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Proteomic Identification of α-Amylase Isoforms Encoded by *RAmy3B/3C* from Germinating Rice Seeds

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We isolated and identified 10 α-amylase isoforms by using β-cyclodextrin Sepharose affinity column chromatography and two-dimensional polyacrylamide gel electrophoresis from germinating rice (*Oryza sativa* L.) seeds. Immunoblots with anti-α-amylase I-1 and II-4 antibodies indicated that 8 isoforms in 10 are distinguishable from α-amylase I-1 and II-4. Peptide mass fingerprinting analysis showed that there exist novel isoforms encoded by *RAmy3B* and *RAmy3C* genes. The optimum temperature for enzyme reaction of the *RAmy3B* and *RAmy3C* coding isoforms resembled that of α-amylase isoform II-4 (*RAmy3D*). Furthermore, complex protein polymorphism resulted from a single α-amylase gene was found to occur not only in *RAmy3D*, but also in *RAmy3B*.

Key words: α-amylase; germinating rice seed; *RAmy3B*; *RAmy3C*; proteomics

α-Amylase (EC 3.2.1.1) plays an important role in breaking down the starch molecules stored in the starchy endosperm of cereal seeds during germination and subsequent seedling growth. Multiple isoforms of α-amylase are present in cereal seeds. These isoforms have been shown to be encoded by a multiple gene family.[1)] In rice, several α-amylase isoforms have been detected by isoelectric focusing (IEF) gel separation.[2)] Previously, 10 α-amylase isoforms (A, B, E, F, G, H, I, J, Y, and Z) were purified and characterized from rice suspension-cultured cells. The isoform molecules of α-amylase are classified into two classes from its physicochemical and serological properties, class-I (A, B, Y, Z) and class-II (E, F, G, H, I, J), and further into four subgroups, group1 (A, B), group-2 (Y, Z), group-3 (E), and group-4 (F, G, H, I, J). Furthermore, α-amylase I-1 (A, B), II-3 (E), and II-4 (F, G, H, I, J) have been shown to be encoded by *RAmy1A*, *RAmy3E*, and *RAmy3D*, respectively.[2)] In germinating rice seeds, α-amylase isoforms A, B, and D have been identified and characterized.[2,3)] Recently, we have demonstrated the induction of α-amylase II-4 expression by gibberellic acid (GA_3) in germinating rice seeds.[4)] Although it has been reported that at least 10 distinct α-amylase genes exist in rice,[5–7)] the exact corresponding relationship between genes and isoform proteins of rice α-amylase is still largely undetermined.

In this communication, we isolated and identified 10 α-amylase isoforms, which were designated A, B, E_1, E_2, K_1, K_2, K_3, K_4, K_5, and L from germinating rice seeds. These corresponding genes were identified by peptide mass fingerprinting with matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF-MS).

Materials and Methods

Plant materials. One kg of rice seeds (*Oryza sativa* L.) were sterilized in 1% sodium hypochlorite for 20 min and thoroughly rinsed in sterile water. These seeds were let to imbibe with sterile water and incubated at 30°C in the dark for up to 5 days. The germinating rice seeds were used for purification of α-amylase.

Enzyme purification. α-Amylase isoforms were purified following the procedure reported previously.[8)] The germinating rice seeds were extracted with 20 mM acetate, 1.2 mM $CaCl_2$, and 1.6 mM NaCl (pH 5.3). The homogenate was filtered through 2 layers of gauze and centrifuged at 20,000 × *g*, for 20 min. The supernatant was concentrated by 70% $(NH_4)_2SO_4$, and dialyzed with the above buffer, then applied to a β-cyclodextrin Sepharose 6B column (φ15 × 150 mm). After washing with 20 mM acetate, 1.2 mM $CaCl_2$, and 0.3 M NaCl (pH 5.3), the enzyme was eluted with 8 mg/ml β-cyclodextrin, 20 mM acetate, 1.2 mM $CaCl_2$, and 1.6 mM NaCl

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Abbreviations: α-amylase isoform Ks, α-amylase isoform K_1, K_2, K_3, K_4, K_5; CBB, Coomassie brilliant blue; ESI, electrospray ionization; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; GA_3, gibberellic acid; IEF, isoelectric focusing; MALDI TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrometry

(pH 5.3). An α-Amylase assay was done as described previously.[2] Active fractions were examined by 2D-PAGE separation.

2D-PAGE. Purified α-amylases were put through native IEF-PAGE with 4% polyacrylamide disc gels (pH 4–7, $\phi 3 \times 110$ mm) according to the procedure described earlier.[2] The first-dimensional gels were then put through SDS-PAGE.[9] After electrophoresis, 2-D gels were stained with CBB R-250.

