Characterization of Chimeric Enzymes Constructed between Two Distinct α-Amylase cDNAs from Cultured Rice Cells

Rei Abe,1 Kensuke Yoshida,1 Masanobu Aoyagi,1 Shin Kasahara,1 Eiji Ichishima,2 and Tasuku Nakajima1†

1Laboratory of Enzymology, Division of Life Science, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai-shi 981-8555, Japan
2Department of Biotechnology, Faculty of engineering, Soka University, 1-26 Tankicho, Hachioji, Tokyo 192-8577, Japan

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Cultured cells of rice (Oryza sativa cv Sasanshikiki) produce two α-amylase isoymes, AMY-I and AMY-III. Using a bacterial expression system, eight chimeric genes constructed with various combination of AMY-I and AMY-III cDNA fragments were expressed, and each recombinant chimeric protein was characterized. Four of the eight recombinant enzymes having region c (one of the four regions having unconserved base sequences between AMY-I and AMY-III cDNAs) of AMY-I showed the same enzyme characteristics as that of native AMY-I, which had high temperature optimum at 35°C. The other four chimeric proteins carrying region c of AMY-III showed the AMY-III type characteristics, which are a low temperature optimum at 25°C and susceptibility to a higher maltooligosaccharide (G17) substrate. The unconserved region c is involved in the decision of the characteristic of AMY-I or AMY-III.

Key words: α-amylase; gene expression; chimeric protein; suspension-cultured rice cells

Alpha-amylase (1,4-gulcan glucanohydrolase; EC 3.2.1.1) catalyzes the hydrolysis of internal α-glucosidic linkages in starch and other related oligo and polysaccharides. These enzymes are widespread among plants, mammals, insects, fungi and bacteria and a number of their cDNAs have been cloned. In higher plants, α-amylases important in germinating seeds5 and usually are present as multiple isoforms. Mitsui et al. have characterized ten α-amylase isoforms from suspension-cultured rice cell, and the isozymes were separated into two major classes and moreover these classes were separated into four subgroups.23

We had found two distinct α-amylase isoforms AMY-I and AMY-III in suspension cultured rice cells. AMY-I has shown unique enzymatic properties when compared to the other usual α-amylases including AMY-I. It had a low temperature optimum at 25°C and an abnormal curve for Arrhenius plots indicating great conformational change of the enzyme structure occurring around 25°C,5 and the AMY-III also showed great reduction of its activity when raw corn starch or a higher maltooligosaccharide of DP 17 (G17) was added to the assay mixture.41

AMY-III has been shown to be identical to the isoform E obtained from cultured rice cells reported by Mitsui et al., with respect to the low temperature optimum. On the other hand, AMY-I was found to be identical to the isoform G and H.36

A number of recombinant rice α-amylase isoforms5 and barley α-amylase isoforms6 have been produced and characterized using yeast expression systems. Two rice α-amylase isoforms, Amy1A and Amy3D, and two chimeric enzymes of those two isoforms were expressed in yeast and these recombinant enzymes were studied for kinetics and substrate specificities.7 The recombinant Amy1A and Amy3D were significantly different in many kinetic parameters and optimal pH, and the chimeric enzyme had both characteristic properties of Amy1A and Amy3D.8 The Amy1A corresponds to our AMY-I, while the Amy3D is different from AMY-III.

In this paper, to understand which part of the protein module is responsible for the unique properties of AMY-III, we constructed various combinations of AMY-I and AMY-III cDNAs, and characterized these chimeric gene products expressed in Escherichia coli.

Materials and Methods

Materials. A suspension culture of rice (Oryza sativa cv Sasanshikiki) cells was initiated from callus tissue retrieved from whole seeds. The cell suspension was grown on 150 ml of R2 medium with shaking at 26°C. Harvested cells were stored at –70°C. AMY-I and AMY-III were purified from the cells as described previously.6 The microbial strains used were Escherichia coli XLI-Blue and BL21 (DE3) pT-groE. DNA restriction and modification enzymes were obtained from Takara Shuzo Co. (Kyoto). The polyclonal antibodies obtained from rabbits immunized by injection (three times) of 1 mg of purified AMY-I or AMY-III with complete Freund’s adjuvant.

