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Partial β-Amylolysis Retards Starch Retrogradation in Rice Products

YUAN YAO,* JINGMIN ZHANG,† AND XIAOLIN DING

Department of Food Science, Wuxi University of Light Industry, Wuxi, Jiangsu 214036, People's Republic of China

Starch retrogradation is the main cause of quality deterioration of starch-containing foods during storage. The current work investigated the effect of partial β-amylolysis on the retrogradation of rice starch and the potential of β-amylase in preparing rice products with extended shelf life. Isolated amylopectin, whole rice starch, and rice flour from a regular rice cultivar were partially hydrolyzed by either reagent-grade or food-grade β-amylase. The degree of β-amylolysis was expressed as average external chain length (ECL) for isolated amylopectin or the degree of hydrolysis (%) for other starch systems. Pulsed nuclear magnetic resonance was used to monitor starch retrogradation during storage at 4 °C. The results indicated that partial β-amylolysis using reagent-grade β-amylase retarded amylopectin retrogradation by shortening the ECL of amylopectin. When ECL was below DP 11.6, the amylopectin retrogradation was essentially inhibited. Partial β-amylolysis had a similar effect on the amylopectin retrogradation in the whole starch system. The maltose produced in β-amylolysis might slightly attenuate the retrogradation-retarding effect of partial β-amylolysis. The effect of food-grade β-amylase on starch retrogradation was also evident, although less effective than that of reagent-grade β-amylase. The retrogradation-retarding effect of food-grade β-amylase was also demonstrated in rice flour system, indicating a potential method for controlling the starch retrogradation of rice products.

KEYWORDS: Amylopectin; retrogradation; partial β-amylolysis; rice starch; rice flour; PNMR

INTRODUCTION

Rice products are staple foods, especially in Oriental countries. Sharing the market of instant foods, fresh-cooked rice products, such as cooked rice grains, rice noodles, rice cakes, and rice pasta, have profound commercial potential. However, the aging of rice products results in the deterioration of desirable qualities and is partly caused by the retrogradation of rice starch during storage. Therefore, the technology of preparing rice products with extended shelf life requires effective retarding of rice starch retrogradation.

Using PNMR, Yao and Ding (*1*) showed that the rice starch retrogradation could be eliminated by heating the retrograded starch gel at 90 °C for 20 min. Klucinec and Thompson (*2*) indicated that the starch retrogradation observed at 50−60 °C using DSC was mainly caused by the recrystallization of amylopectin. Factors affecting starch retrogradation include the structure of amylopectin (*3*−*7*), the content of amylose (*8*, *9*), and the presence of nonstarch components including protein (*10*, *11*), lipids (*12*−*15*), and oligosaccharides (*16*).

It was shown that a minimum chain length of DP 10 was required for the formation of double helices in model systems (*17*). Lu et al. (*6*) studied the relationship between the fine structure and the retrogradation of rice amylopectin. Their results indicated that the portion of short chains with DP 6−9 appeared to inhibit retrogradation. On the other hand, Wursch and Gumy (*18*) indicated that partial β-amylolysis of waxy corn starch and potato amylopectin could inhibit retrogradation; in their experiment, the maltose produced during partial β-amylolysis was removed from the starch systems.

Because most works were done on purified amylopectin (*6*, *18*), we considered that it would be necessary to perform further investigations on whole starches and starch-containing food systems. Such a study would help us to understand the role of amylopectin structure in determining the starch retrogradation in various systems and would benefit the technological development of rice products with extended shelf life.

In the current work, isolated rice amylopectin, whole rice starch, and rice flour were treated using reagent- or food-grade β-amylase. The objectives of this study were as follows: As the starting point, the relationship between the retrogradation behavior and the external chain length of rice amylopectin was investigated. Second, because maltose is an inevitable product of β-amylolysis, its influence on the retrogradation-retarding effect of partial β-amylolysis was addressed. Third, the retro-

* To whom correspondence should be addressed. Current address: 302 Wartik Laboratory, Huck Institute for Life Sciences, The Pennsylvania State University, University Park, PA 16802. Tel: (814)863-7958. Fax: (814)-863-6132. E-mail: yoy1@psu.edu.

