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Key aromatic residues at subsites +2 and +3 of glycoside hydrolase family 31 α-glucosidase contribute to recognition of long-chain substrates

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ABSTRACT

Glycoside hydrolase family 31 α-glucosidases (31AGs) show various specificities for maltooligosaccharides according to chain length. *Aspergillus niger* α-glucosidase (ANG) is specific for short-chain substrates with the highest k_{cat}/K_m for maltotriose, while sugar beet α-glucosidase (SBG) prefers long-chain substrates and soluble starch. Multiple sequence alignment of 31AGs indicated a high degree of diversity at the long loop (N-loop), which forms one wall of the active pocket. Mutations of Phe236 in the N-loop of SBG (F236A/S) decreased k_{cat}/K_m values for substrates longer than maltose. Providing a phenylalanine residue at a similar position in ANG (T228F) altered the k_{cat}/K_m values for maltooligosaccharides compared with wild-type ANG, i.e., the mutant enzyme showed the highest k_{cat}/K_m value for maltotetraose. Subsite affinity analysis indicated that modification of subsite affinities at +2 and +3 caused alterations of substrate specificity in the mutant enzymes. These results indicated that the aromatic residue in the N-loop contributes to determining the chain-length specificity of 31AGs.

1. Introduction

α-Glucosidase (EC 3.2.1.20) hydrolyzes terminal α-glucosidic linkage at the non-reducing ends of the substrate with release of α-glucose. Almost all organisms have one or more α-glucosidases, but their substrate specificities vary between species. α-Glucosidases can be divided into three types according to differences in substrate specificity: type I enzymes hydrolyze heterogeneous substrates more efficiently than maltose; type II enzymes prefer maltose and maltooligosaccharides [Gn, where n represents the degree of polymerization (DP)] and have low activity toward aryl glucosides; type III enzymes have the same specificity as type II and show high levels of activity toward polysaccharides such as amylose and soluble starch [1].

Among 130 glycoside hydrolase families, α-glucosidases are found in glycoside hydrolase families 4, 13, and 31 [2]. Most glycoside hydrolase family 31 α-glucosidases (31AGs) show either type II or type III substrate specificity. That is, 31AGs can be classified into the short-chain-specific group and long-chain-specific group. Short-chain-specific 31AGs, including enzymes from *Aspergillus niger* (ANG) [3], *Schizosaccharomyces pombe* [4], and barley (*Hordeum vulgare*) [5], exhibit higher k_{cat}/K_m values for G2 and G3 than those for longer Gn. Long-chain-specific 31AGs include enzymes from buckwheat (*Fagopyrum esculentum*) [6] and sugar beet (*Beta vulgaris*) [7], which show 8- and 50-fold higher k_{cat}/K_m for G7 than for G2, respectively. Moreover, the sugar beet α-glucosidase (SBG) shows a 90-fold higher k_{cat}/K_m value for soluble starch than for G2. It is of interest that these enzymes with significant sequence similarity have such divergent substrate specificities.

The present study was performed to identify the structural element that determines the diverse chain-length specificity of 31AGs. A few studies related to the chain-length specificity have been reported. In the C-terminal subunit of human maltase-glucoamylase (CtMGAM), its preference for longer substrate is explained by an extra segment of 21 amino acids, which forms subsites +2 and +3 [8]. Sim et al. suggested that a phenylalanine residue located in the β→α loop 7 of the $(\beta/\alpha)_8$ barrel domain of CtMGAM is associated with the high affinity for long-chain substrates [9]. However, the long-chain-specific enzymes, including SBG

Abbreviations: ANG, *Aspergillus niger* α-glucosidase; *AOX*1, alcohol oxidase 1 gene; BWG, buckwheat α-glucosidase; CtMGAM, C-terminal subunit of human maltase-glucoamylase; DP, degree of polymerization; *GAP*, glyceraldehyde-3-phosphate dehydrogenase gene; Gn, maltooligosaccharides; G2–G7, Gn with DP = 2–7, respectively; G18, maltodextrin with average DP = 18; NtMGAM, N-terminal subunit of human maltase-glucoamylase; rANG, recombinant *A. niger* α-glucosidase; rSBG, recombinant sugar beet α-glucosidase; SBG, sugar beet α-glucosidase; 31AG, glycoside hydrolase family 31 α-glucosidase; 3D, three-dimensional

