RESEARCH LETTER

Molecular characterization and antifungal activity of a family 46 chitosanase from *Amycolatopsis* sp. CsO-2

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Abstract

An actinomycete strain, *Amycolatopsis* sp. CsO-2, produces a 27-kDa chitosanase. To reveal the molecular characteristics of the enzyme, its corresponding gene *ctoA* was cloned by a reverse genetic technique, based on the N-terminal amino acid sequence of the protein. The encoded CtoA protein was deduced to be composed of 286 amino acids, including a putative signal peptide (1–48), and exhibited 83% identity in the amino acid sequence with the family 46 chitosanases from *Streptomyces* sp. N174 or *Nocardioides* sp. N106. The active recombinant CtoA protein was successfully overproduced in *Escherichia coli*. The mutant protein E22Q, in which the glutamic acid residue 22 was replaced with glutamine, abolished the chitosanase activity, showing that the Glu22 residue is required for the enzymatic activity. CtoA exhibited antifungal activity against *Rhizopus oryzae*, which is known to produce chitosan probably as a cell wall component. In contrast, E22Q did not inhibit the growth of the fungus, suggesting that chitosan-hydrolyzing activity is essential for the antifungal activity. It is noteworthy that the antifungal effect of CtoA against *R. oryzae* was drastically enhanced by the simultaneous addition of the family 19 chitinase ChiC from *Streptomyces griseus*.

Introduction

Chitosan is a polymer of glucosamine linked by β-1,4 bonds, while its acetylated compound, a polymer of *N*-acetylglucosamine, is called chitin. In contrast to chitin, which is the second most abundant biopolymer after cellulose in nature, chitosan has been found in very few organisms, such as Zygomycota: *Mucor rouxii* (White *et al*., 1979), *Absidia coerulea* (McGahren *et al*., 1984) and *Rhizopus oryzae* (Hang, 1990). Recently, fungi other than Zygomycota were also shown to produce chitosan (Pochanavanich & Suntornsuk, 2002). Chitosan produced in ecosystems by fungi is assumed to be decomposed and utilized as a carbon, nitrogen, and energy source by chitosan-degrading microorganisms, which hydrolyze chitosan by producing an enzyme called chitosanase (EC 3.2.1.132).

We previously reported that a chitosan-degrading actinomycete, *Amycolatopsis* sp. CsO-2, produces a 27-kD chitosanase (Okajima *et al*., 1994a). The N-terminal amino acid sequence of the chitosanase (Saito & Ando, 1996) exhibits a high level of identity with the family 46 chitosanase from *Streptomyces* sp. N174 (N174 chitosanase), which is the most intensively studied chitosanase for its structure and function, by means of site-directed mutagenesis and X-ray crystallography (for a review, see Fukamizo & Brzezinski, 1997). In the N174 chitosanase, two acidic amino acid residues, Glu22 and Asp40, the corresponding amino acid residues of which are conserved among family 46 chitosanases, act as active centers (Boucher *et al*., 1995).

Here, we report the gene structure of the 27-kD chitosanase from *Amycolatopsis* sp. CsO-2. The recombinant form of the chitosanase not only exhibited chitosan-hydrolyzing activity but also antifungal activity against Zygomycota strains. It is noteworthy that the antifungal activity was drastically enhanced by the simultaneous addition of a bacterial chitinase ChiC from *Streptomyces griseus*

HUT6037, which is known to have antifungal activity (Watanabe *et al.*, 1999).

Materials and methods

Bacterial strains, plasmids, and media

Amycolatopsis sp. CsO-2, which was isolated from soil (Okajima *et al.*, 1994b), was used as the source of chromosomal DNA. The strain was maintained on Bennet agar slants (Okajima *et al.*, 1994b). *Escherichia coli* JM109 (Yanisch-Perron *et al.*, 1985) and pUC19 (Vieira & Messing, 1982) were used in routine gene manipulation. A plasmid vector pET15b (Novagen, Darmstadt, Germany) was used to overproduce the recombinant CtoA protein in *E. coli* BL21(DE3) (Novagen). Luria–Bertani medium containing 100 μg mL^{-1} of ampicillin was used to cultivate *E. coli* transformants carrying pUC19 and pET15b derivatives.

