

Characterization of the functional module responsible for the low temperature optimum of a rice α -amylase (Amy 3E)

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Cultured cells of rice produce two α -amylase isozymes, AMY-I (Amy 1A) and AMY-III (Amy 3E). Eight chimeric genes, constructed from various combination of AMY-I and AMY-III cDNA fragments, were expressed using a bacterial expression system, 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 those of native AMY-I, which had a high temperature optimum at 50°C. The other four chimeric proteins carrying region c of AMY-III showed AMY-III type characteristics and exhibited a low temperature optimum at 25°C. These results suggest that the region c (Phe179–Trp205: α -helix-4, β -sheet-5, α -helix-5) is responsible for the property of the low temperature optimum of AMY-III.

Key words: plant α -amylases, cultured-rice cells, chimeric enzymes.

Abbreviations: AMY-I and AMY-III, α -amylase (EC 3.2.1.1) isozymes I and III from cultured rice cells; *AMY*, gene encoding AMY; G17, maltoheptadecasaccharide.

Introduction

Alpha-amylases (1,4- α -D-glucan glucanohydrolase; EC 3.2.1.1) are widespread among plants, mammals, insects, fungi and bacteria, and a number of their cDNAs have been cloned. In higher plants, α -amylases are important in germinating seeds and usually are present as multiple isoforms. In cultured cells of rice, ten α -amylase isoforms have been found (MITSUI et al., 1996).

We have found two distinct α -amylases, AMY-I and AMY-III in suspension-cultured rice

cells (*Oryzae sativa* cv Sasanishiki). One of these, AMY-III, showed unique enzymatic properties when compared to the other usual α -amylases including AMY-I (CHIBA et al., 1990, 1991). It had a low temperature optimum at 25°C and an abnormal curve for the Arrhenius plots, indicating great conformational change of the enzyme structure occurring around 25°C. 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.

A number of recombinant rice α -amylase

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AMY-I	1:M-KNTS SLCLLLVWLCSLTCNSGQAQVLFQGFNWE SWKQGGWYNMLKGQVDDIAKAGV	59
AMY-III	1:MGKHHVTLCCVVFVAVLC-LAS SLAQAQVLFQGFNWE SWRKQGGWYNFLHEKVEEIASTGA	59
		a
AMY-I	60:THVWLPPPSHSVAPQGYMPGRLYDLDA SKYGTAAE LK SLIAAFHGKGVQCVAQVVDIHRC	119
AMY-III	60:THVWLPPPSHSVAPQGYMPGRLYDLDA SKYGTAAE LK SLIEAFHDKNVEC LADIVDHR	119
		b A
AMY-I	120:A EKKDARGVYCVFEGGTPD-R LDWGP GMICSDDTQYSEG TGRD TG EGFGAAPD IDHLNP	178
AMY-III	120:ADYKDSRGVYCVFEGGTPDGR LDWGP DMICSDDTQY SNGRGRD TGAGFGAAPD IDHLNP	179
AMY-I	179:R VQR EL TDWLNWLK SDVGFDSWR LDFAKGYS TDIAKMYVESCKP GFVVAEIVNSLSYNGD	238
AMY-III	180:R VQR EL TDWLNWLR TD LGFDSWR LDFAKGYS AP LAR IYVDN TNP TFVVG EIWSS LIYNGD	239
		B c C
AMY-I	239:GKPAANQDQGRQELVNWVAVGGP AMTFD FTTRG LLQAGVQGELVRLRD GNGKAPGMDGW	298
AMY-III	240:GKPS TNQADRQELVNWVWVGGVGP ATAFD FTTRG ILLQAAVQGELVRLHD GNGKAPGLMGW	299
AMY-I	299:LPEKAVTFVNDHDTGS TQK LWPFP SDKVMQGYAYIL THPGVPCI FYDHMFDVNLKQ EIT	358
AMY-III	300:MPDQAVTFVNDHDTGS TQS LWPFP SDKVMQGYAYIL THPGIPIFYDHYFDVNLQH EIA	359
		D
AMY-I	359:LA AIERENGINAGSKLRIVVADADAYVAVVD EKVMYKIG TRYDVGNAMP SD FHQ TVHGD	418
AMY-III	360:LA EIRSRNGIHAES TLDILK AEGDIYVAMIDGKVIITKLGPR YDAGG IIP SD FHVVAHGND	419
		d
AMY-I	419:Y SVWERGSLRYPAGR-HL	435
AMY-III	420:Y CVWEREGFRYPAGRKH	437

Fig. 1. Deduced amino acid sequences of AMY-I and AMY-III from rice cultured cells. A, B, C, and D are highly similar regions in all α -amylases (known as conserved sequence regions I, II, III, and IV; NAKAJIMA et al., 1986). Small letters a, b, c, and d are unconserved regions with low amino acid sequence similarity between AMY-I and AMY-III.

isozymes (TERASHIMA et al., 1995) and barley α -amylase isozymes (SOGAARD & SVENSSON, 1990) have been produced and characterized using yeast expression systems. The nucleotide sequence analyses of AMY-I and AMY-III indicated that the AMY-I had an identical sequence to that of the cDNA clone of pOS137(Amy1A) which was obtained from germinated rice seed (O'NEILL et al., 1990), and the AMY-III was identical to that of a genomic clone without introns of Ramy-3E obtained from rice (M202) DNA (HUANG et al., 1990).

