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Synthesis and High Expression of Chitin Deacetylase from *Colletotrichum lindemuthianum* in *Pichia pastoris* GS115

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A gene, *ClCDA*, encoding chitin deacetylase from *Colletotrichum lindemuthianum*, was optimized according to the codon usage bias of *Pichia pastoris* and synthesized *in vitro* by overlap extension PCR. It was secretorily expressed in *P. pastoris* GS115 using the constitutive expression vector pHMB905A. The expression level reached the highest with 110 mg/l culture supernatant after 72 h of methanol induction, which comprised 77.27 U/mg chitin deacetylase activity. SDS-PAGE, mass spectrometry, and deglycosylation assays demonstrated that partial recombinant protein was glycosylated with an apparent molecular mass of 33 kDa. The amino acid sequences of recombinant proteins were confirmed by mass spectrometry.

Keywords: Chitin deacetylase, *Colletotrichum lindemuthianum*, high expression, *Pichia pastoris*, synthesis

Chitin deacetylase (CDA, E.C. 3.5.1.41) is an enzyme that hydrolyses the *N*-acetamido groups of *N*-acetyl-D-glucosamine (GlcNAc) residues in chitin to produce chitosan [25]. It is one of the members of the carbohydrate esterase family 4 according to the CAZY database (http://www.cazy.org/) [5]. CDAs have been reported to occur in several fungi, some insect species, and marine bacteria [29]. The most well-studied CDAs are those from the fungi, such as *Mucor rouxii* [2, 13], *Absidia coerulea* [9], *Aspergillus nidulans* [1], *Saccharomyces cerevisiae* [7], *Schizosaccharomyces pombe* [16], and *Colletotrichum lindemuthianum* [19, 22].

Chitosan, the hydrolysis product of chitin by CDA, has great potentials in many industrial applications, such as materials science, biomedicine, food ingredients, cosmetics, and pharmaceuticals [8]. Compared with the presently used chemical procedure, the use of CDA for the conversion of chitin to chitosan has advantages, such as easily controlled, environmentally safe, and non-degradable process, resulting in the production of novel, well-controlled chitosan polymers and oligomers [12].

To the present, although some CDAs have been reported to be heterologously expressed in *Escherichia coli* [21, 23] and *P. pastoris* [18, 10], the proteins yields were not high. Therefore, it limits the application of CDAs in large-scale biotechnology.

In this study, in order to obtain CDA possessing high yields for high-scale production of chitosan or deacetyl *N*-chitooligosaccharides, the CDA gene from *C. lindemuthianum* (*ClCDA*) was optimized based on the codon usage bias of *P. pastoris* and synthesized with overlap extension PCR, and the putative signal peptide was deleted from *ClCDA*. We described the optimal DNA sequence synthesis, recombinant strain construction, and secretory expression in *P. pastoris*. To our knowledge, this is the first report of a high production of CDA through gene synthesis and codon optimization.

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MATERIALS AND METHODS

Reagents

Chitosan was purchased from Sigma (USA). The BCA protein assay kit was purchased from Beyotime Institute of Biotechnology (P. R. China). All other chemicals were of the highest purity commercially available.

Strains, Plasmids, and Media

E. coli XL10-GOLD and *P. pastoris* GS115 were from Invitrogen (USA). The vector pMD18-T was from Invitrogen (USA) and pHBM905A is stored in our laboratory. Buffered glycerol-complex medium (BMGY), buffered methanol-complex medium (BMMY), and minimal dextrose medium (MD) were prepared as described in the instruction manual of the Invitrogen *Pichia* expression kit (USA).