† Current address: 302 Wartik Laboratory, Huck Institute for Life Sciences, The Pennsylvania State University, University Park, PA 16802.

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gradation-retarding effect of food-grade β-amylase was compared with that of reagent-grade β-amylase and was studied in an actual rice flour system.

In our study, the PNMR was used to monitor starch retrogradation. The advantage of PNMR lies in its higher sensitivity to starch samples with low retrogradation. The PNMR has also proven to be nondestructive and highly reproducible (*1*, *19*).

MATERIALS AND METHODS

Milled Rice, Rice Starch, and Rice Amylopectin. The milled regular rice was purchased from a local market in Wuxi, Jiangsu, China. Rice flour suspensions were prepared from milled rice soaked in five times (w/w) of deionized water using an electric homogenizer until the suspension passed through a 100 mesh sieve. The precipitate after centrifugation was then dried at 40 °C until the moisture content was lower than 8%. For rice starch, rice flour was soaked in five times (w/w) of 0.4% NaOH solution at room temperature for 48 h and washed repeatedly with deionized water until the pH reached 7. The starch precipitate was then dried at 40 °C until the moisture content was lower than 6%. Amylopectin from rice starch was prepared according to the method of Takeda et al. (*20*). For the measurement of moisture content, a sample was first dried at 40 °C in an oven for 48 h to remove most of the water and then was heated at 105 °C to constant weight when the sample was weighed at an interval of 12 h.

Enzymes and Polysaccharide Standards. Isoamylase (EC 3.2.1.68, from *Pseudomonas amylodermosa*) and pullulanase (EC 3.2.1.41, from *Klebsiella pneumoniae*) purchased from Sigma (St. Louis, MO) were used to prepare the debranched starch dispersions. Two types of β-amylase were used. Reagent-grade β-amylase (EC 3.2.1.2, from sweet potato) was purchased from Sigma. The activity unit was indicated, as that one unit would liberate 1.0 mg of maltose from starch in 3 min at pH 4.8 at 20 °C. Food-grade β-amylase (crude powder extracted from soybean, labeled as 200 000 unit/g) was purchased from Wuxi Enzyme Corporation (Current Genenco-Wuxi Corporation, Wuxi, China). The activity unit was indicated, as that one unit would liberate 1.0 mg of maltose per hour using 1.1% soluble starch as the substrate at pH 5.5 and 60 °C.

The polysaccharide standards were used to determine the standard curve of HPSEC analysis. Maltosaccharide with an average DP of 18 was a product of Hayashibara Institute (Okayama, Japan). Maltotriose, maltoheptaose, and dextrans (M_w of 41 000 and 580 000) were purchased from Sigma.

Amylose Content of Rice Starch. The amylose content of rice starch after defatting was measured as described by Yao et al. (*21*).

$\overline{ECL}$ for Amylopectin and the DH for Whole Rice Starch and Rice Flour. The $\overline{ECL}$ of amylopectin after partial β-amylolysis was calculated using the following equation (*18*):

$$\overline{ECL} = \overline{ECL}_0 - (\beta/\beta_0) \times (\overline{ECL}_0 - 2) \quad (1)$$

Because

$$\overline{ECL}_0 = \beta_0 \times \overline{CL} + 2 \quad (2)$$

We have

$$\overline{ECL} = (\beta_0 - \beta) \times \overline{CL} + 2 \quad (3)$$

$\overline{ECL}_0$ and $\overline{CL}$ are the average external chain length and the average chain length of intact amylopectin, respectively. β is the degree of β-amylolysis, and β_0 is the β-amylolysis limit. β or β_0 was calculated as [reducing sugar (expressed as maltose)]/[total sugar (expressed as maltose)] after partial or limit β-amylolysis. The reducing sugar was analyzed using the DNS method (*22*). $\overline{CL}$ was calculated as [total sugar]/[number of reducing end in the debranched amylopectin dispersion]. The total sugar was measured as described by Dubois et al. (*23*). The reducing end was analyzed using the Somogyi−Nelson method (*24*).

