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A unique post-translational processing of an exo-β-1,3-glucanase of *Penicillium* sp. KH10 expressed in *Aspergillus oryzae*

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ABSTRACT

To characterize an exo-β-1,3-glucanase (ExgP) of an isolated fungal strain with high laminarin degradation activity, identified as *Penicillium* sp. KH10, heterologous secretory expression of the ExgP was performed in *Aspergillus oryzae*. Deduced amino acid sequence of the *exgP* gene possibly consisted of 989 amino acids which showed high sequence similarity to those of fungal exo-β-1,3-glucanases belonging to the glycoside hydrolase (GH) family 55. Notably, the purified recombinant ExgP showed a single protein peak in the native state (by gel-permeation chromatographic analysis), but showed two protein bands in the denatured state (by SDS–polyacrylamide gel electrophoresis). These two polypeptides exhibited activity in a coexisting state even under reducing conditions, suggesting that non-covalent association of both polypeptides took place. Taken together with the nucleotide sequence information, the ExgP precursor (104 kDa) would be proteolytically processed (cleaved) to generate two protein fragments (42 and 47 kDa) and the processed products (polypeptide fragments) would be assembled each other by a non-covalent interaction. Moreover, one of the matured ExgP polypeptides was N-glycosylated by the post-translational modification.

Introduction

β-1,3-Glucanases are widely distributed among bacteria, fungi, and higher plants and many of them have been purified and characterized [1]. Depending on the sugar cleavage manner, they can be classified into exo-β-1,3-glucanases (EC 3.2.1.58) and endo-β-1,3-glucanases (EC 3.2.1.6 or 3.2.1.39). Endo-type of enzyme randomly hydrolyzes linear substrates, whereas exo-type enzyme releases glucose residues from the non-reducing ends. On the other hand, this type of enzyme belongs to several glycoside hydrolase (GH)[1] families based on structural hydrophobic cluster analysis [2]. The corresponding β-1,3-glucanase genes from the above-mentioned organisms have also been isolated and characterized [1]. As for the physiological functions of β-1,3-glucanases, it mainly depends on their originating source. For example, in plants, these types of enzymes are thought to be involved in a defense system against fungal pathogens [3,4]. In bacteria, which possesses no β-1,3-glucan, a nutritional role has been assigned [5]. Especially, fungal β-1,3-glucanases possess different functions. First, a physiological role in morphogenetic-morpholytic processes during fungal development and differentiation has been indicated [6]. Second, β-1,3-glucanases have been related to the mobilization of β-glucans under conditions of carbon and energy source exhaustion, functioning as autolytic enzymes [7,8]. β-1,3-Glucanases are also involved in fungal pathogen–plant interactions, degrading cellulose in the host's vascular tissues during pathogen attack [9].

Here we focused our interest on *Penicillium* sp. as the first source for investigation of new member of GH family 55. As expected, an active fraction for β-1,3-glucanase could be detected in the liquid culture of the fungus, *Penicillium* sp. KH10, isolated by us, but hardly recovered owing to its too small amount of production. Thus, we attempted to isolate the β-1,3-glucanase encoding gene and its over-expression in *Aspergillus oryzae* for enzymatic characterization. During the course of purification steps, we found an interesting phenomenon that the recombinant enzyme would be post-translationally processed as two polypeptides and both polypeptides exhibit enzymatic activity by complex formation. We will discuss this unique protein modification in association with the structural feature of the cloned relevant gene.

Materials and methods

Isolation of β-glucan degrading fungus

In order to isolate β-glucan utilizing-fungus from soil samples, enrichment culture was performed at 30 °C in the medium containing β-1,3-glucan, prepared from the fruiting body of *Pleurotus cornucopiae* [10] as a sole carbon source. Medium A used here contained 0.5% β-1,3-glucan, 0.01% yeast extract, 0.1% ammonium

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[1] *Abbreviations used:* GH, glycoside hydrolase; ExgP, exo-β-1,3-glucanase.

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sulfate, 0.01% $MgSO_4 \cdot 7H_2O$, 0.05% KH_2PO_4 (pH 6.0). Enrichment culture was repeated three times, and then an aliquot of the cultures showing efficient growth and the decrease of total sugar were plated in the agar plate. Fungal colonies were isolated and cultivated on the medium A instead of the β-1,3-glucan to laminarin as a sole carbon source for monitoring the efficient utilization of laminarin.

Enzyme assay

β-1,3-Glucanase activity was determined by measuring the amount of reducing sugar released from laminarin (Nakarai tesque, Kyoto, Japan) as a substrate. The standard reaction mixture (0.5 ml) contained enzyme solution and 2.5 mg laminarin in 100 mM sodium acetate buffer (pH 6.0) and was incubated at 30 °C for 10 min. One unit of enzyme activity was defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per min. Sugar were measured with Somogyi-Nelson method for reducing sugar and the phenol–sulfuric acid method for total sugar. Protein concentration was measured with the Bio-Rad protein assay, using bovine serum albumin as a standard protein.

PCR amplification and Southern blot analysis

A primer pair (ExgP-F: 5′-CGCAACGTCAAGGACTTTGGCG-CCAA GGG-3′ and ExgP-R: 5′-TTGAAGGTCCAGGCCCAGTTCCAGTTCAT-3′) was designed from the nucleotide sequences of fungal β-1,3-glucanase genes belonging to the GH family 55 and used for PCR amplification with KOD plus DNA polymerase (Toyobo Co, Tokyo, Japan). The following PCR conditions were employed: 94 °C for 30 s, 55 °C for 10 s, and 74 °C for 30 s (30 cycles), with a final extension of 74 °C for 5 min.

