Appl Biochem Biotechnol (2010) 162:843–854
DOI 10.1007/s12010-009-8772-z

Cloning of a Heat-Stable Chitin Deacetylase Gene from *Aspergillus nidulans* and its Functional Expression in *Escherichia coli*

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Received: 19 July 2009 / Accepted: 3 September 2009 /
Published online: 17 September 2009

Abstract A gene encoding chitin deacetylase was cloned by polymerase chain reaction from *Aspergillus nidulans*. Sequencing result showed 40% homology to the corresponding gene from *Colletotrichum lindemuthianum*. The complete gene contains an open reading frame of 747 nucleotides encoding a sequence of 249 amino acid residues. The chitin deacetylase gene was subcloned into a pET28a expression vector and expressed in *Escherichia coli* BL21 and then purified by metal affinity chromatography using a His-bind column. The purified chitin deacetylase demonstrated an activity of 0.77 $U ml^{-1}$ for the glycol chitin substrates, and its specific activity was 4.17 $U mg^{-1}$ for it. The optimal temperature and pH of the purified enzyme were 50 °C and 8.0, respectively. When glycol chitin was used as the substrate, K_m was 4.92 mg ml^{-1}, and K_{cat} showed 6.25 s^{-1}, thus the ratio of K_{cat} and K_m was 1.27 ml $s^{-1} mg^{-1}$. The activity of chitin deacetylase was affected by a range of metal ions and ethylenediaminetetraacetic acid.

Keywords *Aspergillus nidulans* · Chitin deacetylase · Gene cloning · Expression

Introduction

Chitin, a homopolymer comprising β-(1-4)-linked *N*-acetyl-D-glucosamine residues is one of the most abundant, easily obtained and renewable natural polymers, second only to cellulose [1]. Up to now, no important industrial applications have been found due to its insolubility in aqueous and organic solvents. Chitosan, which is obtained by the *N*-deacetylation of chitin, is biodegradable, non-toxic to animals, soluble in acidic solutions, available in various physical forms, and much more tractable than chitin [2]. Thus, chitosans have several applications in areas such as biomedicine, food ingredients, cosmetics, and pharmaceuticals [3]. In addition to harsh thermochemical procedure, chitosan can be obtained by refined enzymatic procedure resulting in the production of novel, well-defined chitosan oligomers and polymers [4].

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Chitin deacetylase (CDA), the enzyme that catalyzes the conversion of chitin to chitosan by the deacetylation of *N*-acetyl-D-glucosamine residues, was first identified and partially purified from extracts of the fungus *Mucor rouxii* [5]. Chitin deacetylase (EC 3.5.1.41) has been recently classified in carbohydrate esterase family 4 (CE4) [6]. So far, chitin deacetylases have been isolated from the fungi *M. rouxii* [7], *Colletotrichum lindemuthianum* [3], *Absidia coerulea* [8], *Aspergillus nidulans* [9], *Metarhizium anisopliae* [10], *Scopulariopsis brevicaulis* [11], and *Rhizopus nigricans* [12]. In most cases the optimum temperature for enzyme activity is 50°C while optimum pH varies from 4.5 to 8.5. All enzymes are active on chitin oligomers, but they are not effective in deacetylating water-insoluble chitin substrates. Genes encoding CDA from *M. rouxii*, *Saccharomyces cerevisiae*, *C. lindemuthianum*, *Vibrio alginolyticus*, and *Mucor racemosus* have been cloned and characterized [13–18].

Chitin deacetylase from *A. nidulans* is a glycoprotein with 28% (wt/wt) carbohydrate content. The enzyme was stable in the pH range 4.0–7.5. Its optimum temperature of reaction was 50°C and it was stable from 30°C to 100°C after 1 h of preincubation [9]. One interesting property with a potential biotechnological application for the enzymes from *A. nidulans* is that, apart from its thermal stability, it is not inhibited by acetate, a product of the deacetylation reaction.

Cloning of the chitin deacetylase gene from *A. nidulans* has not been reported. We succeeded in cloning the gene and overexpressing it in *Escherichia coli* cells, resulting in a large-scale production of the recombinant enzyme as inclusion bodies, whose activity could be recovered with a simple renaturation step. The enzyme was further purified and characterized.

Materials and Methods

Strains, Vectors, and Chemicals

The fungal strain *A. nidulans* AF93062 was obtained from China Center for Type Culture Collection (CCTCC). *E. coli* TOP10 and BL21 (DE3) were used as the host strains. pMD18-T was purchased from Takara (China), and pET-28a was stored in our lab. His-bind column was purchased from Novagen (Madison, WI, USA). Glycol chitosan and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Sigma (St Louis, USA). Molecular weight markers were purchased from Fermentas (China). D-Glucosamine–HCl was purchased from Zhejiang Aoxing Biotechnology Co. Ltd (Taizhou, China). All other chemicals used in this study were of commercially available analytical grade.