For whole rice starch or rice flour systems, the DH (%) was used to characterize the degree of β-amylolysis. DH was expressed as [reducing sugar (expressed as maltose)]/[total starch (expressed as maltose)] after hydrolysis by reagent- or food-grade β-amylase. The DNS method was used to analyze the reducing sugar.

For the quantification of total starch, 10.0 mL of 5.0% (w/w) aqueous suspension of rice starch or rice flour was heated (20 min, 121 °C) and then cooled to room temperature. An aliquot of 1.0 mL was mixed with 4.0 mL of sodium acetate buffer (0.05 M, pH 3.65) containing 10 000 units of isoamylase and incubated at 25 °C for 24 h. After the isoamylase was deactivated by heating the mixture for 5 min in a boiling water bath, the pH was adjusted to 6.0. The mixture was then diluted to 10.0 mL using sodium acetate buffer (0.05 M, pH 6.0) containing 10 units of reagent-grade β-amylase. The mixture was then incubated at 25 °C for 48 h before it was heated in a boiling water bath for 5 min to deactivate the enzyme. The product was analyzed for its reducing sugar by the DNS method with maltose solutions as the standards. The HPSEC test showed that the starch was completely converted into maltose and maltotriose using this procedure.

Amylopectin Chain Length Distribution. The amylopectin chain length distribution was analyzed as described by Yao et al. (*21*). The intact amylopectin (50 mg) was treated using isoamylase (10 000 units) at 25 °C for 24 h, while amylopectin after partial β-amylolysis (50 mg) was further treated by pullulanase (5 units) at 25 °C for 24 h following isoamylase treatment.

PNMR Used To Follow the Starch Retrogradation. The starch retrogradation was monitored using the relative solid content (S', %) of PNMR as described by Yao and Ding (*1*).

Retrogradation Analysis of Starch Systems after Partial β-Amylolysis. Five starch−β-amylase reaction systems were designed to investigate the influence of starch system, maltose, or the grade of β-amylase on the retrogradation-retarding effect of partial β-amylolysis. The starch systems studied included isolated amylopectin, whole starch, and rice flour. The retrogradation behavior of rice starch after partial β-amylolysis was also investigated with or without maltose removal. Both reagent- and food-grade β-amylase were applied to rice starch, and their effects were compared. Finally, the food-grade β-amylase was applied to rice flour to examine the retrogradation-retarding effect of partial β-amylolysis in practical application.

1. Partial Hydrolysis of Rice Amylopectin Using Reagent-Grade β-Amylase with Maltose Removal. Amylopectin, isolated from regular rice starch, was dispersed at room temperature (22 °C) in the sodium acetate buffer (0.01 M, pH 6.0) under a constant gentle stirring. Two hundred units of reagent-grade β-amylase was added to the dispersion (880 mL, 4.5%, w/w) at 30 min intervals for 2 h. Meanwhile, a 100 mL aliquot of dispersion was sampled every 30 min, from which 5 mL was used to analyze the degree of β-amylolysis and $\overline{ECL}$ of amylopectin. In the sample remaining (95 mL), 2 volumes of ethanol (95%, w/w) was added to precipitate the amylopectin. After it was centrifuged, the pellet collected was washed using 95% and nonaqueous ethanol and then dried in a vacuumed $CaCl_2$ desiccator. The β-amylolysis limit was obtained after incubating the dispersion left for 24 h.

The 30.0% (w/w) amylopectin gels were used for the analysis of retrogradation behavior. For each amylopectin after partial β-amylolysis, 1.50 g of dry powder was mixed with 3.50 g of sodium acetate buffer (0.001 M, pH 6.0) and injected into a PNMR test tube. The tube was sealed and heated in a boiling water bath for 5 min and then stabilized at 4 °C for 30 min. Then, the initial relative solid content S' (S_0') was measured using PNMR. For each sample, the S_t' values during 4 °C storage were measured after 1, 2, 3, 4, 5, 6, 8, 9, 12, 14, 16, and 19 days. Samples were prepared and analyzed in duplicate. The mean values of the change of S' ($\Delta S'$, $\Delta S' = S_t' - S_0'$) were used for further analysis.