Antibody preparation and immunoblotting. Preparation and characterization of polyclonal antibodies, Poly anti-AB (anti-α-amylase I-1) and Poly anti-H (anti-α-amylase II-4), and monoclonal antibody Mono H-B35 (anti-α-amylase II-4) were described previously.[2] Protein spots excised from the CBB-stained 2-D gels were put through SDS-PAGE and western blotting.[2] The blotted nitrocellulose sheets were reacted with specific antibodies, followed by [^{35}S]-labeled anti-rabbit IgG or anti-mouse IgG (Amersham), and then analyzed by a radioisotope imaging analyzer (BAS-5000, Fuji Film).[10] The blotted protein bands were made visible by staining with Amido Black solution consisting of 0.1% (w/v) Amido Black 10B and 30% (v/v) ethanol.

Optimum temperature identification. Each active α-amylase isoform was excised and isolated from the native IEF gels, and extracted with 20 mM acetate, 1.2 mM $CaCl_2$, and 1.6 mM NaCl (pH 5.3). The gel suspension was centrifuged at 20,000 × *g* for 20 min at 4°C. A portion of the supernatant was assayed for the enzyme activity at different temperatures.

Peptide mass fingerprinting. The protein spots excised from the CBB stained 2-D gels were washed with 25% (v/v) methanol/0.7% (v/v) acetic acid for 2 h, and soaked 3 times in 50 mM NH_4HCO_3/50% methanol for 1 h at room temperature. The solution was removed, and the gel pieces were dried in a vacuum centrifuge. The dried gel pieces were swollen in 50 μl of 10 mM dithiothreitol and 50 μl of 100 mM NH_4HCO_3 for 1 h at 60°C. After removing the solution, the gel pieces were dried and re-swollen in 50 μl of 50 mM iodoacetamide/100 mM NH_4HCO_3 for 30 min at room temperature in the dark. The swollen gels were washed 3 times with 1 ml of pure water for 10 min, crushed by a Pipetman chip, and incubated with 50 μl of trypsin solution consisting of 5 ng/μl trypsin (Promega) and 50 mM NH_4HCO_3 for 12 h at 37°C. After addition of 0.1% (v/v) trifluoroacetic acid (TFA) in acetonitrile (100 μl), the gel suspension was sonicated in a sonicator (27 kHz, 150 W; UC-0515, Tocho) for 10 min and centrifuged at 10,000 × *g* for 10 min to collect the supernatant. The precipitated gels were resuspended and sonicated with 0.1% TFA (100 μl), 0.1% TFA in 50% (v/v) acetonitrile (50 μl), and 0.1% TFA in acetonitrile (80 μl), sequentially. The combined supernatants were concentrated to approximately 20 μl in a vacuum centrifuge. The peptide sample was further concentrated and desalted by $ZipTip_{C18}$ (Millipore). The obtained peptides were subjected to MALDI-TOF-MS (AXIMA-CFR, Shimadzu). The matrix solution was prepared by mixing a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA/100% acetonitrile (1:1, v/v). The peptides were further analyzed by electrospray ionization (ESI) Q-TOF MS/MS (Q-Tof Micro, Micromass) according to the manufacturer's protocol. The above peptide samples were dried up and then re-suspended with 1% (v/v) formic acid/60% acetonitrile. The obtained peptide mass fingerprints were analyzed by a Mascot search system (Matrix Science, http://www.matrixscience.com).

Results

We purified α-amylases from germinating rice seeds by β-cyclodextrin Sepharose 6B affinity column chromatography. The active enzyme specifically bound to the affinity column and eluted with β-cyclodextrin (data not shown). The purified enzyme preparation was put onto 2D-PAGE. Ten protein spots with different pI values (pI 4.60–6.20) and molecular sizes (41–44 kDa) were detected on the 2-D gel (Fig. 1). We designated these α-amylase isoforms as K_1, L, K_2, K_3, K_4, E_1, K_5, E_2, B, and A, from the high pI side (Fig. 1, Table 1). To identify their epitope characteristics, the protein spots excised were put through SDS-PAGE and immunoblotting analysis with anti-α-amylase I-1 and II-4 antibodies[2] (Fig. 2). Anti-α-amylase I-1 antibodies recognized isoform A and B, and anti-α-amylase II-4 antibodies bound to the isoform H prepared from a rice-cell culture. In contrast, Ks, L, and $E_{1,2}$ were scarcely recognized by both anti-α-amylase I-1 and II-4 antibodies, suggesting that Ks, L, and $E_{1,2}$ are differed from α-amylase I-1 and II-4, which are encoded by *RAmy1A* and *RAmy3D*, respectively.

