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Cloning, Expression and Enzymatic Characterization of Chitin Deacetylase 4 from *Hyphantria Cunea* (Drury)

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Abstract. A novel chitin deacetylase (CDA), *HcCDA4*, was identified from the American white moth, *Hyphantria cunea*. The full-length cDNA sequence of *HcCDA4* was identified,and the cDNA is 1494bp in length. The *HcCDA4* was shown as a 56 kDa protein in *E. coli* BL21 by SDS-PAGE analysis and the specific antibodies reacting to HcCDA4 were obtained by immunizing rabbits. The insect cells secreting expressed HcCDA4 proteins were used to study enzymatic properties. Results showed that HcCDA4 possessed catalytic activity, and the optimum temperature was 50℃ and the optimum pH was 8.0. Under the optimum reaction conditions, the enzyme activities of HcCDA4 protein was 3.44 $U \cdot mL^{-1}$. The enzyme activity is enhanced in the presence of Zn^{2+}, Mn^{2+} and Fe^{2+} ions, but inhibited in the presence of Mg^{2+} and Co^{2+} ions, Ca^{2+} showed a trend of increasing first and then decreasing on HcCDA4 enzymatic activity.

Introduction

In order to grow, insects must undergo periodic molts during which they shed their old cuticle and acquire a new, larger one. The three main constituents of the cuticle are proteins, lipids and chitin, the latter 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 and it is commonly found in many invertebrates and in the cell walls of most fungi and algae [1-3]. In the last few years, research shows that the enzymes in chitin metabolism are the critical target for insecticides [4-6].

Chitin deacetylase (CDA) is a kind of chitin-degrading enzyme which can turn chitin into chitosan as well as modify chitin [7]. Previously, CDAs were thought to be restricted to fungi and bacteria until Aruchami reported their presence in arthropods [8] and Guo identified TnPM-P42 from the cabbage looper, *Trichoplusia ni* [9]. Fungal CDAs have been studied more widely than those from bacteria and insects, which play an important role in fungal growth, being involved in formation of the fungal cell wall, and in most cases the optimum temperature for fungal enzyme activity is 50℃ while optimum pH varies from 4.5 to 8.5 [10]. At present, the study of CDA gene in insects is mostly concentrated in Diptera, Coleoptera, Lepidoptera and Hemiptera, such as *Drosophila melanogaster* [11], *Mamestra configurata* [12], *Tribolium castaneum* [13], *Choristoneura fumiferana* [14], *Bombyx mori* [15], *Nilaparvata lugens* [16]. DIXIT used bioinformatics methods in four kinds of insects made a systematic study, CDAs

are divided into five groups [17]. Group I and II CDAs display three functional domains: a chitin-binding/peritrophin-A domain (CBD), a low-density lipoprotein receptor class A domain (LDla), and a polysaccharide deacetylase-like catalytic domain (CDA). Group I and II CDAs are involved in molting, possibly by modifying cuticular and tracheal chitin in the embryonic stages as demonstrated in *D. melanogaster* [11]. Group III and IV CDAs also encode the CBD domain, but lack the LDla domain. Group III CDA seems to be gut-specific and no observable morphological phenotype or interstructural abnormality in the gut was detected after dsRNA injection, whereas Group IV CDAs may be associated with moulting as demonstrated in *N. lugens* [16]. Group V only encode the catalytic CDA domain. At present, the research on chitin deacetylase mainly concentrates on the discussion of its biological function, while there are few reports on the activity of chitin deacetylase in vitro. In 2014, the *B. mori* midgut PM protein BmCDA7 was expressed in yeast and displayed chitin deacetylase activity [15]. In 2017, Zhao found that the CDA genes cloned from *Locusta migratoria* revealed enzyme activity [18].

In this paper, we report the identification of a novel chitin deacetylase, *HcCDA4*, from the American white moth, *H. cunea*. *HcCDA4* belongs to Group III CDA and its enzymatic activity was studied, which was of great significance for the subsequent study of chitin deacetylase.