2. Partial Hydrolysis of Rice Starch Using Reagent-Grade β-Amylase with Maltose Removal. Rice starch was suspended in the sodium acetate buffer (0.01 M, pH 6.0) at a concentration of 6.6% (w/w). A 1000 mL suspension was heated in a boiling water bath for 20 min and then instantly cooled to room temperature. The procedure followed for partial

β-amylolysis was the same as that of amylopectin indicated in the above paragraph, whereas the DH rather than $\overline{ECL}$ was analyzed.

For the analysis of retrogradation behavior, 2.0 g of each sample (dry powder of rice starch after partial β-amylolysis) was mixed with the sodium acetate buffer (0.001 M, pH 6.0) for a dispersion of 28.0% (w/w) starch and then injected into a PNMR test tube. The tube was sealed and heated in a boiling water bath for 5 min and then stabilized at 4 °C for 30 min. Then, S_0' was measured using PNMR. For each sample, the S_t' values during 4 °C storage were measured after 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, and 16 days. Samples were prepared and analyzed in duplicate. The mean values of $\Delta S'$ were used for further analysis.

3. Partial Hydrolysis of Rice Starch Using Reagent-Grade β-Amylase without Maltose Removal. Rice starch was suspended in the sodium acetate buffer (0.001 M, pH 6.0) at the concentration of 32.0% (w/w). A suspension of 200 mL was heated in a boiling water bath for 20 min and cooled to room temperature. The gel of rice starch was evenly distributed into 10 beakers. More buffer solution with reagent-grade β-amylase (10−100 units) was added to the gel for a final rice starch concentration of 30.0% (w/w).

After they were incubated for 2 h at room temperature, the samples were injected into PNMR test tubes. The tubes were sealed and heated in a boiling water bath for 5 min and then stabilized at 4 °C for 30 min. Then, S_0' for each sample was measured. The S_t' values during 4 °C storage were measured after 1, 2, 3, 4, 5, 6, 8, 9, 12, 14, 16, and 19 days. Samples were prepared and analyzed in duplicate. The mean values of $\Delta S'$ were used for further analysis. The DH value of each sample was analyzed after the storage.

4. Partial Hydrolysis of Rice Starch Using Food-Grade β-Amylase without Maltose Removal. The procedures of partial β-amylolysis and retrogradation analysis were the same as in the previous paragraph, except that food-grade β-amylase was used instead of reagent-grade. The activity load was 200−2000 units for 20 mL of starch gel.

5. Partial Hydrolysis of Rice Flour Using Food-Grade β-Amylase without Maltose Removal. The procedures of partial β-amylolysis and retrogradation analysis were the same as in the previous paragraph, except that 35.0% of rice flour was used instead of 30.0% of starch.

RESULTS AND DISCUSSION

Partial β-Amylolysis Shortens the $\overline{ECL}$. Figure 1 shows the chain length distribution of amylopectin with different average external chain length ($\overline{ECL}$) after partial β-amylolysis. The average chain length ($\overline{CL}$) measured was DP 20.2. The β-amylolysis limit measured was 59%. The $\overline{ECL}_0$ calculated was 13.9. After 30, 60, and 90 min of β-amylase treatment, the degree of β-amylolysis was 3.7, 5.9, and 11.5%, corresponding to $\overline{ECL}$ of DP 13.2, 12.7, and 11.6, respectively. During β-amylolysis, the chain length of peaks corresponding to long chains (DP_1) and short chains (DP_2) decreased slightly, together with the shortening of $\overline{ECL}$. It is understood that external chain population is comprised of both short chains (A and B1 chains) and long chains (B2 and B3 chains) (*25*) and that these chains are all available to the trimming effect of β-amylase. β-Amylase is an exoamylase, releasing maltose from the nonreducing ends of a long chain substrate composed of the linear α-1,4-glucosidic bonds (*26*). By multiple attacks on the substrate, β-amylase produces several maltose molecules from a single enzyme−substrate complex without dissociating (*27*, *28*). Using the Monte Carlo simulation of multiple attack process in an β-amylase-catalyzed reaction, Nakatani calculated that approximately 2.4 maltoses (corresponding to DP 4.8) were produced from a single enzyme−substrate complex formation if the chain length of the substrate was infinite (*29*). Therefore, we considered that in an actual reaction system, it was unlikely that upon forming an enzyme−substrate complex, the β-amylase would trim the same external chain repetitively until a stub of DP 2 or 3 was left. Indeed, what was observed in **Figure 1** was a gradual shift of the bimodal distribution curve to the low DP region, rather than an abrupt increase in the amount of chains with DP 2 or 3.