The protein spots were further analyzed by peptide mass fingerprinting with MALDI TOF-MS. The MASCOT homology search indicated that the mass maps obtained from the isoform A and B match to the predicted peptide sequences of *RAmy1A* (score 145–168, coverage 32–35%), Ks match to *RAmy3B* (score 118–170, coverage 28–45%), L to *RAmy3C* (score 135, coverage 32%), and $E_{1,2}$ to *RAmy3E* (score 89–93, coverage 22–27%) (Table 1). Figure 3 shows typical mass spectra of isoform A, E (E_1), and K (K_1). The patterns of peptide mass fingerprinting were able to distinguish each other. The peptide fragments of isoforms K_2, A, and B were also put through ESI Q-TOF MS/MS. It was confirmed that the isoform K_2 and isoforms A and B are encoded by *RAmy3B* and *RAmy1A*, respectively (Table 2).

Optimal temperature for the enzymatic activity of each α-amylase isoform was found. As shown in Table 3, the isoform A and B had a high optimum temperature

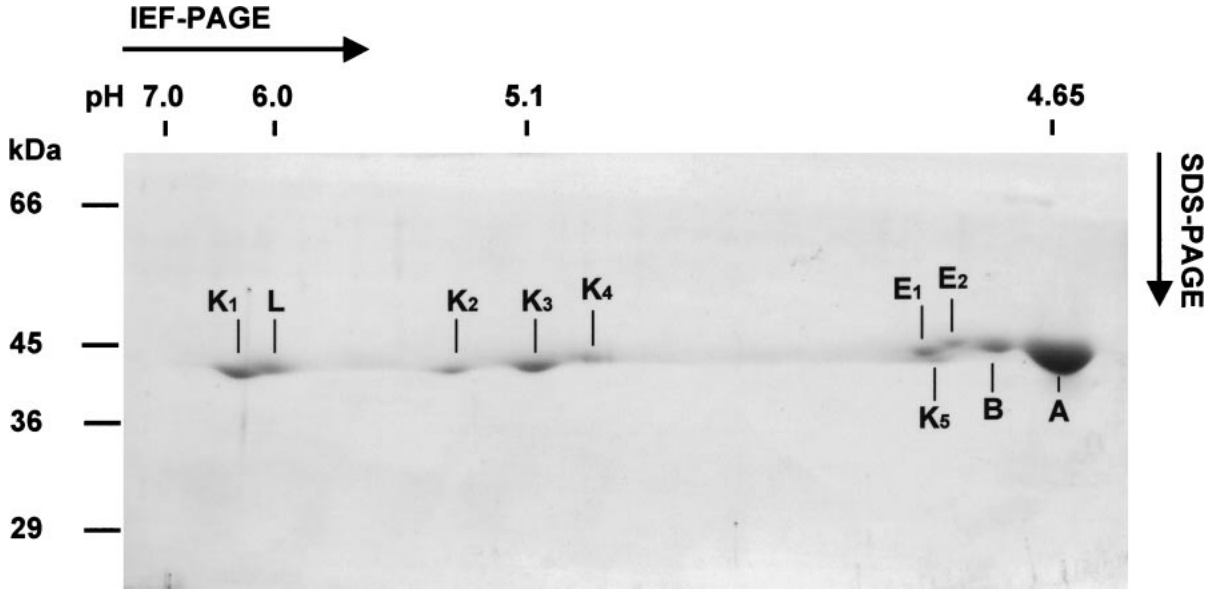

Fig. 1. Separation Profile of α-Amylase Isoforms from Germinating Rice Seeds by 2D-PAGE.

Purified α-amylase sample (40 μg) was put through 2D-PAGE, followed by CBB staining. The indicated pI values and molecular sizes were measured by IEF standards (Bio-Rad). K_1, L, K_2, K_3, K_4, E_1, K_5, E_2, B, and A indicate individual isoform protein spot.