Maltooligosaccharides substrates (G-7 and G-17) were from Hayashibara Biochemicals (Okayama) and soluble starch and α- or β-cyclodextrin were from Nakalai

1 To whom correspondence should be addressed. Fax: +81-22-717-8778; E-mail: tasuku@biochem.tohoku.ac.jp

Abbreviations: AMY-I and AMY-III, α-amylase (EC 3.2.1.1) isoyme I and III from cultured rice cells; AMY, gene encoding AMY; G17, malto-heptadecasaccharide; IPTG, isopropl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis.
Tesque Inc. (Kyoto). The α- or β-cyclodextrin-Sepharose conjugates were synthesized.

Isolation of RNA. Three g of 10-day-old cultured-rice cells were disrupted under liquid nitrogen and total RNA was isolated from the disrupted cells by the method of Chirgwin et al. Poly(A)⁺ RNA was purified by using oligo(dT)-Latex (Oligotex-dT30 obtained from Takara Shuzo Co.).

cDNA Cloning. cDNA library was prepared using Mo-MLV reverse transcriptase (Superscript: GIBCO BRL), which was followed by synthesis of the second strand using DNA polymerase I. EcoR I-Nor I adaptors (Pharmacia) were added to the cDNA, ligated to the EcoR I cleavage site of Bluescript II KS, and the resulting plasmids were used to transfect E. coli DH5α competent cells. The library was screened with a probe synthesized by RT-PCR method using the internal amino acid sequence of AMY-III as a primer (5’-ATGGGGTGAATGCAGCAAGGC-3’). Double stranded plasmid DNA carrying the deleted insert were prepared and bidirectionally sequenced using a Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems, Inc.). The sequences were analyzed using a DNA sequencer (model 373A, Applied Biosystems, Inc.).

Westernblot analysis. Western blot analyses were done with the antibody against AMY-I, because it cross-reacted with AMY-III and was useful to detect various chimeric α-amylases between AMY-I and AMY-III. On the other hand, anti-AMY-III serum was specific to AMY-III and was not useful to detect the chimeric proteins.

Construction of expression plasmids encoding AMY-I, AMY-III and chimeric genes between AMY-I and AMY-III. For expression in a bacterium, an E. coli expression vector, pET-12a (obtained from Novagen) was used as shown in Fig. 2. The mutagenic oligonucleotides (I-Nde I; 5’-CTCGGGTATCATGCTAGGTCCTC-3’, III-Nde I; 5’-CTTAGACATATGCAAGTGGCTC-3’) were synthesized using an oligo1000 DNA syntheser (Beckman) and were purified using oligonucleotide purification cartridges. For site-directed mutagenesis, a cDNA fragment encoding AMY-I and AMY-III were inserted into M13mp18 RF DNA (M13AMY-I or M13AMY-III). These plasmids were used to construct chimeric amylase genes by the restriction fragment replacement technique using existing restriction sites, MuI, SacI, NaeI, and SalI (Fig. 6). All mutants and chimeric genes were confirmed by DNA sequencing.

Enzyme purification. E. coli BL21 (DE3) pT-gro E harboring the expression plasmid, pETAMY-I or pETAMY-III, were grown at 30°C in Luria broth supplemented with Ampicillin (50 µg/ml) and Chloramphenicol (38 µg/ml). When OD600 reached 0.4–1, IPTG was added to a final concentration of 0.4 mM and incubated at 18°C for 18 hr. The E. coli cells harvested from the above culture were suspended in 20 mM Hepes buffer (pH 7.5) containing 5 mM CaCl2, and disrupted by sonication. After centrifugation, the cell lysate was put on an α- or β-cyclodextrin-Sepharose 6B column and washed with 20 mM Hepes, pH 7.5, 5 mM CaCl2 and the α-amylase retained on the column was eluted with α- or β-cyclodextrin. The enzyme-containing fraction were pooled and dialyzed against the Hepes buffer. The N-terminal sequence was analyzed using a protein sequencer (model 473A, Applied biosystems, Inc.)

