Different Distributions of α−Glucosidases and Amylases in Milling Fractions of Rice Grains

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The distribution of starch-degrading enzymes in dormant rice grains was investigated with specific antibodies against α-glucosidases, α-amylases, and β-amylases. The α-glucosidases were predominantly localized in the inner endosperm. The α-amylases were mainly localized in the outer layers, and the β-amylases were distributed in whole grains. We propose a model to demonstrate how these enzymes degrade starch during rice cooking.

Keywords: α-glucosidase, α-amylase, β-amylase, immunoblot, rice cooking

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 reported that physical properties such as stickiness and hardness (Okabe, 1979; Maruyama, 1991; Kainuma, 1992; Okadome et al., 1996; Okadome et al., 1999), 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). However, it remains unclear which enzyme isoforms play a pivotal role in the sequential degradation of starch granules during rice cooking.

The isoforms that exist in dormant rice and degrade starch granules during germination are 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), pullulanases (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). The α-amylase is an endoamylase that catalyzes the random hydrolysis of internal 1,4-α-glucans of starch and releases oligosaccharides. Two studies demonstrated that rice grains contained 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 temperatures: α-amylase I (37℃) and α-amylase II (70℃) (Mitsui et al., 1996). These α-amylase isoforms are remarkably induced during germination (Alpi and Beevers, 1983; Perata et al., 1992; Perata et al., 1993) and play a major role in degrading native starch to oligosaccharides during rice seed development. The 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 isoforms of α-glucosidases (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).

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Enzyme assays in the dormant rice revealed the distribution of enzyme activity: α-glucosidases in the inner endosperm, α-amylases in the outer layers, and β-amylases in whole grains (Awazuahara et al., 2000; Iwata et al., 2002). However, the particular distribution of each enzyme isoform in dormant grains is unclear.

In this study, we focused on the distribution of α-glucosidases, α-amylases, and β-amylases. Additionally, we attempted to visualize the localization of these enzymes in dormant grains with a specific antibody against each enzyme. We analyzed how the localization of enzymes and their sequential activation affect the production of reducing sugars during rice 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 horseradish peroxidase-labeled anti-rabbit IgG antibody (HRP) and chemiluminescence reagent from GE Healthcare (Buckinghamshire, UK). Others were purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise noted. Polyclonal antibodies against α-amylase H, α-amylase A + B, and β-amylase were kindly provided by Dr. Mitsui (Niigata University, Japan) and Dr. Yamaguchi (Hokkaido University, Japan).

Preparation of rice flour Brown rice (Oryza sativa L., cv. Koshihikari) was purchased from Uonuma in Niigata in 2008 and stored at 4°C until use. Brown rice flour samples (hereafter designated as whole rice flour) were prepared by grinding rice grains with an electric mill (WB-1, Osaka Chemical, Japan). The brown rice was fractionated into four parts (100 to 90% rice flour, 90 to 80% rice flour, 80 to 70% rice flour, and 70 to 0% rice flour) by dry-milling. The 100 to 90% rice flour was obtained by milling brown rice up to 90% with a polishing machine (MC-90A, Toyo Rice Cleaning Machine, Japan). The remaining polished rice grain was scraped off up to 70% using a grain testing mill (TM05, Satake Engineering Co., Ltd., Tokyo, Japan). The first 10% fractions were designated as 90 to 80% rice flour, and the later 10% fractions were designated as 80 to 70% rice flour. The 70 to 0% rice flour was prepared by milling the remaining endosperm of the grains with the electric mill. Each material was sieved through a 500 μm mesh.

Preparation of crude enzyme extracts Each rice flour (3 g) was suspended in 30 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM dithiothreitol (DTT) and 100 mM NaCl (PBS medium), and extracted by shaking the mixtures for 15 h at 4°C. The solution was centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatants were then filtered through filter paper (No. 2, Advantec, Tokyo, Japan). 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 pellets were washed twice with ethanol, and the precipitated proteins were dissolved into 50 mM sodium phosphate buffer (pH 7.0) and used as crude enzyme extracts. Purified α-glucosidase was obtained as previously described (Mabashi et al., 2009).

