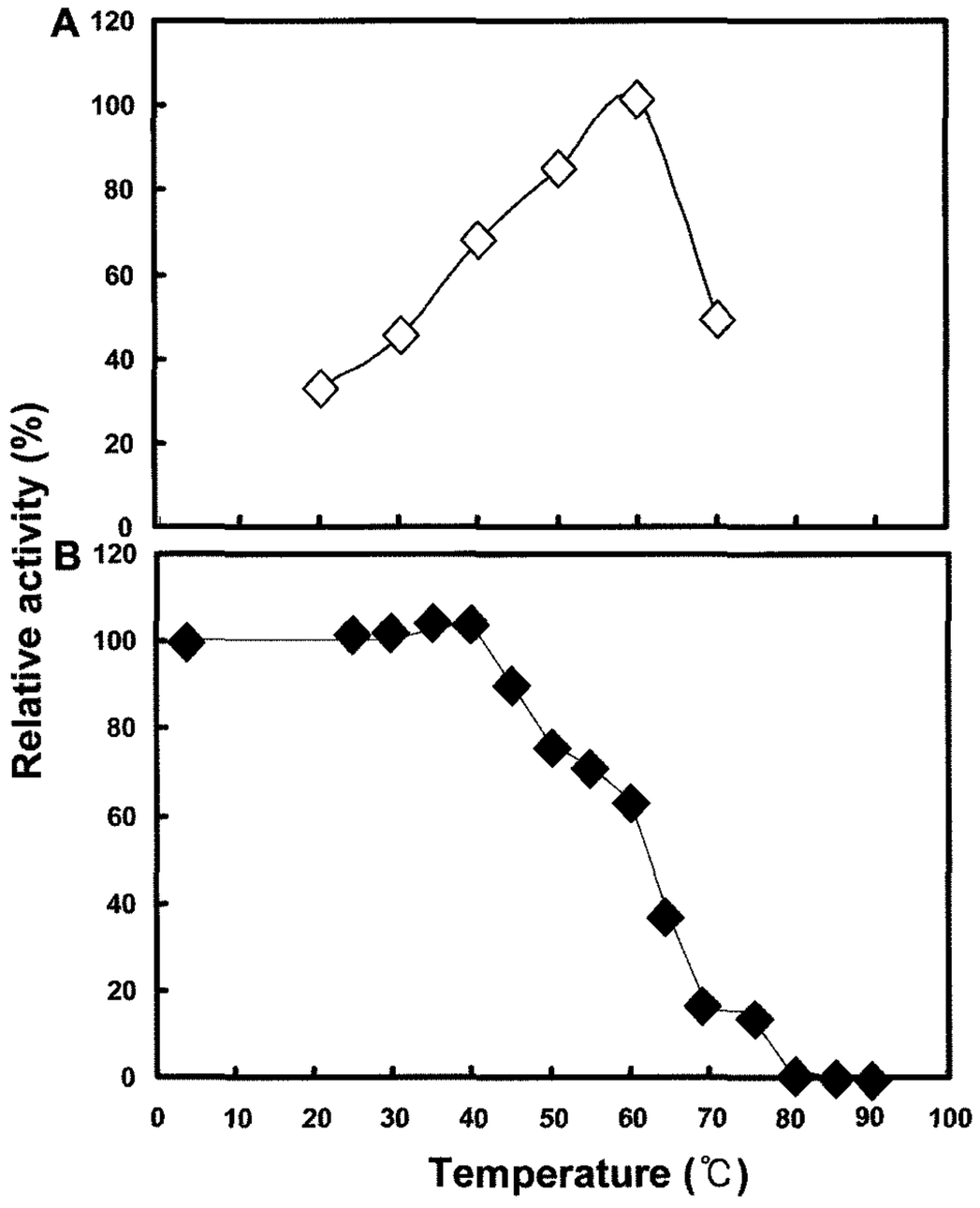

Fig. 7. Effect of temperature on the activity (**A**) and stability (**B**) of the CDA.

isozymes is under way for further study at the molecular level of the CDA.

