Production of a recombinant chitin deacetylase in the culture medium of *Escherichia coli* cells

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Abstract With the aid of a signal sequence of a chitinase from *Streptomyces lividans*, a recombinant chitin deacetylase, whose gene originated from a Deuteromycete, *Colletotrichum lindemuthianum*, was produced in the culture medium of *Escherichia coli* cells, existing as a highly active form without the signal peptide. During the production of the recombinant chitin deacetylase, both a slight increase in the value of $OD_{600\ nm}$ in the culture medium and a drastic decrease in viable cell number were observed. When penta-*N*-acetyl-chitopentaose was used as the substrate, the recombinant chitin deacetylase had comparable kinetic parameters to those of the original enzyme from the fungus. The addition of a C-terminal six histidine sequence to the recombinant enzyme caused a slight decrease in the k_{cat} value, and the further addition of a 12 amino acid sequence at its N-terminus caused a further decrease in the value. This production system allowed us to easily produce in the culture media the recombinant chitin deacetylases possessing as good properties as the original enzyme, without any disruption steps of the *E. coli* cells.

Key words: Chitin deacetylase; Recombinant enzyme; *Colletotrichum lindemuthianum*; *Escherichia coli*

1. Introduction

Amino sugars are widely distributed in a variety of organisms in all kingdoms, and they are mainly found in cell wall components such as chitin and peptidoglycans, or in extracellular matrices such as heparan sulfate and hyaluronic acid. Most of these residues in the extracellular polymers are *N*-acetylated, however, in some cases the cells drastically modify the functions of the polymers by removing the *N*-acetyl groups by themselves, in order to acquire resistance against attack by a lysozyme [1] and dispose free amino groups in the cell surface for binding to a negatively charged compound [2]. Polysaccharide *N*-deacetylases play crucial roles for these modifications, and several kinds have been investigated intensively [3–6].

Chitin is a linear polymer of *N*-acetylglucosamine residues, and is a component of fungal cell walls or arthropod integuments. Chitin deacetylases are the key enzymes for converting chitin into chitosan, the *N*-deacetylated form of chitin, and it has been suggested that the enzymes from Deuteromycetes are secreted out of the cells in the course of their invasion into host plants [7]. However, the significance of their production has not yet been elucidated, which is of particular interest from the viewpoints of biochemistry, physiology, and plant pathology.

We have been studying an extracellular chitin deacetylase from a Deuteromycete, *Colletotrichum lindemuthianum*, a pathogen of beans, for the purpose of understanding the significance of *N*-acetyl groups in chitin. We have purified and characterized the enzyme from the culture filtrate, but the yield was too low for further characterization, particularly of its structure [8]. Recently 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 [9]. However, the specific activity of the renatured enzyme was much lower than that of the original one from the fungus, and it was not a satisfactory system for further studies on the characterization of the reaction mechanisms with kinetics.

Herein we report the production of the recombinant chitin deacetylase in the culture medium of *E. coli* cells, with the aid of a signal sequence from *Streptomyces lividans*. The production of recombinant proteins in culture media of *E. coli* cells is rare [10], and it has been exclusively studied for the production of antibodies [11,12], a cytotoxin [13], and an insulin-like growth factor binding protein [14]. The recombinant chitin deacetylases produced in the culture media of *E. coli* had comparable kinetic parameters to those of the original enzyme from the fungus, indicating that the expression system can be applied for further characterization studies of the enzymes, and the system is quite desirable for efficient recovery of the recombinant chitin deacetylase because no disruption steps of the cells are required.

2. Materials and methods

2.1. Recombinant DNA

The PCR amplification of the chitin deacetylase gene from *C. lindemuthianum* was performed as described previously [9], using the cDNA as the template and a set of primers 1 (5′-GAATTC-CAGGTTCCCGTGGGCAC-3′, *Eco*RI site was incorporated at its 5′ end; underlined) and 2 (5′-AGATCTCGCCTTGTACCAGTTC-TCCG-3′, *Bgl*II site was incorporated at its 5′ end; underlined), or primers 1 and 3 (5′-AAGCTTACGCCTTGTACCAGTTCTCCG-3′, *Hin*dIII was incorporated at its 5′ end; underlined). PCR amplification of the N-terminal sequence of chitinase C from *S. lividans* [15] containing a signal sequence was also performed under the same reaction conditions, using the genomic DNA as the template and a set of primers 4 (5′-CCATG**G**GGCTTCAGACACAAAGCCG-3′, one base was modified from cytosine to guanine (in bold type) in order to introduce a *Nco*I site (underlined), which resulted in the substitution of Gly for Arg at the second amino acid residue of the N-terminus) and 5 (5′-GAATTCGGCCGCCTGGGCCGGGC-3′, *Eco* RI

