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Alanine 310 is important for the activity of 1,4-α-glucan branching enzyme from *Geobacillus thermoglucosidans* STB02

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ABSTRACT

1,4-α-Glucan branching enzyme (GBE) catalyzes the formation of α-1,6 branch points in starch or glycogen by hydrolyzing α-1,4-glucosidic linkages and then synthesizing α-1,6-glucosidic linkages. In the GBE from *Geobacillus thermoglucosidans* STB02, alanine 310 (Ala310) is located in conserved region II. An analysis of the amino acid sequence shows that Ala310 is highly conserved in the prokaryotic GBE subfamily. Site-directed mutagenesis was used to determine the function of Ala310 in GBE. Replacement of Ala310 with glycine, aspartate, asparagine, isoleucine, glutamate, or glutamine resulted in mutant enzymes with less than 10% to 25% of wild-type activity when amylopectin or amylose was used as substrate. Studies using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) showed that A310G mutant had no effect on the transfer pattern, but the branching activity had been repressed to a large extent. Kinetic analysis also showed that mutations of Ala310 had an effect on the K_M value that changed the preferred substrate from amylopectin to amylose. These results show that Ala310 is important for the catalytic activity of the GBE from *G. thermoglucosidans* STB02.

1. Introduction

Numerous 1,4-α-glucan branching enzymes (GBEs, EC 2.4.1.18) have been characterized from various sources, including plants [1], microorganisms [2,3], animal tissues [4]. These enzymes catalyze a transglycosylation reaction that cleaves an α-1,4-glucosidic bond and introduces a new α-1,6-glucosidic linkage. Three different modes of action have been reported. Intra-chain transfers occur when the donor and the acceptor chain are the same [5], inter-chain transfers occur when the donor and acceptor chains are different [6], and intra-chain cyclizations have also been demonstrated [7,8].

From a structural perspective, most GBEs belong to glycoside hydrolase family 13 (GH13); a minority belongs to glycoside hydrolase family 57 (GH57) [9,10]. The GBE from *G. thermoglucosidans* STB02 belongs to GH13, which also includes α-amylases (EC 3.2.1.1), pullulanases (EC 3.2.1.41), isomylases (EC 3.2.1.68) and cyclodextrin glucanotransferases (EC 2.4.1.19) of similar structure. The crystal structures of the GBEs can divided into three domains: an amino-terminal domain, a carboxyl-terminal domain, and a central $(\alpha/\beta)_8$-barrel catalytic domain [10–14]. Many mutagenesis studies have been conducted to determine the roles played by the three domains in GBE activity. These studies have shown that the carboxyl-terminal domain is involved in substrate preference and catalytic capacity, the amino-terminal domain determines the size of the chain transferred [15,16], and the central $(\alpha/\beta)_8$ barrel catalytic domain is responsible for catalysis and product specificity. Based on the length of their amino-terminal domains, GBEs can be divided into two groups. The GBE from *G. thermoglucosidans* STB02 belongs to the short group, possessing only one common module, which was recently classified as a family 48 carbohydrate-binding module (CBM48) [15,17,18].

The central $(\alpha/\beta)_8$ barrel catalytic domain of the GBE from *G. thermoglucosidans* STB02 contains four regions (I–IV) that are highly conserved among GBEs. These regions include seven amino acids that are highly conserved among members of GH13 [14]. These residues are most likely to be involved with catalysis and substrate-binding. While the roles played by most of these residues in GBE activity have been investigated, the relationship between structure of the GBE from *G. thermoglucosidans* STB02 and its catalytic mechanism remains unclear. Ala310 is located in conserved region II,

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just after the conserved catalytic residue (aspartate 309) that corresponding to Asp405 in *E. coli* GBE. This residue is located near the active center cleft in the $(\alpha/\beta)_8$-barrel, suggesting it may play a role in substrate binding. In the GBEs of eukaryotes, this alanine residue is replaced by glycine. In this study, we used site-directed mutagenesis to investigate the influence of Ala310 of the GBE from *G. thermoglucosidans* STB02 on the efficiency of catalysis and the preferred substrate for branching.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli JM109, which was used for recombinant DNA manipulations was purchased from Qiagen (Valencia, CA, USA). *E. coli* BL21(DE3), which was used as GBE(mutants) proteins expression host, was purchased from Qiagen (Valencia, CA, USA). Plasmid pET-20b(+), which was used for site-directed mutagenesis, sequencing, and expression of the GBE proteins, was purchased from Novagen (Madison, WI, USA). The *gbe* gene encoding wild-type GBE was from *G. thermoglucosidans* STB02 (GenBank accession no. KJ660983).

2.2. Construction of site-directed mutants

The desired mutations in GBE were introduced using a one-step PCR method in which plasmid pET-20b(+) containing the *gbe* gene from *G. thermoglucosidans* STB02 was used as the template. Sets of two complementary oligonucleotide primers (Supplemental Table 1) were used for each mutation. All PCR products were cloned separately into a fresh sample of pET-20b(+) using *Nde* I and *Xho* I restriction sites. Use of these sites removes the plasmid sequence encoding the pelB leader peptide, but retains the sequences encoding a C-terminal His tag. The presence of each intended mutation was confirmed by DNA sequencing.

