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Article

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

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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 $(\beta/\alpha)_8$ 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

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The abbreviations used are: GH, glycoside hydrolase; CBM, carbohydrate-binding module, MALDI-TOF MS, matrix-assisted laser desorption ionization/time-of flight mass spectrometry.

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

Primer code	Nucleotide sequence (5'→3')
40F1	gcgaattcggccgccgcggcagcgctcgcc
40F2	ccgaattcgcgcccggcgacctcctgtg
40F3	cggaattcggcgagatcgacatcatggag
40F4	gcgaattcggcaagtgcctcgacgtgcgc
40R1	ccggatccctccatgatgtcgatctcgcc
40R2	ccggatccgcggacggtcccgtcggagc
40R3	gaggatccgcgcacgtcgaggcacttgcc
40R4	gcggatcctcagagcgtccactgctgggc
40R5	ccggatccgtgccccgcccgcgcggccggt
40R6	tcggatccgagccaggtcagccgggtg

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 27,696.0 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.

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   1  CCGCGACGTCGGCCGCCGCCGCACCCGGCGACCTCCTGTGGTCCGACGAGTTCGACGGC
        A  T  S  A  A  A  A  P  G  D  L  L  W  S  D  E  F  D  G   13
                          ↑ N-terminus
  60 GCGGCGGGCTCGGCGCCGAACCCCGCCGTCTGGAACCACGAGACCGGTGCGCACGGGTGG
     A  A  G  S  A  P  N  P  A  V  W  N  H  E  T  G  A  H  G  W   33
 120 GGCAACGCCGAGCTGCAGAACTACACGGCCTCGCGCGCCAACTCCGCGCTCGACGGCCAG
     G  N  A  E  L  Q  N  Y  T  A  S  R  A  N  S  A  L  D  G  Q   53
 180 GGCAACCTCGTCATCACCGCGCGCCGCGAGGGCGACGGGTCGTACACGTCGGCGCGCATG
     G  N  L  V  I  T  A  R  R  E  G  D  G  S  Y  T  S  A  R  M   73
 240 ACGACCCAGGGGAAGTACCAGCCGCAGTACGGGCGCATCGAGGCGCGCATCCAGATCCCG
     T  T  Q  G  K  Y  Q  P  Q  Y  G  R  I  E  A  R  I  Q  I  P   93
 300 CGGGGCCAGGGGATCTGGCCGGCGTTCTGGATGCTCGGCGGGAGCTTCCCCGGGACGCCG
     R  G  Q  G  I  W  P  A  F  W  M  L  G  G  S  F  P  G  T  P  113
 360 TGGCCGTCGTCGGGCGAGATCGACATCATGGAGAACGTCGGGTTCGAGCCGCACCGCGTG
     W  P  S  S  G  E  I  D  I  M  E  N  V  G  F  E  P  H  R  V  133
 420 CACGGCACGGTGCACGGCCCGGGGTACTCCGGCGGCTCCGGCATCACGGGCATGTACCAG
     H  G  T  V  H  G  P  G  Y  S  G  G  S  G  I  T  G  M  Y  Q  153
 480 CACCCGCAGGGCTGGTCGTTCGCGGACACGTTCCACACGTTCGCGGTCGACTGGAAGCCG
     H  P  Q  G  W  S  F  A  D  T  F  H  T  F  A  V  D  W  K  P  173
 540 GGGGAGATCACCTGGTTCGTCGACGGCCAGCAGTTCCACCGCGTCACGCGCGCGAGCGTC
     G  E  I  T  W  F  V  D  G  Q  Q  F  H  R  V  T  R  A  S  V  193
 600 GGCGCGAACGCCTGGGTGTTCGACCAGCCGTTCTTCCTCATCCTCAACGTCGCGGTCGGC
     G  A  N  A  W  V  F  D  Q  P  F  F  L  I  L  N  V  A  V  G  213
 660 GGGCAGTGGCCGGGCTACCCCGACGGCACGACCCAGCTCCCGCAGCAGATGAAGGTCGAC
     G  Q  W  P  G  Y  P  D  G  T  T  Q  L  P  Q  Q  M  K  V  D  233
 720 TACGTGCGCGTCTACGACAACGGCTCGGGCTCGTCGAACCCGGGGAACCCCGGCACCGGC
     Y  V  R  V  Y  D  N  G  S  G  S  S  N  P  G  N  P  G  T  G  253
 780 CTGCCGACGGGGACCGGCGCGGTGCGCGCCGCGAACGGCATGTGCGTGGACGTCCCGTGG
     L  P  T  G  T  G  A  V  R  A  A  N  G  M  C  V  D  V  P  W  273
 840 GCGGACCCGACCGACGGGAACCCGGTGCAGATCGTCACGTGCAGCGGCAACGCCGCCCAG
     A  D  P  T  D  G  N  P  V  Q  I  V  T  C  S  G  N  A  A  Q  293
 900 ACCTGGACGCGTGGCTCCGACGGGACCGTCCGCGCGCTCGGCAAGTGCCTCGACGTGCGC
     T  W  T  R  G  S  D  G  T  V  R  A  L  G  K  C  L  D  V  R  313
 960 GACGGCTCGACGACGCGCGGGGCGGCCGTGCAGGTGTGGACGTGCAACGGGACGGGCGCG
     D  G  S  T  T  R  G  A  A  V  Q  V  W  T  C  N  G  T  G  A  333
1020 CAGCTGTGGGCCTACGACGCGGGGAGCAAGGCGCTGCGCAACCCGCAGTCCGGGCTCTGC
     Q  L  W  A  Y  D  A  G  S  K  A  L  R  N  P  Q  S  G  L  C  353
1080 CTCGACGCCACGGGCGGCGCGCCCCTGCACGACGGCCAGCGGCTGCAGACCTGGACGTGC
     L  D  A  T  G  G  A  P  L  H  D  G  Q  R  L  Q  T  W  T  C  373
1140 AACGGCACGACCGCCCAGCAGTGGACGCTCTGAC
     N  G  T  T  A  Q  Q  W  T  L  383
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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.

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