Design and Synthesis of *ClCDA* Gene

A DNA-coding sequence, suiting the triple codon preference of *P. pastoris*, was modified from the *CDA* gene of *C. lindemuthianum* (GenBank Accession No. AY 633657). In order to synthesize *ClCDA*, 22 oligonucleotides (Table 1) were designed with the DNAWorks program (http://helixweb.nih.gov/dnaworks/) for overlap extension PCR. For designation of the oligonucleotides, several parameters should be defined. These parameters include the target genome (currently limited to *P. pastoris*), annealing temperature (currently limited to be within 58−62°C), and the oligonucleotides length (currently limited to be within 48−52), and the other parameters were kept at default settings. Subsequently, the amino acid sequence of the protein was input into the "Sequence(s)" column, and then the oligonucleotides were designed. The PCR process was performed according to previous reports [4, 6]. The synthesized *ClCDA* fragment was cloned into pMD18-T for DNA sequencing.

Construction of Expression Plasmid for the Expression in *P. pastoris* GS115

The full-length *ClCDA* fragment was amplified with primers p-clcd-1/p-clcd-22 (Table 1), and then digested with T4 DNA polymerase supplemented with dTTP to form overhangs compatible with the sticky ends of the pHBM905A previously digested with *Cpo*I and *Not*I [28]. After this, these two fragments were ligated, and the recombinant plasmid, named pHBM-*ClCDA,* was confirmed by PCR and DNA sequencing. Transformation of *P. pastoris* GS115 and expression of the recombinant protein were carried out according to the manual of the *Pichia* expression kit (Invitrogen).

Table 1. Primers used in this study for PCR.

Primers	Sequence (5'→3')
clcd-1	gtcacaggttccagttggactccaattt
clcd-2	cctggttgagtacattgcaaaattggagtaccaactggaacctg
clcd-3	tgcaatgtactcaaccaggtctggttgctctaacttacgacgatggtccattt
clcd-4	gcttcaaaatatccaacaactgtggagtaaaagtaaatggaccatcgtcgtaa
clcd-5	cagttgttggatattttgaagcagaacgatgttagggctaccttctttgttaa
clcd-6	aaccagcttcaatgttagcccagttattaccgttaacaaagaaggtagcccta
clcd-7	gctaacattgaagctggttctaacccagatactattagaagaatgagggctga
clcd-8	gagcgtaagtatgagaaccaaccaaatgaccatcagccctcattcttctaata
clcd-9	tggttctcatacttacgctcatccagatttgaacactttgtcttccgctgaca
clcd-10	cgtagcttcttcaacatgcctcatttgagaaattctgtcagcggaagacaaag
clcd-11	gcatgttgaagaagctacgaggagaattgatggttttgctccaaagtacatga
clcd-12	acaaccagcatcacaagacaagtatggagctctcatgtactttggagcaaaac
clcd-13	tcttgtgatgctggttgtcaaggtgatttgggcggtttgggttaccatattat
clcd-14	tttcgtaatccttagtatccaagttagtgtcgataatatggtaacccaaaccg
clcd-15	cttggatactaaggattacgaaaacaataagccagaaactactcatctatctg
clcd-16	tctgcactcaattcgttattgaacttttcagcagatagatgagtagtttctgg
clcd-17	caataacgaattgagtgcagatgttggagctaactcttacattgttctttctc
clcd-18	agaaacaacagtttgttcatggacatcgtgagaaagaacaatgtaagagttag
clcd-19	catgaacaaactgttgtttctttgactcaaaagttgattgatactttgaagtc
clcd-20	tcaccaacagtaacagctctgtatcccttagacttcaaagtatcaatcaactt
clcd-21	gagctgttactgttggtgaatgtctaggtgatgccccagagaactggtacaag
clcd-22	ggccattaagccttgtaccagttctctggg

The underlined parts of p-clcd-1 and p-clcd-22 were attached with part of the *Cpo*I and *Not*I restriction sites, respectively.

Yeast Transformation

The recombinant yeast expression plasmid was linearized with the restriction enzyme *Sal*I, and then transformed into *P. pastoris* GS115 by electroporation (7,500 V/cm, 25 lF, 400 X; Bio-RadGene Pulser, USA). The positive His^+ transformants were selected on MD plate, and then confirmed by colony PCR.

