FUNCTIONAL ANALYSIS OF A NOVEL C-TERMINAL DOMAIN FOUND IN A CHITOSANASE FROM THE KOJI MOLD *ASPERGILLUS ORYZAE*

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Abstract

The koji mold *Aspergillus oryzae* secretes chitosan-degrading enzymes, chitosanase and exo-β-D-glucosaminidase, in medium constitutively. Three genes (*csnA, csnB,* and *csnC*) coding for chitosanases had been isolated and characterized. *csnC* encodes a major chitosanase secreted in medium, which is composed of 385 amino acid residues with a molecular mass of 37.3 kDa. It has a multi-domain structure from the N-terminus, composed of a signal peptide, a catalytic domain classified into family 75 of glycosyl hydrolases, Thr- and Pro-rich linker, and three times tandemly-repeated peptides (R3 domain) that are rich in Cys, Glu and Asp residues. The R3 domain bound to insoluble powder chitosan, however, it did not promote a hydrolysis rate of the catalytic domain to any extent. On the other hand, R3 domain increased a solubility of recombinant proteins in host cells of *Escherichia coli* when fused with green fluorescent protein (GFP). This solubilizing effect was also evident for a recombinant protein fused with chitosanase from the fungus, *Fusarium solani*.

Introduction

Chitosanases (E.C.3.2.1.132), which catalyze an endo-type cleavage of chitosan, are produced by a large number of microorganisms including bacteria and fungi. In most cases, bacterial chitosanases are inducible by the substrate chitosan, and play a role in the degradation and utilization of exogenous chitosan. In contrast, a physiological role of fungal chitosanases is unclear, since exogenously added chitosan could not be used efficiently as carbon source by most fungi [1, 2]. Chitin is an important component in the cell wall of fungi and its deacetylated form, chitosan, is also found in a limited group of fungi [3]. Therefore, one plausible explanation for a physiological role of fungal chitosanases is that they would be involved with a process of cell division or autolysis through a degradation of deacetylated portion of chitin polymer in their cell walls [4].

We previously isolated a chitosanase gene from the plant pathogenic fungus, *Fusarium solani*, and determined its nucleotide sequence, which is the first report on a chitosanase of fungal origin [5]. Primary sequences of fungal chitosanases have been reported from various species to date, and they show significant similarity with each other but have no similarity to those of bacterial chitosanases. These results suggest that a fungal chitosanase might have an evolutionary origin distinct from that of bacterial counterparts. At present, most fungal chitosanases have been classified into family 75 of glycosyl hydrolases based on their conservative amino acid sequences in catalytic regions.

The koji mold, *Aspergillus oryzae* has been long used for a production of rice wine (sake), soy sauce (shoyu), and soybean paste (miso) in Japan, because the fungus is a strong producer of amylases and proteases. We reported that *A. oryzae* strain IAM2660 also secreted a large amount of chitosan-degrading enzymes in culture fluid in apparently constitutive manner. Chitosanase and

exo-β-D-glucosaminidase were purified and characterized from culture fluid of the fungus as two major chitosanolytic enzymes [6]. In this paper, we report an isolation of a gene coding for chitosanase and a functional analysis of a unique domain composed of three times tandemly-repeated peptides located in the C-terminus.

Material and Methods

Strains and plasmids

Cells of *A. oryzae* IAM2660 were grown in Czapek-Dox (CD) medium (K_2HPO_4, 1 g; KCl, 0.5 g; $NaNO_3$, 2 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.02 g; *N*-acetyl-D-glucosamine, 20 g; and deionized water, 1000 ml, pH 5.5), and were used to prepare chromosomal DNA and total RNAs. An expression plasmid vector pPICZαA (*Pichia* expression kit, Invitrogen) was used to express chitosanase (*csnC*) cDNA with a host strain of *Pichia pastoris* X-33. The constructed plasmid was introduced into cells of *P. pastoris* X-33 by an electroporation method described in a supplier's protocol. To express *csnC* cDNA in *E. coli* BL21(DE3), an expression plasmid vector pCold I (Takara, Kyoto, Japan) was used.