2.3. Production and purification of GBE (mutants) proteins

A single colony of *E. coli* BL21 (DE3) cells harboring a pET-20b(+) variant encoding a wild-type or mutant GBE was used to inoculate 50 mL of Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7) supplemented with ampicillin (50 μg/mL), which was then incubated overnight, with shaking, at 37 °C. A 1 mL portion of this overnight culture was then diluted into 50 mL of Terrific Broth (TB) medium (12 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4, pH 7.5) supplemented with ampicillin (50 μg/mL) and incubated on a rotary shaker (200 rpm) at 37 °C until the absorbance of the culture at 600 nm (A_{600}) reached approximately 0.6. At this point, the temperature was adjusted to 25 °C and expression of the recombinant enzyme was induced by the addition of 0.01 mM isopropyl β-D-thiogalactopyranside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA). After 24 h of induction, the cells were harvested by centrifugation at 10,000 x *g* for 10 min, resuspended in 50 mM sodium phosphate buffer (pH 7.5), and disrupted by sonication. The enzyme was purified using His tag affinity chromatography with a HiTrap Chelating™ column (5 mL, GE Healthcare, Little Chalfont, UK), which was eluted with a linear gradient of 20–500 mM imidazole on an Akta purifier 10 purification system (GE Healthcare). Fractions containing the protein of interest were pooled and stored at −80 °C.

2.4. Enzyme activity assays

Iodine assay–The method was performed as previously described by Palomo, et al. [10,19], with minor modifications. The substrate was 0.25% potato amylopectin type III (Sigma-Aldrich, St. Louis, MO, USA) or 0.1% potato amylose type III (Sigma-Aldrich) dissolved in 50 mM sodium phosphate buffer (pH 7.5). Suitably diluted enzyme (100 μL) was incubated with substrate solution (900 μL) at 50 °C for 15 min. The reaction was terminated by boiling for 15 min. A 5 mL aliquot of iodine reagent was added to the solution (300 μL), and the absorbance at 530 nm (A_{530}) or 660 nm (A_{660}) was measured. One unit of enzyme activity was defined as the amount of branching enzyme that decreased the A_{530} or A_{660} by 1% per minute. The absorbance spectra and the wavelength of maximum absorption were analyzed using a wavelength scan from 500 to 800 nm [5,20].

Branching assay–Takeda [21] described a quantitative assay method, based on direct measurement of the number of α-1,6-glucosidic linkages synthesized, that allows determination of the branching activity by measuring the difference in the number of reducing ends before and after debranching of the product by isoamylase. This method was performed as described by Takata, et al. [2,22] with some modifications. The enzyme (100 μL) was incubated at 50 °C for 30 min with 0.25% potato amylose type III (Sigma-Aldrich). After termination of the reaction by heating at 100 °C for 15 min, 1 M sodium acetate buffer, pH 3.5 (100 μL), and isoamylase (100 μL; 10,000 U/g, Sigma-Aldrich) or water (100 μL) were added. The solution was incubated at 37 °C for 20 h. The debranching reaction was stopped by boiling for 15 min. Reducing ends in the solution were measured using the modified 3,5-dinitrosalicylic acid method described by Luchsinger and Cornesky [23]. One unit of branching activity is defined as 1 μmol of α-1,6-glucosidic linkages synthesized per minute.

2.5. Analysis of chain length distribution

Amylose was incubated with GBE (wild-type or A310G) in 50 mM sodium phosphate buffer (pH 7.5) at 50 °C. After 4, 8, or 12 h of incubation, the reaction was stopped by heating at 100 °C for 15 min. The samples were dried in vacuum freeze-drying equipment for 3 days. The control was amylose treated without GBE. Each sample was suspended in 50 mM sodium acetate buffer, pH 3.5, to a final concentration of 0.5% (w/v), and the mixture was divided into two parts. One part was not debranched. The other was incubated with isoamylase (10,000 U/g) at 37 °C for 24 h. The reaction was stopped by boiling for 15 min. The solutions were diluted 5-fold (to 300 μL) with water and analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using an Dionex ICS-5000 system equipped with a CarboPac PA-200 column (ThermoFisher Scientific, Waltham, MA, USA) [24].The areas of the peaks corresponding to oligomers containing a fraction of the original linear chain length are presented as a percentage of the total peak area, up to a degree of polymerization (DP) of 44.

2.6. Analysis using proton nuclear magnetic resonance spectroscopy

The α-1,4-and α-1,6-glycosidic linkage of starch samples was determined using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy with an Avance III 400 MHz spectrometer (Bruker Co., Germany). The analysis was performed according to the method of Li, et al. [25], with some modifications. The starch samples were treated as described in section 2.5. The control was amylose treated without GBE. Starch samples were dissolved in deuterium oxide containing sodium hydroxide (final starch concentration, 20 mg/mL [w/v]) and then boiled, with stirring, for 30 min. The ^{1}H NMR spectra were obtained at 60 °C. The chemical shift of the anomeric protons of the α-1,4-glycosidic linkages was 5.30 ppm, while the chemical shift of the anomeric protons of the α-1,6-