

PCR Cloning and Sequencing of the β -Amylase cDNA from Barley

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Received for publication, June 24, 1993

Polymerase chain reaction (PCR) amplification of mRNA from developing barley (cultivar Haruna two-rows) endosperm was used to clone and sequence full-length cDNA encoding β -amylase. The β -amylase cDNA was 1,775 bp in length. The β -amylase was deduced to be composed of 535 amino acid residues and its molecular weight was calculated to be 59,610. Kreis *et al.* reported that the β -amylase cDNA from barley (cultivar Hiproly) was 1,754 bp in length and coded for a polypeptide of 535 amino acids [*Eur. J. Biochem.* (1987) 169, 517–525]. A comparison of the β -amylase sequences from Haruna two-rows and Hiproly barleys revealed nine differences in the nucleotide sequence which resulted in three changes in the amino acid residues and 21 additional nucleotides at its 3'-end in the cultivar Haruna two-rows. The three changes were as follows: Ala-233, Ser-347, Met-527 (Haruna two-rows) and Val-233, Met-347, Ile-527 (Hiproly). Lundgard and Svensson pointed out that 23 amino acid residues of the peptide fragment derived from the COOH-terminal region of barley (cultivar Gula) β -amylase were in agreement with the deduced amino acid sequence reported by Kreis *et al.*, with the exception of a single position (Met-527 compared to Ile) [*Carlsberg Res. Commun.* (1986) 51, 487–491]. Our findings described above showed Met-527 is reasonable. In the cases of β -amylases from soybean and sweet potato, the positions that corresponded to those at 233 and 347 in the amino acid sequence of β -amylase from barley were Ala and Ser, respectively. Therefore, Ala-233 and Ser-347 in the amino acid sequence of barley β -amylase were thought to be reasonable. Sequence homology of barley β -amylase with the enzymes from soybean and sweet potato amounted to 66.7 and 59.2%, respectively.

Key words: β -amylase, barley, PCR cloning.

β -Amylase (α -1,4-glucan maltohydrolase; EC 3.2.1.2) catalyzes the liberation of β -maltose from the nonreducing ends of starch and related 1,4- α -glucans (1). The enzyme has been purified from various higher plants, most notably from barley, soybeans, and sweet potato and microorganisms (2). The β -amylase is a major protein in the starchy endosperm of ungerminated barley seeds (3). The primary structure of barley (cultivar Hiproly) β -amylase has been deduced from the nucleotide (nt) sequence of a cloned full-length cDNA and it was shown that the mRNA was 1,754 nt long [excluding the poly(A) tail] and coded for a polypeptide of 535 amino acids (aa) with a relative molecular mass of 59,663 (4). It has already been reported that 23 aa residues of the peptide fragment derived from the COOH-terminal region of barley (cultivar Gula) β -amylase were in agreement with the deduced aa sequence from the β -amylase cDNA (4), with the exception of a single position (Met-527 compared to Ile) (5).

This study was aimed to clone a β -amylase cDNA from the barley endosperm and ascertain the primary structure of the barley β -amylase.

MATERIALS AND METHODS

Materials—A cDNA synthesis kit, a cDNA cloning system- λ gt11, and nitrocellulose filters (Hybond-C) for immunoscreening were obtained from Amersham and were used according to the respective protocol. Restriction enzymes, trypsin (*N*-tosyl-L-phenylalanyl chloromethyl ketone treated), and an RNA-PCR kit were purchased from Takara Shuzo and were used as recommended by the supplier. All other chemicals were commercial products of analytical grade.

Assay of β -Amylase Activity and Protein Content—The β -amylase activity was measured at 37°C using a 2,4-dichlorophenyl β -maltopentaoside kit (DIACOLOR AMY) obtained from Toyobo and the β -amylase activity was calculated according to the instruction manual. One unit of the β -amylase activity was defined as the amount of the enzyme required to provide 1 μ mol of 2,4-dichlorophenol per min. Protein content was measured by the method of Lowry *et al.* with bovine serum albumin as the standard (6).