Experimental Section

Insects and Insect Cell Lines

Fall webworm larvae (*Hyphantria cunea*; Lepidoptera; Arctiidae) were purchased from the Chinese academy of forestry science and reared on an artificial diet under a 16h light / 8h dark photoperiod at 26 (±1) ℃ with 75 (±10) % relative humidity.

The insect cell line used in this study was Sf9 from *Spodoptera frugiperda*. Sf9 cells were donated by Professor Li Guoxun of Qingdao Agricultural University, and cultured at 27 ℃ in Grace's medium supplemented with 10% fetal bovine serum (FBS).

Total RNA Isolation and First-strand cDNA Synthesis

Total RNA was isolated from the fifth-instar larvae with Tiangen RNAprep Pure Tissue Kit (Beijing, China), and the first-strand cDNA Synthesize Kit (Promega, America) was used to synthesize first-strand cDNA with 1.2μg total RNA in a 20μl reaction following the manufacturer's instructions.

Cloning of a Chitin Deacetylase CdNA Coding for *HcCDA4*

Based on the *H. cunea* transcriptome database, a complete cDNA sequence of *HcCDA4* was obtained. The gene specific primers was designed using the DNAMAN software. The full-length gene was amplified by PCR and sequenced in Beijing Huada company, primers details shown in Table 1.

Prokaryotic Expression and Preparation of Polyclonal Antibodies

The PCR product contained restriction enzyme sites (*Kpn* I & *Not* I) was cloned into the prokaryotic expression vector pET30a, and then the recombinant expression vector pET30a-*HcCDA4* was transformed into *E. coli* BL21 (DE3). The recombinant HcCDA4 protein was expressed abundantly as an insoluble inclusion body by induction with 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 8 h at 37 ℃ and confirmed by SDS-PAGE. The recombinant proteins were used to injected into

rabbits to generate polyclonal antibodies (by Hebei Sciences Academy Research Institute of Biology).

Expression of *HcCDA4* in Insect Cells Sf9

To express *HcCDA4* in insect cells, a recombinant baculovirus was constructed using the Bac-to-Bac® baculovirus expression systems (Invitrogen, Carlsbad, CA, USA). The cDNA for the *HcCDA4* was digested with *Sal* I & *Xho* I and cloned into the vector pFastBacHTA, generating recombinant pFastBac-*HcCDA4*. The plasmid pFastBac-*HcCDA4* was transformed into *E. coli* DH10Bac followed the instruction provided by the manufacturer. *HcCDA4* would be transposed into Bacmid and constructed Bacmid-*HcCDA4* (Bac-*HcCDA4*), then cellfectin Ⅱ reagent was used to help Bac-*HcCDA4* infect Sf9 cells. The first generation of the recombinant baculoviruses was named P1 virus, and continue infect Sf9 cells to get P2 virus and P3 virus respectively, collect P3 virus supernatant to obtain recombinant HcCDA4 proteins and detected by western blot analysis. The empty carrier transfected Sf9 cells supernatant was used for comparison.

Enzymatic Characterization of the Chitin Deacetylase

Recombinant protein HcCDA4 was crude purified by ammonium sulfate, and the method of chitin deacetylase activity was described by Zhong *et al* [15], in which the chitin deacetylase catalyzes the p-nitroacetanilide to p-nitroaniline. The enzyme assay of recombinant HcCDA4 was analyzed by measuring the absorbance at A_{400} in a spectrophotometer and the inactivated recombinant HcCDA4 proteins were used as control.

The optimal temperature was determined by incubating the recombinant HcCDA4 proteins at the temperatures ranging from 30 ℃ to 80 ℃ in Tris-HCl buffer (pH8.0) for 15 min. The enzyme activity in different temperature conditions were measured. The highest enzyme activity was regarded as 100%, and the rest for the relative enzyme activity, respectively. Three test repeats were set for each group.