Cloning and Sequencing of the Chitin Deacetylase Gene

The fungus was cultured in liquid Czapek medium and mycelia were harvested by vacuum filtration followed by washing with distilled water and subjected to DNA isolation [19]. To induce chitin deacetylase gene expression, mycelia was cultivated on PDA slants for 96 h at 28°C. The cells were harvested into sterile water. This suspension was inoculated into a 500-ml Erlenmeyer flask containing 80 ml liquid medium ($NaNO_3$, 0.16 g; K_2HPO_4, 0.08 g; KCl, 0.04 g; $MgSO_4$, 0.04 g; chitin, particle size less than 100 mesh, 0.4 g). The flask was shaken at 200 rpm and 28°C for 96 h [11]. For the amplification of the complementary deoxyribonucleic acid (cDNA) sequence of the chitin deacetylase gene, total RNA was extracted from mycelia of *A. nidulans* using Trizol reagent (Invitrogen) and digested with DNaseI (Promega, Madison, WI, USA).

The *M. Rouxii cda* was used to search for homologous sequences in *A. nidulans* predicted proteins and in *A. nidulans* genomic sequences at the TIGR website [20]. Primer design was based on the nucleic acid sequence alignment of *cdas* from published databases and primers P1: 5'-ATCTTCTCGCTAGACTCC-3' and P2: 5'-ACCAACCCTAACTATTCAC-3' were selected for polymerase chain reaction (PCR) amplification from DNA. P3: 5'-ATGTTCG CAACGTTGGCTCTCG-3' and P4: 5'- TCAATGATACCACGCAATCTCTCCAT-3' were designed for PCR amplification from cDNA including completed CDS.

Polymerase chain reaction consisting of 30 cycles amplification was performed denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 1.5 min. An additional extension step was performed at 72°C for 10 min. The PCR products were purified by UNIQ-10 Column Collection Tube Kit (Sangon, Shanghai, China) and ligated with the pMD18-T vector, and the ligation products were transformed into *E. coli* TOP10-competent cells. Transformants were screened by colony PCR using P1, P2 and P3, P4, respectively. Positive transformants were further confirmed by sequencing using the dideoxy chain-termination method [21].

Construction of Expression Plasmid

The fourth nucleic acid (deoxythymidine acid) of the full chitin deacetylase gene was replaced by guanylic acid using the forward primer (P5) to form the *Nco*I site. The terminator (TGA) was removed by TGG using the reverse primer (P6) to express the His tag in the pET-28a. Recombinant pMD18-T plasmid which contained the cDNA was amplified by PCR with the primers by adding *Nco*I and *Eco*RI sites at two ends of the chitin deacetylase gene. The primer sequences were designed as follows: P5: 5'-CATG CCATGGTCGCAACGTTGGCTCTCG-3' (*Nco*I site is underlined) and P6: 5'-CGGAATTCCAATGATACCACGCAATCTCTCCATC-3' (*Eco*RI site is underlined). PCR amplification was performed with 29 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. A final extension step was performed at 72°C for 10 min. The amplified chitin deacetylase gene was purified using the UNIQ-10 Column Collection Tube Kit and digested with *Nco*I and *Eco*RI (Promega, Madison, WI, USA) and ligated with similarly digested pET28a. The ligation product was transformed into *E. coli* TOP10 followed by incubation overnight at 37°C on LB plate supplemented with 100 μg/ml kanamycin (Invitrogen, San Diego, CA, USA). Transformants were screened by colony PCR using P5 and P6 primers. Positive transformants were further confirmed by sequencing using dideoxy chain-termination method [21].

Expression, Denaturation, Purification, and Renaturation

Recombinant expression plasmid was transformed into *E. coli* BL21 (DE3). A transformant from a single colony was inoculated in 5 ml kanamycin selection LB medium and cultivated overnight at 30°C. The cell culture was diluted in 100 ml LB medium to an initial OD_{600} of 0.02 and grown until OD_{600} reached 0.2, then 0.5 mmol l^{-1} isopropyl-β-D-thiogalactoside (IPTG; Sangon, Shanghai, China) was added to induce chitin deacetylase gene expression for 5 h at 28°C.

Cells were harvested by centrifugation, and the pellet was resuspended in 20 ml PBS buffer. Ten units of lysozyme solution was added and incubated at room temperature for 10 min. After ultrasonication the misfolded chitin deacetylase protein was released in inclusion bodies. The inclusion bodies were collected by centrifugation at 5,000×*g* for 15 min, followed by washing with 20 ml binding buffer (containing 2 mol l^{-1} urea) twice.

