Cloning and Sequencing of a cDNA Encoding \( \alpha \)-Glucosidase from Sugar Beet

Hirokazu Matsui,¹ Shunsuke Iwanami, Hiroyuki Ito, Haruhide Mori, Mamoru Honma, and Seiya Chiba

Faculty of Agriculture, Hokkaido University, Kita-9, Nishi-9, Sapporo 060, Japan

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A cDNA encoding sugar beet \( \alpha \)-glucosidase was cloned from a library constructed from mRNA of suspension-cultured cells. The cDNA, 3056 bp in length, had an open reading frame encoding a polypeptide of 913 amino acid residues with a molecular mass of 102,078 Da, included only one of four regions which were conserved in the \( \alpha \)-amylase family of enzymes. The deduced amino acid sequence from the analysis of the cDNA contained the sequences of the proteolysis peptides and the active site region peptide of sugar beet \( \alpha \)-glucosidase. The primary structure indicated relatively high homology in the range of 28.2 to 54.3% to those for other \( \alpha \)-glucosidases. The highest homology was found in barley \( \alpha \)-glucosidase.

Key words: \( \alpha \)-glucosidase; sugar beet; cDNA cloning; nucleotide sequence

\( \alpha \)-Glucosidase [EC 3.2.1.20, \( \alpha \)-D-glucoside glucohydrolase] is a typical exo-carbohydrase, that catalyzes the splitting of an \( \alpha \)-glucosyl residue from the non-reducing terminals of various substrates to liberate \( \alpha \)-glucose. Many \( \alpha \)-glucosidases have been purified from microorganisms, plants, and mammals, and their substrate specificities differ depending on the source of enzyme. Plant’s and mammal’s \( \alpha \)-glucosidases can attack not only maltoligosaccharides but also \( \alpha \)-glucans, such as soluble starch or glycogen. Sugar beet \( \alpha \)-glucosidase is very characteristic, that is, this enzyme shows the highest hydrolytic activity toward \( \alpha \)-glucan among \( \alpha \)-glucosidases so far reported. We have found that the enzyme hydrolyzes maltose and soluble starch at single active site, and has seven subsites where glucosyl units can be bound.

The difference in substrate specificities of \( \alpha \)-glucosidases is dependent on their fine structure. Recently the primary structures of several \( \alpha \)-glucosidases have been reported. However, most of the information on amino acid sequences is from mammals9-13 and microorganisms. Studies of \( \alpha \)-glucosidases from plant sources have been very limited. Recently the cDNA from barley \( \alpha \)-glucosidase was isolated and showed a high degree of homology with human acid \( \alpha \)-glucosidase.14

In our previous paper,15 we reported the amino acid sequence of the active site in sugar beet \( \alpha \)-glucosidase, based on a technique using the mechanism-based inhibitor conduritol B epoxide (CBE). In this study, we describe the isolation of an \( \alpha \)-glucosidase cDNA clone from sugar beet suspension-cultured cells, and a comparison of the deduced amino acid sequence with those of other \( \alpha \)-glucosidases and \( \alpha \)-amylases.

Materials and Methods

Plant and cell culture conditions. Sugar beet seed (Beta vulgaris L. cv. NK-152) was kindly supplied from the Hokkaido National Agricultural Experiment Station. Sugar beet callus was obtained from a sterilized leaf of a plant grown for 4 weeks on Murashige-Skoog agar (0.7%) medium containing 0.25 mg/ml of 6-benzylaminopurine (Wako Pure Chemicals Ind., Osaka, Japan) at 25°C in the dark. Suspension-cultured cells were derived from this callus in 200-ml conical flask containing 30 ml of MS medium on a rotary shaker at 100 strokes/min at 25°C under 16-h-night conditions and the cells were subcultured at 2-week intervals.

Amino acid sequencing. \( \alpha \)-Glucosidase was purified to homogeneity, as evidenced by ultracentrifugation and SDS-PAGE, from sugar beet seeds by the method described previously. The enzyme (8 nmol) was digested at 37°C for 24 h with lysyl endopeptidase (80 pmol; Wako Pure Chemicals Ind.) in 0.05 M Tris-HCl buffer containing 6 M urea (pH 8.9). The resultant peptides were separated by HPLC using a Finepak C8-P column (Asahi Chemical Ind., Tokyo, Japan) and sequenced on an Applied Biosystem 477A protein sequencer (Applied Biosystems Inc., CA, U.S.A.).