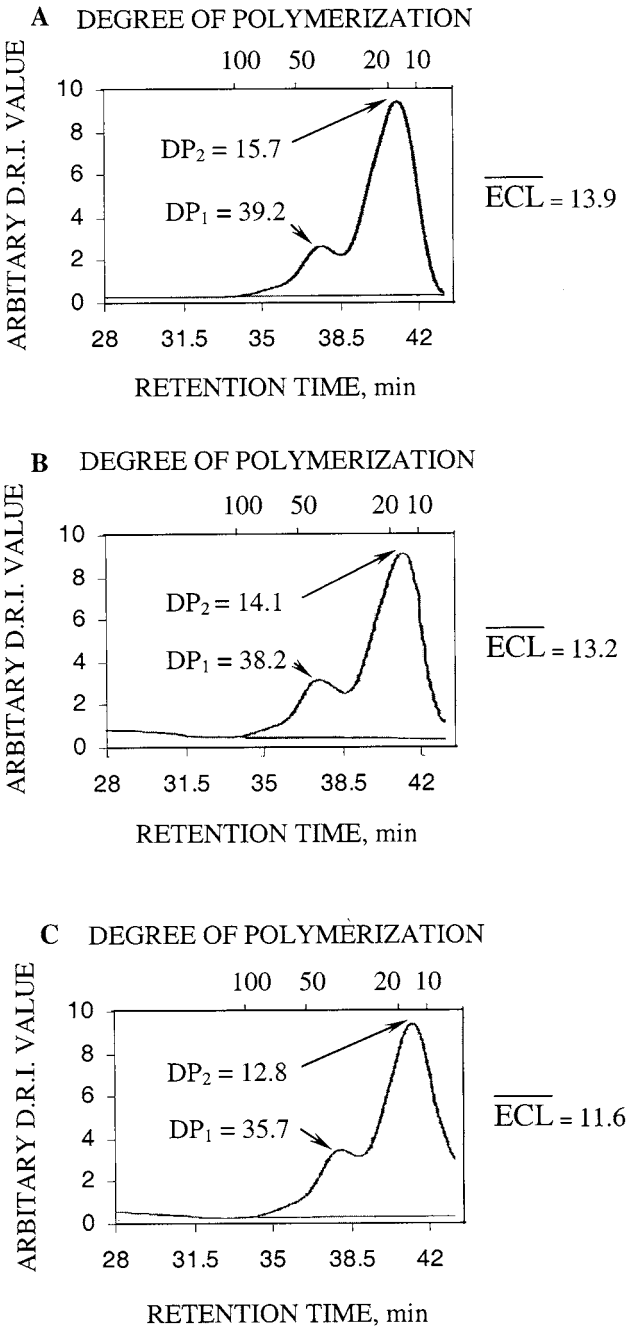

Figure 1. HPSEC of debranched amylopectin before (**A**) and after partial β-amylolysis (**B**, 30 min; **C**, 90 min treatment) using reagent-grade β-amylase. $\overline{ECL}$ and chain length at the peaks corresponding to long chains (DP_1) and short chains (DP_2) are labeled in individual chromatograms.

