Presence and Activity of Various Amylases in Rice: Effect on Texture and Leached Sugar Composition during Cooking

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Amylase isozymes from non-glutinous japonica and indica rice types showed various band patterns, strengths and intensities based on zymography. Major bands were presumed to be α - and β -amylase according to their molecular weights; other amylases, 270 and 200 kDa in size, were also detected. Three debranching enzymes (DBEs) were detected from the dye pattern as well. Amylase activities were observed at pH 7.0 during rice cooking. However, the α -amylase activity at pH 7.0 was 50% lower than that at pH 5.0; DBE, β -amylase, and α -glucosidase values were similarly reduced by 25%, 90% and 99%, respectively. α -Amylase activity and DBE activities correlated positively with both the amount of malto-oligomer leached during rice cooking and the adhesiveness of cooked rice. Thus, it is considered that the activity of amylase isozymes affects the palatability of cooked rice. Therefore, enzymes from rice endosperm may represent a new and highly useful biomarker for evaluating the palatability of cooked rice.

Keywords: amylase, debranching enzyme, leached sugars, cooked rice, zymogram

Introduction

In general, the palatability of Japanese cooked rice is greatly affected by its texture; thus, sensory evaluation has long been used in assessing rice quality. The texture of cooked rice is affected by the chemical composition of the rice, mainly the amylose and protein content (Eksittikul *et al.*, 1993). However, it is difficult to compare the taste of new rice and old rice on the basis of chemical composition. The lipid content of raw rice can be used as a biomarker of freshness. Lipid deterioration is the main factor responsible for odor generation and the inhibition of gelatinization, which has an effect on the taste of cooked rice (Chikubu, 1995). However, it is difficult to compare the taste among rice types on the basis of lipid characteristics. In order to determine the palatability of rice, PCR-based DNA analysis has been carried out and a new taste biomarker for cooked rice is expected to be determined using this technique (Ohtsubo *et al.*, 2003).

Moreover, enzymes from milled rice endosperm are expected to function as a new biomarker for rice palatability.

For example, α - and β -amylases have been purified from milled rice; these enzymes are responsible for the production of oligosaccharides during rice cooking (Maruyama *et al.*, 1981a,b). However, no detailed analysis of the synergy between enzymes from the endosperm has yet been performed.

These enzymes (i.e., α - and β -amylases, debranching enzymes (DBEs), and α -glucosidase) are responsible for the conversion of biological materials and the generation of energy necessary for growth (Erwin and Paul, 1989). Multiple isoforms of α -amylase are present in rice. There are at least 10 α -amylase genes in rice (Huang *et al.*, 1992; Kim and Wu 1992; Yu *et al.*, 1996; Mitsui and Itoh, 1997). More than 20 native α -amylase isoforms have been identified and characterized in rice. Amylase from the endosperm of glutinous rice was reported to exhibit characteristics and activity that differ among rice cultivars (Tsuji *et al.*, 2007). β -Amylase is only synthesized in the aleurone cells, and its production in embryo-less half-seeds is not under hormonal control (Yamaguchi *et al.*, 1999). There are two types of DBEs, namely isoamylase and pullulanase, which have been purified from the developing rice endosperm (Fujita *et al.*, 1999; Nakamura *et al.*, 1996). These enzymes remove unnecessary α -1,6-glucosidic linkages at improper positions apart from the

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densely branched regions of a cluster of amylopectin (Nakamura, 2002). It was shown that the DNAs amplified by PCR, using primers designed for debranching enzymes (DBE), revealed marked differences between japonica and indica subspecies of rice (Nakamura *et al.*, 2004). α -Glucosidase has been purified from various rice cultivars (*Oryza sativa* L.), namely Nipponbare (Nakai *et al.*, 2007), Koshihikari (Awazuhara *et al.*, 2000), Onnemochi (Ito *et al.*, 1989), Shinsestsu (Takahashi *et al.*, 1971), and Yashiromochi (Yamasaki and Suzuki, 1997), and from the germinating seeds of Thai rice (Eksittikul *et al.*, 1993). The action of α -glucosidase may affect the accumulation of glucose during rice cooking (Mashashi *et al.*, 2010).

