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A β-*N*-acetylhexosaminidase from *Symbiobacterium thermophilum*; gene cloning, overexpression, purification and characterization

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Abstract

An ORF encoding the gene (*nah*A)-related carbohydrate hydrolase was found in chromosomal DNA of the symbiotic thermophile, *Symbiobacterium thermophilum*. BLASTX results indicated that the product of this structural gene is β-glucosidase or β-*N*-acetylhexosaminidase. To investigate details of the *nah*A gene product, cloning of the gene, overproduction of the gene product in *Escherichia coli*, and purification and characterization of the resulting protein were conducted. The *nah*A gene was amplified by PCR using fragmented chromosomal DNA of *S. thermophilum* as a template, sequenced, and then ligated into the *Bam*HI and *Nde*I sites of plasmid pET-25b(+) to construct the expression vector pST-BNAH-A for overproduction of the gene product. Results of studies on the hydrolytic activity of cell-free extracts against *p*NP-β-Glc, *p*NP-β-GlcNAc and *p*NP-β-GalNAc, obtained by disruption of cultured *E. coli* cells harboring pST-BNAH-A, suggested that the *nah*A gene product was β-*N*-acetylhexosaminidase. Comparison of the amino acid sequence of the recombinant protein with those of other β-*N*-acetylhexosaminidases indicated that the β-*N*-acetylhexosaminidase of *S. thermophilum* is a member of the 3-glycoside hydrolase family. The recombinant enzyme was purified to homogeneity from cell-free extract in an overall yield of 24%. This purified β-*N*-acetylhexosaminidase possessed thermostability, was stable in alkaline solution, and exhibited greater hydrolytic activity against chitin oligosaccharides than against *p*NP-β-GlcNAc.

Keywords: *Symbiobacterium thermophilum*; β-*N*-acetylhexosaminidase; Gene cloning; Enzyme purification; Enzyme characterization

Symbiobacterium thermophilum is a thermophilic bacterium, which was isolated from compost while screening heat-stable enzymes such as tryptophanase [1–3] and β-tyrosinase [4,5]. Results of phylogenetic analyses indicate that this bacterium belongs to a novel phytogenetic branch at the outermost position of the Gram-positive bacterial group without belonging to any other known genus [6]. Growth of *S. thermophilum* is dependent on the coexistence of an associated thermophilic *Bacillus* sp., strain S [1]. Therefore, the proliferation of this bacterium independently in culture medium was considered to be difficult. Recently, independent growth of this bacterium was accomplished by dialyzing culture physically separated from *Bacillus* strain S with a cellulose membrane [7,8]. Independent growth of *S. thermophilum* was also managed by adding conditional medium prepared from the culture filtrate of the *Bacillus* strain [7]. Recently, the entire nucleotide sequence of the chromosomal DNA of *S. thermophilum* has been determined [9], which contains several ORFs associated with carbohydrate hydro-

Abbreviations: ORF, open reading frame; PCR, polymerase chain reaction; BAP, bacterial alkaline phosphatase; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide-gel electrophoresis; LB, Luria–Bertani; TLC, thin-layer chromatography; GH, glycoside hydrolase; *p*NP-β-GlcNAc, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide; *p*NP-β-GalNAc, *p*-nitrophenyl-β-acetyl-β-D-galactosaminide; *p*NP-β-Glc, *p*-nitrophenyl-β-D-glucoside; $(GlcNAc)_n$, chitin oligosaccharide; $(GlcNAc)_2$, di-*N*-acetylchitobiose; $(GlcNAc)_3$, tri-*N*-acetylchitotriose; $(GlcNAc)_4$, tetra-*N*-acetylchitotetraose; $(GlcNAc)_5$, penta-*N*-acetylchitopentaose

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lase. Hydrolases involved in carbohydrate degradation have not yet been isolated from *S. thermophilum.* We cloned these genes in the chromosomal DNA of *S. thermophilum,* and produced, purified, and characterized the gene products. Initially, we chose the *nah*A gene, which seemed to encode the β-*N*-acetylhexosaminidase, for this study.

β-*N*-Acetylhexosaminidases fulfill an important vegetative function in heterotrophic microorganisms. They release *N*-acetylglucosamine from *N*-acetylchitoorigosaccharides obtained by the action of chitinases on insoluble chitin. The β-*N*-acetylhexosaminidase genes were cloned from some microorganisms, and they were produced, purified, and their characters were investigated [10].

Here, we report the results of cloning the *nah*A gene of *S. thermophilum,* overproduction of the recombinant protein in *Escherichia coli,* purification from *E. coli* cell extract, investigation of substrate specificity and physiological properties of the purified enzyme.