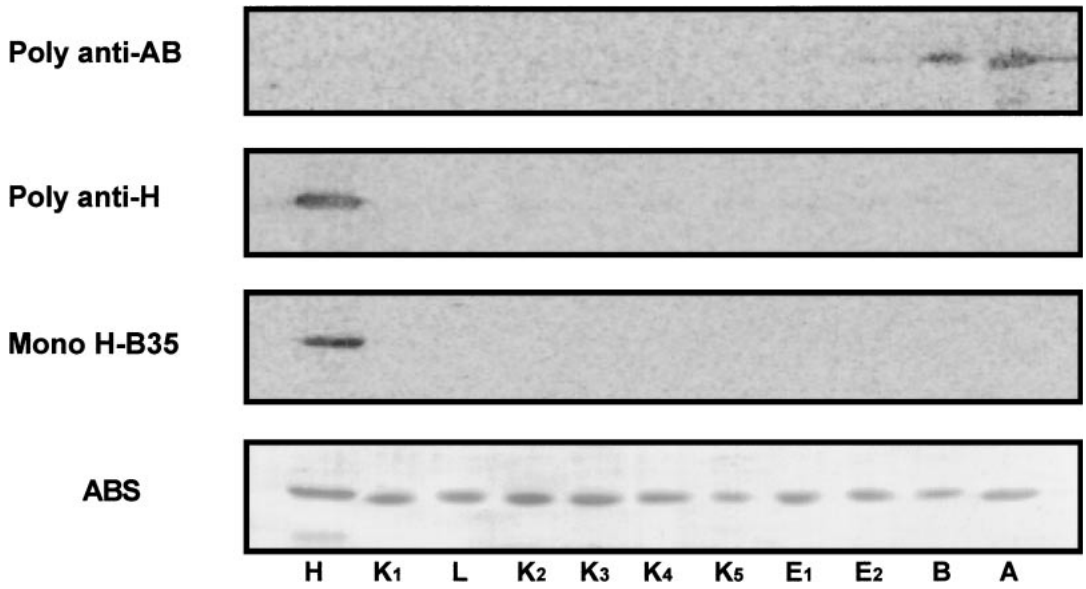

Fig. 2. Immunoblot Analysis with Anti-α-amylase I-1 and II-4 Antibodies.

Each isoform spot on the 2-D gels were excised and put onto SDS-PAGE, followed by immunoblotting with Poly anti-AB (anti-α-amylase I-1), Poly anti-H (anti-α-amylase II-4), and Mono H-B35 (anti-α-amylase II-4) antibodies. ABS indicates Amido Black staining. Lane H: the isoform H from rice cell culture.

(70°C), while $E_{1,2}$ (26°C), Ks (37°C), and L (37°C) had lower optimum temperatures. Based on the physicochemical properties, the isoform Ks and L were classified to Class II, and subdivided to group-5 and -6, respectively. Rice α-amylase isoforms and their corresponding genes are summarized in Table 4.

Discussion

The α-amylase isoforms (Ks and L) encoded by *RAmy3B* and *RAmy3C* were found to be synthesized in germinating rice seeds (Fig. 1, Table 1), particularly the *RAmy3B* coding isoform was highly expressed comparable to the isoform A and B encoded by *RAmy1A* (Fig. 1). Previously, mRNA of *RAmy3B* and *RAmy3C* has been detected in both embryo and aleurone layer of germinating rice seed.[7,11] This study demonstrated the native forms of these gene products. The degree of amino acid sequence similarity among *RAmy1A*, *RAmy3B/3C*, and *RAmy3E* was calculated to be 83.7 to 90.4%. The results presented in Tables 1, 2, and Fig. 3 indicated that the peptide mass fingerprinting analysis

Table 1. Peptide Mass Values of Specific Sequences of α-Amylase Isoforms by MALDI TOF-MS Analysis