Isolation of a gene (csnC) coding for the purified chitosanase

Two oligonucleotides were designed based on the partial amino acid sequences determined from the purified chitosanase [6] and used for PCR primers: Sense primer used was 5'-AAYCAYGGNACNAARGG-3' and antisense primer was 5'-GCGAAGCTTARRTCYTCYTTNGGRAA-3'. A DNA fragment corresponding to the partial *csnC* was PCR-amplified using *A. oryzae* chromosomal DNA as a template. This DNA fragment was used as a probe to isolate recombinant phage clones containing a full-length *csnC* from an *A. oryzae* genomic library [7]. The *csnC* cDNA corresponding to the entire ORF was amplified by reverse transcription (RT)-PCR.

Expression of chitosanase cDNA in Pichia pastoris

csnC cDNA corresponding to the whole ORF without a putative signal peptide (CsnC) and its truncated from devoid of R3 domain (CsnCΔR3) were PCR-amplified, and inserted into a *Pichia pastoris* expression plasmid vector pPICZαA. To adjust a reading frame in joint regions, suitable restriction sites were added at the 5' termini of the PCR primers. *P. pastoris* X-33 cells possessing the recombinant plasmids were grown in the BMMY medium, and an expression of recombinant proteins was induced by feeding methanol as described in the protocol of a *Pichia* expression kit (Invitrogen). Recombinant proteins fused to polyhistidine tag were purified from crude proteins in culture using a His Trap HP Kit (Amersham Bioscience). Chitosanase was assayed by measuring the reducing sugars liberated during the hydrolysis of chitosan with a degree of deacetylation of 70% (D.D. 70), as described in our previous paper [8]. One unit (U) of activity was defined as the amount of enzyme catalyzing the production of 1 μmol of the reducing sugar per min using glucosamine (GlcN) as the standard.

Binding test of the recombinant chitosanase toward insoluble substrates

Three mg of powder chitosan (D.D. 100%) was mixed with 500 ng of the recombinant proteins in 30 μl of 20 mM Tris-Cl buffer (pH7.0). The mixture was kept in ice for 2 hr with occasional shaking. After insoluble chitosan was removed by centrifugation, the supernatant was used as unbound protein fraction. The resulting precipitate was washed with 20 mM Tris-Cl buffer (pH7.0) and then boiled for 3 min in the presence of 1% SDS. After removing insoluble chitosan by centrifugation, the resulting supernatant was used as bound protein fraction. Proteins contained in each fraction were analyzed by SDS-PAGE.

The cDNA corresponding to CsnC R3 domain was amplified by PCR and fused to a gene coding for green fluorescent protein (GFP) or *Fusarium solani* chitosanase (*Fs-csn*) in frame. These constructs were inserted into an expression vector pCold I and the resulting recombinant plasmid was introduced into *E. coli* BL21(DE3). Cells of the transformant were grown in LB medium, and a production of recombinant proteins was induced with addition of isopropyl-β-thiogalactopyranoside (IPTG). Polyhistidine-tagged recombinant proteins were purified from crude extract by a His Trap HP Kit (Amersham Bioscience).

Results and Discussion

Cloning of a third chitosanase gene from A. oryzae IAM2660

Two genes coding for chitosanase (*csnA* and *csnB*) had already been isolated and characterized from *A. oryzae* IAM2660 [7]. Determination of partial amino acid sequences from a major chitosanase purified from culture fluid indicated neither of the two genes encoded the purified chitosanase. We could not detect any chitosanases encoded by *csnA* or *csnB* in culture fluid, and a cellular localization of these two chitosanases is still unknown. Then, we isolated a third chitosanase gene (*csnC*) starting from a design of oligonucleotides based on the amino acid sequences determined from the purified chitosanase as described in Material and Methods. Thus, *A. oryzae* strain IAM2660 possesses three genes coding for chitosanase, and they have been deposited in the EMBL/GenBank/DDBJ database under accession nos. AB038996 (*csnA*), AB090327 (*csnB*), and AB159785 (*csnC*). A whole genome sequence has been recently reported from *A. oryzae* RIB40 [9]. A comparison of the sequences reveals that the *csnA, B* and *C* genes locate on chromosome 6, 7, and 5, respectively, and that no other homologous gene is found in the genome.