Biochemical methods. α-Amylase activity was measured by the following method in a 100-µl reaction mixture consisting of 100 mM sodium acetate buffer (pH 4.5) containing 5 mM CaCl2, 50 mM, 5% soluble starch, 20 µl, and enzyme solution, 30 µl. The mixture was incubated at 25°C or 50°C for an appropriate time and the released reducing sugar was measured by the method of Nelson and Somogyi.

Results

Nucleotide sequences of AMY-I and AMY-III

The nucleotide sequence of the 1.7-kb EcoR I fragments in pBluescript II were detected. Two α-amylase cDNA clones corresponding to AMY-I and AMY-III contained 1686 and 1688 base pairs, respectively. From the initiation codon ATG to the stop codon TGA, the AMY-I contains an open reading frame consisting of 1305 bp coding for 435 amino acids with a calculated molecular weight of 45,316 daltons, while the AMY-III contains an open reading frame of 1311 bp coding for 437 amino acids with molecular weight of 46,123 daltons. Two consensus polyadenylation-like signals, AATAAG and AGTAA are found in the 3’ untranslated nucleotide sequence of AMY-I, while in AMY-III, one polyadenylation-like signal, AATAAG is found upstream from the ATG codon. From the deduced amino acid sequence (Fig. 1), both AMY-I and AMY-III have no putative N-glycosylation sites. The degree of similarity in nucleotide sequences between the two enzymes is estimated to be 74%. The partial amino acid sequences of tripic peptides (underlined sequences as shown in Fig. 1) obtained from native AMY-I and AMY-III were identical with those deduced from the nucleotide sequences of AMY-I and AMY-III. This indicates that the cDNAs encode AMY-I and AMY-III. AMY-I had an identical nucleotide sequence to the cDNA clone of pOS137, which was obtained from germinated rice seed. AMY-III had also shown to be identical in sequence to a genomic clone without introns of RAMy-3E obtained from rice (M202) DNA reported by Huang et al.

Expression of AMY-I and AMY-III

To express α-amylase as a mature form in E. coli, the restriction enzyme Nde I cleavage site was introduced to AMY-I and AMY-III between signal sequence and mature sequence. Nde I-EcoR I fragments encoding mature sequences of AMY-I and AMY-III were inserted into the E. coli expression vector, pET12a (Fig. 2). The resulting vectors (pETAMY-I and pETAMY-III) containing AMY-I and AMY-III transferred to E. coli BL21
Fig. 1. Deduced Amino Acid sequences of AMY-I and AMY-III from Cultured Rice Cells.

A, B, C, and D were highly similar regions in all α-amylases. a, b, c, and d were unconserved regions with low amino acid sequence similarity between AMY-I and AMY-III. Underlined showed the partial amino acid sequences of trptic peptides.

(3E3) pT-groE having the T7 RNA polymerase gene, and expression of AMY-I and AMY-III was induced by the addition of 0.4 mM IPTG. Expression of them in E. coli were detected by western blot analysis which showed a single band of 45 kDa and one at 42 kDa comigrating with native AMY-I and AMY-III. To find the optimum temperature for this expression system, the gene products in the soluble or the insoluble fraction were detected by western blot analysis and their activity was analyzed. The activity of AMY-I was detected at all incubation temperatures and the highest activities were obtained at 25°C. On the other hand, AMY-III, at 37°C, was detected in the insoluble fraction (Fig. 3A) and showed very low activity (Fig. 3B). The AMY-III activity was increased in proportion to the lowered incubation temperature. At 18°C the AMY-III activity increased to about 26-fold greater than that obtained at 37°C.

