Elution Behavior Analysis of Starch Degrading Enzymes During Rice Cooking with Specific Antibodies

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Rice grains contain starch degrading enzymes, including α-glucosidases, α-amylases, β-amylases, pullulanase and isoamylases. To investigate the elution behavior of these enzymes from rice grains into cooking water during rice cooking, we separated cooking water from rice grains after soaking (20°C) and when the temperature reached 40°C and 60°C during cooking. Immunological detection of these enzymes in rice grains and cooking water was carried out with SDS-PAGE and immunoblot. Bands corresponding to pullulanase, α-glucosidase, isoamylase 1, and α-amylase E were detected in both rice grains and cooking water at each temperature. Bands corresponding to α-amylases A + B and H were not detected in rice grains, but were detected in the cooking water at each temperature. The β-amylase band was detected in rice grains but was not detected in cooking water. These results suggest that the amount of enzyme eluted into cooking water depends on enzyme localization and quantity in rice grains.

Keywords: rice, starch degrading enzymes, immunoblot, rice cooking, elution

Abbreviations: PBSD medium, sodium phosphate buffer (pH 7.0) containing DTT and NaCl; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Rice (Oryza sativa) is a major food in Asian countries, including Japan. Extensive studies have been carried out to determine how a preferable taste can be achieved in rice cooking. It was found that physical properties such as stickiness and hardness (Maruyama, 1991; Kainuma, 1992), and chemical components such as sugars and amino acids contribute to the taste of cooked rice (Matsuzaki et al., 1992; Tajima et al., 1992; Ikeda, 2001). The amounts of sugars (reducing sugars and oligosaccharides) and amino acids correlate with the activities of starch-degrading enzymes and proteases, which are activated during cooking (Maruyama et al., 1981; Kasai et al., 2000; Maruyama, 2002).

The enzyme isoforms present in dormant rice, which degrade starch granules during germination, include: α-amylases (EC 3.2.1.1) (Mitsui et al., 1996; Yu et al., 1996), β-amylases (EC 3.2.1.2) (Matsui et al., 1975; Yamaguchi et al., 1999), isoamylases (EC 3.2.1.68) (Fujita et al., 1999), pullulanase (EC 3.2.1.41) (Takeuchi et al., 1999), and α-glucosidases (EC 3.2.1.20) (Takahashi et al., 1971; Matsui et al., 1988). Two studies demonstrated that rice grains contain more than 10 types of α-amylase isoforms (Daussant et al., 1983; Mitsui et al., 1996). These isoforms are classified into two groups on the basis of their optimum temperature: α-amylase I (70°C) and α-amylase II (37°C) (Mitsui et al., 1996). These α-amylase isoforms are remarkably induced during germination (Alpi and Beevers, 1983; Perata et
al., 1992, 1993) and play a major role in degrading native starch to oligosaccharides during rice seed development. β-Amylases catalyze the liberation of maltose from the non-reducing ends of 1,4-α-glucans in starch. β-Amylase activity is found in dormant and germinating rice grains (Shinke et al., 1973; Matsui et al., 1977; Okamoto and Akazawa, 1978). α-Glucosidases are enzymes that produce glucose from the non-reducing ends of 1,4-α-glucans in starch. At least three α-glucosidase isoforms (ONG1, ONG2-I, and ONG2-II) are present in the rice cultivar Nipponbare (Nakai et al., 2007). Nakai et al. also demonstrated that ONG2-II is the major isoform in dormant rice and is a post-translational isoform of ONG2-I. The existence of an ONG2-II-like α-glucosidase is implicated in Koshihikari (Awazuhara et al., 2000; Mabashi et al., 2009) and Yamadanishiki (Iwata et al., 2003). Isoamylases and pullulanase are enzymes that hydrolyze the α-1,6-glycosidic linkages of amylepectin. Both enzymes differ in their substrate specificity. Isoamylases can debranch glycogen, but rarely attack pullulan, while the pullulanase shows a reverse specificity. Several studies revealed that starch-debranching enzymes not only hydrolyze amylepectin in storage starch during germination, but also play essential roles in amylepectin biosynthesis (Ball et al., 1996; Nakamura 1996; Nakamura et al., 1996; Kubo et al., 1999).