The amino acid sequences of the two chitosanases (CsnA and CsnB) show significant similarity to those of fungal chitosanases belonging to family 75 of glycosyl hydrolases. On the contrary to this, the similarity of CsnC is fairly low and restricted in the region nearby catalytic residues (Fig. 1) [10, 11]. Moreover, CsnC has a unique domain at its C-terminus, which is joined to catalytic domain by a linker rich in Thr- and Pro-residues (Fig. 1). This domain (named R3) is composed of short peptides rich in Glu, Asp, and Cys residues, which are tandemly-repeated three times. This peptide sequence was used as a query sequence to search for similar sequences in the database available, but no similar sequence had been deposited. We examined a function of this unique R3 domain that is located in *A. oryzae* CsnC but not found in other fungal chitosanases.
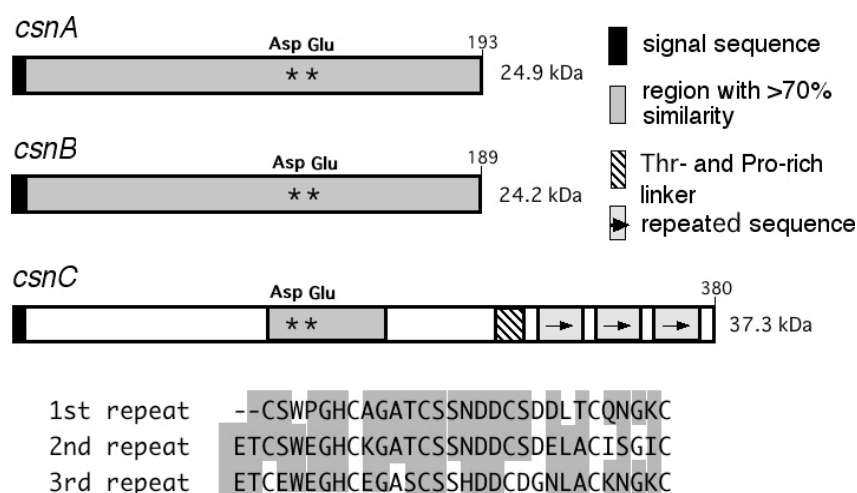

Figure 1 :   Domain structure of three chitosanases from *A. oryzae*.   Asterisks denote amino acid residues essential for catalytic activity.   The sequences of peptides tandemly-repeated in R3 domain are shown.

## Expression of csnC in the yeast Pichia pastoris

A. oryzae csnC cDNA and its truncated forms (CsnCΔR3 and R3) were expressed in the yeast *Pichia pastoris*. Each of the purified recombinant proteins was analyzed by SDS-PAGE. A migration distance of CsnC and R3 was much different in the presence or absence of reducing reagent, 2-mercaptoethanol, while that of CsnCΔR3 was almost identical in both cases (data not shown). Since the R3 domain is rich in Cys residues, S-S bonds likely formed within the domain and resulted in a shift of migration distance in SDS-PAGE.