Construction of cDNA library. Total RNA was isolated from 10 g of 9-day-old sugar beet cell suspensions, harvested by filtering, using the SDS/phenol method.16 Double-stranded cDNA was synthesized from poly(A)+ RNA purified by OligoTex-dT30 (Takara Shuzo Co., Ltd., Kyoto, Japan) using a cDNA synthesis kit (Amersham Japan, Tokyo, Japan). Blunt-ended double-stranded cDNA was EcoRI-methylated before ligation with EcoRI linkers (5'-pCCGGAAATTCGCG-3'; Takara Shuzo). Double-stranded cDNA was size-fractionated by NaCl gradient centrifugation. Fractions containing cDNA in excess of about 500 bp were used to construct a cDNA library in zgt10 phase vector (Stratagene Cloning Systems, CA, U.S.A.). The recombinant DNAs were packaged into bacteriophage particles using packaging extract (GIGA pack GoldIII, Stratagene) and grown on E. coli NM514.

Polymerase chain reaction (PCR). Two DNA primers consisting of 28 and 29 bases, designated P2 and AS primers, were synthesized on the basis of the partial amino acid sequences of sugar beet \( \alpha \)-glucosidase. The sequences of the P2 sense primer and the AS antisense primer were 5'-ATGTATTGCGCGTGTTCGTTGCA-3' and 5'-GCTCCATTGCTATCTCCTCC-3', respectively (underlined amino acid sequences marked P-2 for P2 sense primer and AS for AS antisense primer in Fig. 3). The PCR amplification reaction mixture consisted of 1 \( \mu \)g cDNA as a template, 50 pmol of the P2 primer, 50 pmol of the AS primer, 1 mM each dNTP, 1.5 mM MgCl2, 50 mM KCl, and 2.5 U Taq polymerase (Life Technologies, Inc., MD, U.S.A.) in 10 mM Tris·HCl (pH 8.3). The DNA-denaturing step was set at 94°C for 1 min, the primer-annealing step at 50°C for 2 min, and the primer-extension step at 72°C for 3 min. The reaction was allowed to run for 30 cycles.

Isolation of cDNA clone. The cDNA library was screened using a PCR amplified DNA fragment as a probe. The probe was labeled with 1.85 MBq of [\( ^{32}P \)]dCTP using a random primer labeling kit (Takara Shuzo). About 1 x 10⁵ plaques from the library were transferred onto duplicate nylon membranes (Hybond N⁺, Amersham). The membranes were treated with the \( ^{32}P \)-labeled probe in hybridization buffer at 50°C and then rinsed with 0.1 x SSC and 0.5% SDS at 50°C. The membranes were then

¹ To whom correspondence should be addressed.
exposed to X-omat AR film (Kodak, CT, U.S.A.) for autoradiography with an enhancer screen (Lighting Plus: DuPont, DE, U.S.A.).

**Nucleotide sequencing.** Nucleotides were sequenced by the dideoxy chain-terminal method, but α-deaza-dGTP was used instead of dGTP. Various fragments of the putative genes for α-glucosidase were prepared from the subcloned plasmids and cloned into appropriate sites of the vectors pBS SKI1 (+) or KSII (−) to provide templates. DNA sequences were analyzed by an automated sequencer (Applied Biosystems, Model 373A) using the cycle sequencing protocol and the fluorescence-labeled primer by the procedure provided by the manufacturer (Applied Biosystems). The sequences were deduced from the results from both strands. The DNA sequences and the predicted amino acid sequences were analyzed using the Gene/Protein sequence database of a DNANIS computer (Hitachi). The complete nucleotide sequences of the cDNAs for the sugar beet α-glucosidase have been submitted to the DDBJ, EMBL, and GenBank international nucleotide sequence databases under the accession number D89615.

**Enzyme and protein assays.** α-Glucosidase activity was assayed as the amount of glucose liberated from 0.2% maltose at 37°C in 0.04 M sodium acetate buffer (pH 4.5), using a glucose AR-II Test (Wako Pure Chemical) as described previously. The enzyme concentration and amount were estimated using 13.6 of A_{410\mu m} at 280 nm and 91 kDa as the molecular mass.