Cloning of the chitosanase gene

To amplify a part of the gene for the 27-kD chitosanase of *Amycolatopsis* sp. CsO-2, a set of primers (5′-CCGGTGAA GAAGGACATCG-3′ and 5′-CCTTCGCCCAGTCCTTGG TG-3′) was designed based on the N-terminal amino acid sequence of the protein (Saito & Ando, 1996). PCR was performed using *Amycolatopsis* sp. CsO-2 total DNA as the template. The PCR product of the expected size (*c.* 250 bp) was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), sequenced, and used as a probe to clone the *ctoA* gene. Labeling and detection of the cloned PCR product were carried out using ECL random prime labeling and detection systems II (GE Healthcare, UK) according to the manufacturer's instructions. The PstI fragment, which hybridized to the probe, was cloned, based on a colony hybridization technique. A positive *E. coli* clone was obtained and the plasmid pPCSO2 carrying the whole chitosanase gene was used in further subcloning and sequence analysis.

Overproduction and purification of the recombinant chitosanase

To overproduce the CtoA protein in *E. coli*, an expression plasmid that carries a gene encoding the CtoA protein without the signal sequence, and with six histidine residues at the N-terminus, was constructed as follows: primers CSO2-N (5′-TCTAGACATATGGCGTCCGTCGGGCTG-3′) and CSO2-C (5′-GAATTCCTCGAGTCAGCCGTTGATC TG-3′), in which NdeI and XhoI sites are underlined, respectively, were designed to amplify part of the *ctoA* gene, which encodes the part of the CtoA protein without the putative signal peptide (48 amino acids from the N-terminus). The PCR product, which was amplified using pPCSO2 as the template, was cloned in the plasmid vector pGEM-T Easy (Promega) to obtain the plasmid pGEM/CSO2. The nucleotide sequence of the cloned fragment was ascertained, and the NdeI–XhoI fragment of pGEM/CSO2 was then ligated to the corresponding sites of pET15b (Novagen). The resulting plasmid pET15b/CSO2 was introduced into *E. coli* BL21(DE3) (Novagen). The recombinant CtoA protein was overproduced as a soluble form and purified in accordance with Novagen's instructions.

Site-directed mutagenesis

The EcoRI *ctoA* fragment in pGEM/CSO2 was inserted into the corresponding sites within pKF19K (Takara Bio), to construct the plasmid pKF19K/CSO2, which was used as the template for point mutation within the gene. Point mutation was introduced using a Mutan-Super Express Km (Takara Bio) according to the manufacturer's instruction, with a primer 5′-TCCTCGGCGCAGAACTCCTCG-3′, to replace the codon for Glu22 with that for Gln. Following the confirmation of the site-directed mutation by DNA sequencing, the mutated *ctoA* genes were integrated into pET15b to obtain the plasmid pET15b/E22Q. The mutant recombinant CtoA protein was overproduced in *E. coli* and purified as described above.

Chitosanase assay

Chitosanase activity was measured by a modification of Schales' procedure (Imoto & Yagishita, 1971), using chitosan 7B (Funakoshi, Tokyo, Japan). Chitosanase assay was routinely performed in 100 mM sodium acetate buffer (pH 5.3) at 37 °C, unless otherwise mentioned. One unit of chitosanase activity was defined as the amount of enzyme that produces 1 μmol of reducing sugars equivalent to glucosamine min^{-1}.

Protein analysis

Proteins were separated with 12.5% polyacrylamide gels (PAG) containing 0.1% sodium dodecyl sulfate (SDS) (Laemmli, 1970) and were visualized by staining SDS-PAGs with Coomassie Brilliant Blue R-250.

Antifungal assay

Conidia of *R. oryzae* IAM6252 were aseptically suspended in potato dextrose broth (BD, Franklin Lakes, NJ) to yield a final conidial density of 1×10^4 mL^{-1} medium. Aliquots (80 μL each) of the suspension were dispensed into wells on a sterilized 96-well microplate. Twenty microliters of the recombinant CtoA protein or its mutant E22Q dissolved in 20 mM acetic acid buffer (pH 5.3) was added to each of the aliquots, so that each well contained 360 pmol of the wild type or mutant enzyme. The acetic acid buffer was added for comparison. The suspension in the microplate, which was

covered with a lid, was incubated at 25 °C. Growth was monitored by measuring the $OD_{595\,nm}$ with a microplate reader model MTP-32 (Corona Electric, Hitachinaka, Japan). The antifungal activity was also measured against *Rhizopus stolonifer* var. *stolonifer* IAM6021, *Mucor javanicus* IAM6087, *Mucor racemosus* IAM6123, *Aspergillus awamori* var. *kawachi*, and *Penicillium expansum* IAM13777. IAM strains were purchased from the Center of Bioinformatics, Institute of Molecular and Cellular Bioscience, The University of Tokyo. To observe the synergism of a chitinase with CtoA, the recombinant ChiC of *S. griseus* HUT6037 was overproduced in *E. coli* and purified as reported previously (Watanabe *et al*., 1999).

