Article

Cloning and sequencing of endo-1,3-β-glucanase from Cellulosimicrobium cellulans

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Received August 26, 2008; Accepted September 23, 2008

A gene encoding endo-1,3-β-glucanase was cloned from Cellulosimicrobium cellulans DK-1 genomic DNA. Analysis of the deduced amino acid sequence revealed that the full-length enzyme has 383 amino acids composing of two functional domains, a catalytic domain classified as glycoside hydrolase family 16 and a carbohydrate-binding module (CBM) classified as CBM family 13. The mass spectrometry analysis revealed that the enzyme purified from a crude enzyme preparation originated from Cellulosimicrobium cellulans DK-1 (Pang et al., J. Biol. Macromol. 4, 57-66, 2004) corresponds to the catalytic domain which should be liberated from the CBM during the crude enzyme preparation.

Keywords: catalytic domain, carbohydrate-binding module, Cellulosimicrobium cellulans, mass spectrometry, primary structure

Introduction

Endo-1,3-β-glucanases (EC 3.2.1.6 and EC 3.2.1.39) are widely distributed among bacteria and higher plants. In plants, 1,3-β-glucanases are thought to be important to protect against fungal invasion through the ability to hydrolyze 1,3-β-glucan, which is a major cell wall component [1]. In bacteria, they are used as cell-wall degrading enzymes for growing on viable yeast and fungal cells [2]. Although both enzymes catalyze the same hydrolytic reaction, bacterial and plant enzymes belong to different glycoside hydrolase (GH) families, GH16 and GH17, respectively, based on their amino acid sequences [3]. The bacterial enzyme has a β-sandwich architecture, while the plant enzyme adopts a (β/α)8 TIM-barrel fold [4, 5]. Some glucanases are multi-domain proteins that include not only a catalytic domain but also a carbohydrate-binding module (CBM). For the catalytic function, CBMs are considered to play a role in maintaining the substrate in proximity with the enzyme [6], but details about its contribution to the catalytic activity, especially for type C CBMs, remain to be elucidated.

We purified endo-1,3-β-glucanase from a yeast cell wall lytic enzyme, Tunicase®, a commercially available crude enzyme preparation originated from Cellulosimicrobium cellulans DK-1 (previously classified as Arthrobacter sp.) [7]. The N-terminal amino acid sequence of this enzyme was found to be Ala-Pro-Gly-Asp-Leu-Leu-Trp-Ser-Asp-Glu-, which is the same as that of endo-1,3-β-glucanase from Oerskovia xanthineolytica LL G109 [8]. The first 63 amino acid residues encoded on the gene from the initiation-codon was not present on the mature enzyme from Oerskovia xanthineolytica LL G109, suggesting that the region is removed by proteolytic cleavage [8]. Pang et al. reported that the molecular mass of the enzyme purified from Tunicase® was estimated to be 32.5 kDa by SDS-PAGE and 21.7 kDa by gel-filtration analysis [7]. The enzyme was crystallized, and the diffraction The abbreviations used are: GH, glycoside hydrolase; CBM, carbohydrate-binding module, MALDI-TOF MS, matrix-assisted laser desorption ionization/time-of flight mass spectrometry.
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data were collected to a resolution of 1.66 Å [9]. The primary structure of this enzyme was needed to determine the crystal structure. So far, the crystal structure available for bacterial endo-1,3-β-glucanases is the enzyme from alkaliphilic Nocardiopsis sp. [5].

Materials and Methods

Gene cloning

Genome DNA was prepared from the culture of Cellulosimicrobium cellulans DK-1 as described previously [10]. Based on the nucleotide sequence of endo-1,3-β-glucanase from Oerskovia xanthineolytica LL G109, primers were designed to amplify the endo-1,3-β-glucanase gene by the PCR on the genome DNA (Table 1). The respective PCR fragments were sequenced.

Table 1. Oligonucleotides used as primers in PCR

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<th>Primer code</th>
<th>Nucleotide sequence (5’→3’)</th>
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<tr>
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<td>gcgaattcgcgcgcggcagcgctcgcc</td>
</tr>
<tr>
<td>40F2</td>
<td>ccgaattcgcgcccggcgacctcctggt</td>
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Mass spectrometry

MALDI-TOF MS analysis was carried out using a REFLEX III mass spectrometer (Bruker Daltonics) equipped with a 337 nm nitrogen laser, and pulsed ion extraction. Spectra were obtained in linear- or reflector-positive mode with an accelerating voltage of 20 kV. Sinapinic acid (Fluka) was prepared as a saturated solution in a 2:1 (v/v) mixture of 0.1% trifluoroacetic acid/acetonitrile and used as the matrix. A 1-μl aliquot of 1:1 (v/v) matrix/sample mixture was deposited onto the MALDI plate and dried up at room temperature.