To unfold the misfolded protein, the pellet was resuspend in 5 ml binding buffer (containing 8 mol l^{-1} urea) overnight. The supernatant was passed through a 0.45-μm membrane filter and applied to His-bind column (Novagen, Madison, WI, USA) equilibrated with binding buffer (containing 8 mol l^{-1} urea). After washing with washing buffer (containing 8 mol l^{-1} urea), the recombinant protein was eluted with elution buffer (containing 8 mol l^{-1} urea). By dialysis against a series of buffers of 6, 4, and 2 mol l^{-1} urea solutions and sodium tetraborate buffer (0.05 mol l^{-1}, pH8.0), urea was removed from the solution and the protein reassumed its refolding. The purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The protein concentration was determined by Bradford assay using bovine serine albumin as a standard [22]. Purified protein was used for its characterization. Protein purity was determined by high-performance liquid chromatography (HPLC) analysis using reverse phase column system (C-18 column, Agilent) and the chromatogram was analyzed by UNICORN V3.20 software.

Enzyme Activity Assay

Chitin deacetylase activity was estimated using ethylene glycol chitin as the substrate prepared according to the method of Araki and Ito [5]. Ethylene glycol chitosan (EGC, 40 mg) was treated at 4 °C in a mixture containing 400 mg of $NaHCO_3$ and 4.5 ml of acetic anhydride and kept at 4 °C for 24 h. After dialysis, acetylated ethylene glycol chitosan (1 mg/ml) was used as a substrate for the assay of chitin deacetylase. The substrates could be stored at −20 °C for months until to use.

The assay for chitin deacetylase was carried out according to MBTH method [23, 24] with 100 μl of 10 mM sodium tetraborate buffer, pH8.0, 100 μl of 1 mg/ml ethylene glycol chitin, and 50 μl enzyme incubated at 50 °C for 30 min. The reaction was terminated by the addition of 250 μl 5% (*w*/*v*) $KHSO_4$. For color development, 250 μl of 5% (*w*/*v*) $NaNO_2$ was added and allowed to stand for 15 min, and 250 μl 12.5% (*w*/*v*) ammonium sulfamate ($N_2H_6SO_3$) was added. After 5 min, freshly prepared 250 μl 0.5% (*w*/*v*) MBTH was added and the mixture was heated in boiling water for 3 min. The tubes were cooled in tap water and 250 μl 0.5% (*w*/*v*) $FeCl_3$ was added and estimated spectrophotometrically at 650 nm. One unit of enzyme released 1 μmol of acetate from ethylene glycol chitin per minute. Standard curves were prepared with D-glucosamine-HCl standard.

Characterization of the Chitin Deacetylase

The substrate was diluted in the range of 0.004–0.1% (*w*/*v*) in 0.01 mol l^{-1} Na_2HPO_4–KH_2PO_4 buffer (pH8.0). The values of the Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) were determined using Lineweaver–Burk plot. The kinetic parameters, turnover number (K_{cat}) and catalytic efficiency value (K_{cat}/K_m), were calculated from initial velocities using concentrations of 0.1% (*w*/*v*) of ethylene glycol chitin.

The optimal pH of the chitin deacetylase was determined over various pH ranges of HAc–NaAc acid buffer (pH4.0–6.0), Na_2HPO_4–KH_2PO_4 buffer (pH7.0–8.0) and glycine–NaOH buffer (pH9.0–10.0). For pH stability determination, the chitin deacetylase was incubated in different buffer solutions for 1 h at 4 °C, and the remaining activity was measured under the standard assay condition.

The optimal temperature was determined by incubating the enzyme at the temperatures ranging from 30 °C to 100 °C at pH8.0. For determination of the thermostability, the chitin deacetylase was incubated at the temperatures ranging from 30 °C to 100 °C for 1 h.

Samples were withdrawn and immediately cooled on ice. The residual activity of each sample was determined as described above.

Effect of Ions and Chemicals on Chitin Deacetylase Activity

To study the effects of different metal ions and chemical reagents on the chitin deacetylase activity, different metal ions or chemicals, $CaCl_2$, $ZnCl_2$, $CoCl_2$, $MgSO_4$, $MnCl_2$, $CuSO_4$, $(NH_4)_2SO_4$, and ethylenediaminetetraacetic acid (EDTA) were individually added to the reaction solution to a final concentration of 40 mmol l^{-1}. Chitin deacetylase activities were determined under the standard assay. The reaction mixture without the metal ions or chemicals was used as control.

It is reported that the activity of chitin deacetylase from *A. nidulans* was not inhibited by acetate [9]. To determine whether the acetate ions affected the recombinant chitin deacetylase activity, 0–50 mmol l^{-1} acetate ions were individually added to the buffer. The activities were determined under the standard assay.

Nucleotide Sequence Accession Number

The nucleotide sequence of *A. nidulans* chitin deacetylase gene was assigned to the GenBank accession no. EU734183 (http://www.ncbi.nlm.nih.gov/nuccore/190576801).