In this study, we determined the texture of cooked rice, the amount of leached sugars during rice cooking, and α -amylase, β -amylase, α -glucosidase and DBE activities from the zymograms, and reported on the relationships among them.

Materials and Methods

Rice samples and preparation Different varieties of non-glutinous short-grain japonica rice (i.e., Koshihikari, Nipponbare, Takitate and Chinese 294 (C294)), non-glutinous medium-grain japonica rice (i.e., M401 and Calrose), and non-glutinous long-grain indica rice (Thailand White Rice, TWR) were used. Rice samples were stored at 4°C until analysis. Before enzyme extraction, rice was milled using a mixer-type rice-milling machine (MR-D500 Twinbird Co., Tsubame, Japan) to obtain a 90% (degree of polish) milling yield. Rice samples were obtained directly from a wholesaler in Tokyo, Japan (Kitoku Shinryo Co., Ltd.). In this study, a variety of rice commonly consumed in Japan (i.e., Koshihikari, Nipponbare (standard) and Takitate (low amylose content)), USA (M202 and L205), China (Chinese 294) and Thailand (TWR) were selected.

Extraction of crude enzyme Milled rice was added to water in a ratio of 1:1.8 and the mixture was left at room temperature for 1 h. The mixture was homogenized (15,000 rpm, 3 min; NS-51K, Microtec Co., Chiba, Japan) and placed in a reciprocal shaker for 1 h. Then, the precipitate was removed by centrifugation at $18,000 \times g$ for 30 min. The supernatant was used as the crude enzyme for the determination of enzyme properties. All the processes were carried out at 4°C. Total protein content was measured by the Bradford assay (Bradford, 1976).

Detection of amylase on zymograms Amylase activity was detected by SDS-PAGE using a 10% polyacrylamide gel containing 1.0% soluble starch (Nacalai Tesque, Inc., Kyoto, Japan, following the procedure described by Laemmli (Laemmli, 1970), with some modifications. Electrophoresis

was carried out at 4°C, and SDS was removed using 2.5% Triton X-100 (Yamagata *et al.*, 1988). The gels were incubated at 37°C overnight in 100 mM McIlvaine buffer (pH 5.0) containing 10 mM CaCl_2 , and then stained with 0.01% (w/v) $\text{I}_2/0.1\%$ (w/v) KI solution. A clear band indicated amylase activity and a blue band indicated DBE activity.

Enzyme assay α -Amylase activity was assayed following the cereal α -amylase assay reagent method (Megazyme International Ireland, Wicklow, Ireland.) (McCleary and Sheehan, 1987; Sheehan and McCleary, 1988). After 60 min of incubation at 37°C (pH 5.0 and 7.0), the reaction was stopped. One unit (U) of α -amylase activity is defined as the amount of enzyme required to release one micromole of *p*-nitrophenol in 1 min under the defined assay conditions.

β -Amylase activity was assayed using a 500 μL reaction mixture containing 1.0% soluble starch, 200 mM McIlvaine buffer (pH 5.0 and 7.0), and an appropriate dilution of the enzyme preparation. After 30 min of incubation at 37°C, the reaction was stopped and the amount of residual sugars was measured by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952). One unit (U) of β -amylase activity is defined as the amount of enzyme required to release one micromole of reducing sugars in 1 min under the defined assay conditions.

α -Glucosidase activity was assayed using a 500 μL reaction mixture containing 5 mM *p*-nitrophenyl- α -D-glucopyranoside (Sigma-Aldrich Corp. St. Louis, MO, USA), 200 mM McIlvaine buffer (pH 5.0 and 7.0), and an appropriate dilution of the enzyme preparation. After 30 min of incubation at 50°C, the reaction was stopped by adding 100 mM Na_2CO_3 and the amount of *p*-nitrophenol released was monitored. One unit (U) of α -glucosidase activity is defined as described previously for α -amylase activity.

