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Vibrio vulnificus glycogen branching enzyme preferentially transfers very short chains: N1 domain determines the chain length transferred

Hye-Jin Jo [a,1], Sunghoon Park [b,1], Hee-Gon Jeong [a], Jung-Wan Kim [c], Jong-Tae Park [a,*]

[a] Department of Food Science and Technology, Chungnam National University, Daejeon 305-764, Republic of Korea
[b] Research Institute of Food and Biotechnology, SPC Group, Seoul 151-742, Republic of Korea
[c] Division of Bioengineering, Incheon National University, Incheon 406-771, Republic of Korea

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ABSTRACT

The glycogen branching enzyme from *Vibrio vulnificus* (VvGBE) transfers short side chains (DP 3–5) significantly greater than any other bacterial glycogen branching enzyme (GBE). To elucidate the role of the N-domain of VvGBE in the unique branching pattern, domain-truncated (N1 and N) and N1-domain-swapped (with VvGBE N1 replacing the counter part of *Escherichia coli* GBE) mutants were constructed. The truncation mutants synthesized branched products with a greatly reduced proportion of short chains. The swapping mutant exhibited a branching pattern of the short chain region similar to that of VvGBE. We conclude that the N1-domain of VvGBE has a crucial role in the determination of the branching pattern of glycogen.

1. Introduction

Glycogen is a major polysaccharide of energy reservoir in animals and microorganisms. It is a highly branched polysaccharide, in which glucose residues are linked by α-1,4 glycosidic bonds to from linear chains and at every 10 residues, other linear chains are linked by α-1,6-glycosidic bonds to form sides chains [1–3]. Formation of side chains in glycogen is catalyzed by glycogen branching enzyme (GBE) or branching enzyme (BE) (EC 2.4.1.18). GBE catalyzes formation of α-1,6-glycosidic linkage by cleaving α-1, 4 linkages of substrate and transferring the non-reducing end of the chain to an acceptor [4]. BEs are carbohydrate-active enzymes (CAZY) and found in glycoside hydrolase family 13 (GH13) that include amylolytic enzymes such as α-amylase, α-glucosidase, isoamylase, pullulanase, cyclodextrin glycosyltransferase [5] and GH57 as well [6,7]. Up to date, GBEs have been cloned and characterized from many bacteria and hyperthermophilic archaea [6–10]. GBEs of GH13 are generally categorized into two major groups based on their amino acid sequences. One group of GBE (type I) including *Escherichia coli* GBE contains an additional N-terminal stretch of 100–150 amino acids, while the other group (type II) including GBEs from *Bacillus subtilis* and *Deinococcus geothermalis* does not [8].

GBEs of GH13 mainly consist of three domains: N-domain, C-domain, and an $(\alpha/\beta)_8$ barrel catalytic domain [11]. Each domain has been characterized for its function by mutagenesis of the enzymes: C-domain may be involved in substrate preference and catalytic capacity, N-domain in determining the size of the chain transferred. N-domain is a typical for most of the GH13 enzymes and is involved in substrate binding and active site forming [12,13]. *E. coli* GBE was reported that the length of its N-domain affected the chain transfer pattern [14]. N-domain of type I GBE consists of N1- (~130 amino acids) and N2-domain, the latter contains carbohydrate-binding module 48 (CBM48) [8]. The CBM48 has been found at N-terminal region of pullulanases and cyclodextrinases as well [12,13,15]. The CBM48 is known as one of the starch-binding domain and exhibits similar tertiary structures and binding functions to CBM20 [15–17]. In contrast to the CBM20, the CBM48 showed binding affinity only to soluble substrates [18]. Crystal structures of *E. coli* GBEs were solved without

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* Corresponding author at: Department of Food Science and Technology, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 305-764, Republic of Korea. Fax: +82 42 821 8785.
E-mail address: jtpark@cnu.ac.kr (J.-T. Park).
[1] Authors contributed equally.

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its N1-domain [11]. Previously, crystalline structure of *Mycobacterium tuberculosis* GBE was determined and showed a full domain structure. However, the role of N1-domain in branching pattern was not investigated [19].

In this report, we cloned and characterized a GBE from *Vibrio vulnificus* MO6-24/O (VvGBE), and investigated unknown function of VvGBE N1-domain by constructing N1- and N-domain-truncated mutants and swapping N1-domain of VvGBE into *E. coli* GBE. We report that this enzyme transfer shortest chain (degree of polymerization 3–5) among GBEs, and N1-domain of VvGBE has a crucial role in the determination of the branching pattern.

2. Materials and methods

2.1. Bacterial strains and plasmids

Genomic DNA of *V. vulnificus* MO6-24/O, *E. coli* K12, and *D. geothermalis* was isolated using a genomic DNA extraction kit (Intron, Korea) and used for cloning the GBE genes. *E. coli* BL21 (DE3) or MC1061 was used as hosts for the recombinant plasmids and their gene expression. Bacterial strains and plasmids used in this study were listed on Supplementary materials.

2.2. Phylogenetic analysis of GBEs

Amino acid sequences and conserved domains of various GBEs were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) according to the previous reports [20]. Multiple alignment was performed using the CLUSTAL W (http://www.clustal.org/clustal2/) and a phylogenetic tree was constructed using MEGA version 6.06 [21]. Proteins compared in the phylogenetic analysis were chosen by experimental data from previous studies or based on their sequence similarity [7,8,11,19,22,23]. More information on the GBE gene sequences are provided in the Supplementary materials.