Accession number

The nucleotide sequence of the *ctoA* gene is accessible under no. AB041775.

Results and discussion

Cloning and structure of the *ctoA* gene

To clone the gene for the CtoA protein, which is the 27-kD chitosanase from *Amycolatopsis* sp. CsO-2 (Okajima *et al*., 1994a), part of the gene was amplified by PCR using a set of primers, which was designed based on the N-terminal amino acid sequence of CtoA (see Materials and methods for the nucleotide sequences of the primers). Using the amplified product, a PstI fragment (about 2.0 kb) that hybridized to the labeled probe was cloned. The 0.4-kb PstI–BamHI fragment and the neighboring 0.9-kb BamHI fragment within the plasmid were found to carry the *ctoA* gene. The whole structure of the putative *ctoA* gene was obtained by connecting the sequences of the two regions. The gene structure, including the BamHI junction, was ascertained by amplifying the intact *ctoA* gene by PCR, using pPCSO2 as a template. The encoded protein consisted of 286 amino acids and included the amino acid sequence (ASVGLDDPA), which was identical to the N-terminal amino acid sequence of the purified CtoA protein (Saito & Ando, 1996). The molecular mass of the mature protein was deduced to be 26 493 Da, coinciding with the approximate size (27 kDa) of the purified protein (Okajima *et al*., 1994a). The deduced amino acid sequence of the mature CtoA protein was novel and had high levels of identity with those of family 46 chitosanases from *Streptomyces* sp. N174 (83%) and *Nocardioides* sp. N106 (83%) (Masson *et al*., 1994, 1995). A possible promoter region was present 72–101 upstream of the putative translation initiation codon coding a methionine. An inverted sequence (5′-GGTTAGGAA AGTTTCCTAACC-3′), which might be involved in transcriptional regulation of the *ctoA* gene, was located between the possible promoter and the putative translation initiation site.

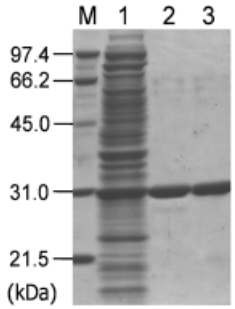

Fig. 1. Recombinant CtoA proteins electrophoresed on SDS-PAG. Lane 1, cell lysate of the *Escherichia coli* (pET15b/CSO2) producing the recombinant wild-type CtoA protein; lane 2, purified recombinant wild-type CtoA protein; lane 3, purified mutant CtoA protein E22Q; M, protein size markers. The sizes of the protein markers are indicated. The gels were stained with Coomassie Brilliant Blue R-250.

To ensure that the cloned *ctoA* gene encodes a chitosanase, the gene was heterologously overexpressed in *E. coli*. CtoA was produced in soluble form and purified to homogeneity (Fig. 1). The approximate size of the His-tagged protein on the SDS-PAG (30 kDa) coincided with the calculated molecular mass (28 657 Da). The purified protein showed chitosan-hydrolyzing activity (88 U mg^{-1} protein). It was thus concluded that the cloned *ctoA* gene encodes a chitosan-hydrolyzing enzyme.

Identification of an active center

To investigate active centers within the CtoA protein, its amino acid sequence was aligned with that of N174 chitosanase (Masson *et al*., 1994), together with other family 46 chitosanases from *Nocardioides* sp. N106 (Masson *et al*., 1995), *Bacillus circulans* MH-K1 (Ando *et al*., 1992), and *Pseudomonas* sp. A-01 (Ando *et al*., 2008). The amino acid residues corresponding to Glu22 and Asp40 of the N174 chitosanase, in which those acidic amino acid residues (Glu22 and Asp40) act as a proton donor and a residue mediating the deprotonation of a water molecule, respectively (Boucher *et al*., 1995; Marcotte *et al*., 1996), were conserved in the aligned chitosanases (data not shown). To investigate the essential nature of the Glu residue in the CtoA protein, Glu22 was replaced with Gln. The resulting CtoA mutant E22Q, which was produced in *E. coli*, did not show chitosan-hydrolyzing activity, as the E22Q and E23Q of the N174 and the A-01 chitosanases, respectively (Boucher *et al*., 1995; Ando *et al*., 2008). The data suggest that Glu22 acts as an active center in the CtoA protein.