The CDA was inducible by addition of glucose and chitin, even though it was also secreted into the YPD medium without glucose or chitin (Fig. 2A), which may be explained as that the other components like yeast extract in the cultivation medium functioned as carbon sources for this fungus. In addition, glucose in the cultivation medium was much easier to be used by this fungus compared with insoluble chitin powder and swollen chitin. The highest CDA activity was produced at pH 5.0 (Fig. 3A), a temperature of 28°C (Fig. 3B), and a speed of 150 rpm (Fig. 3C). The CDA production dropped sharply to 50% of the maximum at 30°C, suggesting that the controlling of temperature is important for the production of CDA. The optimum temperature was 30°C for *A. coerulea* [28], *Scopulariopsis brevicaulis* [5], and *Alcaligenes* sp. [22]. As shown in Fig. 4, the CDA level was maximal (20 U/mg) after 3 days of culture at the optimum condition. This rapid production of CDA by DY-52 has advantages for industrial application by recycling of the fermenters, bulky production, and cost-lowering.

Table 2. Substrate specificity of the CDA with various chitinous substrates.

Substrate	Relative activity (%)
WSCT-50 (Crab, DD 50%)*	100
Swollen chitin (DD 8%)	0
Colloidal chitin (DD 8%)	0
Glycol chitin (DD 8%)	35
CM chitin (DD 8%)	0
Regenerated chitin (DD 1%)	0
Crystal β-chitin (DD 8%, 45 μm)	0
Crab chitin (DD 8%, 45 μm)	0
Crab chitin (DD 8%, 150 μm)	0
Crab chitin (DD 8%, 250 μm)	0
Crab chitosan (DD 71%, 150 μm)	49
Crab chitosan (DD 80%, 150 μm)	7
Crab chitosan (DD 88%, 150 μm)	9
Crab chitosan (DD 99%, 150 μm)	0

*Each substrate (5 mg) was pre-incubated briefly in 0.5 ml of 50 mM sodium glutamate buffer (pH 4.5) at 50°C, and then 0.1 ml of crude enzyme preparation (0.7 U/ml) was added and incubated at 50°C for 1 h.

Table 3. Deacetylation of *N*-acetylchitooligosaccharides.

Substrate	Substrate concentration (mg)	Acetic acid released (nmol/min)	Relative activity (%)
$(GlcNAc)_2$	5.5	65	24
$(GlcNAc)_3$	5.5	66	35
$(GlcNAc)_4$	5.5	70	37
$(GlcNAc)_5$	5.5	79	42
$(GlcNAc)_6$	5.5	146	77
$(GlcNAc)_7$	5.5	189	100
WSCT-50	2.5	141	75

*Each substrate (5.5 mg) or WSCT-50 (2.5 mg) was incubated with crude enzyme preparations (70 mU) in a total volume of 0.6 ml of 50 mM sodium glutamate buffer at pH 4.5 for 20 min at 50°C.

Table 4. Effect of metal ions on the CDA from *Mortierella* sp. DY-52.

Metal ions	Relative activity (%)	
	1 mM	10 mM
Control	100	100
Ag^+	72	37
Ca^{2+}	93	126
Co^{2+}	146	160
Cu^{2+}	86	42
Fe^{2+}	54	35
Hg^{2+}	0	0
Mg^{2+}	57	56
Mn^{2+}	16	44
Ni^{2+}	111	49
Pb^{2+}	12	17
Sn^{2+}	34	14
Zn^{2+}	22	0
EDTA	55	0

*Reaction mixture contained 5 mg of WSCT-50, 70 mU crude enzyme preparations, and 1 or 10 mM metal ion in 0.7 ml of 35 mM sodium glutamate buffer (pH 4.5).

Table 5. Effect of Co^{2+} on the CDA from *Mortierella* sp. DY-52.

Concentration (mM)	Relative activity (%)
0	100
0.01	140
0.1	133
1.0	146
10.0	161

*Reaction mixture contained 5 mg of WSCT-50, 70 mU crude enzyme preparations, and 0–10 mM Co^{2+} ion in 0.7 ml of 35 mM sodium glutamate buffer (pH 4.5).

For the crude CDA, optimal pH and temperature were found to be 5.5 (Fig. 6A) and 60°C (Fig. 7A), respectively. Most CDAs have an optimum temperature of 50°C [7, 12, 16]. The CDA from *C. lindemuthianum* showed the optimum activity of 60°C [25].

The optimum pH of the extracellular CDA of DY-52 was acidic pH 5.5, a novel pH property that is different from that of other extracellular CDAs. According to the results reported so far, the optimum pH of extracellular CDAs were neutral [1] or in the alkaline range from pH 11 to 12 and they were not inhibited by acetate [1, 25], whereas the optimum pH of intracellular CDAs were in the acidic range from pH 4.0 to 5.8 and they were inhibited by acetate [7, 12]. However, the optimum pH of the extracellular CDA from *Mortierella* sp. DY-52 was acidic and inhibited by acetate at 10 mM. This may suggest a different mechanism between DY-52 CDA and other extracellular or intracellular CDAs.