Results

Cloning of the Chitin Deacetylase Gene

Complete cDNA sequence of chitin deacetylase gene was amplified from *A. nidulans* by PCR. As shown in Fig. 1, the gene is composed of 714 bp, which encodes a deduced protein of 237 amino acids with a molecular weight of 26 kDa and a theoretical isoelectric point of 4.6 predicted at http://au.expasy.org/tools/pi_tool.html. The 18 amino acids located at the N terminus of the deduced amino acid showed the typical attributes of a signal peptide. The chitin deacetylase promoter was predicted using BDGP (http://www.fruitfly.org/seq_tools/promoter.html).

Conserved polysaccharide deacetylase domain analysis revealed that a 124-residue functional domain is encoded in the middle part of the gene, sharing high similarity with CDAs of fungi as well as bacterial deacetylases including the nodb-like protein from *Sinorhizobium meliloti* and *Bacillus subtilis*, xylanase D from *Cellulomonas fimi*, and acetylxylan esterase A from *Streptomyces lividans*. No other domain, such as a substrate-binding domain is encoded in the sequence, suggesting that CDA may act primarily in catalyzing the deacetylation of chitin.

Multiple sequence alignment within a CDA family (Fig. 2) demonstrates that the sequences are not similar at the amino and carboxyl termini (data not shown). CDA shares conserved amino acids at the polysaccharide deacetylase domain located in the middle portion of the amino acid sequence. Numerous conserved residues were observed and they may play a common role in the functional catalytic reactions of deacetylases. The sequence is most homologus to the CDA of *C. lindemuthianum* (40% identity and 54% similarity), followed by the CDA of *B. graminis* (28% identity and 40% similarity). On the contrary, 12% and 22% identities with 11% and 22% similarities were observed when the sequence was compared with those of CDAs from *M. rouxii* and *Phycomyces blakesleeanus*, respectively.

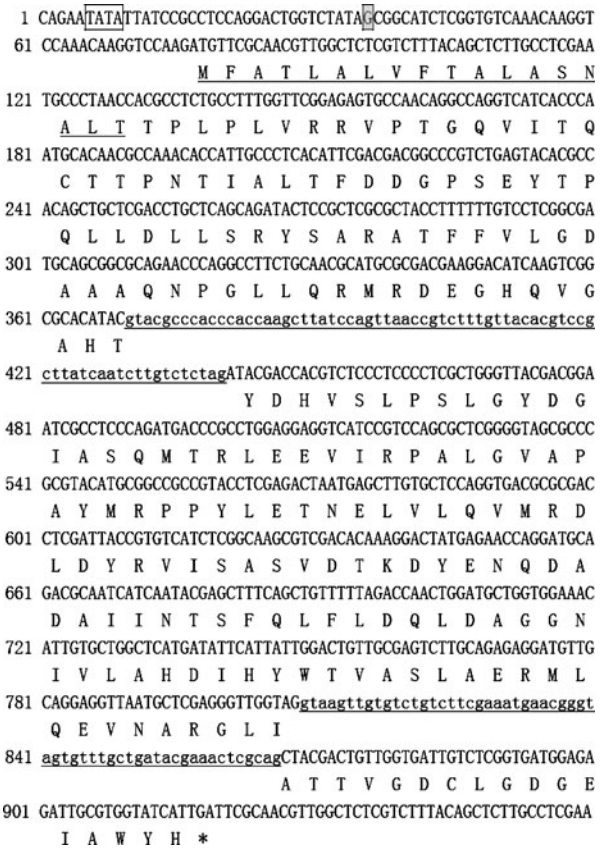

Fig. 1 Nucleotide and deduced amino acid sequences of the chitin deacetylase from *A. nidulans* containing the ORF and 5' and 3' flanking regions. The putative TATA box are *boxed*; the putative transcription initiation site nucleotide (*G*) is *boxed and shaded*; the signal peptide is *underlined*; the intron is *underlined in lowercase*; the stop codon is *indicated with an asterisk*

Expression, Purification, and Enzyme Assay of the Recombinant Protein

As shown in Fig. 3, IPTG induction of *E. coli* BL21 resulted in chitin deacetylase gene expression of the transformants but no expression of the bacteria containing pET-28a. the expressed protein was 26.9 kDa according to the estimated protein size. This difference in molecular mass from the deduced protein is tentatively interpreted as caused by the additionally fused 6 His-tag encoded by a fragment from pET-28a.

The transformed *E. coli* BL21 expressed the exogenous chitin deacetylase in inclusion bodies under our experimental conditions. The recombinant protein purified with His-bind column was refolded by dialysis. The enzyme concentration was 0.185 mg ml^{-1}. As shown in Fig. 4, HPLC analysis demonstrated the purity of the refolded protein was approximately 99.4%. The exogenous expressed chitin deacetylase in *E. coli* BL21 showed the activity of 4.17 Umg^{-1} when ethylene glycol chitin was used as the substrate.