2.3. Cloning of the VvGBE gene and construction of mutants

The VvGBE gene (VVMO6_03058) was amplified from the genomic DNA of *V. vulnificus* MO6-24/O by PCR using the oligonucleotides (see Supplementary materials). The amplified DNA fragment was purified using a purification kit (MEGA Quick Spin Total Fragment DNA Purification Kit, Intron Biotechnology, Seoul, Korea) and digested with *Nde*I and *Pst*I. The DNA fragment was cloned into p6xHis119 at the corresponding restriction sites, and the resulting clone was designated as p6xHisVvGBE. The truncated mutants were constructed by deleting the N-terminal amino acid sequences of 1–129 or 1–240 residues for the truncation of N1- or N-domain, respectively. For the construction, DNA fragments carrying the truncated genes were amplified by PCR using p6xHisVvGBE as a template. The domain-swapping enzyme, in which the N1- or N-domain of VvGBE replaced the counterpart of *E. coli* GBE, was constructed as follows. The N1- (1–129 residues) or N-domain (1–240 residues) of VvGBE amplified by PCR was ligated to p6xHis119. Then, the *E. coli* GBE gene lacking the N1- (1–116 residues) or N-domain (1–211 residues) was fused to the N1- or N-domain of VvGBE, respectively. More information on the cloning strategy and resulting recombinant plasmids is provided in the Supplementary materials. *B. subtilis* GBE clone was obtained as described previously [24].

2.4. Expression and purification of the recombinant proteins

E. coli MC1061 or *E. coli* BL21 (DE3) carrying one of the recombinant plasmids was cultured in 1 L LB broth supplemented with antibiotics (ampicillin 200 μg/mL, kanamycin 50 μg/mL) at 30 °C and the cells were harvested by centrifugation (10,000×g) for 20 min at 4 °C. Soluble proteins were extracted and purified using Ni–NTA affinity chromatography. Protein concentration was determined by Bradford assay [16]. The purity and molecular mass of purified proteins were analyzed using sodium dodecyl sulfate polyacrylamide–gel electrophoresis (SDS–PAGE).

2.5. Activity assay of glycogen branching enzyme

The activity of VvGBE was measured by the iodine assay. The mixture of 0.04% (w/v) potato amylose (or 0.05% maize amylopectin) in 50 mM MOPS buffer (pH 7.5) and VvGBE was incubated at 30 °C. An aliquot (0.1 mL) of the reaction mixture was mixed with 0.1 N NaOH (0.05 mL) to stop the reaction, then neutralized by adding 0.1 N HCl (0.05 mL) and mixed with 1 mL of 0.02% iodine/potassium iodide solution. Absorbance at 620 nm (530 nm for amylopectin) was measured immediately. One unit of the enzyme activity was defined as the amount of enzyme reducing 1 mg of amylose or amylopectin per min under the assay conditions. Assays of other GBEs and the VvGBE mutant enzymes were carried out by the same procedure but at pH and temperature appropriate for each enzyme.

2.6. Branching pattern analysis

Potato amylose or maize amylopectin (0.5%, w/v) was completely dissolved in 90% dimethyl sulfoxide by boiling and stirring at 100 °C for 3 h. The substrates were treated with 0.1 unit/mg of VvGBE in MOPS buffer (50 mM, pH 7.5) at 30 °C for 24 h. Then, the reaction products were used for analyses of branching patterns. Purification, debranching, and high performance anion-exchange chromatography (HPAEC) were conducted as described previously [22].

3. Results and discussion

3.1. Protein sequence and phylogenetic analyses

VvGBE is an enzyme of 742 amino acids with a molecular mass of 85 kDa, which is similar to that of *E. coli* GBE (728 amino acids), but having more than 100 residues than type II branching enzymes. Phylogenetic analysis showed that VvGBE is grouped with other *Vibrio* GBEs and closely related to GBEs of enteric bacteria (Fig. 1A). VvGBE and *E. coli* GBE are type I bacterial GBEs and share 57% sequence similarity. Four regions of conserved sequences were also observed in VvGBE with identical residues especially at those constituting the catalytic site in other bacterial GBEs (Fig. 1B). Domain architectures of type I and II GBE enzymes are different from each other (Fig. 1C). Type I enzymes carry an N-terminal domain of N1 and N2, making it longer than that of type II enzymes. VvGBE shared overall homology higher than 45% with type II GBEs from *D. geothermalis* and *B. subtilis*.

3.2. Cloning and characterization of various GBEs

The gene for VvGBE was cloned and two VvGBE truncation mutants (N1_t and N_t) and two swapping mutants (N1_s and N_s) were derived from VvGBE and *E. coli* GBE as shown in Fig. 2. VvGBE and its mutants were successfully purified and used for further analyses (Fig. 3A), except N-domain swapping mutant (N_s), which consists of the N-domain of VvGBE and the catalytic and the C-domain of *E. coli* GBE, that formed inclusion body during expression. The GBEs from *E. coli*, *D. geothermalis*, and *B. subtilis* were expressed and purified as well.