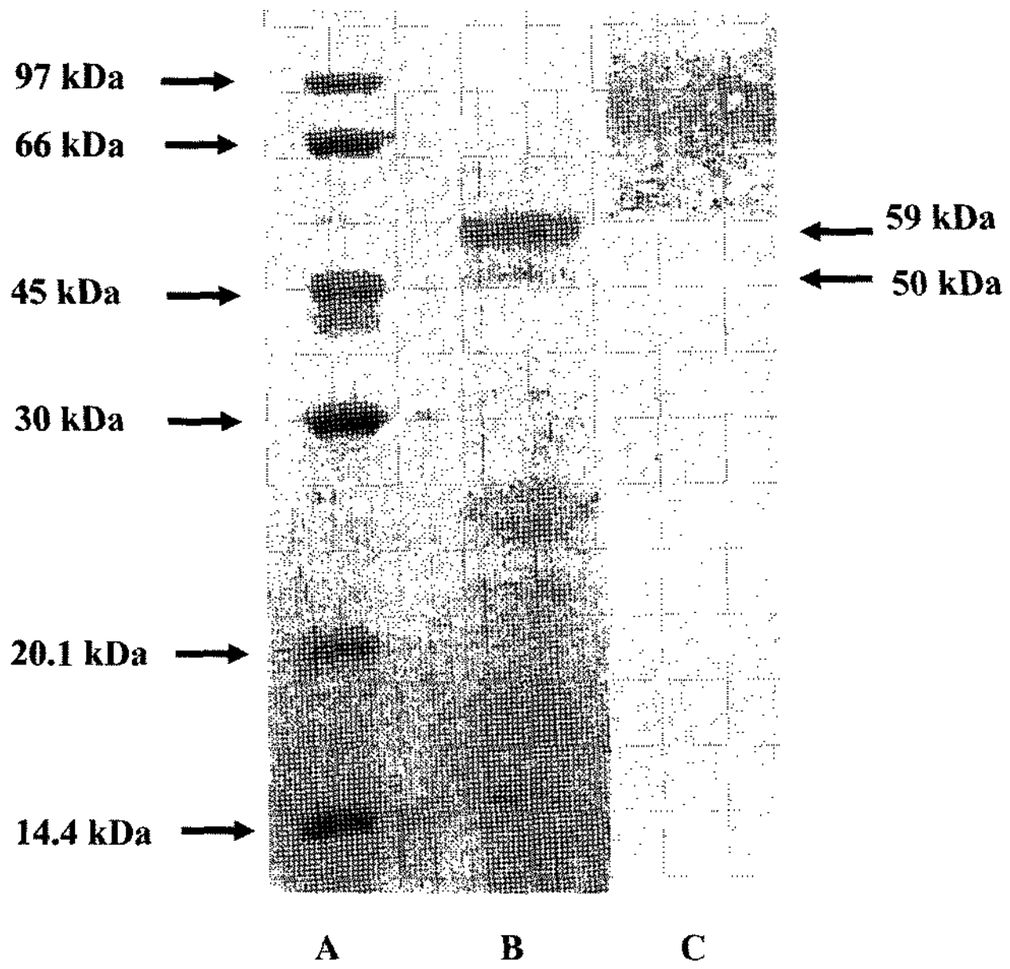

Fig. 8. Visualization of chitin deacetylase activity on a 12.5% SDS-PAGE gel.
A. Protein markers; **B**. Crude enzyme; **C**. Activity staining.

The CDA activity was strongly inhibited by metal ions Hg^{2+}, Zn^{2+}, and EDTA, but activated by Ca^{2+} and Co^{2+} at 10 mM. It has been previously reported that the CDA activity from *C. lindemuthanum* ATCC 56676 was slightly promoted by Co^{2+} at 1 mM, but strongly inhibited by it at 10 mM [25]. However, *Mortierella* sp. DY-52 CDA activity was increased greatly by Co^{2+} in a range of 0.01–10 mM (Table 5). This property has not been reported for other CDA enzymes [8, 12, 25].

As shown in Fig. 5, the process of enzymatic deacetylation was biphasic, rapid at the early stage, and slow at the later stage. The second phase seemed to start when about half of the acetyl groups were liberated. It resulted in part from the assumption that the concentration of the substrate chitin might be lower than saturated for the enzyme after 30 min of reaction.