**Results and Discussion**

**Amino acid sequences and preparation of probe**

Before isolation of cDNA clones, some partial amino acid sequences were analyzed. Digestion of α-glucosidase purified from sugar beets with lysyl endopeptidase gave more than 30 peptide fragments on a reverse-phase HPLC. The sequences of five peptides (P-1, 28 amino acids; P-2, 34 aa; P-3, 22 aa; P-4, 35 aa; P-5, 31 aa) were analyzed. As reported previously, the peptide containing the active site was identified by chemical modification using labeled CBE, a mechanism-based inhibitor, and sequenced (30 aa).

These data on six partial sequences, the amino acids of which are equivalent to about 20% of the whole polypeptide, serve not only to develop strategies for molecular cloning, but also to confirm the amino acid sequence deduced from cDNA nucleotide.

The inner sequence of P-2 peptide, MPYWAFGGFHQ, and that of the active site peptide, DGIWIDMNEA, were chosen to synthesize PCR primers. The 28-mer oligonucleotides, 5′-ATGCCITATTGGGCI1TTTGCATC-3′ (sense primer), and the 29-mer oligonucleotides, 5′-GCTTCACTATCATCIAITCCGTC-3′ (antisense primer) were used, and a 470 bp fragment was amplified by PCR. The product was named P2AS probe.

**Isolation and characterization of cDNA clones for α-glucosidase**

Since flowering of sugar beets is not uniform in time, the growth of seeds is also extremely variable. This means that it is almost impossible to harvest the seeds at a definite growth rate at regular days after flowering. In this study, therefore, mRNA for preparation of cDNA library was isolated from suspension-cultured cells. Figure 1 shows the courses of enzyme activity and proteins after subculture. Both were assayed on culture medium and crude extract prepared from cells by homogenation and centrifugation. α-Glucosidase activity in the medium reached the maximum at approximately 14 days, and in cytosol, at 12 days. A cDNA library was constructed using mRNA isolated from 9-day cells after subculture.

**Fig. 1. Courses of α-Glucosidase Activity and Protein of Suspension Cultured Cells.** ○, culture medium; △, cytosol.

The cDNA library was screened twice by plaque hybridization using the 32P-labeled P2AS probe, and 6 positive clones from 1 x 10^5 plaques were obtained. Each clone was confirmed by PCR using the forward and reverse primers for αglt10, resulting in fragments of 2.3-kb to 3-kb. The longest clone was named 2SBAG. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 2. The longest open reading frame from the start codon ATG at position 127 to a stop codon TAG at position 2866 encodes a polypeptide of 913 amino acid residues with a calculated molecular mass of 102,078 Da. A putative polyadenylation signal, AATAAA, at position 2918 to 2923 was found in 139 bp upstream from the 3′-end of the cDNA. Amino acid sequences of five peptides (P-1 to P-5) and the active site-peptide were found on the deduced polypeptide. The molecular mass of the mature enzyme was estimated to be 91,000 by SDS-polyacrylamide gel electrophoresis, and so about one hundred of the deduced amino acid sequence would be removed to form the mature protein. However, the N-terminal amino acid of mature sugar beet α-glucosidase still remains unidentified, because it is modified.

**Amino acid sequence homology**

The deduced amino acid sequence of sugar beet α-glucosidase was compared with those of several α-glucosidases. α-Glucosidases of plant and mammals showed homologies of 54.3% (barley), 31.5% (human α), 33.3% (human intestinal isomaltase), 31.4% (rabbit intestinal isomaltase), and 28.2% (rabbit intestinal sucrase). Microbial α-glucosidases showed homologies of 29.4% (Asp. niger) and 29.2% (Schwanniomyces occidentalis).

Figure 3 shows the alignment of the amino acid sequence of sugar beet α-glucosidase with those of four other α-glucosidases. Tibbot and Skadsen isolated a cDNA clone as one of mRNAs increased by gibberellin A3. They had one open reading frame encoding a polypeptide of 877 amino acids, the 680 amino acid region without N- and C-terminal regions of which was 43% identical to human lysosomal acid α-glucosidase. Therefore, they concluded that it encodes barley α-glucosidase. Our results are consistent with their conclusion, since 476 amino acids of the barley putative α-glucosidase are identical in the sequence to those of sugar beet enzyme. Notable is the fact
Fig. 2. Nucleotide and Deduced Amino Acid Sequences of cDNA Encoding Sugar Beet α-Glucosidase.