Genomic DNA was isolated from *Penicillium* sp. KH10 mycelium, using DNAzol reagent (Invitrogen Corp. Carlsbad, CA USA). Genomic DNA (10 μg) was digested with several restriction enzymes at 37 °C for 3 h. Digested DNA samples were electrophoresed and blotted onto a positively nylon membrane (Hybond N^+, GE Healthcare UK Ltd. England). A DIG-labeled DNA fragment was amplified from the genomic DNA with the primer pair as described above. Hybridization and visualization was according to the manufacturer's instruction (Roche) with the DIG-labeled probe.

Construction of genomic DNA library

Genomic DNA of *Penicillium* sp. KH10 was digested with EcoRI. The fragments of about 7 kb were gel purified and cloned into pUC 19 and then transformed into *Escherichia coli* JM109. This library was screened by colony-directed PCR using the primer pair (ExgP-F and ExgP-R). About 0.7 kbp DNA fragment from plasmid clones that amplified with the primers were analyzed to obtain full length *exgP* genes.

Expression of the exgP gene and cultivation of recombinant A. oryzae

The XbaI fragment containing *exgP* was inserted into the XbaI site at downstream of the modified *enoA* promoter of the pNEN142 plasmid [11]. The resulting plasmid was introduced into *A. oryzae* niaD300 [12]. Transformation of *A. oryzae* was performed using a protoplast-polyethylene glycol method [13]. Transformants were selected using CD medium containing $NaNO_3$ as a sole nitrogen source [14]. A CD medium containing maltose as a sole carbon source was used for induction culture for the enzyme preparation. Approximately 10^7 conidia of the transformant were inoculated into the 100 ml CD medium and were grown at 30 °C for 3 days by shaking culture.

Purification of recombinant ExgP

The culture supernatants of a recombinant *A. oryzae* was passed through filter paper and was directly applied to a DEAE-cellulose column (37 × 260 mm) equilibrated with 50 mM sodium acetate buffer (pH 6.0). The enzyme was eluted from the column with a linear gradient of 0–600 mM NaCl at a flow rate of 2 ml/min. The fractions showing laminarin-hydrolyzing activity were pooled, brought to 60% saturation with solid $(NH_4)_2SO_4$, and applied to a Butyl-Toyopearl 650 column (24 × 220 mm, Tosoh, Tokyo, Japan) equilibrated with 50 mM sodium acetate buffer, pH 6.0, containing $(NH_4)_2SO_4$ (60% saturation). Recombinant ExgP was eluted from the column with a liner reverse gradient of 60–0% $(NH_4)_2SO_4$ saturation. The fractions containing ExgP were concentrated with a Q100 ultrafilter membrane (Advantec, Tokyo, Japan). The enzyme solution was applied to a Sephacryl S-200 HR column (28 × 900 mm, GE Healthcare, UK) equilibrated with 50 mM sodium acetate buffer, pH 6.0. The fractions were pooled and then applied to a Phenyl-Toyopearl 650 column (24 × 220 mm, Tosoh, Tokyo, Japan) equilibrated with 50 mM sodium acetate buffer, pH 6.0 containing $(NH_4)_2SO_4$ (60% saturation). The enzyme was eluted with a liner reverse gradient of 60–0% $(NH_4)_2SO_4$ saturation at a flow rate of 1 ml/min. Active fractions were pooled and stored at −20 °C until use.

Protein analysis

The purified protein was separated on 10% SDS–PAGE gel and visualized by Coomassie brilliant blue R-250 staining. The protein bands were transferred to a PVDF membrane (Bio-Rad Laboratories, Inc., CA, USA) and analyzed with an Applied Biosystem 470A protein sequencer.

Determination of ExgP molecular mass

The molecular mass of the purified native ExgP was determined in 50 mM potassium phosphate buffer (pH 6.8) containing 100 mM NaCl on a TSK-gel G300SW (Tosoh, Tokyo, Japan) column (7.5 × 600 mm) calibrated with the following standard proteins: thyroglobulin (670 kDa), γ-globlin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa). It was also analyzed using 10% SDS–PAGE with standard proteins: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

Effect of pH and temperature on the enzymatic activity

The optimum pH for native ExgP was tested in 100 mM McIlvaine buffer (pH 3.0–6.5). The optimum temperature for the activity was investigated at 30–70 °C in 100 mM sodium acetate buffer (pH 5.0).

Preparation of whole cell and crude cell-free extracts

The recombinant *A. oryzae* was cultivated at 30 °C for 3 days with shaking in the CD medium, and mycelia were harvested by filtration and washed twice with ice-cold 20 mM acetate buffer (pH 5.5). The mycelium was suspended with the 1 ml of same buffer and the whole cell extract was prepared by disrupting the mycelia below 5 °C for 6 min with glass beads using a Multi-beads shocker (Yasui Kikai Co., Osaka, Japan). Cell-free extract was prepared from the whole cell extract by centrifugation at 12,000*g* for 10 min.

Deglycosylation of ExgP

Deglycosylation of the purified recombinant ExgP was carried out with glycopeptidase F (Takarabio Co., Osaka, Japan) as recom-