Preparation of germinating rice seeds and their crude enzyme extracts Crude enzyme extracts from germinating seeds were prepared as follows. Brown rice seeds were soaked in a 5% NaClO solution for 15 min. After extensive washing in distilled water, the seeds were germinated on filter paper moistened with distilled water in a plastic dish in the dark at 30°C for 8 days. The germinating seeds were homogenized in PBS medium using a polytron homogenizer. Concentrated crude enzyme extracts were obtained using the procedure described above. These extracts were used as positive controls for α-amylases and β-amylases in immunoblots.

Protein assay The total protein of the crude extract was assayed using a Bio-Rad protein assay kit with bovine serum albumin solution as a standard (Bradford, 1976).

Polyclonal antibody production An antibody against α-glucosidase was raised in rabbits with a synthetic peptide (QDVIPRPSPDSFLA), which corresponded to the 99th through 112th amino acid residues for ONG2 α-glucosidase (Nakai et al., 2007). This peptide was conjugated to keyhole limpet hemocyanin through an extra cystein residue at its N-terminus. The antibody was purified by affinity chromatography with the immobilized synthetic peptide onto Sepharose (T-K craft, Gunma, Japan).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot Sodium dodecyl sulfate polyacrylamide gel electrophoresis (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 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 membrane was incubated in 20 mM Tris buffered saline (TBS, pH 7.5) containing 0.1% Tween-20 and 5% skim milk for blocking. The transferred membrane was re-
acted with specific antibodies: anti-rice α-glucosidase ONG2 (1:5000 dilution), anti-α-amylase isoform A + B (1:300 dilution) and isoform H (1:500 dilution) (Mitsui et al., 1996), and anti-β-amylase (1:100 dilution) (Yamaguchi et al., 1999). An HRP-labeled antibody and a chemiluminescence reagent were used to detect immunoreactive bands with a LAS-4000 molecular imager (Fujifilm, Tokyo, Japan).

Results

Localization of α-glucosidases in milling fractions of rice grains The distribution of α-glucosidases in rice grains was analyzed with SDS-PAGE and immunoblot. For this analysis, 10 mg of crude enzyme extracts from each fraction of rice flour was loaded and separated on 7.5% polyacrylamide gels. Figure 1 presents the results of SDS-PAGE and the immunoblot experiment with an anti-α-glucosidase antibody. The purified α-glucosidase antibody recognized 1 μg of the purified α-glucosidase (lane 7), which had an estimated molecular weight of 95 kDa. Bands of the same molecular weight were detected in the lanes of crude enzyme extracts from each fraction (arrows in Fig. 1B). The visualized bands on each crude enzyme extract were consequently identified as α-glucosidases in rice grains.

We found that the inner endosperm layer of rice grains (70 to 0% rice flour) had the highest amounts of α-glucosidases in each fraction (Fig. 1B), and the amount gradually decreased toward the outer layers in grains. In the outer layer of 100 to 90% rice flour (rice bran), α-glucosidases were hardly detected in this experiment.

Localization of α-amylases in milling fraction of rice grains The distribution of α-amylases in rice grains was analyzed with anti-α-amylase antibodies. For this analysis, 15 mg of enzyme extracts from each fraction of rice flour was loaded and separated on 10% polyacrylamide gels. Figure 2 presents the results of SDS-PAGE and the immunoblot experiment with α-amylase antibodies (anti-A + B and anti-H). The α-amylases, which are hardly detected in dormant rice grains, are reportedly induced during germination (Mitsui et al., 1996). We thus prepared positive control samples from germinating seeds. The anti-α-amylase antibodies recognized the band from the germinating seeds (lane 7), which had an estimated molecular weight of 44 kDa. Bands of the same molecular weight were detected in the lanes of crude enzyme extracts from each fraction (arrows in Figs. 2B and 2C). The visualized bands on each crude enzyme extract were consequently identified as α-amylases in rice grains.