Partial β-Amylolysis Decreases the Retrogradation of Amylopectin. It was indicated that the relative solid content S' of the starch sample measured by PNMR is comprised of the contributions from both amylose and amylopectin (*1*). The contribution of amylose remains constant during the entire retrogradation process, whereas the contribution of amylopectin increases during storage. Therefore, the change of S' ($\Delta S'$, $\Delta S' = S_t' - S_0'$) reflects the retrogradation of amylopectin in either amylopectin or whole starch system.

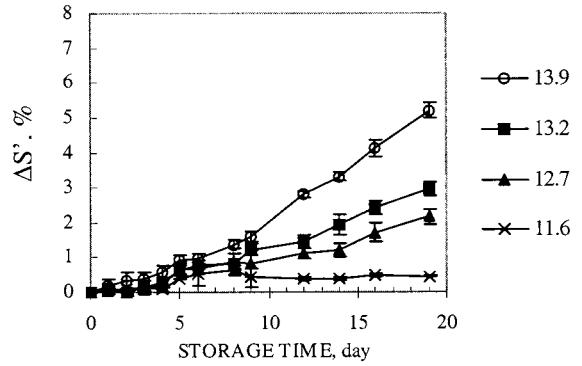

Figure 2. Change of relative solid content (Δ*S*′, %) measured by PNMR during 4 °C storage for isolated amylopectin after partial β-amylolysis using reagent-grade β-amylase. The maltose produced was removed from the amylopectin systems. ECL of each sample is labeled in the figure. Δ*S*′ values shown were the averages of duplicate tests with standard deviation (SD) shown as error bars.

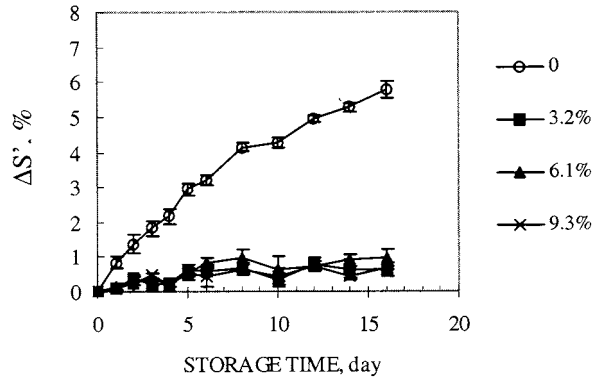

Figure 3. Δ*S*′ measured by PNMR during 4 °C storage for rice starch after partial β-amylolysis using reagent-grade β-amylase. The maltose produced was removed from the starch systems. The DH of each sample is labeled in the figure. Δ*S*′ values shown were the averages of duplicate tests with SD shown as error bars.

Figure 2 shows the retarding effect of partial β-amylolysis on the retrogradation of amylopectin. A slight partial β-amylolysis of amylopectin drastically changed the retrogradation behavior, and when the ECL decreased from DP 13.9 to DP 11.58, the retrogradation process was essentially inhibited. This observation is consistent with the work of Wursch and Gumy on waxy corn starch (*18*).

Figure 3 shows the retarding effect of partial β-amylolysis on the retrogradation of amylopectin in the whole starch system with the removal of the maltose produced during β-amylolysis. Strikingly, when the DH was only 3.2% in the whole starch system, the retrogradation was essentially inhibited, while for isolated amylopectin system a higher degree of β-amylolysis (11.5%) was needed to inhibit the retrogradation. We considered that the higher retrogradation-retarding efficiency of partial β-amylolysis in the whole starch system was attributed to the lower amylopectin concentration. In a gelatinized starch system, both amylose and amylopectin are susceptible to β-amylolysis. Because the external chains of amylopectin contribute most nonreducing ends in the whole starch system, we assumed that the DH value measured during the partial β-amylolysis reflected the shortening of amylopectin external chain length. Considering the amylose content (after defatting) of 26.2% in the starch, the amylopectin concentration was 20.7% in the 28.0% starch gel system. In comparison, the concentration of amylopectin gel studied was 30.0%. Because of the high sensitivity of