In a previous study, we revealed the distribution of enzymes in dormant grains by employing a specific antibody against each enzyme, such as α-glucosidase (against an ONG2 specific peptide), α-amylase, and β-amylase (Tsuyukubo et al., 2010) antibodies. Having clarified the activity profiles of enzymes in rice grains (Mabashi et al., 2009), we encountered difficulty in measuring enzyme activity in cooking water, possibly due to a low abundance of enzymes. In this study, we separated cooking water protein from rice grain protein by trichloroacetic acid precipitation and investigated, using a specific antibody against each enzyme, whether these enzymes were eluted from rice grains into the cooking water during cooking.

Materials and Methods

Materials
Reagents and chemicals were purchased from the following commercial sources: acrylamide and protein marker from Daiichi Pure Chemicals (Tokyo, Japan), bis-acrylamide from Sigma (St. Louis, MO, USA), a protein assay kit from Bio-Rad (Richmond, CA, USA), and horse-radish peroxidase-labeled anti-rabbit and anti-mouse IgG antibodies and chemiluminescence reagent from GE Healthcare (Buckinghamshire, UK). All others were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise noted. Polyclonal antibodies against β-amylase and α-amylase A + B were kindly provided by Dr. Yamaguchi (Hokkaido University, Japan). The polyclonal antibody against isoamylase 1 was kindly provided by Dr. Nakamura (Akita Prefectural University, Japan).

Rice Sample
Brown rice (Oryza sativa L., cv. Koshihikari) was purchased from Uonuma in Niigata in 2008 and stored at 4°C until use. The brown rice was milled to 90 % with a polishing machine (MC-90A, Toyo Rice Cleaning Machine, Japan).

Preparation of crude enzyme extracts from rice grains and cooking water during rice cooking
Milled rice grains (100 g) were soaked in distilled water (1 rice:1.5 water) without washing for 1 h at 20°C. The samples were cooked in an electric rice cooker (SR-03F, Matsushita, Japan) connected to a volt slider (V-130-5, Yamabishi, Japan) to control the heating power, ensuring that the rice was brought to boil in 11 min. Figure 1 shows the temperature course during rice cooking and the temperature range at which starch degrading enzymes are activated (Matsui et al., 1996; Matsui et al., 1975; Fujita et al., 1999; Takeuchi et al., 1999; Takahashi et al., 1971) during cooking. After soaking at 20°C for 1 h and turning off the heat when the temperature reached 40°C and 60°C, the mixture of rice and cooking water was separated into rice grain and cooking water, as shown in Figure 1. Both fractions were freeze-dried. The dried rice grains (2 g as raw rice) were ground and suspended in 20 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM dithiothreitol (DTT), 100 mM NaCl and 0.25 % SDS (PBS medium). The freeze-dried cooking water fraction was suspended in

![Fig. 1. Summary of the temperature course during cooking and starch degrading enzyme activity.](image-url)

Sampling points for analyzing the elution behavior are indicated with arrows. Time course of rice cooking: 0 − 60 min; soaking, 60 min; heat on, 60 − 71 min; heating, 71 − 84 min; boiling, 84 min; heat off. The dotted lines indicate the range of temperatures for enzyme activation: (1) 20 − 30°C; α-amylase II-3, (2) 20 − 40°C; α-amylase II-4, β-amylase, ISA1, and pullulanase, (3) 20 − 60°C; α-glucosidase, (4) 20 − 70°C; α-amylase I.
100 mL of the same buffer. Proteins in both fractions were extracted by shaking the suspended mixtures at 20°C for 1 h. The mixtures were centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatants were subjected to filtration by aspiration. Next, 100 μL of 100% (w/v) ice-cold trichloroacetic acid (TCA) was added to 900 μL of the filtered supernatants, and the mixtures were kept on ice for 30 min. The mixtures were then centrifuged at 12,000 rpm for 15 min at 4°C. The resultant pellets were washed twice with ethanol, and the precipitated proteins were dissolved in 50 mM sodium phosphate buffer (pH 7.0) and used as crude enzyme extracts of the rice grains and cooking water. Additionally, crude enzyme extracts were prepared from the raw brown rice flour sample, 100 to 90% and 90 to 0% rice flour milled fractions, as described previously (Tsuyukubo et al., 2010).