Amino acid sequences of proteolytic peptides (P-1 to P-5) and active site region (AS) are underlined. A putative polyadenylation signal and a stop codon are indicated by double-underline and an asterisk, respectively.
Fig. 3. Alignment of Amino Acid Sequence of Sugar Beet α-Glucosidase with Those of α-Glucosidases From Other Origins. SB, BA, HG, R1 and AN indicate sugar beet, barley, human lysosomal (acid), rabbit intestine (isosialamidase), and *Aspergillus niger* α-glucosidases, respectively. The identical amino acids are represented by reversal letters, and the catalytic residue (Asp) is marked by an asterisk. The reversal numbers mean total amino acids of each α-glucosidase. The upper number is through one to five sequences (Tn). The hyphen on *Aspergillus* α-glucosidase (Tns. 327 and 328) is considered the dipeptide removed after one polypeptide is synthesized to form P1 and P2 subunits.

<table>
<thead>
<tr>
<th>Table</th>
<th>Comparison of Four Conserved Regions of α-Amylases and α-Glucosidases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Consensus regions</td>
</tr>
<tr>
<td></td>
<td>Region I</td>
</tr>
<tr>
<td>x-Amylase</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td></td>
</tr>
<tr>
<td>Human salivary</td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td></td>
</tr>
<tr>
<td>z-Glucosidase</td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>117 DVVANH</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>99 DAVINH</td>
</tr>
<tr>
<td><em>Schwanniomyces occidentalis</em></td>
<td>88 DIVINH</td>
</tr>
<tr>
<td><em>Mucor javanicus</em></td>
<td>106 DLVVNH</td>
</tr>
<tr>
<td>Human acid</td>
<td></td>
</tr>
<tr>
<td>Rabbit intestine (isosialamidase)</td>
<td></td>
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<tr>
<td>Rabbit intestine (sucrase)</td>
<td></td>
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<td>Barley</td>
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<tr>
<td>Sugar beet</td>
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</tr>
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Three α-amylases, from *Aspergillus oryzae*, human salivary, and barley, and nine α-glucosidases, from *Saccharomyces cerevisiae*, *Aspergillus niger*, *Schwanniomyces occidentalis*, *Mucor javanicus*, human acid, rabbit intestine (isosialamidase, sucrase), barley, and sugar beet, were listed.
that both sequences in the middle regions show a high degree of homology. These plant \( \alpha \)-glucosidases have distinct regions, i.e., number (TNo.) 986 to 1046 (803rd to 860th for sugar beet, 769th to 827th for barley). The regions lacking eight to eleven amino acids (TNo. 121–127, TNo. 178–185, TNo. 217–226, TNo. 256–265, TNo. 329–333) were also specific to both plant \( \alpha \)-glucosidases. In contrast, the regions of TNo. 578–642 and TNo. 896–911 were distinct from \textit{Asp. niger} (474th to 538th) and human (779th to 794th) enzymes. The former, adding region in \textit{Asp. niger}, is also found in \( \alpha \)-glucosidase originated from \textit{Schwanniomyces occidentalis} (493rd to 556th). The significance of microbial distinct regions near the active site is unclear.

Most starch hydrolases and related enzymes are known to belong to the \( \alpha \)-amylase family, which has four common conserved regions,\(^{21-22}\) Sugar beet \( \alpha \)-glucosidase was compared to some \( \alpha \)-amylases as well as other \( \alpha \)-glucosidases. As seen in the Table, this plant \( \alpha \)-glucosidase has only one region (Region II), similar to barley and mammal enzymes. Among microbial \( \alpha \)-glucosidases, the enzyme from \textit{Saccharomyces carlsbergensis}\(^{9}\) has four regions, and \textit{Asp. niger} \( \alpha \)-glucosidase,\(^{11}\) three (Regions II, III, and IV). In contrast, \textit{Schwanniomyces occidentalis}\(^{20}\) and \textit{Mucor javanicus} \( \alpha \)-glucosidases have only one (Region II). Thus, Region II is a common conserved among all of the \( \alpha \)-glucosidases, regardless of origin. In the other regions (I, III, and IV), conserved sequences were not observed. Especially sequences which correspond to region III of \textit{A. niger} (442–482) were lacking in plant and mammal \( \alpha \)-glucosidases (Fig. 3). To study the relationship between the structure of the enzyme and substrate specificity, this region, which contains one of the catalytic active residues (Asp), should be targeted in the future.

References