The optimal pH of the recombinant HcCDA4 proteins were determined over various pH ranges of Tris-HCl buffer (pH 3.0-9.0). The recombinant HcCDA4 proteins were incubated in different buffer solutions for 15 min at the optimal temperature. The enzyme activity of different pH conditions were measured. The highest enzyme activity was regarded as 100%, and the rest for the relative enzyme activity, respectively. Three test repeats were set for each group.

Effect of Ions on Chitin Deacetylase Activity

To study the effects of different metal ions on the HcCDA4 protein activity, different metal ions, Ca2+、Mg2+、Zn2+、Fe2+、Co2+、Mn2+,were added to the reaction solution individually with concentration ranges of 0、1.0、2.0、3.0、4.0、5.0 mmol Ł-1, respectively. Chitin deacetylase activities were determined under the optimal temperature and pH above. The reaction mixture without the metal ions was used as control, and the rest for the relative enzyme activity, respectively. Three test repeats were set for each group.

Results and Discussion

Full-length Gene Amplification of *HcCDA4*

The full-length cDNA sequence of HcCDA4 was identified from H. cunea with gene specific primers (Table1), and the gene is 1494 bp in length (Fig. 1) and purified by Tiangen DNA Purification Kit.

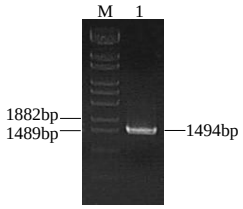

Fig. 1 PCR amplification of *CDA4* gene from *H.cunea* M: DNA Marker λ-*Eco*T14 1: PCR product of *CDA4* gene

Recombinant Expression of *HcCDA4* in *E. coli* BL21

The *HcCDA4* sequence was cloned into the prokaryotic expression vector pET30a with gene specific primers (Table1) contained restriction enzyme sites (*Kpn* I & *Not* I) (Fig. 2A). Then the recombinant expression vector pET30a-*HcCDA4* was transformed into the *E. coli* BL21 and induced with IPTG. SDS-PAGE analysis showed that there was one specific band corresponding to the molecular mass of HcCDA4 (56kDa) as shown in Fig. 2B. Polyclonal antibodies were prepared and detected (1:8000) with HcCDA4 recombinant proteins (Fig. 2C).

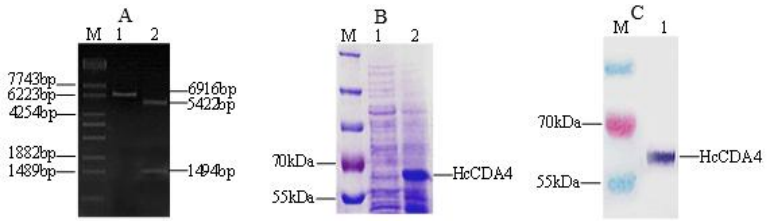

Fig.2 Verification of the recombinant expression vector pET30a-HcCDA4, SDS-PAGE analysis of its expression in E. coli and polyclonal antibodies quality test. A: M: DNA Marker λ-EcoT14; 1: pET30a-HcCDA4/ Not I; 2: pET30a-HcCDA4/ Kpn I&Not I; B: M: Pre dye Marker; 1: pET30a-HcCDA4 uninduced; 2: Expression of pET30a-HcCDA4 after IPTG induced 8 h; C: M: Pre dye Marker; 1: HcCDA4 recombinant proteins expression detected with HcCDA4 polyclonal antibodies.

Recombinant Expression of *HcCDA4* in Insect Cells

The recombinant baculovirus expression vector pFastBac-HcCDA4 was constructed by connection, transformation and identification (Fig. 3A). After that, pFastBac-HcCDA4 was transformed into DH10BacTM competent cells and the PCR amplification was used to identify Bacmid-HcCDA4 (Fig. 3B). Liposome transfection method was used to help Bacmid-*HcCDA4* transfect insect cells Sf9. After transfection for 72 h, compared with empty vector transfected Sf9 cells (Fig. 3D), the cells transfected with recombinant baculovirus were diameter increases, nucleus becomes larger, and a large number of cells were suspended in the culture medium (Fig. 3E). The HcCDA4 expressed in Sf9 cell line was detected by western blot analysis (Fig. 3C).