The α-amylase isoforms recognized by anti-α-amylase isoforms A + B and isoform H were localized in the outer layers of 100 to 90% and 90 to 80% rice flours. The α-amylase isoforms recognized by anti-α-amylase isoforms

![Fig. 1. α-Glucosidases are predominantly localized in the inner endosperm layers.](image-url)

(A) Crude extracts from each fraction of rice grains were separated with SDS-PAGE (lane 1, whole rice flour; lane 2, 100 to 90% rice flour; lane 3, 90 to 80% rice flour; lane 4, 80 to 70% rice flour; lane 5, 70 to 0% rice flour; lane 6, whole rice flour). As a positive control, 1 μg of purified α-glucosidase was loaded in lane 7. Molecular size markers are indicated on the left side. (B) Immunoblot analysis of the α-glucosidase in rice grain fractions. Lane 6 was probed with only a HRP-labeled antibody. An arrowhead indicates bands of α-glucosidases.
The immunoreactive bands of β-amylases were found in all fractions (Fig. 3B). The β-amylases were widely localized from the outer layers to the inner layers of rice grains. The 100 to 90% rice flour contained more β-amylases than other fractions. In Fig. 3B, the bands indicated by arrows are non-specific bands with the HRP-labeled antibody.

Discussion

During rice cooking, glucose is liberated from starch granules by starch-degrading enzymes, including α-glucosidases, α-amylases, and β-amylases. To visualize the distribution of those enzymes in rice grains, we conducted immunoblot analyses of α-glucosidases, α-amylases, and β-amylases.

Three isoforms of α-glucosidases (ONG1, ONG2-I, and ONG2-II) were purified from dormant rice grains (Nakai et al., 2007). Nakai et al. also demonstrated that ONG2-II is dominant in dormant grains (cultivar Nipponbare) and can hydrolyze the α-1,4-glucosidic linkage of maltose and starch to produce glucose. In addition, several studies have demonstrated that other rice cultivars contained ONG2-II-like α-glucosidase, on the basis of their substrate specificity and the N-terminal amino-acid sequence (Takahashi et al., 1971; Matsui et al., 1988; Takeuchi, 2002; Iwata et al., 2003). In our previous study, we purified ONG2-II from the cultivar Koshihikari (Mabashi et al., 2009). In the present study, we prepared an anti-α-glucosidase antibody against the N-termi-
α-glucosidases in dormant grains are predominantly distributed in the inner endosperm. Several studies have found that the outer layer contained a large amount of proteins and that storage compounds such as starch were predominantly located in the inner endosperm of rice grains (Barber, 1972; Kennedy et al., 1974; Koga et al., 1996; Okuda et al., 2007). Abundant localization of α-glucosidases in the inner endosperm might be required to effectively degrade starch to glucose upon germination.

The α-amylases exhibited a different distribution from the α-glucosidases. The α-amylase isoforms were confined to the outer layers of rice grains (Fig. 2). The α-amylase isoforms recognized by anti-α-amylase isoforms A + B were predominantly localized in the 90 to 80% rice flour fraction. The α-amylase isoforms recognized by anti-α-amylase isoform H were mainly localized in the 100 to 90% and the 90 to 80% rice flour fractions. The former isoforms are classified as α-amylase I, which consists of isoforms A, B, Y, and Z; the latter isoforms are classified as the α-amylase II, which consists of isoforms F, G, H, I and J (Mitsui et al., 1996).

The optimum temperature of α-amylase I is 70℃, and that of α-amylase II is 28 to 37℃. Iwata et al. (2002) reported that the α-amylase activity of dormant grains was restricted to the outer layers. Awazuhara et al. (2000) reported that the α-amylase isoforms with high and low optimum temperatures were mainly localized in the outer layer. In addition, they reported that the latter isoform was likely to be isoform α-glucosidases in dormant grains are predominantly distributed in the inner endosperm.