Results and Discussion

The primers were designed for the N-terminal and conserved regions in bacterial endo-1,3-β-glucanase on the enzyme from Oerskovia xanthineolytica LL G109 (GenBank, AF052745), and the gene encoding endo-1,3-β-glucanase was amplified by PCR using genomic DNA from Cellulosimicrobium cellulans DK-1. The nucleotide sequence from the site corresponding to the N-terminus, Ala-Pro-Gly-, revealed by amino-acid sequencing of the purified protein [7] to the stop-codon is shown in Fig. 1. The protein is composed of 383 amino acids and has a molecular mass of 40.8 kDa (Fig. 1). The N-terminal and C-terminal regions correspond to the catalytic domain, GH16, and the carbohydrate-binding domain, CBM13, respectively, both of which are connected by a Gly/Ser-rich linker. In comparison with the amino acid sequence of the corresponding region of endo-1,3-β-glucanase from Oerskovia xanthineolytica LL G109, the three amino acids, Asn246, Leu335, and His363, are different (Fig. 1).

The sequence alignment to other endo-1,3-β-glucanases previously analyzed indicates that the catalytic residues correspond to Glu119, Asp121, and Glu124, which are conserved among GH16 enzymes [11]. In addition, Met123 would be a specific residue in the active site of the GH16 laminarinase subfamily, not present in that of the GH16 lichenase subfamily. Based on the alignment to the CBM13 of xylanase [12], the C-terminal CBM13 would consist of a tandem of three imperfect repeats, in which three disulfide bonds, Cys268-Cys287, Cys309-Cys328, and Cys353-Cys373, play an important role in the three-dimensional structure. The proteins in CBM13 are classified into type C CBMs, small sugar binding CBMs, and would have β-trefoil structures [13].

The MALDI-TOF MS spectrometry analysis showed that the molecular mass of the purified enzyme reported previously [7] was 27,696 Da, which corresponds to Ala1 – Thr256, the catalytic domain. The value was slightly different from those estimated by SDS-PAGE and gel-filtration analysis [7]. The purified enzyme should be liberated from the CBM during the preparation of Tunicase®. Because neutral proteases extracted from Bacillus sp. are included in Tunicase®, they should catalyze the full-length endo-1,3-β-glucanase and generate only the catalytic domain.
Fig. 1. Nucleotide and deduced amino acid sequences of endo-1,3-β-glucanase from *Cellulosimicrobium cellulans* DK-1. All the base sequence determined is shown, and the number is indicated on the left. The N-terminal amino acid of purified enzyme is indicated by vertical arrow, and the CBM region is underlined. The number of amino acid from the N-terminus is indicated in italic on the right. The bases and amino acids different from those of endo-1,3-β-glucanase from *Oerskovia xanthineolytica* LL G109 are shaded and boxed, respectively.

This is supported by the results that the enzyme corresponding to the full-length endo-1,3-β-glucanase was purified from the crude enzyme preparation without addition of the neutral proteases (data not shown).

In the preliminary structure determination for the crystal obtained [9], the deduced primary structure corresponding to Ala1-Ser242 could be well assigned on the electron density map, and the C-terminal region was suggested to be disordered.
The present primary structure information can make it possible to determine the three-dimensional structure of the catalytic domain of endo-1,3-β-glucanase from *Cellulosimicrobium cellulans* DK-1 (manuscript in preparation). In addition, the structure-function analysis of the full-length endo-1,3-β-glucanase will clarify the role of CBM for the glucanase function, which is still controversial [6]. We now construct the overexpression system of the full-length endo-1,3-β-glucanase, the catalytic domain, and the CBM in *E. coli* for further investigation.

**Acknowledgements**

The authors thank Mr. Masashi Minoda and Mr. Kyoji Goto of Daiwa Kasei K. K., Ms. Naoko Sato-Nakamura of Kyowa Hakko Kogyo, Co. Ltd., and Ms. Yoko Nakanishi, Mr. Kodo Otaka, and Dr. Masatake Ohnishi of Kyoto Prefectural University for technical support and helpful discussion.

The nucleotide sequence data reported here have been submitted to the GenBank sequence data bank and are available under accession number EU589324.

**References**


Communicated by Susumu Ito