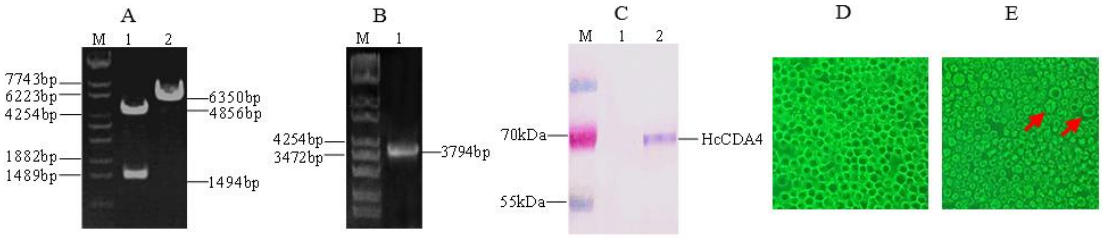

Fig. 3 The expression of *HcCDA4* in insect cells. A: Verification of the recombinant vector pFastBac-HcCDA4. M: DNA Marker λ-EcoT14; 1: pFastBac-HcCDA4/Sal I & Xho I; 2: pFastBac-HcCDA4/Xho I; B: PCR verification of the recombinant bacmid-HcCDA4. M: DNA Marker λ-EcoT14; 1: PCR product of recombinant Bacmid-*HcCDA4* with universal primers M13F/R; C: Western blot analysis of the *HcCDA4* expression in insect cells. M: Pre dye Marker; 1: Contrast; 2: Supernant of insect cells infected by recombinant virus Bacmid-HcCDA4 at 72 h; D: Contrast; E: Insect cells infected by recombinant virus Bacmid-*HcCDA4* at 72 h.

Characterization of the Chitin Deacetylase

Enzyme activity showed that the optimal temperature of HcCDA4 was 50 ℃, and the enzyme activity value was 3.44 U mL^{-1} under the optimal temperature. Relative enzymatic activity decreased rapidly when the reaction temperature was 80 ℃ (Fig. 4A). As shown in Fig. 4B, the optimal pH of HcCDA4 was 8.0, and the enzyme activity value was 3.44 U mL^{-1} under the optimal pH. Relative enzymatic activity decreased rapidly when the pH value was less than 6.0 or more than 8.0 showing that the chitin deacetylase functioned better under weak alkaline pH conditions.

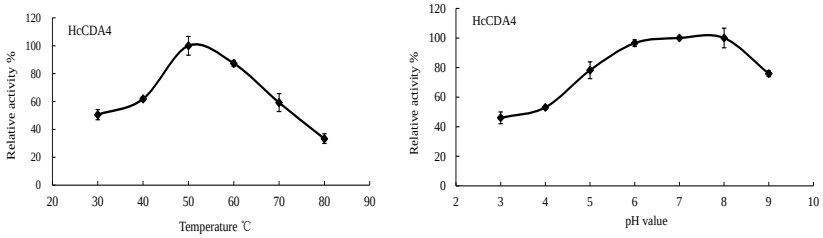

Fig. 4 The impact of temperature and pH on the enzyme activity

Effect of Metal Ions on Enzymatic Activity

As shown in Table 2, under the optimum reaction conditions, the enzyme activities of HcCDA4 protein was 3.44 U mL^{-1}. $Mg^{2+}$and Co^{2+} showed an inhibition on HcCDA4, an increasing Mg^{2+} or Co^{2+} concentration would lead to the enhancement of inhibitory effects on HcCDA4; Ca^{2+} showed a trend of increasing first and then decreasing on HcCDA4 enzymatic activity; Zn^{2+}, Mn^{2+} and Fe^{2+} showed activation on enzyme activities, Fe^{2+} showed activation stably with increasing concentration, while Zn^{2+} and Mn^{2+} showed the activation of decreasing with increasing concentration.