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G, judging from the isoelectric point. In this study, our immunological data confirmed these previous results and visualized the spatial distribution of α-amylase isoforms.

The β-amylases in rice grains were detected in all fractions (Fig. 3). This finding differed from the results for other starch-degrading enzymes (e.g., α-glucosidases and α-amylases). Past studies reported that β-amylases were hardly detected in dormant rice grains (cultivar Arborio) with enzyme assay and with immunoblot analysis (Gulielminetti et al., 1995). Gulielminetti et al. also reported that the amounts of β-amylases were clearly enhanced on germination. Yamaguchi et al. (1999) identified at least four β-amylase-deficient cultivars (Nipponbare, Sachikaze, Chu-kyoasahi, and Shinriki) by amylolytic activity staining after the separation of β-amylases by isoelectric focusing. Several reports suggested the presence of β-amylases in dormant rice grains (e.g., cultivars Shinsetsu, Takanenishiki and Koshihikari) by assaying enzyme activity and zymogram pattern (Shinke et al., 1973; Matsui et al., 1975, Uyen et al., 2001). Awazuhara et al. (2000) detected β-amylase activity in the outer layer of dormant rice grains (cultivar Koshihikari). Shinke et al. (1978) reported that β-amylase was widely distributed in rice grains. In this study, we found that Koshihikari contained β-amylases in every fraction in dormant rice grains and that the 100 to 90% rice flour fraction contained more β-amylase than other fractions. Combining previous findings with our results, we conclude that β-amylase is widely distributed in dormant Koshihikari grains.

Summarizing these results, Fig. 4 describes the relationship between enzyme activity and temperature during rice cooking. The α-amylase II in the outer layers (100 to 80% fraction) and the ubiquitously distributed β-amylase are activated at 37°C (Mitsui et al., 1996; Awazuhara et al., 2000). Oligosaccharide and maltose are consequently produced from starch granules. The α-glucosidases in the inner endosperm are then activated at 60°C (Awazuhara et al., 2000), and glucose is liberated from starch and oligosaccharide. Moreover, α-amylase I in the outer layer is activated at 70°C (Mitsui et al., 1996), degrading starch into oligosaccharide. In addition, gelatinization of rice starch occurs from 58°C to 69°C (Ayabe et al., 2006). The coincidence of gelatinization and enzyme activation facilitates starch degradation into reducing sugars. Additionally, Awazuhara et al. (2000) reported that high levels of α-glucosidase activity contribute to starch degradation into reducing sugars. Kasai et al. (2000) reported that the time required for the temperature to rise between 40 and 80°C is about 5.5 min. This duration seems sufficient to activate the enzymes, judging from the fact that the amounts of reducing sugars increased 55 mg/(100 g raw rice) during this period.

Fig. 4. Timing and location of starch-degrading enzyme activation during rice cooking. The starch-degrading enzymes in rice grains are activated at each optimum temperature during rice cooking and degrade starch into reducing sugars (e.g., oligosaccharide, maltose, and glucose). The activities of α-amylase II, localized in the outer layers (100 to 80%), and β-amylases, ubiquitously distributed (100 to 0%), are enhanced at 37°C. At this temperature, rice starch is degraded into reducing sugars, the main components of which are oligosaccharides. A large amount of reducing sugars (almost glucose) is then produced by α-glucosidases in the inner endosperm (70 to 0%) at 60°C and α-amylase I in the outer layer (90 to 80%) at 70°C. The gelatinization of rice starch occurs from 58°C to 69°C.

*1 Ayabe et al., 2006; *2 Mitsui et al., 1996; *3 Awazuhara et al., 2000.
Previous reports demonstrated the distribution of starch degrading enzymes. In a starch-degrading enzyme assay, we cannot exclude the possibility that enzyme substrates may be processed with another type of enzyme. As far as we know, this is the first report that visualizes the distribution of starch-degrading enzymes in dormant rice grains using specific antibodies.

Abbreviations

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

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