Protein assay The total protein of crude extracts was assayed using a Bio-Rad protein assay kit with bovine serum albumin solution as the standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE was carried out with 7.5% and 10% gels (Laemmli, 1970), and proteins were stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid. For this analysis, crude enzyme extracts from rice grains, which were equivalent to 22.5 mg raw rice grains, were loaded. Since cooking water included less enzymes, crude enzyme extracts from cooking water that were equivalent to 225 mg raw rice grains were loaded. Moreover, 25 mg of whole grains, 2.5 mg of 100-90% rice flour and 22.5 mg of 90-0% rice flour were loaded.

α-Glucosidase and pullulanase purification and antibody production Rice flour (400 g) was extracted with 1,000 mL of 20 mM sodium acetate buffer (pH 5.0) containing 5 mM DTT and 100 mM NaCl at 4°C for 15 h, then centrifuged at 10,000 x g for 30 min at 4°C. α-Glucosidase was isolated from the precipitates by ammonium sulfate fractionation between the limits of 25 to 70% saturation. The precipitates were dialyzed against 20 mM sodium acetate buffer (pH 5.0) containing 1 mM DTT, filtered through a 0.45 μm filter (Advantec, Japan) and loaded on a HiTrap SP FF cation-exchange column (GE Healthcare) equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 1 mM DTT and 0.35% Empigen (Sigma). Absorbed proteins were eluted with 50 mM HEPES-NaOH buffer (pH 8.2) containing 1 mM DTT and 0.35% Empigen, and then with 50 mM HEPES-NaOH buffer (pH 8.2) containing 1 mM DTT, 0.35% Empigen and 1 M NaCl. The NaCl eluted fraction was loaded on a Mono S 5/5 cation-exchange column (GE Healthcare) after buffer exchange against 20 mM Bicine-NaOH buffer (pH 9.0). Fractionation was carried out with a linear gradient between 0 and 0.5 M of NaCl in the same buffer, at a flow rate of 0.4 mL/min, and 20 fractions were collected. The α-glucosidase activity of each fraction was determined by the method using p-nitrophenyl-α-D-glucopyranoside as a substrate. From the 20 fractions, two active fractions were combined and dialyzed against 20 mM sodium phosphate buffer (pH 7.0), and concentrated up to 1 mg/mL with Nanosep 30K Omega (Nihon Pall, Tokyo, Japan). This enzyme solution was used to immunize rabbits at Kojin-Bio services (Saitama, Japan).

The pullulanase was purified using the pH 8.2 eluted fraction from the HiTrap SP column. This fraction was loaded on a Resource Q 5/5 anion-exchange column (GE Healthcare) after buffer exchange against 25 mM Tris-HCl buffer (pH 8.0). The fractionation was conducted with a linear gradient between 0 and 0.5 M of NaCl in the same buffer, at a flow rate of 1.0 mL/min, and 12 fractions were collected. The fractions were analyzed with a SDS-PAGE 10% gel and stained with Coomassie Brilliant Blue. Fractions containing a 100 kDa band were collected and used for antibody production as described above. The 100 kDa band sequence was confirmed by MALDI-TOF/TOF analyzer (ABI 4800 Plus, CA, USA) as previously described (Mabashi et al., 2009).

Immunoblot For immunoblots, the electrophoresed gels were equilibrated with a blotting buffer (50 mM Tris, 5 mM glycine, 0.037% SDS). The proteins were then transferred to nitrocellulose membranes (Bio-Rad) with a transblotting system (NIHON EIDO, Tokyo, Japan) (Towbin et al., 1979). The transferred membranes were incubated in 20 mM Tris buffered saline (TBS, pH 7.5) containing 0.1% Tween-20 and 5% skim milk for blocking. The transferred membranes were reacted with specific antibodies: anti-rice α-glucosidase (1:10,000 dilution), anti-α-amylase {isoforms A + B (1:300 dilution), H (1:500 dilution) and E (1:500 dilution) (Mitsui et al., 1996)}, anti-β-amylase (1:100 dilution) (Yamaguchi et al., 1999), anti-pullulanase (1:10,000 dilution) and anti-isoamylose 1 (ISA1) (1:500 dilution) (Fujita et al., 1999). A horse-radish peroxidase-labeled anti-rabbit or anti-mouse IgG antibody and a chemiluminescence reagent were used to detect immunoreactive bands with a LAS-4000 molecular imager (FUJIFILM, Tokyo, Japan).