To understand which part of the protein modules 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*.

Properties of the rice cell α -amylase isozymes AMY-I and AMY-III

Two groups of α -amylase isozymes were purified and characterized from cultured rice (*Oryza sativa* cv Sasanishiki) cells (Tab. 1). By native PAGE, three activity bands are detected (AMY-I, AMY-II and AMY-III). AMY-I and AMY-II were 45 kDa proteins and were charged isomers belonging to the same group with the same properties. AMY-III was electrophoretically homoge-

neous with a molecular mass of 42 kDa and pI of 5.8. The AMY-III showed a unique property with a low temperature optimum at 25°C, unlike other plant, animal and microbial amylases that generally had optima at around 35–55°C. The Arrhenius plot for the enzyme also gave an unusual curve, i.e. the plot greatly deviated from a straight line above 25°C. This suggests the occurrence of a conformational change in the structure of AMY-III around 25°C in the presence of substrate. The AMY-III also showed a great reduction of its activity when raw starch or a higher maltooligosaccharide of DP 17 was added.

Amino acid sequences of AMY-I and AMY-III

Two α -amylase cDNA clones corresponding to AMY-I and AMY-III contained 1686 and 1688 base pairs, respectively. From the deduced amino acid sequence, both AMY-I and AMY-III have no putative N-glycosylation sites (Fig. 1). The degree of similarity in nucleotide sequences between the two isozymes is estimated to be 74%. AMY-I had a nucleotide sequence identical to that of the cDNA clone of pOS137, obtained from germinated rice seed (O'NEILL et al., 1990). AMY-III was also shown to be identical in sequence to a genomic clone without introns of Ramy-3E obtained from rice (202) DNA (HUANG et al., 1990).

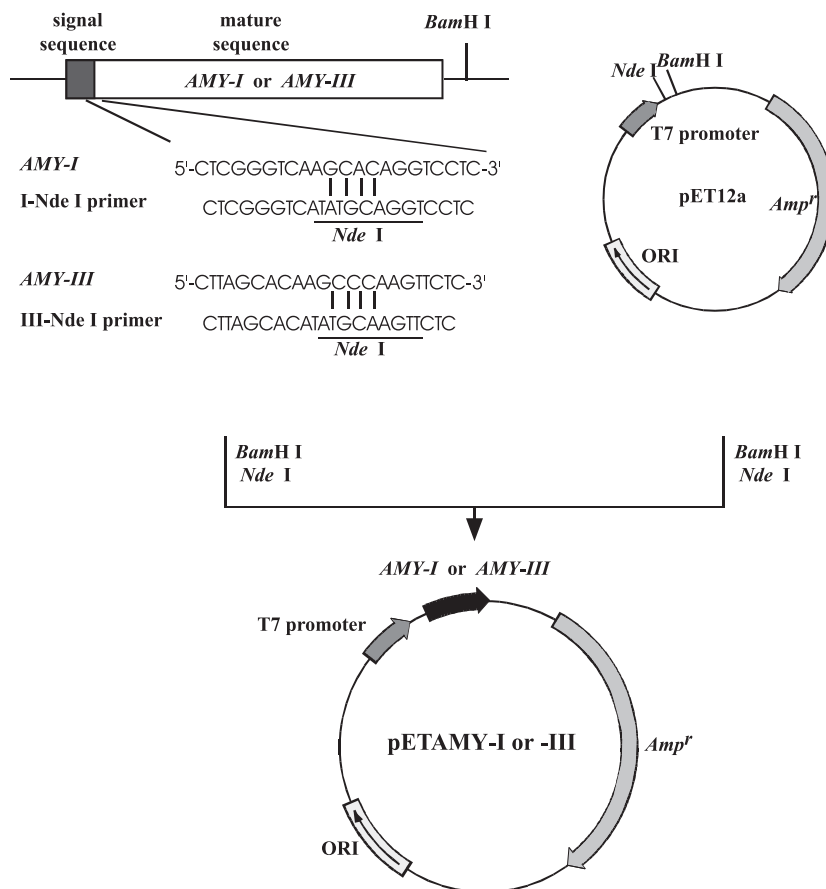


Fig. 2. Construction of expression plasmid, pETAMY-I or pETAMY-III, for *Escherichia coli*. The cDNA sequences of *AMY-I* and *AMY-III* were modified to include the restriction enzyme *Nde* I cleavage site, and were digested with *Nde* I and *Bam*H I. These cDNA inserts, which cover the mature sequences of *AMY-I* and *AMY-III* coding regions, were inserted into the *Nde* I and *Bam*H I site of pET12a. These plasmids were transformed into *E. coli* BL21(DE3)pT-groE.