Expression and Purification of Recombinant ClCDA

These positive colonies were cultured in a 1,000 ml shake flask containing 100 ml of BMGY at 28°C for 2 days. Cells were harvested by centrifugation at 5,000 ×*g* for 10 min at 4°C and recultured in 100 ml of BMMY at 25°C for 7 days, followed by induction with 1.0% methanol every 24 h. To purify the recombinant ClCDA, cultures of the transformants were centrifuged at 15,000 ×*g* for 15 min at 4°C. The cell-free supernatant was concentrated at 75% saturation of ammonium sulfate at 4°C for overnight. The resulting precipitate was collected by centrifugation at 15,000 ×*g* for 15 min at 4°C, and then dissolved in 20 mM Tris-HCl (pH 7.0). The purified ClCDA was used for biological assays.

SDS-PAGE and Protein Concentration Determination

SDS-PAGE was performed according to Laemmli [15]. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. The concentration of the purified ClCDA was determined using a BCA protein assay kit with bovine serum albumin as a standard.

Identification of the Recombinant Protein by Mass Spectrometry

Bands of target proteins on the SDS-PAGE gel were excised, and then identifications were carried out according to the previous report [17]. The peptides were analyzed with an Ultraflex II MALDI-TOF/TOF mass spectrometer. The result of the peptide mass fingerprint (PMF) was processed with Biotools software and searched against the National Center for Biotechnology Information (NCBI) database using the Mascot search engine (http://www.matrixscience.com).

Deglycosylation of ClCDA

Deglycosylation of recombinant protein was performed according to instructions of the manufacturer (New England, Biolabs). The protein was denatured at 100°C for 10 min, and then incubated with Endoglycosidase H (Endo H) at 37°C.

Activity Determination of the Recombinant ClCDA

ClCDA activity was assayed by the method reported by Win and Stevens [27]. Enzymatic activity was measured at 50°C in a heterogeneous incubation mixture containing a suspension of 50 mg of 60% deacetylated chitosan, 1 ml of enzyme preparation, and 5 ml of 50 mM Tris-HCl buffer (pH 8.5). The released acetic acid concentration was measured [27]. One activity unit (U) was defined as the amount of the enzyme required to release 1 μmol acetic acid per minute. All measurements were carried out in triplicate.

Results

Chemical Synthesis of the *ClCDA* Gene

The length of the original *CDA* sequence of *C. lindemuthianum* is 747 bp, and contains a putative signal peptide (27 amino acids) at its N terminus. The optimized *ClCDA* (666 bp),

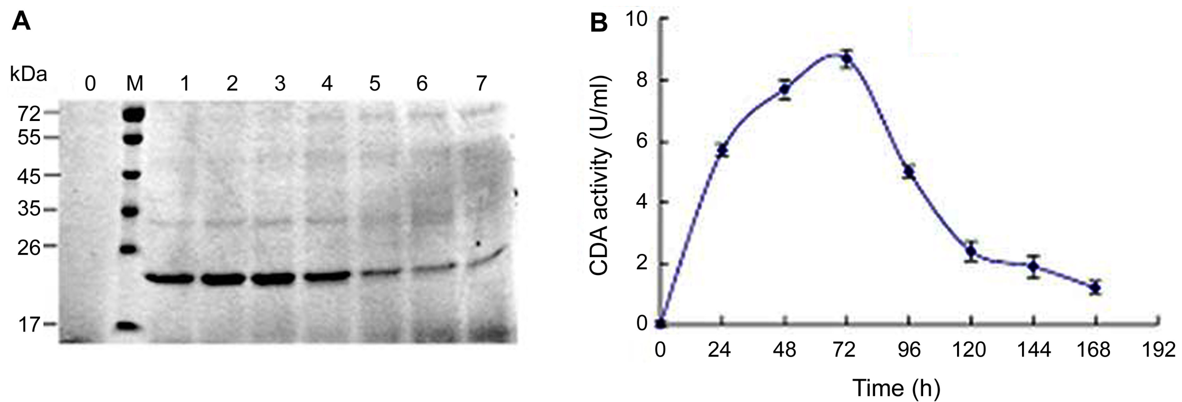

Fig. 1. Analyses of the recombinant ClCDA and the CDA activity.
(**A**) SDS-PAGE analysis of recombinant ClCDA expression at different induction times. Lane 0: the control of *P. pastoris* GS115 transformed with vector pHBM905A. Lanes 1 to 7: the proteins in the supernatant of cell cultures after induction for 1−7 days (samples were collected daily), respectively; Lane M: molecular weight marker of proteins. (**B**) Chitin deacetylase activities of crude supernatants after methanol induction for 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h.