Isoform (pI, gene)	Mr (Expt)	Mr (calc)	Peptide position		Predicted peptide sequence
			Start	End	
K_1 (6.20, *RAmy3B*)	1123.64	1123.57	366–	375	SRNEIHPGSK
	2359.12	2359.16	195–	216	SDVGFDGWRLDFAKGYSAAVAK
	2946.26	2946.39	402–	426	YDVGNLIPSDFHVVAHGNNYCIWEK
L (6.00, *RAmy3C*)	1051.55	1051.55	366–	375	SRNGIHPGSK
K_2 (5.30, *RAmy3B*)	1123.78	1123.57	366–	375	SRNEIHPGSK
	2359.30	2359.16	195–	216	SDVGFDGWRLDFAKGYSAAVAK
	2946.49	2946.39	402–	426	YDVGNLIPSDFHVVAHGNNYCIWEK
K_3 (5.10, *RAmy3B*)	2359.12	2359.16	195–	216	SDVGFDGWRLDFAKGYSAAVAK
	2946.26	2946.39	402–	426	YDVGNLIPSDFHVVAHGNNYCIWEK
K_4 (5.00, *RAmy3B*)	2359.30	2359.16	195–	216	SDVGFDGWRLDFAKGYSAAVAK
	2946.38	2946.39	402–	426	YDVGNLIPSDFHVVAHGNNYCIWEK
K_5 (4.69, *RAmy3B*)	2359.02	2359.16	195–	216	SDVGFDGWRLDFAKGYSAAVAK
E_1 (4.70, *RAmy3E*)	833.57	833.44	208–	215	GYSAPLAR
	1065.64	1065.49	194–	202	TDLGFDGWR
	1344.86	1344.68	184–	193	ELTDWLNWLR
	1377.89	1377.65	40–	50	QGGWYNFLHEK
	1440.00	1439.79	273–	285	GILQAAVQGELWR
	1512.87	1512.68	127–	140	GVYCFEGGTPDGR
	1823.13	1822.86	163–	180	DTGAGFGAAPDIDHLNPR
	2592.47	2592.32	97–	118	SLIEAFHDKNVECLADIVINHR
E_2 (4.68, *RAmy3E*)	833.51	833.44	208–	215	GYSAPLAR
	1065.62	1065.49	194–	202	TDLGFDGWR
	1344.83	1344.68	184–	193	ELTDWLNWLR
	1377.78	1377.65	40–	50	QGGWYNFLHEK
	1439.89	1439.79	273–	285	GILQAAVQGELWR
	1512.83	1512.68	127–	140	GVYCFEGGTPDGR
	1822.99	1822.86	163–	180	DTGAGFGAAPDIDHLNPR
B (4.67, *RAmy1A*)	1157.49	1157.58	256–	264	QELVNWVDR
	1271.60	1271.69	189–	198	ELIGWLDWLK
	1376.59	1376.73	373–	384	NRQGIHPASELR
	1454.69	1454.79	281–	293	GILNVAVEGELWR
	1513.60	1513.69	133–	146	GIYCLFEGGTPDSR
	1826.74	1826.90	87–	102	LYDLDASKYGNEAQLK
	1999.81	1999.10	112–	129	GVQVIADIVINHRTAEHK
	2415.11	2415.34	103–	124	SLIEAFHGKGVQVIADIVINHR
	2984.11	2984.47	409–	434	YDVEHLIPEGFQVVAHGDGYAIWEKI
	3156.16	3156.55	57–	86	VDDIAAAGITHVWLPPPSHSVGEQGYMPGR
A (4.60, *RAmy1A*)	1110.52	1109.50	199–	207	MDIGFDAWR
	1158.63	1157.58	256–	264	QELVNWVDR
	1272.68	1271.69	189–	198	ELIGWLDWLK
	1433.85	1432.81	112–	124	GVQVIADIVINHR
	1455.82	1454.79	281–	293	GILNVAVEGELWR
	1571.75	1570.72	133–	146	GIYCLEGGTPDSR
	2601.06	2600.19	312–	334	ATTFVDNHDTGSTQHLWPFPSDK
	2872.24	2871.39	409–	433	YDVEHLIPEGFQVVAHGDGYAIWEK
	3015.15	3014.33	157–	185	DDPYGDGTGNPDTGADFAAAPDIDHLNKR
	3157.30	3156.55	57–	86	VDDIAAAGITHVWLPPPSHSVGEQGYMPGR

Mr (Exp); experimental mass values, Mr (calc); Registered values in database.
Underlined amino acid indicated difference between Ks (*RAmy3B*) and L (*RAmy3C*).