Characterization of the Chitin Deacetylase

The K_m value of chitin deacetylase was 4.92 mg ml^{-1}, and the maximal velocity was 0.77 μmol $min^{-1}ml^{-1}$ when ethylene glycol chitin was used as the substrate. The turnover

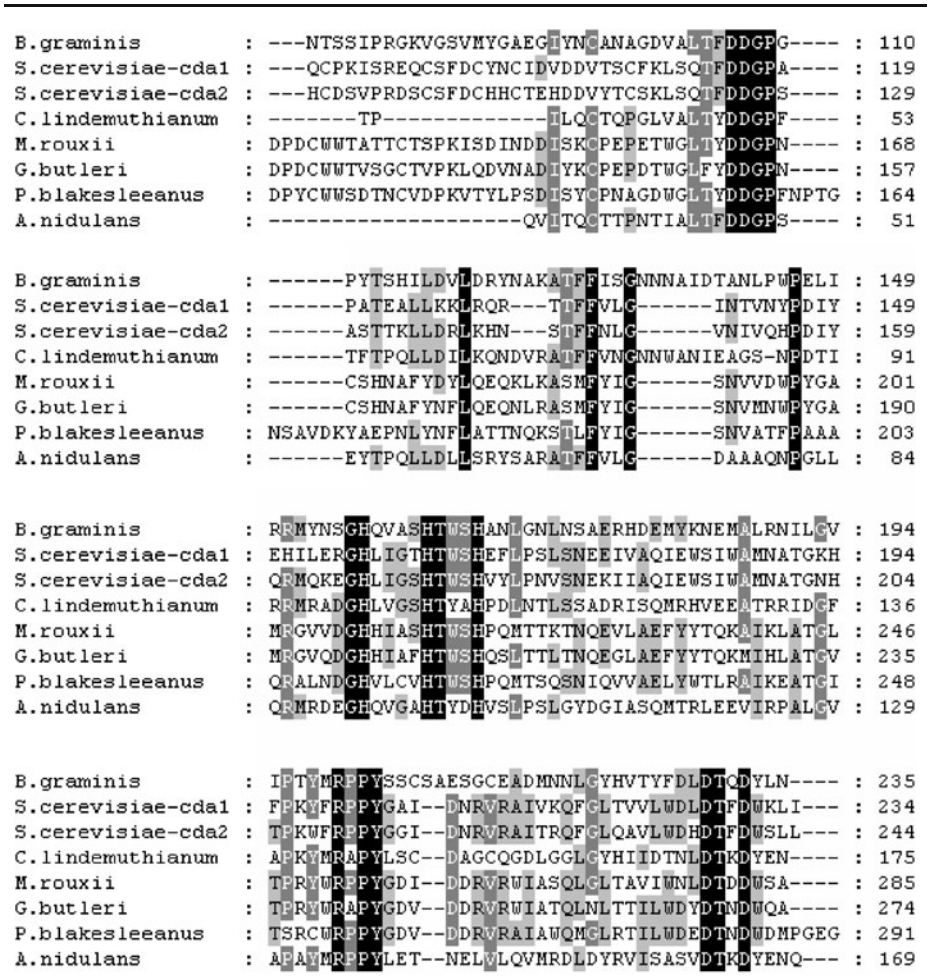

Fig. 2 Comparison of the deduced amino acid sequences of chitin deacetylase from various fungi. GenBank accession nos.: *G butleri*, AF411810; *M. rouxii*, CAA79525; *P. blakesleeanus*, BAB03595; *S. cerevisiae* CDA1, NP013410; *S. cerevisiae* CDA2, NP013411; *B. graminis*, AAK84438. Identical and similar amino acids are indicated by *solid black* and *gray boxes*, respectively

number and the catalytic efficiency value were 6.25 s^{-1} and 1.27 ml $s^{-1}mg^{-1}$, respectively, according to the K_m and V_{max}.

As shown in Fig. 5a, the optimal pH of the chitin deacetylase was 8.0, and chitin deacetylase activity decreased rapidly when the pH value was less than 6.0 or more than 9.0 showing that the chitin deacetylase functioned better under weak alkaline pH conditions. The residual activity remained above 60% when incubated at the pH range from 5.0 to 9.0 for 1 h indicating it was very stable in nearly neutral buffers (Fig. 5b).

As shown in Fig. 5c, 50°C was the optimal temperature for the chitin deacetylase. Relative chitin deacetylase activity at 80°C was less than 32% of the maximal enzyme activity. When the chitin deacetylase was incubated at 80°C for 1 h, the residual activity was about 68% of the maximal enzyme activity (Fig. 5d), which indicated that it was heat stable.

