Note

Cloning, Sequencing and Heterologous Expression of the Gene Encoding Glucoamylase from *Clostridium thermoamylolyticum* and Biochemical Characterization of the Recombinant Enzyme*

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Abstract: *Clostridium thermoamylolyticum* glucoamylase gene was overexpressed in *Escherichia coli* cells. This glucoamylase gene consisted of 2133 bp that encoded a 710-amino-acid protein with a molecular mass of 79,920 Da. The glucoamylase fell into glycoside hydrolase family 15, showing 84% identity and 90% similarity to an amino acid sequence of *Clostridium* sp. G0005 glucoamylase, and showing 82% identity and 87% similarity to that of *Thermoanaerobacterium thermosaccharolyticum* glucoamylase. The corresponding sequence to the mature protein was placed under the control of the T7 promoter as a strong and constitutive promoter. The recombinant glucoamylase was purified by a Ni-NTA column. The molecular mass of the mature glucoamylase was 77 kDa by SDS-PAGE, and it was purified 10-fold with a recovery of 65%. The specific activity was determined to be 1.8 U/mg for maltose. The value of K_m for maltose was determined to be 5.4 mM, and the k_o was 7.1 s^{-1} . The optimum pH of the enzyme was determined to be 4.5, and more than 80% of the enzyme activity remained between pH 3.5 and 9.0. The optimum temperature was 65°C, and more than 80% of the enzyme activity remained up to 65°C.

Key words: glucoamylase, *Clostridium thermoamylolyticum*, sequencing, cloning

Glucoamylase (1,4- α -D-glucan glucohydrolase, E.C. 3.2.1.3, GA) catalyzes the release of β -glucose from the non-reducing end of maltodextrin chains. A number of glucoamylases have been isolated from a variety of eukaryotic and prokaryotic organisms, and they are typified by previous reports.^{1–6)} All of them have been compiled in family 15 of classification of the glycoside hydrolase superfamily.⁷⁾ In this paper, we present the cloning using PCR by synthetic oligonucleotides from a nucleotide sequence of *Clostridium* sp. G0005 glucoamylase,⁸⁾ sequencing, expression of a glucoamylase gene from *Clostridium thermoamylolyticum* (ATCC-39251) and biochemical characterization of the recombinant enzyme.

All chemicals were purchased from NACALAI TESQUE. DNA manipulations were performed in *E. coli* DH5 α (Invitrogen) using standard procedures. Restriction enzymes were from TaKaRa BIOCHEMICALS. Polymerase chain reactions were carried out using LA-Taq polymerase (TaKaRa BIOCHEMICALS) as recommended by the manufacturer. PCR products were sequenced in both orientations with a Li-COR 4000 automated DNA sequencer (Aloka). Chromosomal DNA of *C. thermoamylolyticum* was prepared as described by Ausubel *et al.*⁹⁾ In

the first round amplification, the *C. thermoamylolyticum* glucoamylase gene was amplified by using the above chromosomal DNA as a template with a set of following primers. Two oligonucleotide primers, GA-s (5'-GGG TAG CCG ATT GTA CTG TGG GGC ATT-3') and GA-as (5'-TCG TAT TTA TCG CCG ATG TTG ATG TTT TGC-3'), were designed from the sequence of *Clostridium* sp. G0005 chromosomal DNA (DDBJ accession number D12818). The PCR amplimers were gel-purified and cloned into pCR2.1-TOPO using TA cloning (Invitrogen), yielding pGA, which contained a complete functional glucoamylase gene. The sequence data for the gene displayed in Fig. 1 has been deposited with DDBJ (accession number AB115912). This glucoamylase gene consisted of 2133 bp that encoded a 710-amino-acid protein with a molecular mass of 79,920 Da. The glucoamylase showed 84% identity and 90% similarity to an amino acid sequence of *Clostridium* sp. G0005 glucoamylase,⁸⁾ and showed 82% identity and 87% similarity to that of *Thermoanaerobacterium thermosaccharolyticum* (DSM 572) glucoamylase.¹⁰⁾ The glucoamylase had low similarity, showing 22% amino acid sequence similarity to *Thermoactinomyces vulgaris* R-47 glucoamylase.¹¹⁾ The putative amino acid sequence of the *C. thermoamylolyticum* glucoamylase was compared with the primary structures of glucoamylases from various eukaryotes,^{12–15)} and the five highly conserved regions, CR1, CR2, CR3, CR4 and CR5 (Fig. 1), that have been reported¹⁶⁾ were found in the glucoamylase. The glucoamylase had a primary structure quite different from the structures of eukaryotic gluco-

* Data deposition: The sequence reported in this paper has been deposited in the DDBJ nucleotide sequence database (accession number AB115912).

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Fig. 1. Nucleotide and deduced amino acid sequences of the glucoamylase from *C. thermoamylolyticum*.