DBE activity was assayed using a 500 μL reaction mixture containing 1.0% pullulan, 200 mM McIlvaine buffer (pH 5.0 and 7.0), and an appropriate dilution of the enzyme preparation. After 30 min of incubation at 40°C, the reaction was stopped and the amount of residual sugars was measured by the Somogyi-Nelson method. One unit (U) of DBE activity is defined as the amount of enzyme required to release one micromole of maltotriose in 1 min under the defined assay conditions.

Leached sugars during rice cooking Leached sugars during rice cooking were measured using the method of Chikuu *et al.* (1995). Samples (8 g) in stainless-steel baskets (ϕ 40 $\times$ 75 mm) were boiled in 160 mL of pure water for 20 min, drained for 10 min, and then weighed. The height of each sample was measured immediately after weighing. Cooking water (200 mL) was added and total sugars were measured by the phenol-sulfuric acid method (Dubois *et al.*,

1956); the amount of residual sugar was also measured. The cooking water was freeze-dried and 80% ethanol-soluble carbohydrate was obtained. Oligosaccharides were labeled with 4-aminobenzoic acid octylester (ABOE) using an ABOE labeling kit (J-OIL MILLS, Inc. Tokyo, Japan). ABOE-derivatized oligosaccharides were separated by reverse-phase HPLC according to the manufacturer's instructions. The ABOE-glucose oligomer (J-OIL MILLS, Inc. Tokyo, Japan) was used as the standard (Ikekita *et al.*, 1997; Murata *et al.*, 1997a,b). Area ratio was calculated from the chromatogram of the total carbohydrate to determine the degrees of polymerization (DP), from 1 – 20.

Texture of cooked rice Milled rice samples (10 g) were placed in aluminum cups (ϕ 42 × 40 mm). Prior to cooking, rice was soaked in 16 g of pure water at room temperature (25°C) for 1 h, and then cooked in an ordinary electric rice cooker (SR-3100, National Co., Tokyo, Japan). Cooked rice samples were kept in the rice cooker for about 2 h at room temperature (25°C) before measurements. The hardness and stickiness of cooked milled rice samples were measured with a creep meter (RE2-33005 II, Yamaden Co., Tokyo). The conditions for measurements were as follows: using a stainless steel circular plunger (ϕ 40 mm) at a speed of 0.5 mm/s, samples were compressed at a clearance height of 17 mm. Each measurement was conducted 10 times (20 cups) and expressed as the average result.

Statistical analysis Analysis of variance was conducted using Statcel software (OMS Publishing Inc., Saitama, Japan). Data were subjected to analysis of variance followed by Fisher's PLSD test to compare means at $p < 0.05$. Pearson's correlation coefficient was also calculated (Yanai, 1998).

Results

Electropherogram of amylases in milled rice The amylase isozyme patterns of the different rice samples obtained by SDS-PAGE are presented in Figure 1. The amylase, indicated by a clear band, was resolved into four isoforms (I, II, III and IV) with estimated molecular weights of 270, 200, 140 and 44 kDa, respectively. DBE, which is indicated by a slightly darker band, was resolved into three isoforms (i, ii and iii) with molecular weights of approximately 75, 67 and 57 kDa, respectively. The isozyme pattern differed among samples as follows: DBE isozyme i was not detected in M401. Isozymes IV and I were not detected in TWR. In addition, band intensity varied among the samples. Amylase isozyme IV was only slightly observed in Koshihikari, Calrose and TWR rice varieties, but it was strongly present in all other samples. All amylase-associated bands in TWR were weakly observed compared to other samples.

Enzyme activity The activities of the evaluated en-

Fig. 1. Zymogram of amylases from milled non-glutinous japonica and indica rice varieties. Amylase activity was detected by SDS-PAGE containing 1.0% soluble starch. The gels were incubated at 37°C overnight in 100 mM McIlvaine buffer (pH 5.0) containing 10 mM CaCl_2 . They were stained with 0.01% (w/v) I2 / 0.1% (w/v) KI solution. A clear band indicated amylase activity and a blue band indicated DBE activity.