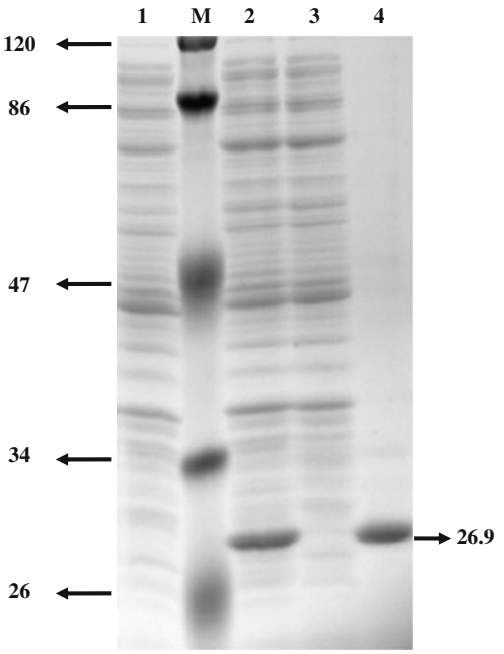

Fig. 3 Analysis of recombinant chitin deacetylase expression and purification on 12% SDS-PAGE; *lane M* protein molecular weight marker, *lane 1* total protein of induced bacteria with PET-28a, *lane 2* total protein of induced recombinant, *lane 3* supernatant of induced recombinant, *lane 4* purified recombinant chitin deacetylase protein

Effect of Metal Ions or Chemicals on Chitin Deacetylase Activity

As shown in Fig. 6a, compared to the activity of the standard chitin deacetylase, the enzyme activity declined to 0.62%, 1.30%, 9.33%, 12.69%, 76.68%, and 90.16% when supplemented with EDTA, $ZnCl_2$, $CoCl_2$, $CuSO_4$, $MnCl_2$, and $(NH_4)_2SO_4$, respectively, in the reaction systems. The values were raised to 106.99% and 108.03% in the presence of $CaCl_2$ and $MgSO_4$ respectively in the reaction mixtures. As shown in Fig. 6b, 0.4 mmol l^{-1} acetate ions inhibited the chitin deacetylase activity notably. The activity decreased as the concentration of acetate ions increased.

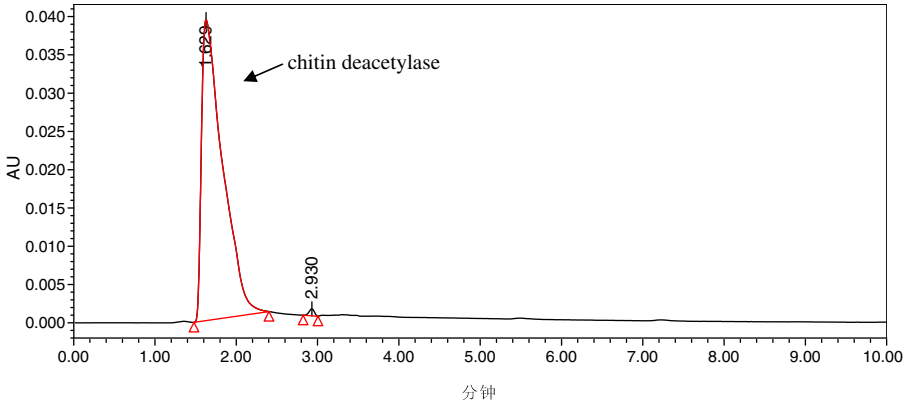

Fig. 4 The purity of the refolded protein. The analysis was performed by high-performance liquid chromatography (HPLC) analysis using C-18 column. The mobile phage was 80% methanol (20% H_2O) with flow rate at 1 ml min^{-1} at 26°C