without the putative signal peptide, does not alter their coding amino acid sequences compared with the original CDA. The sequence alignment between optimized *ClCDA* and original *CDA* was performed with CLUSTALW where 186 nucleotides were changed in the novel ORF. The synthesized *ClCDA* showed 72% identity with the original *CDA* after decreasing the AT-rich region and changing the distribution of the GC content and the optimized codons. A DNA sequence corresponding to *ClCDA* was synthesized with overlap extension PCR. The synthesized *ClCDA* was cloned into the pHBM905A vector by fusing to the downstream of the MF-alpha leader sequence for secretory expression in *P. pastoris* GS115. The resulting recombinant plasmid was named as pHBM-*ClCDA*.

Expression and Purification of Recombinant ClCDA in *P. pastoris* GS115

The recombinant plasmid pHBM-*ClCDA* was linearized with *Sal*I, and then transformed into *P. pastoris* GS115. The positive recombinant strains containing the *ClCDA* sequence were chosen for shake flask expression. The strain transformed with pHBM905A without insert was used as the control. During methanol induction, the supernatant of the cell culture was sampled daily and separated on SDS-PAGE (Fig. 1A).

The production of ClCDA was determined, accumulating from first day after methanol induction, peaking at 72 h, and decreasing thereafter. At 72 h, the activity of the crude supernatant reached the highest level of 8.7 U/ml (Fig. 1B). The result of integral analysis (GeneTools software, USA) showed that the quantity of ClCDA reached 85% in the total secreted proteins of *P. pastoris* GS115/pHMB-*ClCDA*. The purification of ClCDA from the culture supernatant of *P. pastoris* was performed by ammonium sulfate precipitation and details are provided in Table 2. Following purification, about 110 mg of ClCDA was obtained from 1 L of culture medium.

Identification of the Recombinant Protein by Mass Spectrometry

Two bands were detected by SDS-PAGE in the purified ClCDA (Fig. 2A, lane 1, bands a and b). The smaller and prominent band had a molecular mass of 25 kDa, which was consistent with the molecular mass calculated from its deduced amino acid sequence (24.6 kDa). The other band, larger than the calculated molecular mass, had a molecular mass of 33 kDa. Identical amino acid sequences were obtained from the two bands by MALDI-TOF-MS identification (Fig. 2B and Table 3), indicating the existence of potential glycosylation in the recombinant protein.

Table 2. Ammonium sulfate precipitate purification of ClCDA from *P. pastoris*.

Step	Total activity (U)	Total protein (mg)	Specific activity (U /mg)	Purification yield (%)
Supernatant[a]	8,700	129.4[b]	67.23	100
Ammonium sulfate precipitate	8,500	110[c]	77.27	97

One unit of enzymatic activity was defined as the amount of the enzyme required to produce 1 μmol of acetic acid per minute under standard conditions as described in Materials and Methods. [a]The volume of the supernatant was 1 L. [b]The enzyme concentration was crude. [c]The enzyme concentration was purified.