A = Koshihikari, B = Nipponbare, C = Takitate, D = Calrose, E = M401, F = TWR (Thailand white Rice)

zymes are shown in Table 1. Enzyme activity at pH 7.0 was examined during rice cooking. The α -amylase activity of milled rice ranged from 15 to 88 U/g; the Takitate rice variety showed the highest α -amylase activity among all the samples. On the other hand, at pH 5.0 the α -amylase activity ranged from 21 to 160 U/g; the Koshihikari variety presented the highest activity. In general, enzyme activities at pH 7.0 were lower than those at pH 5.0 for all samples. Differences in the taste of the cooked rice samples could be attributed to these results.

A narrow range of β -amylase activity of milled rice was observed: 0.053 – 0.089 U/g and 0.34 – 0.80 U/g at pH 7.0 and 5.0, respectively. The highest β -amylase activity was observed in Takitate and TWR varieties at pH 7.0, and for Koshihikari at pH 5.0. The activity at pH 7.0 was approximately 1/10 of that observed at pH 5.0. At pH 7.0, no difference in activity was observed among samples. A narrow range of α -glucosidase activity of milled rice was observed: 2.5 – 4.7 U/g and 207 – 243 U/g at pH 7.0 and 5.0, respectively. The activities at pH 7.0 were approximately 1/100 of those at pH 5.0, and no significant differences in α -glucosidase activity among samples were observed.

The DBE activity of milled rice ranged from 0.18 – 2.86 U/g at pH 7.0 and from 0.53 – 2.67 U/g at pH 5.0. The TWR variety presented significantly low DBE activity compared

Table 1. Comparison of enzyme activities for samples. Enzyme activity was examined at pH 7.0 during rice cooking and at pH 5.0 near optimum pH of the rice endosperm enzyme. Means in the same column with the same superscript are not significantly different at $p < 0.05$ by LSD test.

TWR = Thailand White Rice

	α -Amylase		β -Amylase		α -Glucosidase		Debranching Enzyme	
	pH5	pH7	pH5	pH7	pH5	pH7	pH5	pH7
Koshihikari	160 ^a	73 ^a	0.800 ^a	0.053 ^a	243 ^a	4.7 ^a	2.67 ^a	1.98 ^a
Nipponbare	121 ^b	67 ^{ab}	0.782 ^{ab}	0.063 ^a	207 ^b	3.2 ^a	1.91 ^b	1.85 ^a
Takitate	113 ^{bc}	88 ^{ac}	0.340 ^c	0.089 ^a	213 ^{abc}	4.3 ^a	2.14 ^{bc}	2.86 ^b
M401	144 ^d	72 ^{abd}	0.443 ^{cd}	0.071 ^a	216 ^{bd}	3.6 ^a	2.08 ^{bc}	2.30 ^c
Calrose	47 ^e	27 ^e	0.636 ^{abc}	0.069 ^a	221 ^{abce}	2.5 ^a	1.12 ^d	0.92 ^d
TWR	21 ^f	15 ^f	0.553 ^{def}	0.089 ^a	225 ^{bcdf}	3.0 ^a	0.53 ^e	0.18 ^e

U/g

to the other rice samples. These results demonstrate a trend similar to that for α -amylase activity. In contrast, the DBE activities of Takitate and M401 rice varieties were higher at pH 7.0 than at pH 5.0.

Composition of leached sugars during rice cooking In this experiment, the amount of leached sugars and texture were assessed for all samples. The results are shown in Table 2. The amounts of total sugars and reducing sugars ranged from 137.8 mg/g (M401) to 64.4 mg/g (TWR), and from 4.54 mg/g (M401) to 1.28 mg/g (TWR), respectively. Weight-average chain length (CLw) varied from 50.3 (TWR) to 20.5 (Nipponbare). Furthermore, the amount of malto-oligomer was measured as the amount of leached sugars from the 80% ethanol-soluble fraction obtained from cooked rice (Fig. 2). DP 1 (number-average degree of polymerization) corresponds to the peak area from each sample and showed a wide range, between 88% (TWR) to 32% (Takitate). The percentage of DP 2 – 5 was 30.8% – 10.3%, that of DP 6 – 10 was 28.8% – 1.4%, and that of DP 11 was 7.5% – 0%. The Takitate rice variety had the largest proportion of malto-oligomer content among all the samples, whereas TWR leached a small amount of malto-oligomer.