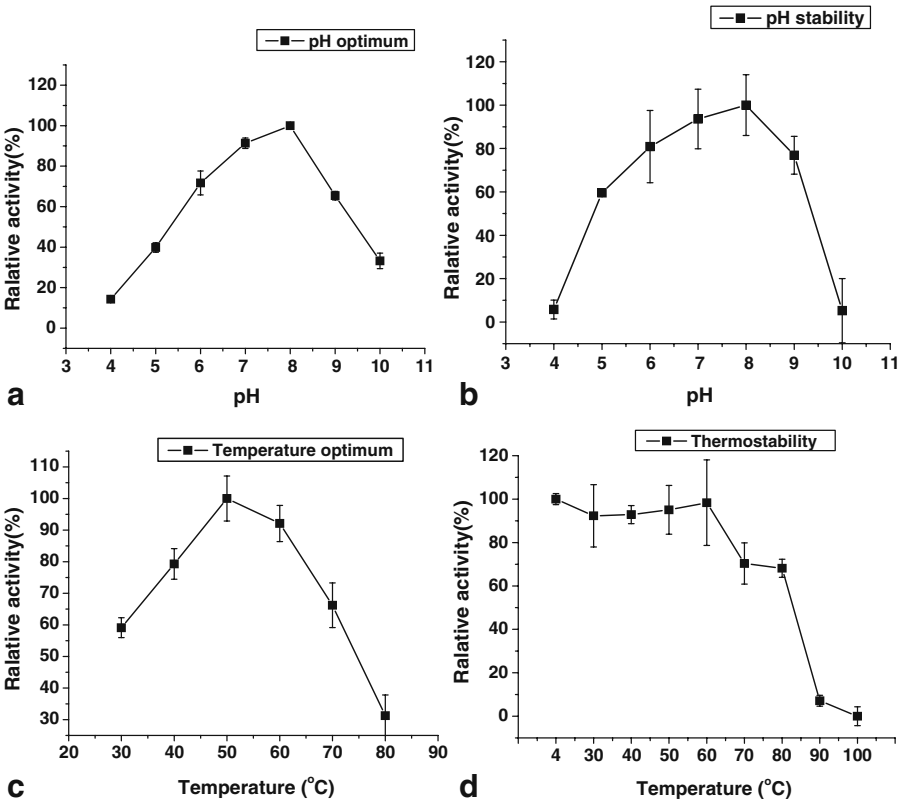

Fig. 5 Activities and properties of recombinant chitin deacetylase. The purified protein of *E. coli* BL21 transformed with the chitin deacetylase gene was used for measuring chitin deacetylase activity. The protein added after incubation for 30 min at 50°C was used as a control. All the experiments were performed three times. **a** Effect of pH on recombinant chitin deacetylase. The enzyme activity was measured in the reaction buffer at different pH values from 4.0 to 10.0 at 1-U interval. **b** pH stability of chitin deacetylase. The chitin deacetylase was incubated at the pH range from 4.0 to 10.0 for 1 h at 4°C. **c** Effect of temperature on recombinant chitin deacetylase. The chitin deacetylase activities were measured at 30°C to 80°C for 30 min at 10°C intervals. **d** Thermostability of the recombinant chitin deacetylase. For determination of the thermostability, the recombinant chitin deacetylase was incubated at temperatures ranging from 30°C to 100°C for 1 h. Samples were immediately cooled in an ice bath before determination

Discussion

In this study, we cloned the chitin deacetylase gene from *A. nidulans*. Having conducted a review of the extant literature, we believe this is the first report of cloning of the chitin deacetylase gene from *A. nidulans*. Sequence analysis showed that it is a member of the polysaccharide deacetylase superfamily. The chitin deacetylase included a putative signal peptide sequence at the N-terminal end, suggesting it is a secreted enzyme. The multiple sequence alignments indicated that the two motifs containing "L(Q)T(F)F(Y)DDGP" and "HTW(Y)S(AD)H" were conserved among the amino acid sequences of the polysaccharide deacetylase superfamily (Fig. 2), that suggested they are essential for chitin deacetylase activity [25].

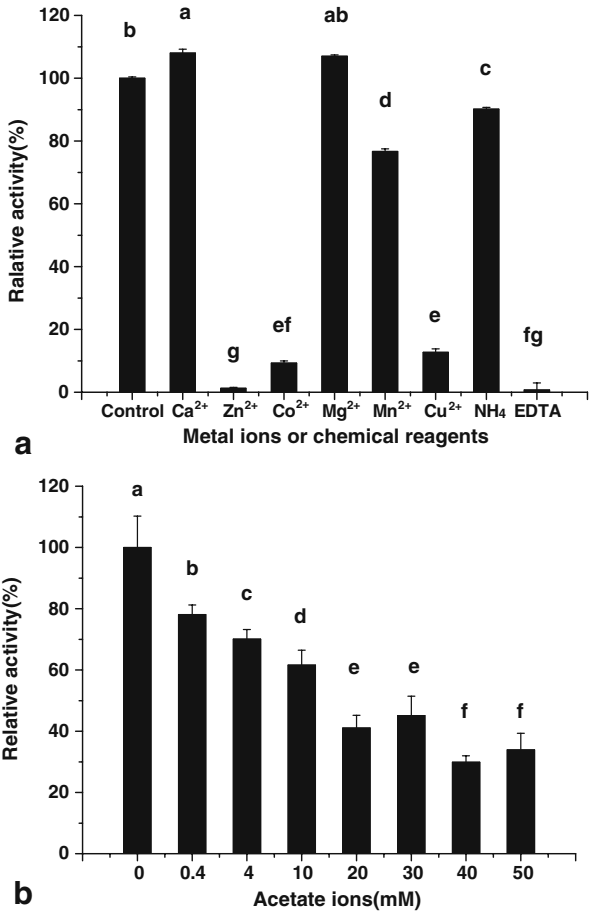

Fig. 6 Effects of metal ions or chemical reagents on the chitin deacetylase activity. **a** Metal ions or chemical reagents. Metal ions or chemicals were individually added up to a final concentration of 40 mmol l^{-1}. Control, the activity of chitin deacetylase was measured under normal reaction conditions without any additional ions. **b** Effects of acetate ions. Acetate ions were individually added up to a final concentration from 0–50 mmol l^{-1}. Experimental data were determined in triplicate. *Different letters* above the columns indicated a significant difference determined by Duncan's multiple comparisons test ($P<0.05$)

Though two strains of *A. nidulans* were used for cloning chitin deacetylase gene, we failed to clone from *A. nidulans* (3.108) obtained from China General Microbiological Culture Collection Center (CGMCC). The two strains were then cultured on the Czapek plates containing 0.05% 4-nitroacetanilide. After 5 days of culture the plates with *A. nidulans* AF93062 (CCTCC) turned yellow because of the generation of 4-nitroaniline obtained by the *N*-deacetylation of 4-nitroacetanilide, but the ones with *A. nidulans* 3.108 (CGMCC) were still colorless (data not shown). The evidence indicated that the expression level of chitin deacetylase was significantly different between the two *A. nidulans* strains.