1. Materials and methods

1.1. Microbial strains and plasmid

S. thermophilum IAM14863 [6] was the genome source for gene cloning. *E. coli* DH5α was the host strain used for gene cloning and propagation of the genetic construction. *E. coli* BL21(DE3) was the host strain used for enzyme production. pGEM-T Easy plasmid (Promega) was used as the cloning vector of the PCR product for DNA sequence analysis. pET-25b(+) plasmid (Novagen) was used as the expression vector for enzyme production.

1.2. General DNA techniques

Cloning, DNA manipulations, and *E. coli* transformation were conducted using standard techniques [11]. Recovery of DNA from agarose gel slices was accomplished using the GENECLEAN II kit (Q•Biogene). Plasmid recovery from *E. coli* cells was achieved using the QIAprep Spin Miniprep Kit (QIAGEN). Ligation was conducted using Ligation Pack (Nippon Gene).

1.3. Cloning of the nahA gene

Chromosomal DNA of *S. thermophilum* was isolated as described previously [3]. After DNA digestion using two types of restriction enzymes (*Bam*HI and *Eco*RV), the *S. thermophilum nah*A gene was amplified by PCR (LA *Taq* DNA polymerase, 1× GC buffer I; Takara), using 100 ng of the resulting DNA fragment mixture and 50 pmol of the synthetic oligonucleotide primers, 5′-C*CAT*ATGTTTG-TTAGTCCTCCAGTATACATTCAGCAGAG-3′ (forward primer) and 5′-C*GGATCC*GCATGCGCACCGATGGTG-AGGATTC-3′ (reverse primer) (underline letteres; *Nde*I site of forward primer and *Bam*HI site of reverse primer. Italic letters; non-complimentary nucleotides added). After 30 amplification cycles (denaturation: 98 °C, 20 s; annealing and elongation: 68 °C, 5 min), the PCR product was incubated at 72 °C for 1 h followed by isolation from agarose gel slice, and was then subcloned into pGEM-T Easy by TA-cloning to yield the plasmid pST-GHLl. This plasmid was used to confirm the nucleotide sequence of the cloned *nah*A gene.

1.4. DNA sequencing

DNA sequencing analysis was performed by the dideoxynucleotide method [12]. The nucleotide sequence of the gene was determined in both orientations using a ThermoSequenase fluorescence-labelled primer cycle sequencing kit (Amersham) and an automated DNA sequencer (DSQ-2000L; Shimadzu).

1.5. Construction of plasmid for enzyme expression

After digestion of pST-GHLl with *Nde*I and *Bam*HI, the resulting DNA fragment containing the *nah*A gene was isolated from agarose gel slice, then ligated into the *Nde*I and *Bam*HI sites of pET-25b(+) to give the plasmid pST-BNAH-A. *E. coli* BL21(DE3) was transformed with this plasmid and the transformants were selected on LB medium-agar plates supplemented with 100 μg/ml ampicillin.

1.6. Production and purification of enzyme

E. coli BL21(DE3) harboring a recombinant plasmid, pST-BNAH-A, was grown at 37 °C in 20 ml LB medium supplemented with 100 μg/ml ampicillin until the OD_{600} reached 0.4. Then, the culture was diluted into one liter of the same fresh medium and cultivated at 37 °C until the OD_{600} reached 0.6. IPTG was added to give a final concentration of 1 mM, and the culture was incubated for an additional 4 h at 37 °C.

The cells were harvested by centrifugation at 4000 × *g* for 10 min, suspended in 80 ml PBS(−) supplemented with 1 mM phenylmethylsulphonylfluoride and 350 μg/ml lysozyme, and the suspension was incubated for 2 h at 30 °C. The cells were disrupted by sonication, and the enzyme extracted by treatment with Triton X-100 (final concentration; 1%). Cell debris and remaining intact cells were removed by centrifugation at 9000 × *g* for 20 min. The resulting cell-free extract was heated at 60 °C for 15 min to remove heat-labile *E. coli* proteins. The protein aggregates were removed by centrifugation at 8000 × *g* for 20 min. After dialysis of the crude enzyme solution in 10 mM sodium phosphate buffer (pH 7.0), the solution was loaded on a DEAE-Toyopearl 650 M (Toso) column (ϕ 2.3 cm × 19 cm) pre-equilibrated with the same buffer, and enzyme protein was eluted with a linear gradient of 0–0.2 M NaCl in a total volume of 320 ml. The active fractions were collected, dialyzed in 10 mM sodium phosphate buffer (pH 7.0), and concentrated to 6 ml by diaflow filtration using an Amicon PM-10