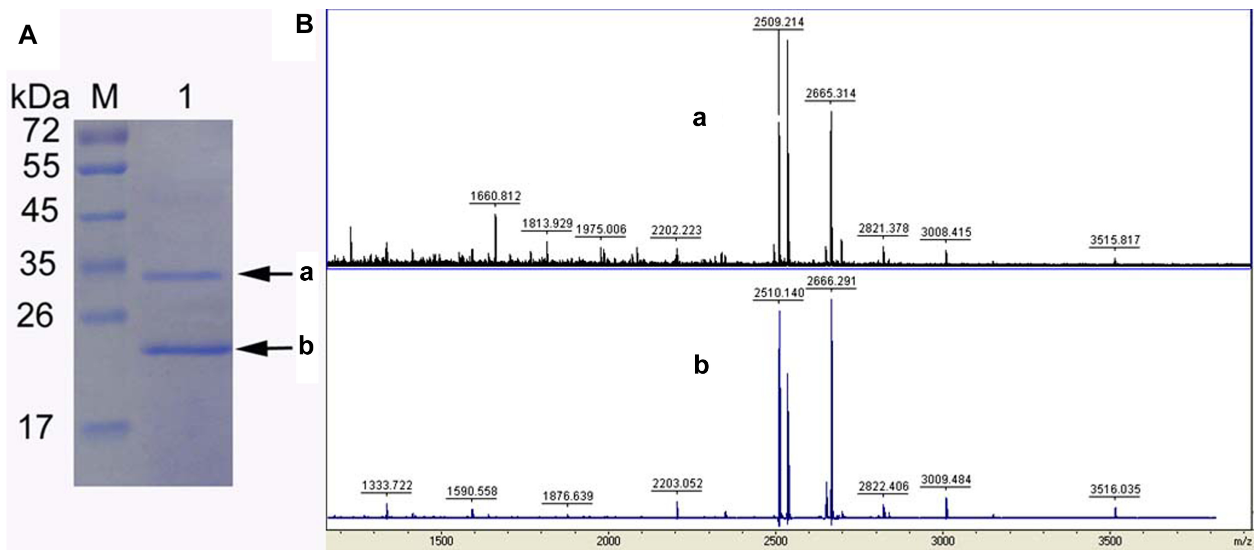

Fig. 2. SDS-PAGE of purified ClCDA and MALDI-TOF-MS peptide mass fingerprint (PMF) of recombinant ClCDA generated by in-gel digestion.

(**A**) SDS-PAGE of purified ClCDA; (**B**) MALDI-TOF-MS PMF of recombinant ClCDA. (a) PMF of protein from band a; (b) PMF of protein from band b.

Deglycosylation of Recombinant ClCDA

Since the two proteins from *P. pastoris* shared the same amino acid sequences, the proteins were analyzed by Endo H. After deglycosylation, the two bands co-migrated with a molecular mass of 25 kDa (Fig. 3), which indicated that the 33 kDa protein (lane 1, band a) was the product of the native 25 kDa protein (lane 1, band b) with some degrees of *N*-glycosylation.

Discussion

Among several identified CDAs, *C. lindemuthianum* CDA has been the most well-studied, including its biochemical properties [3, 22], catalytic mechanism [11, 24], and biological roles [14]. Therefore, the CDA from *C. lindemuthianum* was chosen as the target protein for DNA synthesis, heterologous expression, and further application in our study.

Table 3. Identification results of MALDI-TOF-MS of proteins bands excised from the SDS-PAGE gel shown in Fig. 2.

Band no.	Protein name	Species	Coverage	No. of peptides matched[a]	Peptides sequences of identification	Confidence level[b]
a	Chitin deacetylase	*C. lindemuthianum*	43%	13 (6)	70 – 92 R.ATFFVNGNNWANIEAGSNPDTIR.R 70 – 93 R.ATFFVNGNNWANIEAGSNPDTIRR.M 94 – 119 R.MRADGHLVGSHTYAHPDLNTLSSADR.I 96 – 119 R.ADGHLVGSHTYAHPDLNTLSSADR.I 143 – 171 R.APYLSCDAGCQGDLGGLGYHIIDTNLDTK.D 188 – 219 K.FNNELSADVGANSYIVLSHDVHEQTVVSLTQK.L	76 (73)
b	Chitin deacetylase	*C. lindemuthianum*	51%	19 (7)	70 – 92 R.ATFFVNGNNWANIEAGSNPDTIR.R 70 – 93 R.ATFFVNGNNWANIEAGSNPDTIRR.M 94 – 119 R.MRADGHLVGSHTYAHPDLNTLSSADR.I 96 – 124 R.ADGHLVGSHTYAHPDLNTLSSADRISQMR.Q 143 – 171 R.APYLSCDAGCQGDLGGLGYHIIDTNLDTK.D 172 – 187 K.DYENNKPETTHLSAEK.F 188 – 219 K.FNNELSADVGANSYIVLSHDVHEQTVVSLTQK.L	85 (73)

[a]The number in brackets indicates peptides matched from MALDI-TOF-MS data.