Table 1. Some properties of α -amylase isozymes *AMY-I* and *AMY-III* from rice cultured cells.

Properties	<i>AMY-I</i>	<i>AMY-III</i>
Molecular mass (SDS-PAGE)	45,000	42,000
(Calculated)	45,316	46,123
Isoelectric point	6.0	5.8
K_m for soluble starch (mg/mL)	1.1	5.0
Optimum temperature	55 °C	25 °C
Optimum pH	4.8	4.5

Expression of *AMY-I* and *AMY-III*

To express α -amylase as a mature form in *Escherichia coli*, the restriction enzyme *Nde* I cleav-

age site was introduced to *AMY-I* and *AMY-III* between signal sequence and mature sequence. *Nde*I-*Eco*RI fragments encoding mature sequence 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* were transferred to *E. coli* BL21(DE3) pT-groE having the T7 RNA polymerase gene, and expression of *AMY-I* and *AMY-III* was induced by the addition of IPTG. Expression in *E. coli* was detected by western blot analysis which showed a single band of 45 kDa and one at 42 kDa co-migrating with native *AMY-I* and *AMY-III*, respectively.

The purified recombinant *AMY-I* and *AMY-III* had identical N-terminal amino acid sequences

Table 2. Effects of maltooligosaccharides and corn starch on the activity of AMY-I and AMY-III expressed in *E. coli*.

Enzyme	Host cell	Remaining activity (%)			
		None	G7	G17	Corn starch
AMY-I	rice cell	100	103	92	100
	<i>E. coli</i>	100	90	94	85
AMY-III	rice cell	100	93	13	20
	<i>E. coli</i>	100	110	39	41

Table 3. Effects of maltooligosaccharides and temperature dependency on the activity of chimeric α -amylases expressed in *E. coli*.

Enzyme	Remaining activity (%)		Ratio of specific activity (55°C/25°C)	Type of enzyme
	None	G17		
AMY-I	100	110	1.3	—
AMY-III	100	40	0.3	—
3-1 (<i>Mlu</i> I)	100	84	1.7	AMY-I
3-1 (<i>Sca</i> I)	100	86	1.4	AMY-I
3-1 (<i>Nae</i> I)	100	11	0.4	AMY-III
3-1-3(<i>Sca</i> I, <i>Sal</i> I)	100	101	2.5	AMY-I
1-3 (<i>Mlu</i> I)	100	13	0.3	AMY-III
1-3 (<i>Sca</i> I)	100	10	0.6	AMY-III
1-3 (<i>Sal</i> I)	100	86	2.5	AMY-I
1-3-1(<i>Sca</i> I, <i>Nae</i> I)	100	23	0.2	AMY-III

to those of native AMY-I and AMY-III, respectively. The recombinant enzymes showed the same characteristic properties as the native enzymes with respect to the temperature dependency and the effects of maltooligosaccharides on α -amylase activities. (Fig. 3, Tab. 2).

Construction and characterization of chimeric α -amylases

To discover which part of the primary structure is responsible for the unique properties of AMY-III that has a low temperature optimum and susceptibility to maltooligosaccharide effectors, several chimeric genes were constructed using various combinations of *AMY-I* and *AMY-III* fragments (Fig. 4). Table 3 shows the temperature dependency and effects of maltooligosaccharide effectors on the activity of chimeric α -amylases. Four chimeric α -amylases that carry the region c of AMY-I (3-1 *Mlu* I, 3-1 *Sca* I, 3-1-3 *Sca* I *Sal* I and 1-3 *Sal* I) had a higher optimum temperature and gave residual activities similar to native AMY-I after treatment with maltooligosaccharide effectors (G7 and G17). On the other hand, the other four chimeric enzymes carrying region c of AMY-III showed the same temperature depen-