Purification and characterization of recombinant proteins

Using affinity chromatography on α- or β-cyclodextrin Sepharose 6B, recombinant AMY-I and AMY-III were purified in one step and gave single bands at 45 kDa and 42 kDa in SDS-PAGE by Coomassie blue staining. These proteins reacted with the rabbit polyclonal antibodies against rice AMY-I (Fig. 4). The N-terminal amino acid sequences of recombinant AMY-I and recombinant AMY-III were identical to the deduced amino acid sequences of native AMY-I and AMY-III, respectively. Characteristic properties of the recombinant AMY-I and AMY-III were compared with those of the native enzymes with respect to the temperature dependency and the effects of maltooligosaccharides on α-amylase activities (Fig. 5, Table 1). The recombinant AMY-I and recombinant AMY-III had their optimum temperatures at 55°C and 25°C, respectively. The activity of recombinant AMY-III against soluble starch was decreased to 40% by treatment with maltotetadesacase (G-17) and corn starch. These results indicate that the recombinant enzymes have the same enzyme characteristics as those of native AMY-I and AMY-III.

Construction and characterization of chimeric α-amylases expressed in E. coli

To discover which part of the primary structure is responsible for the unique properties of AMY-III that has low temperature optimum and susceptibility to maltooligosaccharide effectors, several chimeric genes were constructed with various combination of AMY-I and AMY-III fragments. At the beginning, we planned to
select some proper base sequences having a single cleavage site between the two cDNAs, and to exchange the specific base sequences responsible for the characteristics of AMY-I or AMY-III, in other words, the four regions (a, b, c, and d regions as shown in Fig. 1) that had low sequence similarity between the two cDNAs. Following this, the chimera DNAs, 1333, 1133, 1113, 1131, 3111, 3311, 3331, and 3313 were generated (Fig. 6). The chimera 1333, for example, contains a-region of AMY-I connected with AMY-III containing the other three non-conserved regions designated as b, c, and d. On the other hand, 3111 contains a-region of AMY-III to which is connected a part of AMY-I containing regions of b, c, and d. In the same way, 1133 is a combination of a, b-region of AMY-I and the c, d region of AMY-III, as shown in Fig. 6.

At first, four chimeric α-amylases that carry the

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<tr>
<th>Enzyme</th>
<th>Host cell</th>
<th>Remaining activity (%)</th>
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<tr>
<td></td>
<td></td>
<td>H₂O</td>
</tr>
<tr>
<td>AMY-I</td>
<td>rice cell</td>
<td>100</td>
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<td></td>
<td><em>E. coli</em></td>
<td>100</td>
</tr>
<tr>
<td>AMY-III</td>
<td>rice cell</td>
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<td><em>E. coli</em></td>
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Fig. 3. Effects of the Cultivation Temperature on the Expression of AMY-I and AMY-III in *E. coli*.

The cultivation of *E. coli* harboring the expression plasmid was in 50 ml LB medium at various temperatures. The *E. coli* cells were suspended in 5 ml 50 mm Heps, pH 7.5, and followed by disruption in a ultra-sonicator. After centrifugation of the cell lysate, the precipitate was dissolved in 5 ml 50 mm Heps pH 7.5. A; Western blot analysis of α-amylases. The supernatant (soluble fraction) and the precipitate (insoluble fraction) were separated on SDS-PAGE. Protein was transferred from SDS-polyacrylamide gels to PVDF membrane. Membrane was incubated with rabbit polyclonal antibodies against rice AMY-I. B. The specific activity of α-amylases. Specific activity indicated the α-amylases activity in 1 mg protein from the cell lysate. Details are described in the text. The activity measured at 50°C, (full box); at 25°C, (open box).
region c of AMY-I, 3111, 3311, 1113, and 3313, were expressed. On SDS-PAGE, each recombinant chimeric protein showed a single band of 45 kDa that had the same mobility as native AMY-I (Fig. 7A). Table 2 shows the temperature dependency and effects of maltooligosaccharide effectors on the activity of chimeric α-amylases. These four chimeric enzymes had a higher optimum temperature (the specific activity at 50°C is higher than that obtained at 25°C, in other words, ratio
of the specific activity at 50\(^\circ\)C to 25\(^\circ\)C is higher than 1) and gave similar residual activities after treatment maltoligosaccharide effectors (G-7 and G-17) to native AMY-I (Table 2).