Results and Discussion Localization of pullulanase, isoamylase I, α-glucosidase and α-amylase E in rice grains Localization of the pullulanase, isoamylase 1 (ISA1), α-glucosidase and α-amylase E was investigated with each specific antibody in rice grains. Enzyme extracts from rice grain flour fractions were subjected to immunoblot analysis on 7.5 and 10% polyacrylamide gels.
Figure 2A shows the result with the anti-pullulanase antibody. Bands corresponding to pullulanase, which has an estimated molecular weight of 100 kDa, are indicated with an arrowhead. The pullulanase band was detected in all fractions, particularly in the 70–0% fraction (endosperm).

Figure 2B shows the immunoblot result with the anti-ISA1 antibody. Bands corresponding to ISA1, which has an estimated molecular weight of 83 kDa, are indicated with an arrowhead. The ISA1 band was detected in all fractions.

Figure 2C shows the immunoblot result with the anti-α-glucosidase antibody. The band corresponding to α-glucosidase, which has an estimated molecular size of 95 kDa, is indicated with an arrowhead. The α-glucosidase band was detected in all fractions, particularly in the 70–0% fraction (endosperm). This result is consistent with a previous result, in which an antibody against ONG-2-specific peptide was used (Tsuyukubo et al., 2010). In that report, we also showed that α-amylase type I and II-4 isoforms were localized in 100 to 80% flour fractions. In providing reducing glucose ends as an α-glucosidase substrate in rice endosperm, we presumed the presence of another α-amylase isoform in the rice grains. In rice seed maturation, mRNA expression of α-amylase E (Amy3E) and α-amylase D (Amy3D) was augmented at 15 days after flowering by high temperature treatment (Yamakawa et al., 2007). We next investigated whether the α-amylase E isoform remained in mature rice grains or not.

Figure 2D shows the result with the anti-α-amylase E (α-amylase class II-3) antibody. Bands corresponding to α-amylase E, which has an estimated molecular size of 42 kDa, are indicated with an arrowhead. The α-amylase E band was detected in all flour fractions. In contrast with the localization of type A + B and H, we first detected the α-amylases isoforms (type II-3) in mature rice endosperm.

**Elution behavior of pullulanase, isoamylase 1 and α-glucosidase in rice grains during rice cooking**

Elution behavior of the pullulanase, isoamylase 1 (ISA1) and α-glucosidase from rice grains into cooking water at each temperature was investigated with anti-pullulanase, anti-ISA1, and anti-α-glucosidase antibodies. Enzyme extracts from rice grains and cooking water at each temperature were subjected to immunoblot analysis on 7.5% polyacrylamide gels. Figure 3A, 3B and 3C show the results with the anti-pullulanase antibody.

The pullulanase band was detected in both the rice grains (Fig. 3A) and cooking water during cooking (Fig. 3B). Figure 3C shows the result of whole (lane 7), 100-90% (lane 8) and 90–0% flour fractions (lane 9). While pullulanase was mainly localized in the 90–0% inner fraction, elution of pullulanase from rice grains to cooking water was observed at each temperature. The band intensity in Fig. 3A, 3B and 3C suggested that the amount of pullulanase in rice grains was greater than in cooking water.

Considering that pullulanase exhibits optimal activity at 40°C in Nipponbare (Takeuchi, 2002) and is stable around 55°C in Hinohikari (Yamasaki et al., 2008), we suggest that pullulanase hydrolyzes the α-1,6-glycosidic linkages of amyllopectin in rice grains at a temperature range of 40–55°C.

Figure 3D, 3E and 3F show the immunoblot results with the anti-ISA1 antibody. The ISA1 band was detected in both the rice grains (Fig. 3D) and cooking water during cooking (Fig. 3E). Figure 3F shows the immunoblot result of whole (lane

![Fig. 2. Immunolocalization of the starch-degrading enzymes in dormant rice grains.](Image)