Table 1 Primers for PCR amplification and expression

Primer name	Primer sequence (5'-3')	Application of primers
HcCDA4	F1: ATGTCGATACACACAGTG	Gene amplification
	R1: TTACTTCCTTCTGAGGTTTTCTGGTAC	
	F2: CGGGGTACCTTCATTACCGGAAGTTATCAAC	Prokaryotic expression
	R2: ATTTTGCGGCCGCTTACTTCCTTCTGAGGTTTTCTGGTAC	
	F3: GCGTCGACATGTCGATACACACAGTG	Eukaryotic expression
	R3: CCGCTCGAGTTACTTCCTTCTGAGGTTTTCTGGTAC	

The boxes marked the restriction sites.

Table2 The impact of metal ions on the enzyme activity

Ion concentration	HcCDA4 enzyme activity (U·mL^{-1})					
(mmol·L^{-1})	Mg^{2+}	Ca^{2+}	Fe^{2+}	Zn^{2+}	Mn^{2+}	Co^{2+}
0	3.44	3.44	3.44	3.44	3.44	3.44
1	2.64	5.19	3.68	23.91	28.96	1.64
2	1.75	2.83	4.51	16.36	21.51	0.45
3	1.75	2.66	6.59	16.01	18.80	0.42
4	0.76	2.35	9.69	14.54	16.03	0.17
5	0.17	1.39	9.16	13.57	14.84	0.05

Discussion

Chitin deacetylases (CDAs) are metalloproteins that belong to an extracellular chitin-modifying enzyme family, carbohydrate esterase family 4 (CE-4) of the carbohydrate active enzymes (CAZY) database (http://www.cazy.org), which deacetylate chitin to form chitosan [19]. Most of CDA active center contains a metal ions, which play a key role in the catalytic process. Yun Wang found a chitin deacetylase activity in *Aspergillus nidulans* and the activity of chitin deacetylase was affected by a range of metal ions and ethylenediaminetetraacetic acid [10]. Binesh Shrestha found that the enzyme activity of CDA gene from *Colletotrichum lindemuthianum* is enhanced in the presence of Co^{2+} ions [20]. Wang yao found the enzyme activity of CDA from *Micromonospora aurantiaca* was promotied by Ca^{2+}, whereas Cu^{2+}, Zn^{2+} and Mg^{2+} showed inhibitory effect [21]. In 2014, Zhong measured the CDA activity of BmCDA7 with the procedure for measuring CDA activity assay developed by Li [22].

In this paper, the *HcCDA4* gene, belongs to group III CDA, was cloned from *H. cunea*. The active HcCDA4 proteins were obtained and showed catalytic activity *in vitro*, and the optimum temperature and pH were 50℃ and 8.0, same with our previous study of the enzyme activity of HcCDA1 and HcCDA2 [23]. Under the optimum reaction conditions, the enzyme activities of HcCDA1, HcCDA2 and HcCDA4 protein were 2.77 U mL^{-1}, 4.24 U mL^{-1} [23] and 3.44 U mL^{-1}, respectively, and the activity of HcCDA1, HcCDA2 and HcCDA4 showed significant differences. The activity of chitin deacetylase was also affected by a range of metal ions. $Mg^{2+}$and Co^{2+} showed an inhibition on HcCDA4, an increasing Mg^{2+} or Co^{2+} concentration would lead to the enhancement of inhibitory effects on HcCDA4, but Co^{2+} showed activation on HcCDA1 and HcCDA2; Ca^{2+} showed a trend of increasing first and then decreasing on HcCDA4 enzymatic activity; Zn^{2+}, Mn^{2+} and Fe^{2+} showed activation on enzyme activities, while $Zn^{2+}$and Mn^{2+} showed inhibition on HcCDA1 and HcCDA2 [23]. These results indicated that HcCDA1, HcCDA2 and HcCDA4 may contains different metal ions in the CDA active center. This research enriched the study of chitin deacetylase gene, and it was helpful for further study of the role of chitin deacetylase gene in the growth and development of *H. cunea*.

Acknowledgments

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