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and buckwheat α-glucosidase (BWG), must possess different machinery for recognizing long substrates because these enzymes do not possess either the segment of 21 amino acids or the equivalent phenylalanine residue. Several three-dimensional (3D) structures of 31AGs have been solved. The tertiary structure of 31AGs is composed of a catalytic $(\beta/\alpha)_8$ barrel domain and N- and C-terminal β-sandwich domains. An active pocket is present at the rim of the β-barrel as with other typical $(\beta/\alpha)_8$ barrel fold enzymes. The characteristic structure of 31AGs is a long bulging loop (N-loop) from the N-terminal domain that forms one wall of the active pocket. According to the 3D structures of 31AGs, the N-loop is closely related to substrate binding [9]. Nevertheless, their structures and amino-acid sequences are varied (Fig. 1). Thus, this varied segment is likely to be connected to the diverse chain-length specificity. Intriguingly, long-chain-specific enzymes, SBG and BWG, possess an aromatic residue (Phe236 in SBG and Tyr223 in BWG), which often contributes to sugar binding in glycosidases and carbohydrate binding modules [10–12], in the varied segment. Phe236 in SBG and Tyr223 in BWG are, therefore, assumed to contribute to substrate binding and to be connected with long-chain specificity. To address this assumption, we produced two mutant SBG enzymes, in which Phe236 was substituted by Ala or Ser. It was anticipated that these mutations influence the activity of SBG for longer Gn. We also produced a mutant ANG (T228F), which mimics SBG and may exhibit long-chain specificity. The residue, Thr228, is at a similar position with Phe236 in SBG. We investigated the kinetic properties of these mutant enzymes and examined whether the aromatic residue in the N-loop is involved in chain-length specificity in AG31s.

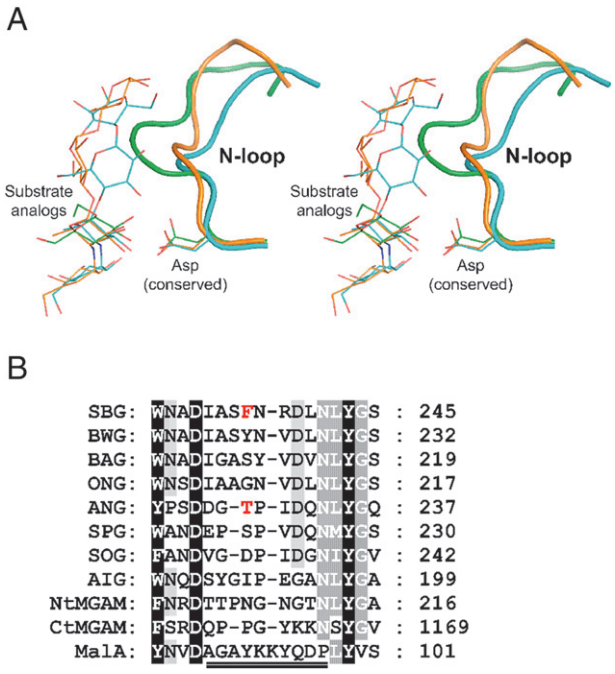

Fig. 1. Structures and sequence alignment of the N-loop of 31AGs. (A) Stereo diagram of superimposition of the N-loop structures with substrate analogs: green, *Sulfolobus solfataricus* α-glucosidase with β-octylglucoside (PDB ID: 2G3N) [26]; cyan, the N-terminal subunit of human maltase-glucoamylase with acarbose (PDB ID: 2QMJ) [9]; orange, the C-terminal subunit of human maltase-glucoamylase with acarbose (PDB ID: 3TOP) [8]. The structural alignment was made by PyMOL ver. 0.99 (http://www.pymol.org). (B) Sequence alignment at the N-loop of 31AGs. Red letters indicate target residues in this study: Phe236 of SBG and Thr228 of ANG. A double underline indicates the divergent segment. SBG, sugar beet α-glucosidase; BWG, buckwheat α-glucosidase (unpublished data); BAG, barley α-glucosidase (Q43763); ONG, rice α-glucosidase (Q653V7); ANG, *Aspergillus niger* α-glucosidase; SPG, *Schizosaccharomyces pombe* α-glucosidase (Q9C0Y4); SOG, *Schwanniomyces occidentalis* α-glucosidase (Q401B1); AIG, *Acremonium implicatum* α-glucosidase (Q75QW0); NtMGAM, N-terminal subunit of human maltase-glucoamylase (O43451); CtMGAM, C-terminal subunit of human maltase-glucoamylase (O43451); MalA, *S. solfataricus* α-glucosidase (D0KQM8). The multiple sequence alignment was produced by ProbCons [27].