Antifungal activity of CtoA

The antifungal activity of the CtoA chitosanase was investigated using a Zygomycota *R. oryzae*, which is known to

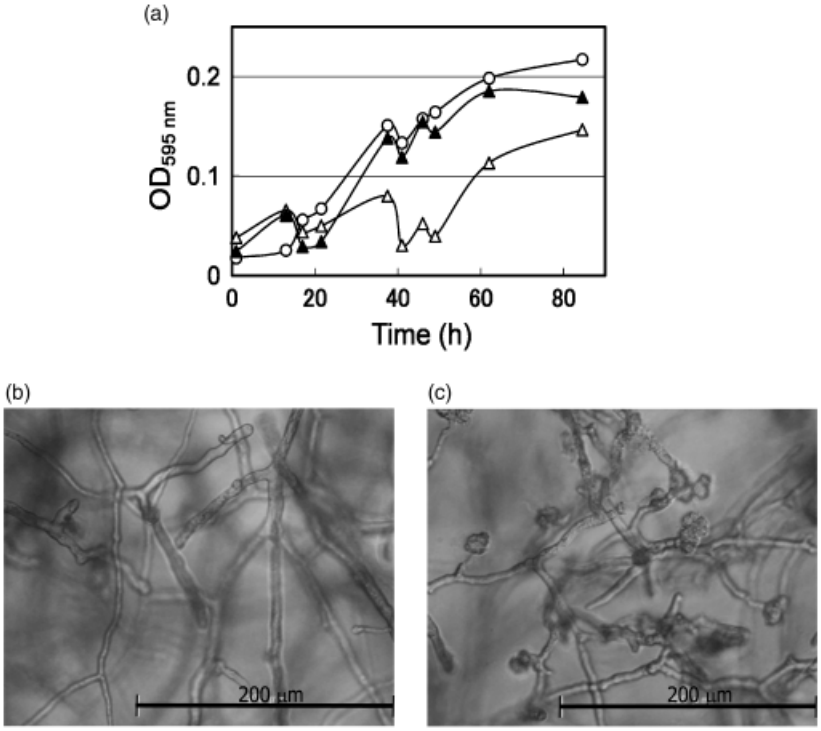

Fig. 2. Growth of *Rhizopus oryzae* in the presence of the CtoA protein. Conidia of *R. oryzae* were inoculated into potato dextrose media, in wells of a microtiter plate, with or without 3.6 nmol mL^{-1} of the wild-type or mutant (E22Q) CtoA protein, and incubated at 25 °C without shaking. (a) Turbidity ($A_{595\ nm}$) of the cultures. The experiments were performed in triplicate and the averages are plotted. ○, Without CtoA proteins; △, with the wild-type CtoA; ▲, E22Q. Morphologies of the *R. oryzae* hyphae in the absence (b) or presence (c) of the wild-type CtoA protein. Hyphae grown for 62 h were observed with a phase-contrast microscope.

produce chitosan (Hang, 1990; Pochanavanich & Suntornsuk, 2002). Conidia of *R. oryzae* were inoculated to potato dextrose broth with or without 3.6 nmol mL^{-1} of the recombinant CtoA and incubated at 25 °C. In the presence of the chitosanase, the turbidity of the culture did not significantly increase until 50 h after starting the incubation, whereas the fungus showed good growth between 20 and 60 h in the absence of the enzyme (Fig. 2a). The hyphae grown in the presence of CtoA were apparently distorted and frizzed, in contrast to those cultivated without CtoA (Fig. 2b and c). The beaded tips of the hyphae, which were observed in the presence of CtoA, seemed to be misshapen rather than forming sporangia. The CtoA chitosanase exhibited antifungal activity against other Zygomycota, *R. stolonifer*, *M. javanicus*, and *M. racemosus*, whereas it did not against other tested fungal species, *A. awamori*, and *P. expansum* (data not shown). A crude chitosanase solution, which was prepared from the culture filtrate of *Sphingobacterium multivorum* KST-009, suppressed the mycelial growth of a fungus *Fusarium oxysporum* (Matsuda *et al.*, 2001). A chitosanase from *Bacillus cereus* D-11 inhibited the mycelia growth of *Rhizoctonia solani* (Gao *et al.*, 2008). These chitosanases might have stronger antifungal activities against Zygomycota strains, which are known to produce chitosan probably as a cell wall component.

