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A novel β-glucanase gene from *Bacillus halodurans* C-125

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Abstract

A novel endo-β-1,3(4)-D-glucanase gene was found in the complete genome sequence of *Bacillus halodurans* C-125. The gene was previously annotated as an "unknown" protein and assigned an incorrect open reading frame (ORF). However, determining the biochemical characteristics has elucidated the function and correct ORF of the gene.

The gene encodes 231 amino acids, and its calculated molecular mass was estimated to be 26743.16 Da. The amino acid sequence alignment showed that the highest sequence identity was only 28% with that of the β-1,3-1,4-glucanase from *Bacillus subtilis*. Moreover, the nucleotide sequence did not match any other known *Bacillus* β-glucanase gene. The member of the gene cluster that includes this novel gene was apparently different from that of the gene cluster including the putative β-glucanase genes (*bh3231* and *bh3232*) from *B. halodurans* C-125. Therefore, the novel gene is not a copy of either of these genes, and in *B. halodurans* cells, the putative role of the encoded protein may differ from that of *bh3231* and *bh3232*.

To examine the activity of the gene product, the gene was cloned as a His-tagged protein and expressed in *Escherichia coli*. The purified enzyme showed activity against lichenan, barley β-glucan, laminarin, and carboxymethyl curdlan. Thin-layer chromatography showed that the enzyme hydrolyzes substrates in an endo-type manner. When β-glucan was used as a substrate, the pH optimum was between 6 and 8, and the temperature optimum was 60 °C. After 2 h incubation at 50 and 60 °C, the residual activity remained 100% and 50%, respectively. The enzymatic activity was abolished after 30 min incubation at 70 °C. Based on the results, the gene encodes an endo-type β-1,3(4)-D-glucanase (E.C. 3.2.1.6).

Keywords: β-glucanase; β-1,3(4)-glucanase; Glycoside hydrolase family 16; *Bacillus halodurans* C-125; Post-genomic study

1. Introduction

β-1,3-1,4-Glucans are linear polysaccharides of cell walls of higher plants that comprise a mixture of both β-1,3- and β-1,4-linked D-glucose [1]. The percentage of the β-1,3-linkage varies from 25% to 30%. The endo-type enzymes hydrolyzing β-1,3-1,4-glucans (β-1,3-1,4-glucanases) are β-1,4-D-glucan 4-glucanohydrolase (EC. 3.2.1.4), β-1,3-D-glucan 3-glucanohydrolase (E.C. 3.2.1.39), and β-1,3-1,4-D-4-glucanohydrolase (lichenase) (E.C. 3.2.1.73). The enzymes hydrolyzing both β-1,3-1,4-glucan and β-1,3-glucan are classified as a β-1,3(4)-glucanase (E.C. 3.2.1.6). The β-1,3-1,4-glucanases from several bacilli, which are classified into the glycoside hydrolase family 16 (GH-16) (http://afmb.cnrsmrs.fr/CAZY/index.html), share a similar amino acid sequence having a conserved "EIDIEF" motif [2]. The two glutamic acid residues in this motif are known to be involved in the hydrolytic activity [3,4]. β-1,3-1,4-glucanases in GH-16 from *Bacillus subtilis* [5–7], *Bacillus amyloliquefaciens* [8], *Bacillus*

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macerans [9], *Bacillus circulans* [10,11], *Bacillus polymyxa* [12], *Bacillus licheniformis* [13], *Bacillus brevis* [14], and *Bacillus* sp. strain N137 [15] have been cloned and characterized.

Bacillus halodurans C-125 is an alkaliphilic bacterium [16] that can grow well at pH 7–10.5 when sufficient sodium chloride is present in the medium. It is well characterized physiologically, biochemically, and genetically [16–19]. The chromosomal DNA of *B. halodurans* C-125 has been sequenced, revealing that the genome contains more than 4000 protein coding sequences (CDSs) [20]. In the CDSs, several lichenase-like genes have been found, and two of the genes are designated *bh3231* and *bh3232*. The proteins translated from these two genes are now classified into GH-16.