2. Materials and methods

2.1. SBG cDNA cloning and construction of expression vector plasmid

The sugar beet line 'NK-185BR2mm-O' used in this study was developed at the National Agricultural and Food Research Organization (NARO), Hokkaido Agricultural Research Center (HARC), Japan. The seeds with pericarp were harvested 1 month after flowering, immediately frozen in liquid nitrogen, and stored at −80 °C before use. Total RNAs were prepared from 10 g of seeds by the sodium dodecyl sulfate (SDS)–phenol method [13], followed by poly(A)$^+$ RNA purification using Oligotex-dT30 Super (Takara Bio, Otsu, Japan). RT-PCR and amplification of SBG cDNA were performed with two gene-specific primers, EXT-I (antisense: 5′-CTAAACACAAATTATGGATTTGGTAATCCG-3′) and EXT-II (sense: 5′-TCCTAAAAGCTCAACATTTATCGAGGGTTT-3′), which were designed according to the reported SBG cDNA sequence (GenBank accession number, BAA20343) [14]. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) with EXT-I. Resultant single-stranded cDNA was used for RT-PCR as the template with the primer sets EXT-I and EXT-II. PCR was performed by using high-fidelity KOD-plus-DNA polymerase (Toyobo, Osaka, Japan). The PCR product was ligated into the *Eco*RV site of pBluescript II SK(+) (Stratagene, La Jolla, CA) using Ligation Kit ver. 2 (Takara Bio). The constructed plasmid was propagated in *Escherichia coli* strain DH5α. The amplified DNA was sequenced using an automated DNA sequencer (Applied Biosystems 310 genetic analyzer and Big Dye Terminator ver. 3.1; Applied Biosystems, Foster City, CA). The nucleotide sequence of the SBG cDNA was deposited in GenBank with the accession number AB698976.

The wild-type recombinant SBG (rSBG) and its two mutant derivatives, F236A and F236S, were produced as fusion proteins, with the N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal and His_6-tag at the C-terminus, in *Pichia pastoris* using pGAPZαA (Invitrogen). A cDNA encoding the predicted mature SBG (Ala29 to Arg913) [15] was amplified by PCR using the sense primer (5′-TTTTCAATTGGCAACAACATCAAAGAATG-3′, the *Mun*I site is underlined) and antisense primer (5′-AAAGCGGCCGCTCTTGTTG CCCCTTCAAAC-3′, the *Not*I site is underlined). The PCR product was digested with *Mun*I and *Not*I and cloned into *Eco*RI-*Not*I sites of pGAPZαA and designated as SBG/pGAPZαA for the production of rSBG.

2.2. ANG cDNA cloning and construction of expression vector plasmid

A. niger AHU7322 was obtained from the culture collection of the Research Faculty of Agriculture, Hokkaido University (Sapporo, Japan). Total RNAs were extracted from *A. niger* AHU7322 cells, cultivated aerobically in YPD medium (1% yeast extract, 2% Bacto Peptone, and 2% glucose) at 26 °C for 2.5 days with rotary shaking, according to the acid guanidinium isothiocyanate–phenol–chloroform method [16]. First-strand cDNA was synthesized by SuperScript II reverse transcriptase with an extension primer (EXT-III: 5′-CCCATGTTGCA TGCTGTAC-3′) from poly(A)$^+$ RNAs. Synthesized single-strand cDNA was amplified using Ex Taq DNA polymerase (Takara Bio) with a pair of synthesized primers: EXT-III (antisense) and EXT-IV (sense: 5′-GTGCCTCGAGCCTCCTTAAG-3′). These two gene-specific primers were designed according to the reported ANG cDNA sequence (accession no. BAA23616) [17]. The PCR product was ligated into the *Eco*RV site of pBluescript II SK(+) using a Ligation Kit ver. 2 and propagated in *E. coli* DH5α, followed by sequencing to determine the entire encoding region. The nucleotide sequence of ANG cDNA was deposited in GenBank with the accession number AB698977. The wild-type recombinant ANG (rANG) and its mutant derivatives