Binding test toward insoluble substrates

Polysaccharide-degrading enzymes often have a substrate-binding domain in their polypeptides. To examine whether R3 domain has a substrate-binding ability or not, two recombinant proteins (CsnC and CsnCΔR3) were used for binding test toward insoluble powder chitosan. CsnC proteins tightly bond to chitosan, while a truncated CsnCΔR3 devoid of R3 domain did not bind to chitosan under similar conditions (Fig. 2). This result clearly indicates that the R3 domain has an ability to bind toward insoluble chitosan. Then the two recombinant proteins were compared for their hydrolyzing activities toward various substrates. We could not detect any differences in reaction rates for the substrates tested, such as soluble chitosan (D.D. 70% and 100%), glycol chitosan, powder chitosan, and flake chitosan (data not shown).
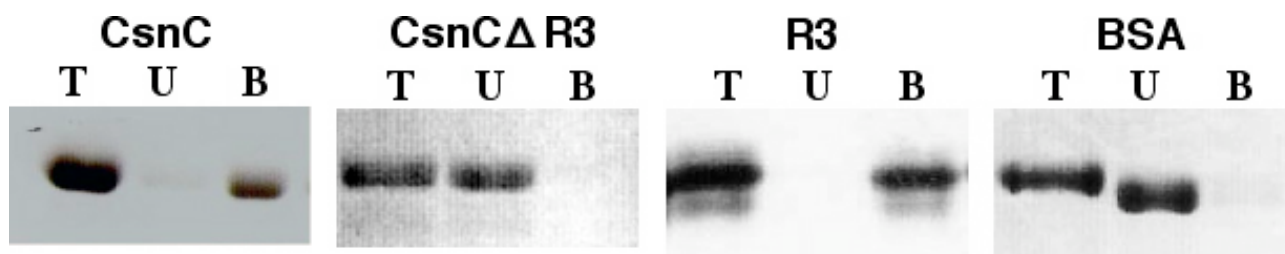
Figure 2 :   Binding of recombinant proteins toward insoluble powder chitosan. Total proteins added initially (T), proteins in unbound fraction (U), and proteins in bound fraction (B) were analyzed by SDS-PAGE. BSA denotes bovine serum albumin used as a negative control.
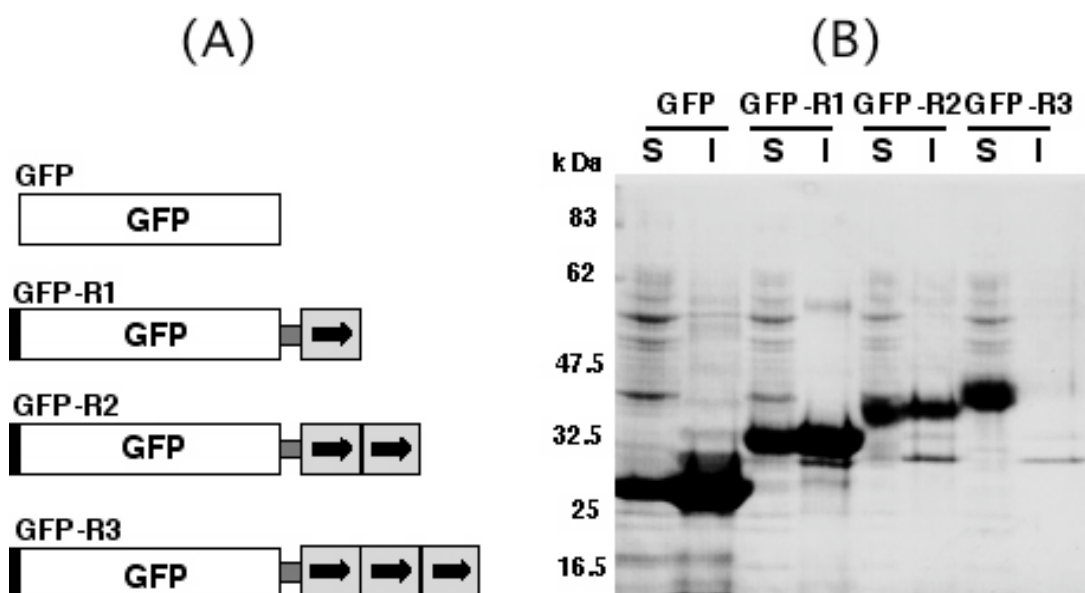
Figure 3 :   Expression of GFP-R3 fusion proteins in *E. coli*. (A) A structure of recombinant GFP proteins. Black box, polyhistidine tag; Box with an arrow, tandemly-repeated peptides in R3 domain. (B) SDS-PAGE analysis of crude proteins prepared from *E. coli* transformant cells. S, soluble fraction; I, insoluble fraction (inclusion bodies).

A solubilizing effect of R3 domain on recombinant proteins in E. coli

In the course of experiment to express *A. oryzae csnC* in *E. coli*, we found that a productivity of recombinant proteins was dependent on the presence of R3 domain. A full-length CsnC was produced in a large amount and its specific activity (633 U/mg-protein) was similar to that of the original CsnC (668 U/mg-protein) purified from the original fungus. On the other hand, a truncated CsnCΔR3 was not produced to any extent (data not shown). This result led us to a notion that a presence of R3 domain in recombinant proteins might help a solubility or stability of the proteins. To confirm this assumption, we examined a productivity of other recombinant proteins when R3 domain was fused to their C-termini.