Purification of β -Amylase for Use as Antigens—Barley (*Hordeum vulgare* L. cv. Haruna) seeds were from the 1990 crop grown at Kasaki in Gunma prefecture. Barley flour (36 g) was extracted three times with 120 ml of 50 mM acetate buffer (pH 5.5) at 4°C for 1 h with shaking. The extract was centrifuged at $4,770 \times g$ for 10 min after each step and the

The GenBank-EMBL-DBJ accession number for *Hordeum vulgare* mRNA for β -amylase is D21349.

Abbreviations: aa, amino acid(s); nt, nucleotide(s); PCR, polymerase chain reaction.

resultant supernatant was used as the starting material for the β -amylase purification. To the starting material (317 ml, 3,424 units), solid $(\text{NH}_4)_2\text{SO}_4$ was added until 30% saturation was reached. The suspension was left for 1 h and centrifuged at $14,000 \times g$ to remove the precipitated proteins. The supernatant (335 ml) was put on a Butyl-Toyopearl 650S column (2.5×18.5 cm) preequilibrated with 50 mM acetate buffer (pH 5.5) containing 30% saturated $(\text{NH}_4)_2\text{SO}_4$. Elution was successively done with 600 ml of 50 mM acetate buffer (pH 5.5) containing 30% saturated $(\text{NH}_4)_2\text{SO}_4$, 500 ml of 50 mM acetate buffer (pH 5.5) containing 10% saturated $(\text{NH}_4)_2\text{SO}_4$, and 500 ml of 50 mM acetate buffer (pH 5.5); the flow rate was maintained at 10 ml (volume of one fraction)/1.92 min. β -Amylase was eluted with 50 mM acetate buffer (pH 5.5). Fractions containing β -amylase were combined (287 ml) and dialyzed against 15 mM Tris-HCl buffer (pH 8.0). The dialyzate (310 ml) was placed on a DEAE-Toyopearl 650S column (2.5×18.5 cm) previously equilibrated with 15 mM Tris-HCl buffer (pH 8.0). Elution was successively done with 500 ml of 15 mM Tris-HCl buffer (pH 8.0), 400 ml of 15 mM Tris-HCl buffer (pH 8.0) containing 0.05 M NaCl [P-1], 400 ml of 15 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl [P-2], and 200 ml of 15 mM Tris-HCl buffer (pH 8.0) containing 1.0 M NaCl [P-3]; the flow rate was maintained at 10 ml/1.92 min. β -Amylase was detected in each of the three fractions (P-1-3). When fractions containing β -amylase activity were pooled and further purified, only the P-2 sample gave a single band of protein on SDS-PAGE. To the P-2 sample, solid $(\text{NH}_4)_2\text{SO}_4$ was added until 40% saturation was reached. After centrifugation, the precipitated proteins were dissolved in and dialyzed against 50 mM acetate buffer (pH 5.5). The dialyzate (1.8 ml) was loaded onto a Toyopearl HW-50S column (1.5×48.5 cm) preequilibrated with 50 mM acetate buffer (pH 5.5) and eluted with the same buffer; the

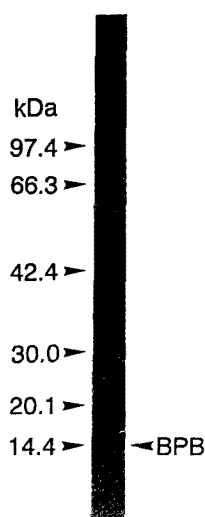


Fig. 1. **SDS-PAGE.** SDS-PAGE was done as described elsewhere (9) and 0.8 μg of the purified β -amylase was used. The protein was stained with Coomassie Brilliant Blue R-250. BPB, Bromophenol Blue. Standard proteins are as follows; phosphorylase *b* (mol. wt. 97,400), bovine serum albumin (mol. wt. 66,267), aldolase (mol. wt. 42,400), bovine carbonic anhydrase (mol. wt. 30,000), soybean trypsin inhibitor (mol. wt. 20,100), and lysozyme (mol. wt. 14,400).

flow rate was maintained at 2 ml/2.52 min. Fractions containing β -amylase were pooled and used as antigens.