Table 2. The amounts of total sugars and reducing sugars leached during rice cooking were determined for all samples. Weight-average chain length (CLw) was obtained by dividing the amount of reducing sugars by the amount of total sugars. Means in the same column by the same superscript are not significantly different at $p < 0.05$ by LSD test.

Samples	Total sugars(mg/g)	Reducing sugars(mg/g)	CLw
Koshihikari	82.4 ^a	3.74 ^a	22.1
Nipponbare	76.6 ^{ab}	3.73 ^{ab}	20.5
Takitate	73.1 ^{bc}	2.22 ^c	32.9
M401	137.8 ^c	4.54 ^c	30.4
Calrose	82.6 ^{ad}	2.77 ^d	29.9
TWR	64.4 ^f	1.28 ^f	50.3

Texture of cooked rice The texture of cooked rice is described in Table 3. The hardness ranged from 1.78×10^2 N/m² for Takitate to 4.93×10^2 N/m² for TWR, while the adhesiveness ranged from 0.84×10^3 J/m³ for TWR to 27.7×10^3 J/m³ for Takitate.

Correlation The correlation coefficients between enzymes activities (pH 7.0), the amount of leached sugars during rice cooking and the texture of cooked rice are sum-

Fig. 2. Malto-oligomer composition in the 80% ethanol-soluble fraction of leached sugars from rice cooking.

A = Koshihikari, B = Nipponbare, C = Takitate, D = Calrose, E = M401, F = Thailand White Rice

*DP = Number-average degree of polymerization

Table 3. Textures of cooked rice samples. Means for each characteristic with the same superscript are not significantly different at $p < 0.05$ by LSD test.

Sample	Hardness ($\times 10^5 \text{ N/m}^2$)	Adhesiveness ($\times 10^3 \text{ J/m}^3$)	CLw
Koshihikari	2.11 ^a	19.2 ^a	22.1
Nipponbare	2.46 ^{ab}	16.1 ^{ab}	20.5
Takitate	1.78 ^{ac}	27.7 ^c	32.9
M401	2.44 ^d	9.8 ^{bde}	30.4
Calrose	2.52 ^{abd}	13.0 ^{abd}	29.9
TWR	4.93 ^e	0.84 ^f	50.3

marized in Table 4. No correlation was observed between the four enzymes and the amount of total sugars or reducing sugars leached during rice cooking. However, α -amylase activity correlated with the proportion of DP 5 – 2 (0.878, $p < 0.05$). DBE activity correlated with the proportions of DP 5 – 2 and DP 10 – 6 (0.909 and 0.829, $p < 0.05$, respectively).

Moreover, α -amylase and DBE activities correlated with rice hardness (-0.810 and -0.891 , $p < 0.05$, respectively).

DBE activity correlated positively with rice adhesiveness (0.817, $p < 0.05$). Rice adhesiveness correlated to the proportions of DP 10 – 6 and DP > 11 (0.889, $p < 0.05$ and 0.943, $p < 0.01$, respectively).

Discussion

In this study, based on the amylase zymogram of milled rice, 4 amylases and 3 DBE isozymes were detected as major bands. Isozyme IV may be the isoform of α -amylase in milled rice. Nanjo (2004) isolated and identified 10 α -amylase isoforms from germinating rice, and Sakamoto and Maruyama (1990) purified α -amylase from milled rice. Isozyme III may be the β -amylase purified from milled rice by Maruyama (1981). In addition, the isozymes i, ii and iii may be the isoamylases or pullulanases purified from the developing endosperm of rice by Fujita *et al.* (1991) and Nakamura *et al.* (1996). However, isozymes I and II are unknown. Thus, it is necessary in the future to examine the endosperm excluding its polymeric protein. The amylase isozymes varied in band pattern and intensity; the amylase bands of TWR were faint and few because the amylase activities were

weak. Shimizu *et al.* (1994) reported differences between brown rice cultivars in the activity of a specific amylase, and indicated that this could explain the relationship between the palatability of cooked rice and proper processing.