The deduced gene products are indicated in the one-letter code under the nucleotide sequence. Box shows the signal peptide region. The five highly conserved regions, CR1, CR2, CR3, CR4 and CR5, are underlined. The location of the two sets of primers, GA-s/GA-as and GA-NdeI-s/GA-XhoI-as, are indicated with arrows.

amylases, but its optimum pH and temperature were similar. The N-terminal sequence was identical to the sequence starting 29 residues, the 28-residue sequence upstream of N-terminal sequence showed the characteristics of prokaryotic signal peptide. The site of cleavage of a signal from the glucoamylase was predicted by PSORT program. The predicted site resembled a cleavage site of *Clostridium* sp. G0005 glucoamylase.⁸⁾ Ohnishi *et al.*⁸⁾ supposed that the 5'-flanking region or the putative region coding the signal sequence for lipoprotein secretion of a glucoamylase was harmful to *E. coli* cells. We constructed an inducible expression plasmid from pGA for the glucoamylase used in this study with N terminus deleted using PCR by synthetic oligonucleotides. The glucoamylase gene was cloned into the pET-23a(+) (Novagen) expression vector with a His₆-tag at the C terminus. This expression plasmid was constructed from the pGA to express the glucoamylase. This gene was amplified by PCR using the following primers: GA-*Nde*I-s (5'-G CTA **CAT ATG** TGC GCT AAC AAT AAT-3') and GA-*Xho*I-as (5'-C TGT **CTC GAG** CTT TGA AGC ATA CCT-3') (Fig. 1). The restriction sites CAT ATG for *Nde*I and CTC GAG for *Xho*I designed in the primers are bold. The resultant products were sequenced to confirm PCR fidelity and cloned as *Nde*I-*Xho*I fragment into the same site of pET-23a(+), yielding an expression construct, pETGA. *E. coli* BL21(DE3)pLysS was used as a host for the expression of the glucoamylase gene. One of the transformants was used for 10 mL seed culture with ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL) at 37°C and 250 rpm. The seed culture was used to inoculate 1 liter LB medium at the same antibiotic concentrations as above. This culture was propagated at 200 rpm and 30°C to a mid-log phase of growth ($OD_{600} = 0.6$ to 0.8), cooled to 4°C for 10 min and induced with 100 µM isopropyl β-D-thiogalactoside. The culture was continued for an additional 9 h at 30°C. Expressed proteins were checked with 10.0%-resolving/4.8%-stacking gel of post Ni-NTA column (5 mL, Novagen) fractions by Coomassie Brilliant Blue stain.

Glucoamylase activity was determined as the amount of glucose liberated from 0.2% maltose at 30°C in 100 mM sodium acetate buffer (pH 5.0), using a Glucose AR-II Test (Wako Pure Chemical), as described previously.¹⁷⁾ The purity of the protein was verified by SDS-PAGE. The molecular mass of the expressed protein was 77 kDa by SDS-PAGE (Fig. 2), which was similar to the calculated molecular mass from the gene. The recombinant enzyme was purified 10-fold with a recovery of 65%. In the purification procedure described above, 100 mg/L of the glucoamylase protein was isolated. The specific activity was determined to be 1.8 U/mg for maltose. The N-terminal sequence was determined according to Edman degradation, using an Applied Biosystems prociseTM 491-HS. The first 7 amino acids (CANNIS) of the recombinant glucoamylase were sequenced. The result revealed that the recombinant glucoamylase from *E. coli* was correctly cut off at the initial methionine residue.

The value of K_m for maltose was determined to be 5.4 mM, and the k_0 was 7.1 s⁻¹ (Fig. 3A). The optimum pH of the enzyme was determined to be 4.5 (Fig. 3B), and more

Fig. 2. SDS-PAGE of the recombinant glucoamylase.

Lane 1, protein marker; 2, extract from *E. coli* harboring pET-23a(+) (negative control); 3, extract from *E. coli* harboring pETGA; 4, purified recombinant glucoamylase.

Fig. 3. Properties of the recombinant glucoamylase.

(A) Lineweaver-Burk plot for hydrolysis of maltose by the purified glucoamylase. The maltose concentrations were 2.0, 2.5, 3.3, 5.0, 10.0 and 20.0 mM. (B) Effect of the pH on the activity of the purified glucoamylase. The initial rates were determined at the pH values in the range of 3.2–7.9 using 100 mM of the buffer citric acid- Na_2HPO_4 .

than 80% of the enzyme activity remained between pH 3.5 and 9.0 at 4°C for 20 h. The optimum temperature was 65°C, and more than 80% of the enzyme activity remained up to 65°C when the enzyme was incubated at various temperatures for 15 min. All the characteristics of the recombinant glucoamylase were similar to those of a glucoamylase from *Clostridium* sp. G0005.⁵⁾

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Clostridium thermoamylolyticum 由来 glucoamylase 遺伝子の単離、発現および組換え酵素の諸性質

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Clostridium thermoamylolyticum 株染色体 DNA を鋳型とし PCR によって 2133 bp の open reading frame をもち、710 アミノ酸残基を翻訳産物とする glucoamylase 遺伝子 (Fig. 1) を取得した。得られた推定 glucoamylase アミノ酸配列は *Clostridium* sp. G0005 由来 glucoamylase に対して 84% 一致し、90% の相同性を示し、*Thermoanaerobacterium thermosaccharolyticum* 由来 glucoamylase に対しては 82% 一致し、87% の相同性を示した。本遺伝子を pET システムを用い大腸菌により発現した。形質転換大腸菌の粗抽出液より Ni-NTA カラムクロマトグラフィーを用い、精製倍率 10 倍、回収率 65%、比活性 1.8 U/mg の精製 glucoamylase を得た。得られた精製酵素は、分子量 77 kDa, maltose に対する K_m は 5.4 mM, k_0 は 7.1 s^{-1} , 至適 pH は 4.5, 至適温度は 65°C であった。本酵素は maltose を基質として用いた場合、上記原核微生物由来 glucoamylase と同様の性質を示した。