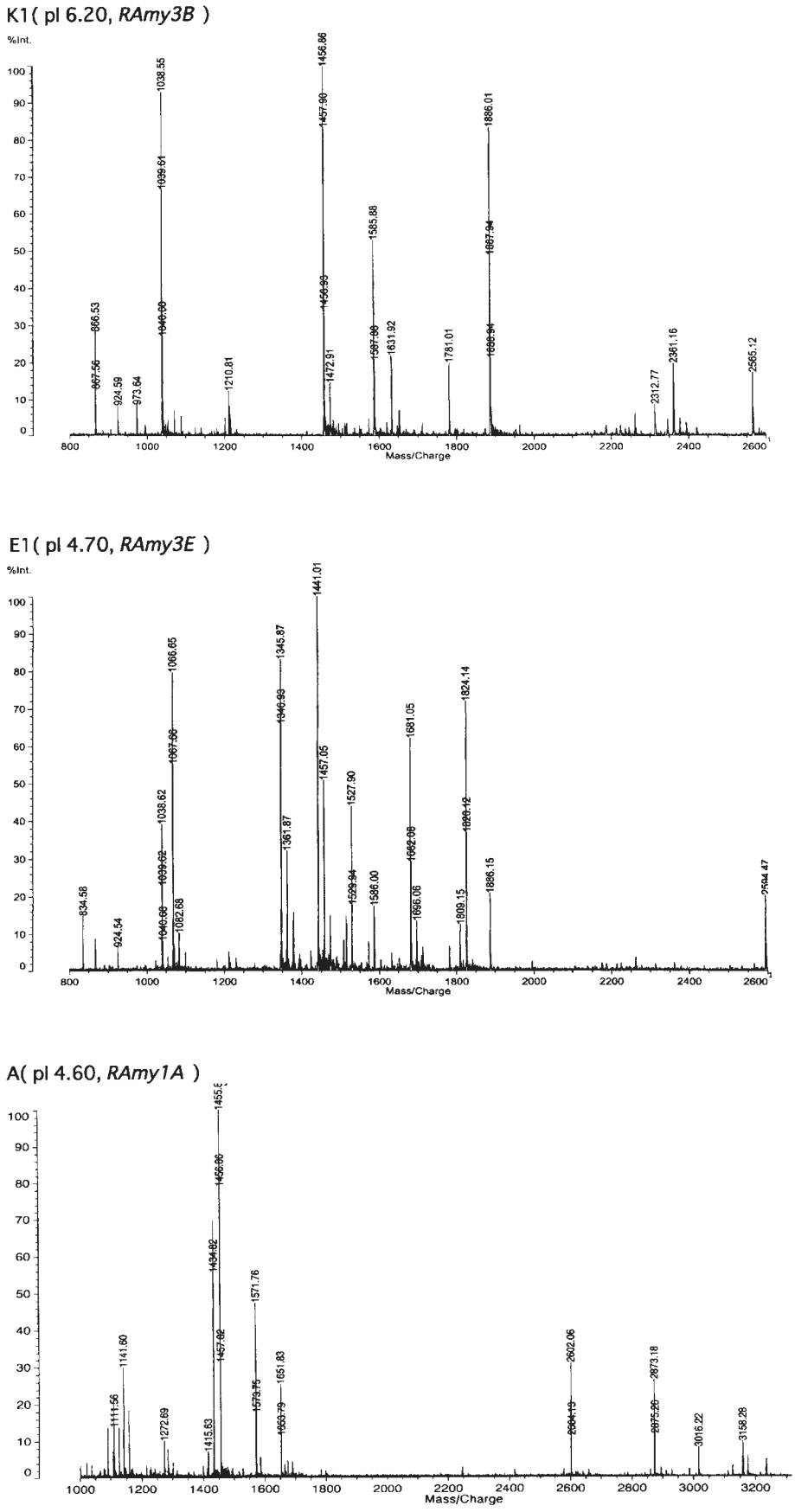

Fig. 3. Typical Peptide Mass Fingerprinting Patterns of α-Amylase Isoform K_1, E_1, and A.

Each protein spot excised from 2-D gels was digested in the gel by trypsin. The resulted peptides were put through MALDI TOF-MS. Details of peptide mass fingerprinting procedure are described in the text.

Table 2. Detected Sequences of α-Amylase Isoforms by ESI-MS/MS Analysis

Isoform (pI, gene)	Peptide position		Detected peptide sequence
	Start	End	
K_2 (5.30, *RAmy3B*)	378–	389	ILAAEGDVYVAM
	219–	231	VDNTDPSFVVAEI
B (4.67, *RAmy1A*)	112–	124	GVQVIADIVINHR
	385–	400	IMEADSDLYLAEIDGK
A (4.60, *RAmy1A*)	112–	124	GVQVIADIVINHR
	281–	293	GILNVAVEGEKWR

Table 3. Physicochemical Properties of α-Amylase Isoforms

Isoform	pI	M.W.	Optimum Temperature
		kDa	°C
A	4.60	44	70
B	4.67	44	70
E_1	4.70	42	26
E_2	4.68	45	26
K_1	6.20	41	37
K_2	5.30	41	37
K_3	5.10	41	37
K_4	5.00	42	37
K_5	4.69	42	37
L	6.00	42	37

with MALDI-TOF-MS could distinguish and identify the genes coding for these isoform proteins easily and efficiently. The similarity between *RAmy3A* and either *RAmy3B* or *RAmy3C* was approximately 87%, indicating that MALDI-TOF-MS must detect the isoform encoded by *RAmy3A* if it was expressed significantly. *RAmy3A*, *RAmy3B*, and *RAmy3C* have been shown to form a multigene cluster within 28 kb of genomic DNA.[11] It is now evident that the *RAmy3B* gene, which bears a gibberellin-response *cis*-element in the promoter region same as *RAmy1A*[12] and *RAmy3E*,[13] is dominantly expressed among the cluster genes at the protein level.