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Abbreviations: CDA, chitin deacetylase

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was incorporated at its 5′ end; underlined), or primers 4 and 6 (5′-GAATTCCGAGGTCTTGGCGAAGGTG-3′, *Eco*RI site was incorporated at its 5′ end; underlined). In order to incorporate six histidine tags at the C-termini of the recombinant proteins, the amplified fragments recovered as double digested fragments were firstly ligated with a *Nco*I-*Bgl*II or *Nco*I-*Hin*dIII digested plasmid vector, pQE60 (Qiagen, USA). Then the plasmids were again digested with *Nco*I and *Hin*dIII, followed by the ligation of the obtained fragments with a *Nco*I-*Hin*dIII digested plasmid vector, pET28a(+) (Novagen, USA). The final constructions called pS1C, pS1CH and pS12CH (Fig. 1) were transformed into *E. coli* BL21 (DE3, pLysS) (Novagen).

2.2. Production and purification of recombinant proteins

The BL21 transformant containing the plasmid pS1C, pS1CH or pS12CH was grown in 100 ml LB medium with kanamycin (Km, 25 μg/ml) and chloramphenicol (Cp, 34 μg/ml) in 500 ml flasks at 200 rpm and 25°C to a cell density of 0.4 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and the cells were further incubated for 18–24 h. Viable cells were determined by colony counts developed in an LB agar medium with Km (25 μg/ml) and Cp (34 μg/ml). The culture medium (800 ml) was then separated from the cells by centrifugation (6500×*g*, 15 min at 4°C), and ammonium sulfate was added to the supernatant fraction (finally brought to 70% saturation) followed by an overnight settlement at 4°C. The sample was centrifuged (6500×*g*, 90 min at 4°C), and the brown pellet was resuspended in 16 ml 20 mM sodium tetraborate/HCl buffer (pH 8.50, buffer A) for dialysis in the same buffer (3 l). Then the ammonium sulfate-precipitated proteins containing a recombinant CDA with a six histidine tag (10 ml) were run on a nickel-loaded Hi-Trap chelating column (5 ml, Pharmacia Biotech, Sweden) with a flow rate of 1 ml/min and a fraction volume of 2 ml. Unbound proteins were eluted with a linear gradient of imidazole (0–50 mM, 25 ml) in buffer A containing 500 mM NaCl, and the recombinant CDA with a six histidine tag was eluted with an additional linear gradient of imidazole (50–250 mM, 25 ml) in the same buffer with NaCl. The fractions with chitin deacetylase activity (fractions 20–22) were recovered, and dialyzed in buffer A (1 l) followed by a concentration using an Ultrafree CL (Millipore Co., USA). As for the ammonium sulfate-precipitated proteins containing a recombinant CDA without a six histidine tag, the enzyme was purified with a hydrophobic interaction column chromatography and an anion exchange chromatography of Q-Sepharose (Pharmacia Biotech) [8]. The original chitin deacetylase from *C. lindemuthianum* was purified as described [8], with a slight modification in the last purification step: a gel filtration column chromatography (Sepharose 12 HR 10/30, Pharmacia Biotech) was used instead of the anion-exchange ion chromatography using Resource Q (Pharmacia Biotech).