The CDS designated *bh2115* was annotated as an "unknown" protein. However, upstream of the CDS, there is an amino acid sequence that is conserved among the enzymes of GH-16. An extended CDS (designated *bgn2115*) has been found at the position from 2242244 to 2242939 (693 base pairs, 231 amino acids) on the complete genome sequence. Although the gene product contains the conserved amino acid motif "EIDIEF", the results of a BLAST search revealed that the maximal identity of the gene product was very low. This suggests that *bgn2115* is a novel protein gene.

Here, we report sequence analyses, protein expression, and enzymatic properties of this novel gene and its product from *B. halodurans* C-125. The biochemical data elucidated the function of this gene product that was previously annotated as "unknown".

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals used were from Wako Pure Chemical. Coomassie Brilliant Blue R-250 (CBB), SDS, and EDTA were products of Bio-Rad. Peptone and yeast extract were purchased from Difco. Lichenan from *Cetraria islandica*, laminarin from *Laminaria digitata*, β-glucan from barley, starch, κ-carrageenan from Irish moss, xylan from birch wood, and *p*-nitrophenyl-β-D-glucopyranoside were purchased from Sigma.

2.2. Sequence analyses

Database analyses were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Amino acid sequence alignment was performed using the CLUSTAL program [21]. The amino acid sequences used were Bgn2115 in this work, lichenase from *B. subtilis* NCIB 8565 (SWISS-PROT Q45691), β-1,3-1,4-endoglucanase from *B. licheniformis* (SWISS-PROT Q8GMY0), lichenase from *B. amyloliquefaciens* (SWISS-PROT P04957), a β-1,3-1,4-glucanase precursor from *Bacillus pumilus* (SWISS-PROT Q8GB49), a β-1,3-1,4-glucanase precursor from *Paenibacillus polymyxa* (SWISS-PROT Q8GB48), lichenase M from *Paenibacillus macerans* (SWISS-PROT Q846Q0), a lichenase from a *Bacillus* sp. (GenBank A00896), a lichenase from *B. brevis* (SWISS-PROT P37073), BH3231 (SWISS-PROT Q9K7X6), and BH3232 (SWISS-PROT Q9K7X5) from *B. halodurans* C-125.

2.3. Cloning the bgn2115 gene

Genomic DNA of *B. halodurans* C-125 was prepared as described by Saito and Miura [22]. The *bgn2115* gene was amplified from the genomic DNA by polymerase chain reaction (PCR) using primers, 5′-GCATAAAATCATATGAAACAACTAG-3′ (*Nde*I site underlined) and 5′-TACAAGTTCTCGAGGTTACAATATG-3′ (*Xho*I site underlined). PCR was performed using Takara Pyrobest DNA polymerase (Takara) in accordance with the manufacturer's instructions. The amplified DNA was purified using a High Pure PCR Product Purification kit (Roche), and then digested using restriction enzymes, *Nde*I and *Xho*I (New England Biolabs). The resulting DNA was inserted into pET-15b (Novagen) using a DNA ligation kit ver. 2, Takara. The plasmid, which encodes an N-terminal Histag with six histidine residues, was introduced by transformation into *Escherichia coli* HB-101 (Takara) by the methods of Hanahan [23] and Chang and Cohen [24]. The plasmid DNA sequencing was performed using an ABI Prism Big Dye Terminator Cycle Sequencing kit and an ABI 377 Sequencer (Applied Biosystems). After the sequence was checked, the desired plasmids were selected (designated pBGN2115).

2.4. Protein expression and purification

To produce the protein (Bgn2115) translated from *bgn2115*, the plasmid pBGN2115 was introduced by transformation into *E. coli* BL21 (DE3) (Novagen). Then, the transformed *E. coli* BL21 (DE3)-pBGN2115 was cultured in 2 × YT medium (1.6% (w/v) peptone, 1.0% (w/v) yeast extract, and 0.5% (w/v) NaCl) containing 100 μg/ml ampicillin at 37 °C until the OD600 reached the level of 0.5. The target protein was induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG) to the medium at a final concentration of 0.1 mM. After 2 h incubation, cells were collected by centrifugation at 8000 × *g* for 15 min. The pellets were suspended in 20 mM sodium phosphate buffer at pH 7.0, followed by sonication. The sonicate was centrifuged, and the supernatant was loaded onto a nickel nitrilotriacetic acid-agarose