Preparation of Polyclonal Antibodies against Barley β -Amylase—For immunization, 10 μg of the antigens in PBS buffer [0.01 M sodium phosphate (pH 7.2), 0.15 M NaCl] was emulsified with an equal volume of complete Freund's adjuvant. A mouse (BALB/c) was given an intraperitoneal injection of the antigens (80 μl). Starting 2 weeks later, the antigens (10 μg) were injected five more times every 1 or 2 weeks. One week after the last booster, the mouse was bled.

Peptide Sequence Analysis of β -Amylase—The N-terminal aa of the purified β -amylase was blocked to Edman degradation. Therefore, peptide sequence analysis was performed by two methods. In one method, about 200 μg of purified β -amylase was dissolved in 100 μl of 70% formic acid containing 1% CNBr. After overnight incubation in the dark, the mixture was dried under N_2 gas, dissolved in 100 μl of 70% formic acid, evaporated to dryness *in vacuo*, and

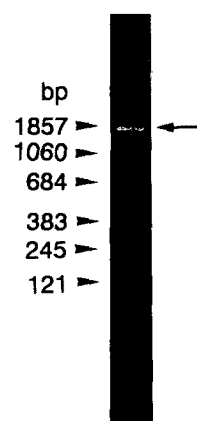


Fig. 2. **Analysis of cDNA product from RNA-PCR.** Numerals on the left margin refer to *Bam*HI+*Mva*I-digested pBR322 DNA markers. An arrow indicates the 1.8-kb DNA band.

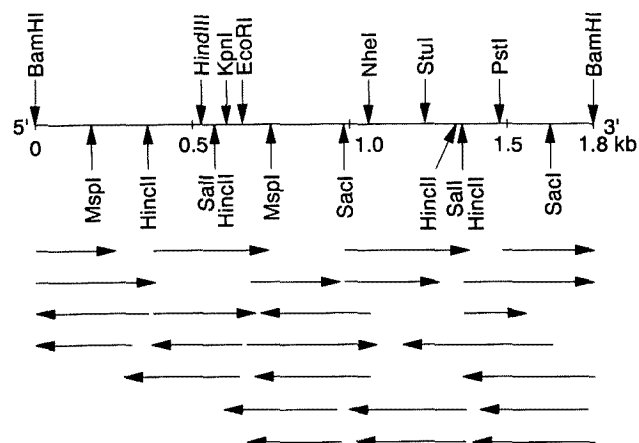


Fig. 3. **Restriction map and DNA sequencing strategy for the cloned 1.8-kb fragment.** The 1.8-kb *Bam*HI fragment was digested with various restriction enzymes, and DNA fragments were subcloned into pUC118 and pUC119 for DNA sequencing. The direction and extent of sequencing determination are shown by horizontal arrows for the subclones used.

Preparation and Purification of mRNA—Barley was grown in the fields of this laboratory. Developing endosperms (1 g) were harvested about 23 d after anthesis and total RNA was extracted using a total RNA extraction kit (Amersham). The final preparation contained 912 μg of total RNA. Poly(A)⁺ RNA (13.1 μg) was enriched by

Construction of the Barley cDNA Library and Cloning of β -Amylase cDNA—The cDNA library was constructed in *Escherichia coli* Y1090 [$\Delta lacU169$ *proA*⁺ Δlon *araD139* *strA* *SupF* (*trpC22::Tn10*) (pMC9)] using 50 ng of cDNA and 1 μ g of λ gt11 *Eco*RI arms. The β -amylase cDNA was immunoscreened with polyclonal antibodies against the purified β -amylase, affinity-purified goat anti-mouse IgG-alkaline phosphatase conjugate, and a picoBlue Immunodetection kit (Funakoshi Pharmaceutical).