Although the chitin deacetylase from *A. nidulans* has been purified and characterized [9], the gene had not been studied. This study is the first to report the gene cloning of chitin deacetylase from *A. nidulans* and expression of the gene in *E. coli*. It has been reported in several cases that by lowering the incubation temperature of *E. coli*, the solubility of recombinant proteins can be improved [26]. We have observed that incubation of *E. coli* at relatively low temperature (16°C) and 0.02 mM IPTG was not effective for the production of the soluble enzyme. The protein formed inclusion bodies that made the signal peptide unable to be recognized by the bacteria. The presence of the signal peptide did not inhibit

the enzyme activity. Primers for amplification of the chitin deacetylase gene without putative signal peptide were designed, but the recombinant protein could not be detected.

The purified recombinant chitin deacetylase demonstrated an activity of 4.17 Umg^{-1} for the glycol chitin substrates and the activity of chitin deacetylase from *A. nidulans* was 0.025 Umg^{-1} [9]. We suspected that the enzyme lose most of its activity after three steps of purification. Due to differences in the conditions of enzyme activity measurements, it is not easy to make a straightforward comparison. The recombinant chitin deacetylase showed high activity at pH6.0–9.0, and the optimal pH was 8.0 (Fig. 5a). Its optimal temperature is 50°C and remains at more than 60% of the enzyme activity with a range of 30–70°C (Fig. 5c). The recombinant chitin deacetylase and the enzyme from *A. nidulans* CECT 2544 have the same optimal temperature but different optimal pH [9]. The chitin deacetylase was stable over a wide pH (5.0–9.0; Fig. 5b) and temperatures range (4–80°C; Fig. 5d) indicating it has good potential for further utilization. This property has previously been described for the chitin deacetylase from *A. nidulans* and *C. lindemunthianum*, respectively [9, 27]. *N*-deglycosylation of Cda2p from *Saccharomyces cerevisiae* resulted in total loss of enzyme activity using glycol chitin as substrates. Reactivation of the inactive deglycosylated form of the enzyme could be effected by $CoCl_2$ (1 mM) resulting in restoration of enzyme activity [28]. The recombinant chitin deacetylase from *C. lindemuthianum* in *Pichia pastoris* was significantly activated by Co^{2+} ions [29]. In this study, chitin deacetylase activity was inhibited when $CoCl_2$ (40 mM) was added (Fig. 6a). The effect of cobalt on the activity of chitin deacetylases needs to be further studied. Alfonso et al. [9] reported that chitin deacetylase form *A. nidulans* was inhibited by a series of ions, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, NH_4^+, and EDTA. Contradictorily the recombinant chitin deacetylase activity was slightly increased by Ca^{2+} and Mg^{2+} (Fig. 6a). Because the enzyme activity was completely inhibited by EDTA we believe that the chitin deacetylase is a metal enzyme. Although chitin deacetylase from *A. nidulans* is non-inhibited by acetate [9], the recombinant chitin deacetylase is inhibited by low concentration of acetate ions notably. The chitin deacetylase from *A. nidulans* is glycosylated in contrast to the recombinant enzyme. It has been previously reported that deglycosylation of chitin deacetylases may change some of the characteristic of the enzyme [30].

The molecular weight of chitin deacetylase from *A. nidulans* CECT 2544 was 19.5 kDa after deglycosylation [9], and the molecular weight of the putative mature chitin deacetylase in this study was 24.2 kDa. They have different K_m and optimal pH, so we suspected the chitin deacetylase gene cloned was not the one that was studied by Alfonso C. There may exist other chitin deacetylase in the *A. nidulans*.

The partial properties of the recombinant enzyme were studied. Expression in *E. coli* inevitably promotes the large-scale production of this enzyme for industrial applications. However, heterogenous proteins expressed by *E. coli* easily forms inclusion bodies, which are inactive. Expression system of *P. pastoris* has been widely used in recent years. Further study will focus on expression of soluble heterologous proteins in *P. pastoris*.

Acknowledgments This research was supported by the projects of the Nature and Technology Heilongjiang province of China (C200609) and the Natural Scientific Research Innovation Foundation in Harbin Institute of Technology (HIT. NSRIF. 2008-18).

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