On the other hand, the other four chimeric enzymes, which carrying region c of AMY-III, 1333, 3331, and 1131, were accumulated within the cell and only a small amount of the expressed protein was detected. Using cell lysates, we measured the general properties of the chimeric enzymes. Western blot analysis showed clearly a 42-kDa protein band that corresponds to the native AMY-III (Fig. 7B). These chimeric enzymes showed the same temperature dependency as AMY-III, the activity at 25\(^\circ\)C was higher than at 50\(^\circ\)C (the ratio of the specific activity at 50\(^\circ\)C to 25\(^\circ\)C is lower than 1 as shown in Table 2). Moreover, the remaining activity after treatment of G-17 maltoligosaccharide showed a significant reduction of soluble starch hydrolyzing activity while G-7 treatment had no effect (Table 2). All these results suggest that the chimeric protein carrying region c of AMY-III was to be classified as an AMY-III type enzyme.

### Discussion

Although the AMY-III amino acid sequence showed 74\% similarity to that of AMY-I, the AMY-III showed unique properties with an abnormal low temperature optimum and susceptibility to the maltoligosaccharide effectors and these are very different from those of the AMY-I and also from other known cereal \(\alpha\)-amyloses.\(^{2,15,16}\) Comparison of the amino acid sequences of various \(\alpha\)-amyloses\(^{17}\) indicated the four highly similar regions namely, regions I, II, III, and IV, conserved in all amyloses examined. Both AMY-I and AMY-III contain all of these four conserved regions, designated as regions A, B, C, and D (Fig. 1). X-ray crystallographic studies of *Aspergillus oryzae* \(\alpha\)-amylase\(^{18}\) and porcine pancreatic \(\alpha\)-amylase\(^{19}\) indicated that these regions may function as catalytic-substrate binding, or calcium binding sites. The characteristic properties of AMY-III may be derived from unconserved regions with low amino acid sequence similarity that are designated a, b, c, and d (Fig. 1).

We have attempted to use *E. coli* for production of recombinant chimeric enzymes. A number of recombinant \(\alpha\)-amyloses from higher plants have been studied using the yeast expression system,\(^{8,20,21}\) but attempts to obtain recombinant enzymes using *E. coli* have been unsuccessful.\(^{22}\) It has been reported that at low cultivation temperatures, the gene products were obtained as a soluble form using the *E. coli* expression system.\(^{23,24}\) In this study, when at first we tried to obtain recombinant AMY-I and AMY-III expressed at 37\(^\circ\)C under the T7 promoter, the gene products were found mostly in the insoluble fraction as inclusion bodies and the activity in
the cell lysate were extremely low (Fig. 3). When the cultivation temperature was shifted to 18°C, high activity of AMY-III was recovered.

One-step purification of the recombinant AMY-I and AMY-III expressed in E. coli was done by affinity chromatography using α- or β-cyclodextrin as ligands. Characterization of eight chimeric α-amylases indicated that region c (Fig. 6) was a critical site for the characteristics of AMY-I or AMY-III. This region is lying between the conserved regions B and C in which the active site Asp and Glu are located. Secondary structural analysis by the Chou and Fasman method indicated that the region c of AMY-I had same α-helix like the other well studied α-amylases, namely, *Aspergillus oryzae* α-amylase (TAA), porcine pancreatic α-amylase (PPA) and barley α-amylase. On the other hand, AMY-III gave β-sheet structure for this region c. This conformational change may influence the characteristics of AMY-III. To identify which amino acids are important for AMY-III characteristics, direct replacement of each amino acid residue in region c by site-directed mutagenesis is now in progress.

References