The activity of individual amylases in milled rice differs, and this affects the enzyme activities of rice grains during cooking. These activities were observed at neutral pH during rice cooking. In particular, the α -amylase activity at pH 7.0 was maintained at 50% to 70% of the activity observed at pH 5.0, and DBE activity was maintained above 80%, except for TWR, whose enzyme activity was approximately 30%. In contrast, the activities of α -glucosidase and β -amylase were approximately 1% and 10%, respectively, of those at pH 5.0. The decreases in α -amylase and DBE activities at pH 7.0 were smaller than those of α -glucosidase and β -amylase activities. Thus, it appears that α -amylase and DBE activities are induced, thereby decomposing milled rice to the state observed during cooking. Therefore, based on these results, α -amylase and DBE activities were significantly correlated with the proportion of malto-oligomer in the leached sugars during rice cooking and to the texture of the cooked rice. In addition, it is thought that endosperm amylases affect the structure of leached starch during rice cooking. Perhaps partially degraded starch affects the adhesiveness of cooked rice. Hanashiro *et al.* (2004) reported that in comparison with raw rice amylose and amylopectin, leached amylose and amylopectin in cooked rice have approximately 1/2 number-average degree of polymerization (DP), and that the chain-length distribution of amylopectin is essentially the same. Moreover, it has been reported that the smaller the amount of leached amylose, the higher the adhesiveness of cooked rice (Hanashiro *et al.*, 2004). Sato and Maruyama (2005) reported that the molecular weight distribution of leached total sugars during rice cooking is lower, and that when a higher concentration of these sugars occurs, the rice taste is evaluated as high.

Conclusion

We reported that the chain polysaccharide matrix structure of the rice endosperm cell wall is decomposed by the

Table 4. The correlation coefficients between the activities of all the enzymes (pH 7.0), amount of leached sugars during rice cooking (TS = total sugars, RS = amount of reducing sugars, CLw = weight-average chain length and >DP11, DP10 – 6, DP5 – 2, DP1 = 80% ethanol-soluble fraction of carbohydrate leached in residual liquid during cooking quality test) and the texture of cooked rice (H = hardness, A = Adhesiveness) are summarized. * = significant at $p < 0.05$, respectively

	TS	RS	CLw	>DP11	DP10 – 6	DP5 – 2	DP1	H	A
α -Amylase	0.326	0.565	-0.628	0.559	0.765	0.878*	-0.833*	-0.810*	0.795
β -Amylase	-0.244	-0.745	0.824*	-0.069	0.018	0.125	-0.054	0.456	-0.165
α -Glucosidase	0.567	0.733	-0.600	0.213	0.299	0.377	-0.340	-0.667	0.469
Debranching Enzyme	0.385	0.543	-0.593	0.658	0.829*	0.909*	-0.891*	-0.855*	0.817*

action of endosperm enzymes during rice cooking. Furthermore, rice freshness or cultivar affects the texture of cooked rice (Tsuji *et al.*, 2009).

In this study, we examined the relationships between endosperm amylases, the amount of sugars leached during rice cooking and the texture of cooked rice. Four isozymes from the rice endosperm, amylase and 3 debranching enzymes (DBEs) were detected by the amylase zymogram. The activity of these enzymes differed among rice cultivars. The endosperm amylases appear to decompose the endosperm starch during rice cooking, thereby producing adhesiveness. Assuming that the conformational change of endosperm starch during rice cooking is a major factor in cooked rice palatability, endosperm enzymes could be considered as a remarkable new biomarker for evaluating the palatability of cooked rice. Results from this study will be applied to the breeding of highly palatable rice.

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