When the E22Q mutant of CtoA, which abolished chitosanase activity, was added to the medium, the inhibition of *R. oryzae* growth was not observed (Fig. 2a). In the N174 chitosanase, whose amino acid sequence is highly similar to CtoA, the mutation E22Q slightly changed its K_m against chitosan (Boucher *et al.*, 1995), suggesting that the affinity of the E22Q mutant for chitosan was comparable to that of the wild-type N174 chitosanase. Chitosan-hydrolyzing activity, and non-chitosan-binding activity, of CtoA would be essential for the antifungal activity, in contrast to the nonenzymatic antifungal, chitin-binding protein from *Streptomyces tendae* (Bormann *et al.*, 1999).

Synergistic effect between chitinase and CtoA in antifungal activity

A GH19 chitinase ChiC from an actinomycete strain *S. griseus* HUT6037 has antifungal activity (Watanabe *et al.*, 1999). Because it was supposed that the cell wall of *R. oryzae* contains chitin as well as chitosan, we investigated the synergism of ChiC with CtoA on the antifungal activity. Individual addition of ChiC (0.40 nmol mL^{-1}) or CtoA (1.8 nmol mL^{-1}) did not severely affect the growth of *R. oryzae*, although CtoA delayed the initiation of fungal growth (Fig. 3). By adding both the enzymes, nevertheless, the increment in turbidity was completely abolished (Fig. 3). It was, thus, concluded that the chitinase and the chitosanase acted synergistically in inhibiting the growth of *R. oryzae*.

Some bacteria produce not only chitinase but also chitosanase. A Gram-negative bacterium *Burkholderia gladioli*

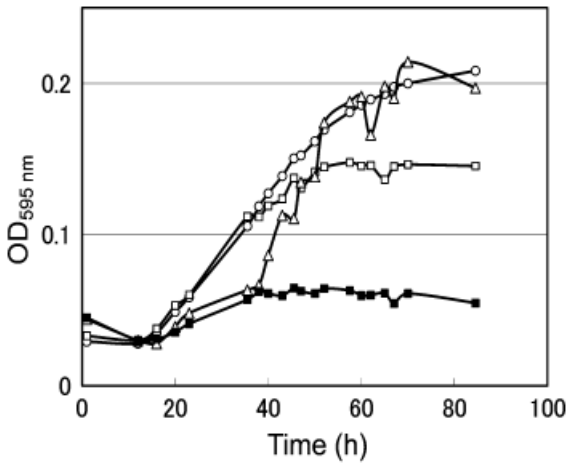

Fig. 3. Growth of *Rhizopus oryzae* in the presence of the *Streptomyces griseus* chitinase ChiC with the CtoA protein. Conidia of *R. oryzae* were inoculated into potato dextrose media, in wells of a microtiter plate, with or without 1.8 nmol mL^{-1} of the wild-type CtoA protein and/or 0.40 nmol mL^{-1} of the family 19 chitinase ChiC from *S. griseus* (Ohno *et al.*, 1996), and incubated at 25 °C without shaking. Turbidity ($A_{595\ nm}$) of the cultures was measured periodically. The experiments were performed in triplicate and the averages are plotted. ○, Without the enzymes; △, with CtoA; □, with ChiC; ■, with CtoA and ChiC.

CHB101 expresses two chitinase genes (one GH18 and one GH19) and one GH46 chitosanase gene (Shimosaka *et al.*, 2000, 2001). The genome of an actinomycete strain *Streptomyces coelicolor* A3(2) (Bentley *et al.*, 2002) contained three ORFs for putative chitosanases (two for GH family 46 and one for GH75) and six for possible chitosanases belonging to GH5, in addition to 13 (putative) chitinase genes *chiA-M* (Saito *et al.*, 1999; Kawase *et al.*, 2006). The GH19 chitinase ChiF, whose gene is one of the chitin-inducible chitinase genes among the 13 *chi* genes in *S. coelicolor* (Saito *et al.*, 2000), exhibits antifungal activity as ChiC of *S. griseus* (Watanabe *et al.*, 1999; Kawase *et al.*, 2006). Chitinase and chitosanase, which are coproduced by chitin- and chitosano-lytic soil bacteria, would act synergistically to degrade partially deacetylated chitin and partially acetylated chitosan, which are believed to be present in terrestrial ecosystems, especially in the cell walls of fungi and in the exoskeleton of insects.

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