[b]Mowse scores are provided for proteins identified by MALDI-TOF-MS. The number in brackets indicates the significance threshold.

Table 4. Comparison of CDAs produced by recombinant expression.

CDA source	Expression host	Special activity (U/mg)[a]	Expression form and level (mg/l)	Source or reference
C. lindemuthianum UPS9	*P. pastoris*	77.27	Soluble form (110)	This study
A. nidulans	*E. coli*	4.17	Inclusion body	26
C. lindemuthianum ATCC56676	*E. coli*	4.22	Inclusion body	23
C. lindemuthianum UPS9	*P. pastoris*	71.67	Soluble form (7.2)	18
R. circinans	*P. pastoris*	965.13	Soluble form (0.62)	10

[a]Special activity means the characterization of purified CDAs.

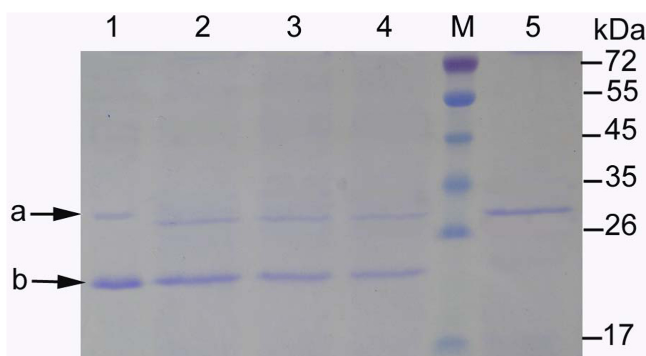

Fig. 3. SDS-PAGE analysis of recombinatant ClCDA after treatment with Endo H.

Lane 1: recombinant ClCDA; lanes 2, 3, 4: recombinant ClCDA after treatment with Endo H for 2 h, 3 h, and 4 h, respectively; lane M: molecular weight marker of proteins; lane 5: the protein of Endo H.

To apply CDA for enzymatic conversion of chitin to chitosan industrially, intensive screening of strong CDA producers and enhancing of expression level are necessary. Although CDAs mainly exist in some fungi, it is difficult to extract CDAs through the traditional preparation methods because of the low content. Therefore, genetic engineering technology is an effective way to increase enzyme expression levels. Cloning and expression of CDAs have great application in large-scale production of the enzyme for industrial use. Many attempts at the expression of CDAs in various systems have been reported, including those of *E. coli* and yeast. Nevertheless, most of them were not very successful because of either the low level of production or the insufficient enzyme activity. Some recombinant CDAs are summarized in Table 4.

Recently, a report indicated that protein expression levels correlate with codon usage bias of yeast, and codons of low usage can reduce the levels of expression [20]. Therefore, in this study, the optimization of the *ClCDA* gene according to *P. pastoris* optimal codons was performed. Corresponding to our expectation, the recombinant protein was expressed from the first day, the crude supernatant had a specific activity of 67.23 U/mg at 72 h, and the yield of the purified recombinant ClCDA was higher than those for reported CDAs in Table 4. In addition, the target protein was fused with the signal peptide of the α-mating factor from *S. cerevisiae*, which caused the ClCDA to be secreted into the growth medium. Furthermore, the native recombinant protein was partially glycosylated, which was not found in other reports of CDAs expressed in *P. pastoris*. Because of good solubility and high quantity, it is convenient to efficiently purify the target protein and get high yield, which provides the valuable material for application to produce some special-performance and high-quality chitosan or chitooligosaccharides and for further studies.

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