Immunoblot analysis of the pullulanase (A), isoamylase 1 (B), α-glucosidase (C) and α-amylase E (D) in each fraction of rice grains (lane 1, whole rice flour equivalent to 2.5 mg raw rice; lane 2, 100–90% rice flour equivalent to 2.5 mg raw rice; lane 3, 90–80% rice flour equivalent to 2.5 mg raw rice, lane 3; 80–70% rice flour equivalent to 2.5 mg raw rice, lane 4; 80–70% rice flour equivalent to 2.5 mg raw rice, lane 5; 70–0% rice flour equivalent to 17.5 mg raw rice). Lane 6 is reacted with only the secondary antibody as a negative control. Purified enzymes are loaded onto lanes 7 as a positive control.
amounts at 40°C (lane 5) and 60°C (lane 6) were less than that at 20°C (lane 4). In our preliminary experiments, recovery of proteins from a rice starchy solution at 70°C was significantly less than that at 60°C. These results suggested that at > 60°C, α-glucosidase become trapped in the gelatinized starch. The band intensity of Fig. 3G, 3H and 3I suggested that the amount of α-glucosidase in rice grains was greater than in cooking water. Considering that ISA1 exhibits optimal activity at 30°C and is inactivated at 50°C (Fujita et al., 1999), we suggest that ISA1 hydrolyzes amylopectin α-1,6-glycosidic linkages in rice grains mainly at a temperature range of 30 – 40°C.

Figure 3G, 3H and 3I show the immunoblot results with the anti-α-glucosidase antibody. The α-glucosidase band was detected both in rice grains (Fig. 3G) and cooking water at each temperature (Fig. 3H). Figure 3I shows the immunoblot result of whole (lane 7), 100 – 90% (lane 8) and 90 – 0% flour fractions (lane 9). While α-glucosidase was predominantly localized in the 90 – 0% fraction, elution of some α-glucosidase from rice grains into the cooking water was observed at each temperature (Fig. 3H); however, the eluted amounts at 40°C (lane 5) and 60°C (lane 6) were less than that at 20°C (lane 4). In our preliminary experiments, recovery of proteins from a rice starchy solution at 70°C was significantly less than that at 60°C. These results suggested that at > 60°C, α-glucosidase become trapped in the gelatinized starch. The band intensity of Fig. 3G, 3H and 3I suggested that the amount of α-glucosidase in rice grains was greater than in cooking water. In a previous study, the amounts of solids and total sugars extracted from rice grains increased with increasing cooking temperature (Ohisi et al., 2006). Awazuhara et al. reported that the optimum temperature of α-glucosidases in Koshihikari was 60°C (Awazuhara et al., 2000). In reference to this information, we suggest that glucose production by α-glucosidase mainly occurs in rice grains when the temperature reaches approximately 60°C.

Elution behavior of α-amylases (I, II-3, and II-4) and β-amylase in rice grains during rice cooking The elution behavior of α-amylases from rice grains into cooking water at each temperature was investigated with anti-α-amylase anti-
bodies. Enzyme extracts from rice grains and cooking water at each temperature were subjected to immunoblot analysis on 10% polyacrylamide gel. Figure 4 shows the results with the anti-α-amylase A + B (α-amylase class I), anti-α-amylase H (α-amylase class II-4), anti-α-amylase E (α-amylase class II-3) and anti-β-amylase antibodies. Bands corresponding to α-amylase A + B and H, which have an estimated molecular size of 44 kDa, are indicated with an arrowhead (Fig. 4C and 4F). The α-amylase E band, which has an estimated molecular size of 42 kDa, is indicated with an arrowhead (Fig. 4I). The β-amylase band, which has an estimated molecular size of 53 kDa, is indicated with an arrowhead (Fig. 4J).

The α-amylase band recognized by the anti-α-amylase A + B antibody was not detected in rice grains (Fig. 4A), but was detected in the cooking water at each temperature (Fig. 4B). These isoforms are classified as α-amylase type I, which consists of isoforms A, B, Y, and Z (Mitsui et al., 1996). In a previous study, we showed that α-amylase type I was predominantly localized in the 90 to 80% rice flour fraction (Tsuyukubo et al., 2010). This fraction corresponds to the outer layer of the 90% milled rice. Since α-amylase type I, found in the outer layer, eluted into the cooking water at each temperature, we suggest that this enzyme contributes minimally to the degradation of starch into oligosaccharides in rice grains.