As shown in Fig. 1 and Table 1, the expression products of *RAmy1A*, *RAmy3B*, and *RAmy3E* were major isoforms rice seeds germinating in 5 days. Chen *et al.* (2002)[13] have recently demonstrated that mRNA of *RAmy1A*, *RAmy1C*, *RAmy3B*, and *RAmy3E* (*αAmy7*, *αAmy10*, *αAmy6*, and *αAmy8*) is actively expressed in the aleurone layer of 5-days germinated rice seeds. The similarity between *RAmy1A* and *RAmy1C* (*αAmy10*) was approximately 91.5% at the N-terminal region (1 to 117), although the full protein sequence of *RAmy1C* is not opened yet. The peptide mass fingerprinting analysis revealed that the isoform A and B contain a *RAmy1A*-specific peptide sequence, Val57 to Arg86. The product of *RAmy1C* was hardly detectable in our proteomic analyses, strongly suggesting that the translational and posttranslational regulation for *RAmy1C* expression may operates in the aleurone layer.

The novel isoform II-5 (Ks) encoded by a single gene *RAmy3B* had a heavy protein polymorphism (Fig. 1 and Table 1) caused by post-translational modifications, which has different pI values (pI 4.69–6.20) and molecular sizes (41–42 kDa). The isoform II-4 encoded by *RAmy3D* was also revealed to have similar heavy polymorphism with different pI (5.1–6.6).[2] However, the nature of these modifications remains to be clarified.

Table 4. Classification of Rice α-Amylase Isoforms and Their Corresponding Genes

Gene Name	Mendel Classification*	Isoform Class and Group	Isoform Name	
			Cell culture**	Germinating rice seeds
RAmy1A, aAmy7	*ORYsa;Amy1;1*	Isoform I-1	A,B	A,B
		Isoform I-2	Y,Z	n.i.***
RAmy1B			n.i.	n.i.
RAmy1C, aAmy10			n.i.	n.i.
RAmy2A	*ORYsa;Amy1;5*		n.i.	n.i.
RAmy3A	*ORYsa;Amy1;2*		n.i.	n.i.
RAmy3B, aAmy6	*ORYsa;Amy1;6*	Isoform II-5	n.i.	K_1,K_2,K_3,K_4,K_5,K_6
RAmy3C	*ORYsa;Amy1;7*	Isoform II-6	n.i.	L
RAmy3D, aAmy3	*ORYsa;Amy1;3*	Isoform II-4	F,G,H,I,J	H****
RAmy3E, aAmy8	*ORYsa;Amy1;4*	Isoform II-3	E	E_1,E_2
Amyc2	*ORYsa;Amy1;8*		n.i.	n.i.

*Price *et al.* (1996), ref. 20.
**Data from ref. 2.
***Not identified.
****Detected by polyclonal anti-H and monoclonal H-B35 antibodies.

N-Glycosylation is well established of an important modification in rice α-amylase,[14] however it does not affect the pI value.[15] It has been shown that the deamination of positive charged residues[16] and C-terminal processing[17] are key factors to result in multiple forms of cereal α-amylase. Calculation of the pI value with a bioinformatics software (GENETYX, Software Development Co., LTD.) indicated that the C-terminal processing of isoform II-5 (C-terminus Tyr438 to Lys427) is not enough to form the isoforms with a wide range of pI values (pI 4.69–6.20), and that one removal of a charged residue changes less than 0.1 pH unit only. Comparison of the peptide mass fingerprinting of isoform Ks showed no significant difference between their mass maps (data not shown). These strongly suggest the other candidates for these pI changes, such as ion binding and salt-bridge forming.[18] Further analyses with MS/MS might clarify the mechanism of isoform formation.

At present, we have identified and characterized more than 20 native α-amylase isoforms encoded by five genes, *RAmy1A*, *RAmy3B*, *RAmy3C*, *RAmy3D*, and *RAmy3E* (Table 4). In addition to the tissue-specific and stage-dependent expression of isoforms, it has been suggested that some α-amylase isoforms are expressed and localized in organelles.[19] To fully identify the corresponding relationship between genes and isoform proteins of rice α-amylase, we continue to investigate tissue-specific and organelle-localized α-amylase isoforms in rice.