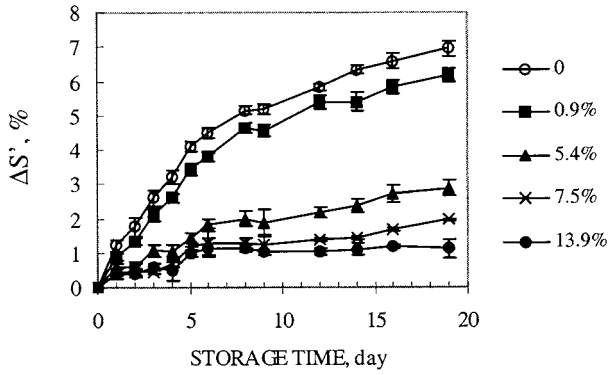

Figure 4. Δ*S*′ measured by PNMR during 4 °C storage for rice starch after partial β-amylolysis using reagent-grade β-amylase. The maltose produced remained in the starch systems. The DH of each sample is labeled in the figure. Δ*S*′ values shown were the averages of duplicate tests with SD shown as error bars.

retrogradation to moisture content (*30*, *31*), the shortening of the external chain length may affect the retrogradation more effectively in a gel system with lower concentration of amylopectin, thus resulting in the higher retrogradation-retarding efficiency of partial β-amylolysis.

As shown in **Figure 4**, when the maltose produced during β-amylolysis remained in the starch system, the retarding effect of partial β-amylolysis was also evident. Biliaderis indicated that when the mass ratio of starch/maltose/water was 1/0.5/1.5, the maltose presented a minor inhibiting effect on the retrogradation of waxy corn starch when monitored by DSC (*16*). In the starch systems shown in **Figure 4**, the highest DH was 13.9%, corresponding to a maltose concentration (4.2%) much lower than that (16.7%) in the system investigated by Biliaderis. It was expected that at such a low concentration, the maltose produced should have little impact on the retrogradation of rice starch. However, while a DH of 3.2% inhibited retrogradation in the starch system without maltose (**Figure 3**), a slight retrogradation could still be observed when DH was 5.4% in systems with maltose (**Figure 4**). Although it appeared that maltose might slightly attenuate the retrogradation-retarding effect of partial β-amylolysis, other factors may be involved. It should be noted that in **Figure 3** the gelatinized starches underwent ethanol precipitation and redispersion, which led to their different heat moisture treatment history as compared to the samples in **Figure 4**. We considered that the difference in sample preparation process might contribute to different retrogradation-retarding effects of partial β-amylolysis.

Retarding Effect of Food-Grade β-Amylase on Retrogradation. As indicated in **Figure 5**, the food-grade β-amylase did demonstrate its ability to retard the retrogradation of rice starch. **Figure 6** compares reagent- and food-grade β-amylase for their effects on retrogradation. At the same DH, the reagent-grade β-amylase tended to retard retrogradation more effectively than food-grade β-amylase. A related observation was the loss of elasticity for the starch gels treated using food-grade β-amylase, indicating damage to the starch gel network. We considered that the decreased retrogradation-retarding efficiency might be attributed to the higher α-amylase activity in the food-grade β-amylase. While the chain length profiles of dextrin products from α-amylolysis display a similar pattern as intact amylopectin (*32*), the dextrin products contributed to the DH values detected. At the same DH value, the ECL of amylopectin would be higher with higher α-amylase activity present in food-grade

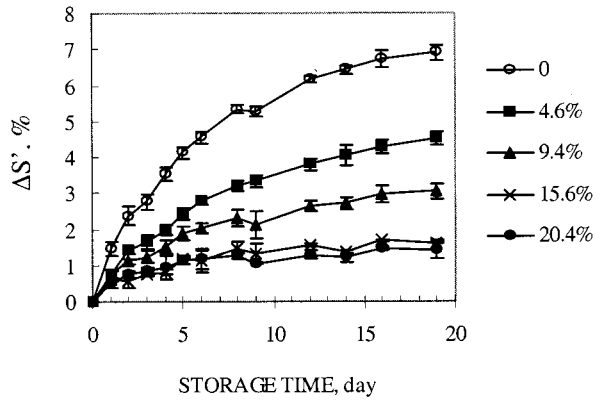

Figure 5. $\Delta S'$ measured by PNMR during 4 °C storage for rice starch after partial β-amylolysis using food-grade β-amylase. The maltose produced remained in the starch systems. The DH of each sample is labeled in the figure. $\Delta S'$ values shown were the averages of duplicate tests with SD shown as error bars.