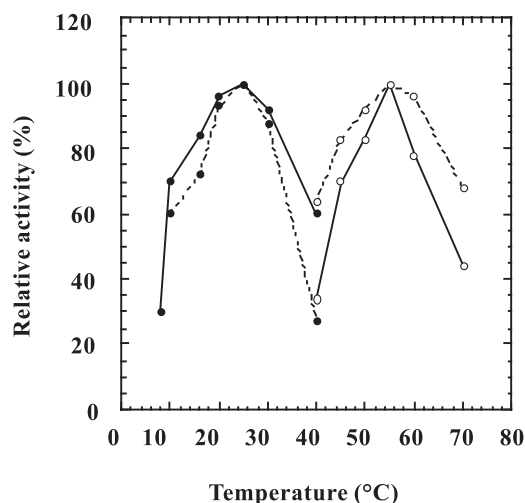


Fig. 3. The temperature dependence of the activity of AMY-I and AMY-III expressed in *E. coli*. The relative activities of AMY-I and AMY-III are shown. The maximum activity of AMY-I and AMY-III at optimum temperature were normalized to 100%. AMY-I (open circles) and AMY-III (filled circles). The activities of intact AMY-I and AMY-III are shown by full lines and those of recombinant enzyme are shown by dotted lines.

A

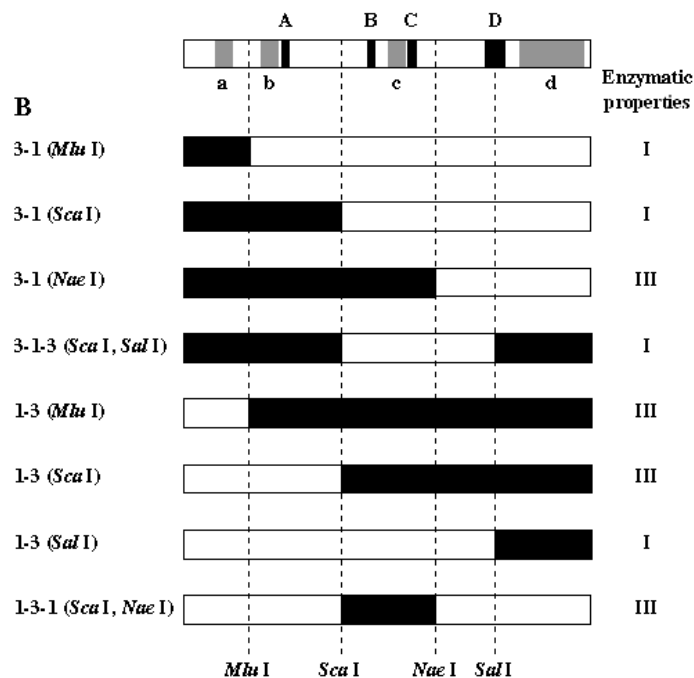


Fig. 4. Structure of chimeric genes and characterization of chimeric α -amylases. (A) Schematic structure of α -amylase. A, B, C, and D are conserved regions in all α -amylases; a, b, c, and d are non-conserved regions between AMY-I and AMY-III. (B) Structure of chimeric genes. AMY-I, (open box); AMY-III, (full box).

gency as AMY-III and the activity at 25°C was higher than at 50°C. Moreover, the remaining activity of these four chimeric enzymes after treatment of G17 maltooligosaccharide showed a significant reduction of soluble-starch-hydrolyzing activity, while G7 treatment had no effect.

Discussion

Although the AMY-III amino-acid sequence showed 74% similarity to that of the AMY-I, the AMY-III showed unique properties with an abnormal low temperature optimum and susceptibility to the maltooligosaccharide effectors. These properties are very different from those of the AMY-I and also other known cereal amylases (CHIBA et al., 1990, 1991; OKAMOTO et al., 1978). Comparison of the amino acid sequences of various α -amylases (NAKAJIMA et al., 1986) indicated that four highly similar regions namely, regions I, II, III and IV, are conserved in all amylases 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* α -amylase (MATSUURA et al., 1984) and porcine pancreatic α -amylase (BUISSON

et al., 1987) indicated that these regions may function as catalytic, substrate-binding, or calcium-binding sites. The characteristic properties of the AMY-III may be derived from unconserve regions with low amino acid sequence similarity that are designated a, b, c, and d (Fig. 1).

Characterization of eight chimeric α -amylases indicated that region c (Fig. 4) was a critical site for the characteristics of AMY-I or AMY-III. This region lies between the conserved regions B (or II) and C (or III) in which the active site Asp and Glu are located.

Secondary structural analysis by the CHOU and FASMAN method (CHOU & FASMAN, 1978) indicated that region c of AMY-I had the same α -helix as other well-studied α -amylases, such as *Aspergillus oryzae* α -amylase (MATSUURA et al., 1980), porcine pancreatic α -amylase (BUISSON et al., 1987) and barley α -amylase (JUGE et al., 1996). On the other hand, AMY-III is predicted to have a 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.

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