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References

1) Mitsui, T., and Itoh, K., The α-amylase multigene family. *Trends Plant Sci.*, **2**, 255–261 (1997).
2) Mitsui, T., Yamaguchi, J., and Akazawa, T., Physicochemical and serological characterization of rice α-amylase isoforms and identification of their corresponding genes. *Plant Physiol.*, **110**, 1395–1404 (1996).
3) Mitsui, T., Ueki, Y., and Igaue, I., Biosynthesis and secretion of α-amylase by rice suspension-cultured cells: purification and characterization of α-amylase isozyme H. *Plant Physiol. Biochem.*, **31**, 863–874 (1993).
4) Nanjo, Y., Asatsuma, S., Mikami, S., Hori, H., Itoh, K., and Mitsui, T., Gibberellin stimulates the posttranscriptional processes of α-amylase II-4 expression in germinating rice seeds. *Plant Cell Physiol.*, **43**, s133 (2002).
5) Huang, N., Stebbins, G. L., and Rodriguez, R. L., Classification and evolution of α-amylase genes in plants. *Proc. Natl. Acad. Sci. USA*, **89**, 7526–7530 (1992).
6) Kim, J.-K., and Wu, R., Nucleotide sequence of a high-pI rice (*Oryza sativa*) α-amylase gene. *Plant Mol. Biol.*, **18**, 399–402 (1992).
7) Yu, S.-M., Lee, Y.-C., Fang, S.-C., Chan, M.-T., Hwa, S.-F., and Liu, L.-F., Sugars act as signal molecules and osmotica to regulate the expression of α-amylase genes and metabolic activities in germinating cereal grains. *Plant Mol. Biol.*, **30**, 1277–1289 (1996).
8) Miyata, S., and Akazawa, T., α-Amylase biosynthesis: signal sequence prevents normal conversion of the unprocessed precursor molecule to the biologically active form. *Proc. Natl. Acad. Sci. USA*, **79**, 7792–7795 (1982).
9) Mikami, S., Kishimoto, T., Hori, H., and Mitsui, T., Technical improvement to 2D-PAGE of rice organelle membrane proteins. *Biosci. Biotechnol. Biochem.*, **66**, 1170–1173 (2002).
10) Mitsui, T., Loboda, T., Itoh, A., and Ikarashi, T., Sugar-controlled Ca^{2+} uptake and α-amylase secretion in cultured cells of rice (*Oryza sativa* L.). *Plant Cell Physiol.*, **40**, 773–783 (1999).
11) Sutliff, T. D., Huang, N., Litts, J. C., and Rodriguez, R. L., Characterization of an α-amylase multigene cluster in rice. *Plant Mol. Biol.*, **16**, 579–591 (1991).
12) Itoh, K., Yamaguchi, J., Huang, N., Rodriguez, R. L., Akazawa, T., and Shimamoto, K., Developmental and hormonal regulation of rice α-amylase (*RAmy1A*)-*gusA* fusion genes in transgenic rice seeds. *Plant Physiol.*, **107**, 25–31 (1995).
13) Chen, P.-W., Lu, C.-A., Yu, T.-S., Tseng, T.-H., Wang, C.-S., and Yu, S.-M., Rice α-amylase transcriptional enhancers direct multiple mode regulation of promoters in transgenic rice. *J. Biol. Chem.*, **277**, 13641–13649 (2002).
14) Terashima, M., Kubo, A., Suzawa, M., Itoh, Y., and Katoh, S., The roles of the N-linked carbohydrate chain of rice α-amylase in the termostability and enzyme kinetics. *Eur. J. Biochem.*, **226**, 249–254 (1994).
15) Mitsui, T., and Akazawa, T., Secondary modification of carbohydrate chains in α-amylase molecules synthesized in rice scutellum. *Physiol. Vég.*, **24**, 629–638 (1986).
16) Sticher, L., and Jones, R. L., α-Amylase isoforms are post-translational modified in the endomembrane system of the barley aleurone layer. *Plant Physiol.*, **98**, 1080–1086 (1991).
17) Søgaard, M., Olsen, F. L., and Svensson, B., C-terminal processing of barley α-amylase 1 in malt, aleurone protoplasts, and yeast. *Proc. Natl. Acad. Sci. USA*, **88**, 8140–8144 (1992).
18) Jensen, M. T., Gottschalk, T. E., and Svensson, B., Differences in conformational stability of barley alpha-amylase isozymes 1 and 2. Role of charged groups and isozyme 2 specific salt-bridges. *J. Cereal Sci.*, **38**, 289–300 (2003).
19) Chen, M.-H., Liu, L.-F., Chen, Y.-R., Wu, H.-K., and Yu, S.-M., Expression of α-amylase, carbohydrate metabolism, and autophagy in cultured rice cells is coordinately regulated by sugar nutrient. *Plant J.*, **6**, 625–636 (1994).
20) Price, C. A., Reardon, E. M., and Lonsdale, D. M., A guide to naming sequenced plant genes. *Plant Mol. Biol.*, **30**, 225–227 (1996).