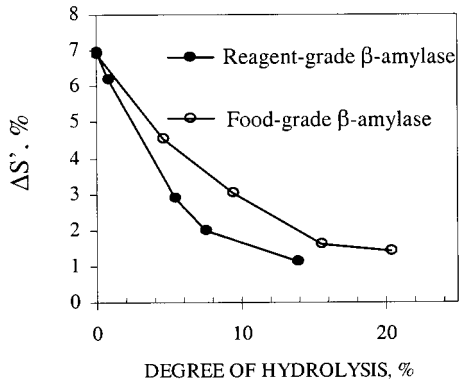

Figure 6. Comparison of retrogradation-retarding effects of reagent- and food-grade β-amylase. The points indicate the $\Delta S'$ values at the end of retrogradation monitoring corresponding to different DH for rice starch treated by reagent- or food-grade β-amylase. At the same DH, the $\Delta S'$ of starch samples treated by reagent-grade β-amylase was much lower than that treated by food-grade β-amylase.

β-amylase used and resulted in a lower retrogradation-retarding effect of enzyme treatment.

Retarding Effect of Food-Grade β-Amylase on Retrogradation in Rice Flour System. As in the rice starch system, the food-grade β-amylase has a retrogradation retarding effect in the rice flour system as well. **Figure 7** shows that when the DH reached 22.7%, the retrogradation could be essentially inhibited. Thus, partial β-amylolysis has potential in preparing rice products including rice cakes, rice noodles, rice pasta, etc. In these systems, the structural integrity of rice grains is destroyed and the β-amylase added is available to most starch molecules in the system. For cooked rice grains, however, a specific procedure is needed to distribute the β-amylase evenly in the system (*33*).

ABBREVIATIONS USED

PNMR, pulsed nuclear magnetic resonance; DP, degree of polymerization; HPSEC, high-performance size exclusion chromatography; $\overline{ECL}$, average external chain length of amylopectin; $\overline{ECL}_0$, average external chain length of intact amylopectin; $\overline{CL}$, average chain length of amylopectin; DH, degree of hydrolysis; DP_1, chain length of the peak corresponding to long chains in HPSEC chromatogram; DP_2, chain length of the peak corresponding to short chains in HPSEC chromatogram; S', relative solid content measured by PNMR; S_0', initial S' during retrogradation monitoring; S_t', S' at storage time t during retrogradation monitoring; $\Delta S'$, change of S' during retrogradation monitoring, $\Delta S' = S_t' - S_0'$.

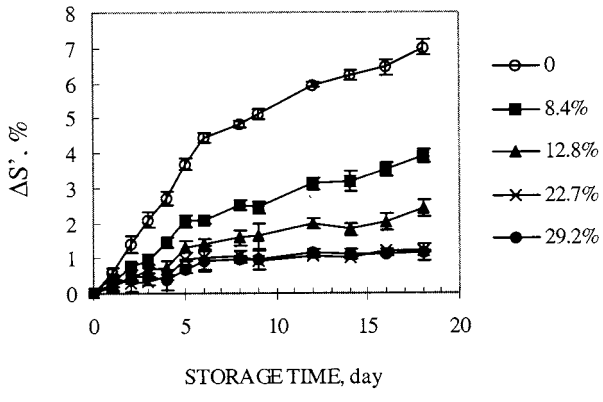

Figure 7. $\Delta S'$ measured by PNMR during 4 °C storage for rice flour after partial β-amylolysis using food-grade β-amylase. The maltose produced remained in the starch systems. The DH of each sample is labeled in the figure. $\Delta S'$ values shown were the averages of duplicate tests with SD shown as error bars.

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