2.3. Properties of the recombinant enzymes

The estimation of chitin deacetylase activities using glycolchitin as the substrate and the determination of kinetic parameters using penta-*N*-acetyl-chitopentaose as the substrate were performed as described previously [8]. The absorption coefficients of the purified enzymes were predicted according to the following equation: $A_{280\,nm}$ = [5800×(No. of tryptophan residues)+1390×(No. of tyrosine residues)]/(the molecular mass of the protein). Protein sequence analyses and SDS-PAGE analyses were performed as described previously [9]. The fused CDAs called S1-CDA, S1-CDAH, and S12-CDAH were designated as follows: S1-CDA, CDA whose N-terminus was fused with the modified signal sequence, a flanking Ala residue and a Glu-Phe sequence (a translated *Eco*RI site); S1-CDAH, S1-CDA fused at its C-terminus with an Arg-Ser sequence (a translated *Bgl*II site) and a flanking six histidine residues; S12-CDAH, S1-CDAH whose Ala-Glu-Phe sequence next to the modified signal sequence was substituted for a sequence consisting of 12 N-terminal amino acid residues of mature chitinase C (Ala-Thr-Ser-Ala-Thr-Ala-Thr-Phe-Ala-Lys-Thr-Ser) and the flanking Glu-Phe sequence.

3. Results

The ORF of the chitin deacetylase gene from *C. lindemuthianum* ATCC 56676 is composed of two parts: the sequences encoding a deduced prepro-domain (81 bp) and a mature chitin deacetylase (663 bp) [9], and in this study we amplified the latter part by PCR for the construction of fusion proteins. The chitinase C from *S. lividans* is known as an enzyme secreted in its culture medium, and its ORF is composed of four parts: the sequences encoding a signal sequence (90 bp), a substrate binding domain (315 bp), a fibronectin type III-like domain (282 bp), and a catalytic domain (1170 bp) [15]. Fig. 1 shows the three plasmids, pS1C, pS1CH, and pS12CH, constructed for the production of the recombinant chitin deacetylases fused with N-termini of chitinase C from *S. lividans* and with or without a six histidine sequence from pQE60 at the C-termini.

Fig. 2 shows the time course of the induction of CDA activity in the culture medium of *E. coli* harboring pS1CH. After the addition of IPTG, the production of CDA immediately started, and the total activity in the medium reached a maximum at 12–18 h after the induction by IPTG. Similar

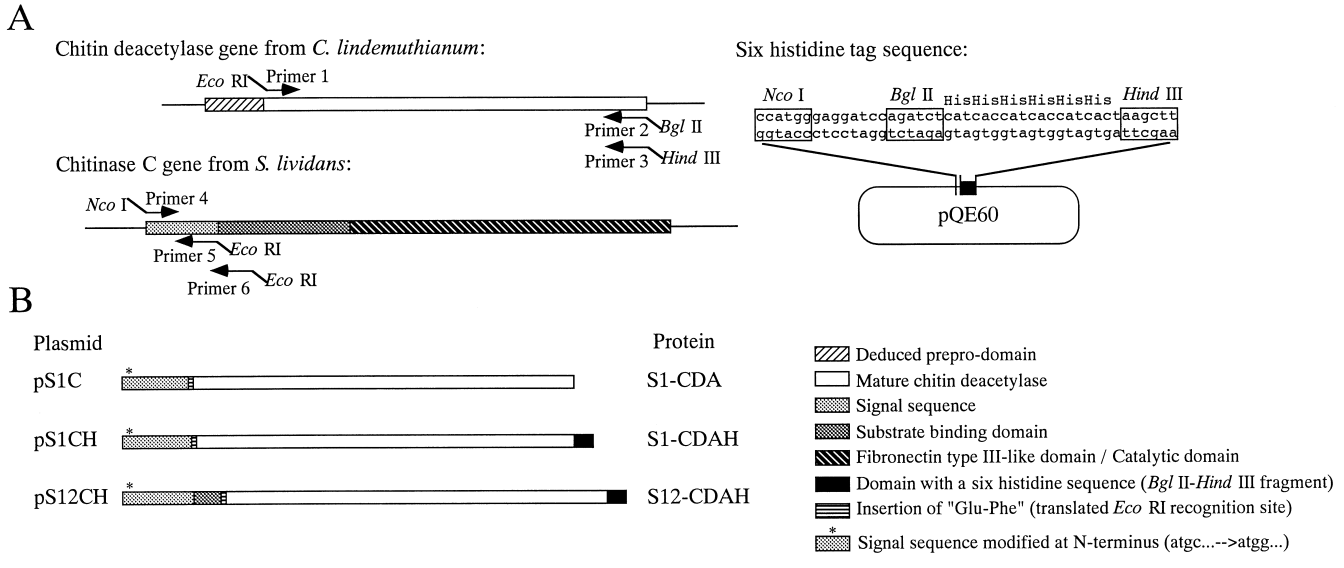

Fig. 1. Construction of recombinant chitin deacetylases. A: Origins of the fragments for the construction of fusion proteins and the positions for PCR amplification. The positions of oligonucleotide primers are indicated by arrows. B: Molecular architecture of the proteins used in this study.