PCR Cloning of β -Amylase cDNA and DNA Sequencing—PCR amplification of mRNA (670 ng) from developing endosperms was used to clone full-length cDNA encoding β -amylase. The primers used in the RNA-PCR were 5'-AAGGATCCGATATCCAACAAACCATTTG and 5'-AAGGATCCCTTTTCAGAACACACAAG. The amplified fragment was digested with *Bam*HI and subcloned into pUC118. DNA sequencing was performed using the dide-

Fig. 4. Nucleotide sequence of the β -amylase cDNA and deduced aa sequence. The positions of the trypsin and the CNBr peptides are underlined and numbered. Nine different nts of the β -amylase cDNA from cultivar Hiproly, which resulted in three changes in the aa residues, are in parentheses. Two asterisks mark the stop codons. The 21 additional nts at the 3'-end of the β -amylase cDNA from cultivar Haruna two-rows are double underlined. The two consensus sequences of polyadenylation (AATAAA) are boxed.

sequence has been determined. However, a few observations are in agreement with our assignment of the beginning of the coding region. The presumed translation initiation codon at positions 56-58 is the first ATG in the sequence and is preceded by a nonsense codon at positions 41-43. The context for initiation is CCACAATGG, with an A in position -3 and a G in position 4; this fits very well with the consensus sequence for known eucaryotic initiation codons (12).

Kreis *et al.* showed that one of the CNBr peptide sequences was identical to the deduced N-terminal aa sequence at positions 2 to 12 and the initial Met was present in the mature β -amylase (4). Therefore, it seemed that the β -amylase from barley did not have a signal peptide and was synthesized as a mature protein from the beginning.

Kreis *et al.* also reported that the β -amylase cDNA from barley (cultivar Hiproly) was 1,754 bp in length and coded for a polypeptide of 535 aa (4). A comparison of the β -amylase sequences from Haruna two-rows and Hiproly barleys revealed nine differences in the nt sequence which resulted in three changes in the aa residues and 21 additional nts at its 3'-end in cultivar Haruna two-rows as shown in Fig. 4. Five of nine nt alterations were conserved aa changes and one of those was in the 3' untranslated region. The three aa changes were as follows: Ala-233, Ser-347, Met-527 (Haruna two-rows) and Val-233, Met-347, Ile-527 (Hiproly). Lundgard and Svensson pointed out that 23 aa residues of the peptide fragment derived from the COOH-terminal region of barley (cultivar Gula) β -amylase were in agreement with the deduced aa sequence reported by Kreis *et al.*, with the exception of a single position (Met-527 compared to Ile) (5). Our previously described findings showed Met-527 is reasonable.

The molecular weight of β -amylase from cultivar Haruna two-rows was calculated to be 59,610. Kreis *et al.* reported that the molecular weight of β -amylase from cultivar Hiproly was calculated to be 59,663. When we calculated it based on the sequence they reported, it was 59,646. Therefore, the difference in the molecular weight of β -amylase between the two cultivars, derived from three changed amino acid residues, was 36.

Sequence homology of barley β -amylase from two cultivars at nt and aa levels were 99.5 and 99.4%. More than two thousand of cultivars have been kept in our company and each brewing barley has its own brewing characteristic. Therefore, it was thought that the nt changes of β -amylase might naturally happen through breeding and selecting steps and the differences in the nt sequence which resulted in changes in the aa residues could be permitted to some

extent except for the highly conservative regions surrounding a catalytic center.

Figure 5 shows the comparison of aa sequences of β -amylases from barley (cultivar Haruna two-rows), soybean (13), and sweet potato (14). In the cases of β -amylases from soybean and sweet potato, the positions that corresponded to those at 233 and 347 in the aa sequence of β -amylase from barley were Ala and Ser, respectively. Therefore, Ala-233 and Ser-347 in the aa sequence of barley β -amylase were thought to be reasonable. Sequence homology of barley β -amylase with the enzymes from soybean and sweet potato amounted to 66.7 and 59.2%, respectively.

Nitta *et al.* concluded that the carboxylate of Glu-186 was a functional group at the catalytic site of soybean β -amylase with 2,3-epoxypropyl α -D-glucopyranoside and its equivalent Glu in other β -amylases was a common essential group for the catalytic action of β -amylase (15). Therefore, Glu-184 was thought to be a functional group at the catalytic site of barley β -amylase.

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