It was found that only partially deacetylated chitin/chitosan could be well handled by CDA [18, 23]. Most of the other chitinous substrates without deacetylation cannot be worked on by CDA even though their particle size is very small [28]. It has been thought that the enzymatic deacetylation is profoundly affected by the physical properties such as crystallinity, degree of deacetylation, particle size, and origin of the substrate.

CDA from *Mortierella* sp. DY-52 appears to exhibit a very narrow specificity, acting on WSCT-50 with full affinity, and glycol chitin and crab chitosans (DD 71, 80, and 88%) with less affinity, similarly to the enzymes from *C. lindemuthianum*, *M. rouxii*, and *A. coerulea* (Table 2). However, DY-52 CDA was effective on $(GlcNAc)_{2-7}$. It is similar to previous reports that CDA from *C. lindemuthianum*, *A. nidulans*, and *Saccharomyces cerevisiae* required at least two *N*-acetyl-D-glucosamine residues [1, 16, 25, 27], but different to the CDA from *M. rouxii* that required at least three *N*-acetyl-D-glucosamine residues for catalysis of the deacetylation process [12]. Similar with the CDA from *M. rouxii* and *A. coerulea*, DY-52 CDA acted with the higher rate toward the longer *N*-acetylchitooligosaccharide as substrate, as shown in Table 3. Accordingly, a heptamer was the most susceptible and a dimer the least susceptible. All these studies seem to suggest a similar mode of deacetylation action for CDAs from *C. lindemuthianum*, *A. nidulans*, *Saccharomyces cerevisiae*, and *Mortierella* sp. DY-52.

However, a recent research indicated that a chitin oligosaccharide deacetylase (COD) from *Vibrio parahaemolyticus* was only effective in catalyzing the deacetylation of GlcNAc-GlcNAc to GlcNAc-GlcN, but not effective for chitooligosaccharide with a DP higher than 3 [11]. Even though the COD from *V. parahaemolyticus* and CDA from *Mortierella* sp. DY-52 come from the same family, they exhibited significantly different forms of deacetylation mechanism.

It has been reported that most CDAs were extracted from fungi in addition to chitinase or chitosanase [9, 18, 28]. In this work, a clear zone was found in chitin solid medium that is especially used for chitinase screening, which suggested that chitinase was present in the crude

enzyme preparation (data not shown). Even though we have little idea about the biological role of the CDA from *Mortierella* sp. DY-52, it would be different from that of *C. lindemuthianum* that may deacetylate chitin oligomers that arise from the fungus cell wall subsequent to the activity of plant chitinase and thereby diminish their elicitor activity [27].

Acknowledgment

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the National Research Laboratory Program funded by the Ministry of Science and Technology (No. M10300000322-06J0000-32210).