First, we constructed a recombinant green fluorescent protein (GFP) fused with R3 domain at the C-terminus (GFP-R3). To check the effect of tandem repeats in the domain, GFP recombinant proteins with 1st repeat alone (GFP-R1) and 1st + 2nd repeats (GFP-R2) were also constructed. These constructed genes were introduced into *E. coli*, and a productivity of recombinant GFPs was analyzed by SDS-PAGE (Fig. 3). When recombinant GFP was expressed alone, most proteins formed inclusion bodies. Addition of tandemly-repeated peptides increased a solubility of recombinant GFP proteins in proportion to the number of repeats, and most GFP-R3 recombinant proteins were obtained in soluble fraction.

Second, we tested a chitosanase gene (*Fs-csn*) from the fungus *Fusarium solani* [5]. We could not obtain any soluble and active recombinant proteins in *E. coli*, because most proteins formed inclusion bodies. Then, the recombinant gene coding for Fs-csn proteins fused with R3 domain at C-terminus was constructed and expressed in *E. coli*. As a result, most recombinant proteins existed in soluble fraction (Fig. 4). The specific activity of the resulting recombinant Fs-csn-R3 (154 U/mg-protein) was comparable to that of the original chitosanase (171 U/mg-protein), indicating that a proper conformation was formed irrespective of a fusion with R3 domain.

In this paper, we reported a functional analysis of the unique R3 domain found at the C-terminus of *A. oryzae* chitosanase. The domain showed a binding ability toward insoluble chitosan. This binding might be partly caused by attraction between positive charge of chitosan and negative charge of Glu and Asp-residues in the domain. This binding did not accelerate a hydrolysis rate of catalytic region at all. We also found that R3 domain would increase a solubility of recombinant proteins in *E. coli*, when it was fused at C-terminus. This solubilizing ability of R3 domain is applicable to express various heterologous genes in *E. coli*, in cases where recombinant proteins easily formed inclusion bodies. It must await further characterization of R3 domain to put this behavior to practical use.
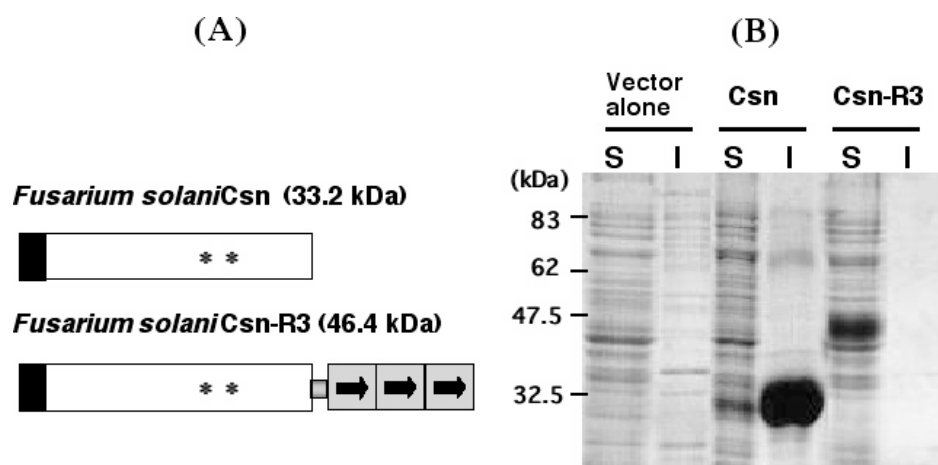
Figure 4 : Expression of Fusarium chitosanase-R3 fusion proteins in *E. coli*. (A) A structure of recombinant proteins. Black box, polyhistidine tag; Box with an arrow, tandemly-repeated peptides in R3 domain. (B) SDS-PAGE analysis of crude proteins prepared from *E. coli* transformant cells. S, soluble fraction; I, insoluble fraction (inclusion bodies).

Acknowledgements

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