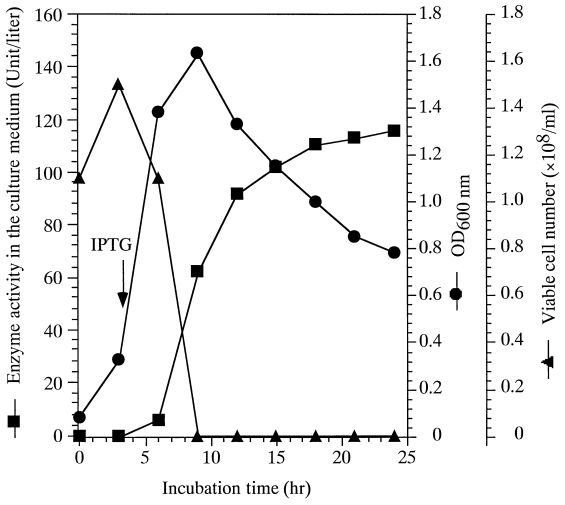

Fig. 2. Time course of the growth of *E. coli* cells and the secretion of S1-CDAH in the culture medium. IPTG was added when the $OD_{600\,nm}$ of the medium was 0.4 (arrow).

induction patterns were observed in the cases with the plasmids pS1C and pS12CH. Although an increase in the value of $OD_{600\,nm}$ for 6 h after the addition of IPTG was observed, the viability of the cells drastically decreased from 1.1×10^8 cells/ml of the culture medium at the incubation time of 6 h to 2.5×10^5 cells/ml at the incubation time of 9 h. The numbers of viable cells after the incubation time of 9 h were in the order of 10^5 cells/ml. Such a drastic decrease in the viability was not observed in the cultures without the addition of IPTG (data not shown). The recombinant proteins were purified to electrophoretic homogeneity as judged by SDS-PAGE analysis (Fig. 3), and to corresponding single peaks in the chromatograms of gel filtration chromatography without any aggregation observed (data not shown). The N-terminal amino acid sequences of S1-CDA and S1-CDAH were identical (Ala-Glu-Phe-Gln), and that of S12-CDAH was Ala-Thr-Ser-Ala, which indicates that their signal peptides were correctly removed in *E. coli* cells in the same way as the chitinase C digested by *S. lividans*. The yields of purified S1-CDA, S1-CDAH and S12-CDAH from 1 l of culture medium were 9.03 mg, 2.96 mg and 5.58 mg, respectively, with recoveries of 39.7%, 32.7% and 23.9%, respectively, of the activities produced in the media.

The kinetic parameters determined using penta-*N*-acetylchitopentaose as the substrate indicate that S1-CDA is highly active and comparable with the original chitin deacetylase (Table 1). The k_{cat} value of S1-CDAH was slightly lower than that of the original one, and a further decrease in the value was observed when the additional 11 amino acid residues were introduced to S1-CDAH at the N-terminus.

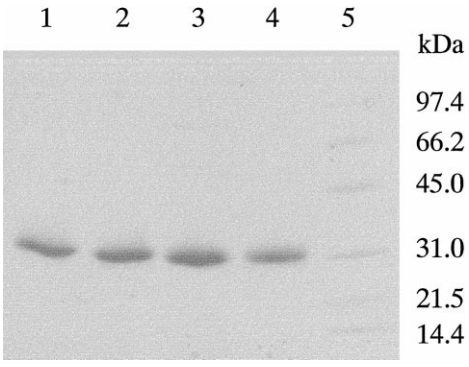

Fig. 3. SDS-PAGE of purified recombinant chitin deacetylases. Lane 1, S12-CDAH; lane 2, S1-CDAH; lane 3, S1-CDA; lane 4, the original chitin deacetylase from *C. lindemuthianum*; lane 5, molecular mass standards (SDS-PAGE Molecular Markers Low-range, Bio-Rad, USA.)