References

1. Alfonso, C., O. M. Nuero, F. Santamaria, and F. Reyes. 1995. Purification of a heat-stable chitin deacetylase from *Aspergillus nidulans* and its role in cell wall degradation. *Curr. Microbiol.* **30:** 49–54.
2. Araki, Y. and E. Ito. 1975. A pathway of chitosan formation in *Mucor rouxii*. *Eur. J. Biochem.* **55:** 71–78.
3. Aruchami, M., N. Gowri, and G. Sundara-Rajulu. 1986. Chitin deacetylases in invertebrates, pp. 263–265. *In* R. A. A. Muzzarelli, C. Jeuniaux, and G. W. Gooday (eds.), *Chitin in Nature and Technology*. Plenum Press, New York, U.S.A.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
5. Cai, J., J. H. Yang, Y. M. Du, L. H. Fan, Y. L. Qiu, J. Li, and J. F. Kennedy. 2006. Purification and characterization of chitin deacetylase from *Scopulariopsis brevicaulis*. *Carbohydr. Polym.* 1–7.
6. Chang, K. L., G. Tsai, J. Lee, and W. R. Fu. 1997. Heterogeneous *N*-deacetylation of chitin in alkaline solution. *Carbohydr. Res.* **303:** 327–332.
7. Gao, X., T. Katsumoto, and K. Onoder. 1995. Purification and characterization of chitin deacetylase from *Absidia coerulea*. *J. Biochem.* **117:** 257–263.
8. Hekmat, O., K. Tokuyasu, and S. G. Withers. 2003. Subsite structure of the endo-type chitin deacetylase from Deuteromycete, *Colletotrichum lindemuthianum*: An investigation using study-state kinetic analysis and MS. *Biochem. J.* **374:** 369–380.
9. Imoto, T. and K. Yagishita. 1971. A simple activity measurement of lysozyme. *Agric. Biol. Chem.* **35:** 1154–1156.
10. Jeuniaux, C. 1982. La chitine dans le regne animal. *Bull. Soc. Zool. Fr.* **107:** 363–386.
11. Kadokura, K., A. Rokutani, M. Yamamoto, T. Ikegami, H. Sugita, S. Itoi, W. Hakamata, T. Oku, and T. Nishio. 2006. Purification and characterization of *Vibrio parahaemolyticus* extracellular chitinase and chitin oligosaccharide deacetylase involved in the production of heterodisaccharide from chitin. *Appl. Microbiol. Biotechnol.* **75:** 357–365.
12. Kafetzopoulos, D., A. Martinou, and V. Bouriotis. 1993. Bioconversion of chitin to chitosan: Purification and characterization of chitin deacetylase from *Mucor rouxii*. *Proc. Natl. Acad. Sci. USA* **90:** 2564–2568.
13. Kauss, H., W. Jeblick, and D. H. Young. 1983. Chitin deacetylase from the plant pathogen *Colletotrichum lindemuthianum*. *Plant Sci. Lett.* **28:** 231–236.
14. Kuk, J. H., W. J. Jung, K. Y. Kim, and R. D. Park. 2005. Enzymatic characteristics and applications of microbial chitin deacetylase. *Kor. J. Microbiol. Biotechnol.* **33:** 9–15.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
16. Martinou, A., D. Koutsioulis, and V. Bouritiotis. 2002. Expression, purification, and characterization of a cobalt-activated chitin deacetylase (Cda2p) from *Saccharomyces cerevisiae*. *Prot. Expr. Purif.* **24:** 111–116.
17. Martinou, A., I. Tsigos, and V. Bouriotis. 1997. Preparation of chitosan by enzymatic deacetylation, pp. 501–505. *In* R. A. A. Muzzarelli and M. G. Peter (eds.), *Chitin Handbook*. Eur. Chitin Soc., Atec, Grottammare, Italy.
18. Nahar, P., V. Ghormade, and V. Deshpande. 2004. The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae*: Possible edge to entomopathogenic fungi in the biological control of insect pests. *J. Invertebr. Pathol.* **85:** 80–88.
19. Ruiz-Herrera, J. 1978. The distribution and quantitative importance of chitin in fungi, pp. 11–21. *In* R. A. A. Muzzarelli and E. R. Pariser (eds.), *Proceedings of the 1st International Conference on Chitin and Chitosan*, Massachusetts Institute of Technology, Cambridge.
20. Sannan, T., K. Kurita, and Y. Iwakura. 1975. Studies on chitin. 1. Solubility change by alkaline treatment and film casting. *Makromol. Chem.* **176:** 1191–1195.
21. Shin, W. S., J. C. Kil, and G. M. Park. 2006. Antiprotozoal activity of deacetylated chitosan oligosaccharide (dp 2-8) on *Trichomonas vaginalis*. *J. Microbiol. Biotechnol.* **16:** 1984–1989.
22. Srinivasan, V. R. 1998. Biotransformation of chitin to chitosan. US Patent 5739015.
23. Stevens, W. F., N. N. Win, C. H. Ng, C. Pichyangkura, and S. Chandrkrachang. 1997. Towards technical biocatalytic deacetylation of chitin, pp. 40–47. *In* A. Domard, G. A. F. Roberts, and K. M. Varum (eds.), *Advances in Chitin Science*, Vol. 2. Jacques Andre, Lyon.
24. Toei, K. and T. Kokara. 1976. A conductometric method for colloid titrations. *Anal. Chem. Acta* **83:** 59–65.
25. Tokuyasu, K., M. O. Kameyama, and K. Hayashi. 1996. Purification and characterization of extracellular chitin deacetylase from *Colletotrichum lindemuthianum*. *Biosci. Biotech. Biochem.* **60:** 1598–1603.
26. Trudel, J. and A. Asselain. 1990. Detection of chitin deacetylase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* **189:** 249–253.
27. Tsigos, I. and V. Bouriotis. 1995. Purification and characterization of chitin deacetylase from *Colletotrichum lindemuthianum*. *J. Biol. Chem.* **270:** 26286–26291.
28. Win, N. N. and W. F. Stevens. 2001. Shrimp chitin as substrate for fungal chitin deacetylase. *Appl. Microbiol. Biotechnol.* **57:** 334–341.
29. White, T. J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. *In* M. Innis, D. Gelfand, J. Sninsky, and T. White (eds.), *A Guide to Methods and Applications*. Academic Press, San Diego, U.S.A.