The α-amylase band recognized by the anti-α-amylase H antibody was barely detected in rice grains, but was detected in the cooking water (Fig. 4D and 4E) at each temperature. These isoforms are classified as α-amylase type II-4, which consists of isoforms F, G, H, I, and J. In a previous study, we showed that α-amylase type II-4 was predominantly localized in the 100 to 90% and 90 to 80% rice flour fractions (Tsuyukubo et al., 2010). Since α-amylase type II-4, found in the outer layer, eluted into the cooking water at each temperature, we suggest that this enzyme contributes minimally to the degradation of starch into oligosaccharides in rice grains.

The α-amylase band recognized by the anti-α-amylase E antibody was detected in both rice grains and cooking water.

Fig. 4. Elution behavior of α-amylases and β-amylase during rice cooking.

Immunoblot analyses of the α-amylase types I (A – C), II-4 (D – F), II-3 (G – I) and β-amylase (J – L). (A, D, G, J) Immunoblot analysis of the α-amylases and β-amylase in rice grains (equivalent to 22.5 mg raw rice) at each temperature during cooking (lane 1, rice grains at 20°C; lane 2, rice grains at 40°C; lane 3, rice grains at 60°C). Molecular sizes are indicated on the left. (B, E, H, K) Immunoblot analysis of the α-amylases and β-amylase in cooking water (equivalent to 225 mg raw rice) at each temperature during cooking (lane 4, cooking water at 20°C for one hour; lane 5, cooking water at 40°C; lane 6, cooking water at 60°C). (C, F, I, L) Immunoblot analysis of the α-amylases and β-amylase in each fraction of rice grains (lane 7, whole rice flour equivalent to 25 mg raw rice; lane 8, 100 – 90% rice flour equivalent to 2.5 mg raw rice; lane 9, 90 – 0% rice flour equivalent to 22.5 mg raw rice). Arrowheads indicate the corresponding bands of α-amylases and β-amylase.
Enzymes in rice grains into cooking water during rice cooking might occur in such a bound form, resulting in minimal elution into the cooking water. Elution of α-amylase type II-3 into cooking water was observed at 20°C (Fig. 4H, lane 1), but the eluted amounts did not significantly increase at 40 and 60°C (Fig. 4H, lanes 2 and 3). The optimal temperature for α-amylase E is reportedly 26°C (Mitsui et al., 1996). This temperature dependence difference may not exclude the possibility of another type of α-amylase (high temperature optimum) in dormant endosperm.

Figure 4L shows the immunoblot result of the whole (lane 7), 100 – 90% (lane 8) and 90 – 0% flour fractions (lane 9). While β-amylase was ubiquitously distributed in rice grains, the β-amylase band recognized by the anti-β-amylase antibody was detected in rice grains, but not in the cooking water, at each temperature in this experiment (Fig. 4J and 4K). Although β-amylase is reported to be widely distributed in dormant rice grains (Tsuyukubo et al., 2010), it was not eluted into the cooking water, remaining in the rice grains. In barley, a β-amylase was reported to occur in bound form on starch granules in dry seeds, and was not released by saline extraction (Hara-Nishimura et al., 1997). The rice β-amylase might occur in such a bound form, resulting in minimal elution into the cooking water.

In this study, we revealed the elution of starch degrading enzymes in rice grains into cooking water during rice cooking. We first uncovered that pullulanase, ISA1, α-glucosidase and α-amylases were eluted from rice grains into the cooking water, while β-amylase was barely eluted during rice cooking. We also found that the elution of enzymes in rice grains occurred during soaking at 20°C, without heating. Considering a previous report that reducing sugars were significantly produced between 40 – 60°C during rice cooking (Kasai et al., 2000), we conclude that the increase in reducing sugars during rice cooking is mainly achieved by α-glucosidase and pullulanase after oligosaccharide formation by α-amylase E, β-amylase, and ISA1 in rice grains. To the best of our knowledge, this is the first report to describe the elution of starch degrading enzymes from rice grains into cooking water during rice cooking using specific antibodies. Knowledge gained regarding the distribution of these enzymes during cooking (in rice grains or in cooking water) will lead to the following future applications:

1. An optimal temperature-time schedule for each rice cultivar.
2. A new delicious cultivar with molecularly arranged starch degrading enzymes.
3. An optimal polishing, water absorption and steaming strategy for delicious sake brewing.

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References
Kubo, A., Fujita, N., Harada, K., Matsuda, T., Satoh, H. and Naka-