4. Discussion

In this work we used modified signal peptide sequences of chitinase C from *S. lividans*, which successfully localized the proteins out of the cells and were cut off at the predicted site by signal peptidases from *E. coli*. Robbins et al. [16] used a signal sequence from chitinase-63 from *Streptomyces plicatus*, which is highly homologous (96% identical) to that of chitinase C, for the expression of the chitinase in *E. coli* cells, which resulted in the secretion of the recombinant chitinase in the periplasmic space, as well as the removal of the signal peptide. Lowering the incubation temperature is a pragmatic technique for improving the solubility of the recombinant proteins, and it may somewhat affect the translational level, which is a critical factor for the secretion in *E. coli* cells [17]. The produced S1-CDA apparently existed in an active form with comparable K_m and k_{cat} values to those of the original enzyme. The addition of Ala-Glu-Phe residues at the N-terminus of CDA for production of S1-CDA did not significantly affect the kinetic parameters. Although the modifications of the N-terminus or the C-terminus of S1-CDA caused a decrease in the activity, neither inactivation nor aggregation was observed, which implies that the construction of fused recombinant chitin deacetylases at either terminus can be performed for the purpose of its efficient application in industry [18].

The production of recombinant proteins in the culture medium of *E. coli* cells has been reported by several groups [11–14], however, it might be rather regarded as an exceptional case with special proteins. In this report we first showed that a polysaccharide-modifying enzyme, chitin deacetylase, was successfully produced in the culture medium of *E. coli*. A drastic decrease in viable cell number was observed during the production of the recombinant CDA in the culture medium (Fig.

Table 1
Kinetic parameters of recombinant chitin deacetylases and the original enzyme from *C. lindemuthianum*

	Molecular mass (kDa)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
S1-CDA	24.7	40.6 ± 3.6	51.6 ± 0.3	1.28 ± 0.11
S1-CDAH	25.7	40.5 ± 5.7	30.3 ± 2.0	0.779 ± 0.050
S1-CDAH	26.8	41.6 ± 3.8	21.5 ± 1.1	0.533 ± 0.044
Original CDA (*C. lindemuthianum*)	24.3	44.6 ± 3.1	47.1 ± 0.9	1.06 ± 0.08

2). The discrepancy between the increase in the value of $OD_{600\,nm}$ and the decrease in the viability of the cells may be due to the simultaneous progress of both cell proliferation and the modification of the cells which causes the decrease in the viability. Although it is still not evident whether the leakage from the outer membrane or the lysis of the cells mainly participates in the production of the enzyme in the culture medium, this production system works well for obtaining recombinant CDAs without any significant proteolysis, judging from the data of SDS-PAGE, N-terminal amino acid sequence analyses, and the behaviors of S1-CDAH and S12-CDAH on a nickel-loaded Hi-Trap chelating column chromatograph. On the other hand, Forsberg et al. [19] reported that the mutation of the structure of the recombinant antibody fragment resulted in a change of its localization from the culture medium to the periplasm, suggesting that the structure of the protein drastically affects its localization. It is likely that the location of original enzymes, whose structures are optimized to be localized as such, is related to the tendency of the localization of corresponding recombinant enzymes in *E. coli* [10]. In general, polysaccharide-modifying enzymes are secreted out of the cells, and it is worth trying to establish new production systems of other enzymes using *E. coli* cells.

N-Deacetylases active on *N*-acetylglucosamine residues, which include chitin deacetylases [3], deduced peptidoglycan deacetylases from *Bacillus* sp. [5], heparan sulfate/heparin *N*-deacetylase/*N*-sulfotransferases [20], and chitooligosaccharide deacetylases [21], are known to show some similarities in amino acid sequence with highly conserved amino acid residues [22,23]. However, because of the lack of sufficient expression systems of the genes [21,22], the reaction mechanism and crystal structures of those *N*-deacetylases are still unclear. This production system for recombinant chitin deacetylases can facilitate the further characterization of the *N*-deacetylases with site-directed mutagenesis and crystallization. Furthermore, the chitin deacetylase is known as a tool in glycotechnology [24,25], and it is speculated that this system will be applicable for producing novel deacetylases with modifications such as catalytic activity and substrate recognition, with the aid of protein engineering techniques.

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