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Environmental Factors that Affect the Ability of Amylose to Contribute to Retrogradation in Gels Made from Rice Flour

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Retrogradation in rice is a trait that describes the hardening of cooked rice after storage or cooling, and it has significant implications for many consumers of rice, since many people cook rice in the morning and consume it several hours later or the next day. Tools to select against retrogradation in breeding programs are yet to be described. Here, we aim to determine the effect on retrogradation of storage time and temperature and the role of starch, protein, and lipids using gels made from Koshihikari grown in either Australia or Japan. Immediately after cooking, cooling from 60 to 40 °C had a minimal effect on firmness, but cooling to 20 °C led to significantly firmer gels. Storing the gels at low temperatures did not have an additional effect on the firmness as compared with storing the gels at 20, 40, or 60 °C. The removal of proteins led to significantly softer gels at all storage treatments but did not affect the change in firmness on cooling. The removal of lipids increased the rate of retrogradation and the firmness of gels significantly for all treatments. Koshihikari grown in Japan retrograded much less than Koshihikari grown in Australia. The amount of amylose that could be washed from gels made from Australian flour was much greater than for gels made from Japanese flour. After storage, only low molecular weight amylose chains were released from the gel and only after rewarming them to 60 °C. Despite the fact that flours from both origins were 18% amylose, the amount of long amylose chains that were complexed with lipids was much greater for the Japanese rice, and the unavailability of the complexed long amylose chains explained the lack of retrogradation in the Japanese rice. Once the long chains were released from amylose−lipid complexes, the Japanese rice retrograded. Thus, the environmental factor affecting retrogradation in this variety is type or amount of lipids synthesized, and the degree of retrogradation was determined by the availability of long chains of amylose.

KEYWORDS: Retrogradation; rice; starch; eating quality; amylose; amylose−lipid complexes

INTRODUCTION

Retrogradation is one of the most important traits defining the sensory and eating quality of rice. The retrogradation properties of rice affect people who cook rice once a day and consume it throughout the day and people who process rice and require particular properties of the gel. There are very few varieties of rice that do not retrograde; therefore, the trait is likely to involve multiple genes. A previous study using quantitative trait loci analysis of a recombinant inbred population reported that retrogradation was controlled by the waxy locus (*1*). The parents of that population were of disparate amylose content, one being of 18% and one being of 26%, so the authors' conclusion is unsurprising given that amylose cross-linking is the first molecular process of retrogradation (*2*). Moreover, the amylose content does not correlate directly with retrogradation since there are varieties that differ in the degree of retrogradation within each amylose class. Thus, factors other than simply the waxy locus must contribute to the amylose component of retrogradation.

Retrogradation is a term that describes the increase in firmness of cooked food following storage or cooling. The composition of the food defines the processes that cause retrogradation. For example, foods that contain starch undergo specific processes as they retrograde. Rapid crystallization of amylose and the slow recrystallization of amylopectin (*3*) are the two mechanisms that are most likely to affect retrogradation of gels from rice flour. These molecular mechanisms are well-characterized. Rapid crystallization of amylose occurs as soon as cooling begins and involves interactions between high molecular weight amylose molecules, leading to a loose network, followed by aggregation

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and crystallization of amylose molecules (*4*, *5*). The processes of retrogradation depend on the amylose content of the rice (*6*); the amount of amylose that is free, rather than complexed with lipids (*7*); and the molecular weight distribution of the amylose (*8*). Amylose−lipid complexes can form during the development of the grain, and others can form during the process of cooking (*9*); as amylose leaches from gelatinized granules, the free amylose can form complexes with particular free lipids (*9*). If amylose is complexed with lipids, it is unable to contribute to the formation of networks since amylose is only released from those complexes at temperatures above 100 °C (*8*). Hot water soluble components of rice starch with high molecular weights promote retrogradation more than lower molecular weight components (*10*), suggesting that the molecular weight distribution of the amylose affects the first phase of retrogradation. Retrogradation due purely to the formation of amylose−amylose crystals is not reversible at temperatures below about 150 °C (*8*).

The second process of retrogradation involves recrystallization of amylopectin chains (*6*, *11*). This process occurs during longer storage terms of a gel (*11*). Several studies suggest that the chain length distribution of amylopectin contributes to differences in the degree of retrogradation by amylopectin. In particular, the proportion of short A chains in the amylopectin lessens the degree of retrogradation (*2*, *10*, *12*, *13*). Furthermore, it has been shown that the interaction of amylose with amylopectin increases the rate of amylopectin retrogradation (*14*), perhaps as amylopectin molecules become embedded within the aggregating amylose gel. Retrogradation due to amylopectin is reversible if the retrograded gel is heated to a temperature beyond the gelatinization temperature of amylopectin crystals in the native starch (*12*). The gelatinization temperature of rice ranges from 60 to 80 °C, so reheating cooked rice beyond 80 °C should melt the amylopectin crystals that formed during cooling and storage.

The crystals formed by retrogradation of amylopectin can be either the A type or the B type. The type depends on crystallization conditions, like water content and storage temperature (*15*). Generally, a high water content and/or low temperatures (in the order of 20 °C) will lead to the formation of B type crystals, which hold 36 molecules of water per unit cell (*16*), and in low water content and/or high temperatures (about 60 °C), A type crystals form (which hold four molecules of water per unit cell) (*16*). It has also been shown that in conditions intermediate between those favoring A or B types of crystal formation, a mixture of A and B types is found (*15*). The extent to which retrogradation can be reversed, by melting the crystals, is likely to depend on the form of the crystals, the mixture of A and B types, and the proportion of amylose that contributes to retrogradation, which is a function of the amount of amylose that is, or that became, complexed with lipids or the amount of amylose−amylose crystals that formed during cooling.

Environmental conditions can affect the synthesis of the components of rice grains and thus should also affect retrogradation properties. The proportion of amylose that accumulates in developing grains is determined to some extent by the environmental conditions in which the rice is grown. In a number of rices, high temperature leads to a decrease in expression of the gene that synthesizes amylose−granule-bound starch synthase, which leads to a decrease in the proportion of amylose synthesized (*17*). High temperatures also alter the chain length distribution of amylopectin by decreasing the activity of starch synthase 1 (SS1) (*18*). SS1 is the enzyme that is most likely to be responsible for the synthesis of short chains on amylopectin (*19*), so a decrease in its activity would result in a longer chain length distribution of the amylopectin (*20*). Thus, high temperatures during grain development is one environmental condition that is expected to alter the degree of retrogradation by altering the proportion and structure of both amylose and amylopectin. The proportion of amylose available for aggregation is a key factor defining the degree to which amylose contributes to retrogradation, and presumably, lipids play a key role in amylose availability. Environmental conditions are known to influence the accumulation of oil in oilseed crops (*21*, *22*); thus, it is possible that environmental conditions affect the synthesis of lipids in rice. Since lipids contribute negatively to the degree of retrogradation by blocking amylose, environmental effects on lipid content could be another condition affecting retrogradation. Here, we investigate the environmental effects on retrogradation and determine the factors affected that contribute to retrogradation using the Japanese variety Koshihikari.

MATERIALS AND METHODS

Samples of milled grain from one variety of rice, Koshihikari (*Oryza sativa*), were obtained in 2003 from two locations: the New South Wales Department of Primary Industries, Yanco Agricultural Institute (NSW, Australia), and the Ni-igata Province in Japan. Milled grain was ground (Cyclotec 1093 sample mill, Tecator) to pass through a 0.5 mm sieve. The amylose content was determined by the standard iodine binding method (*23*), and the protein content was determined by the Dumas method (ASTM E191-64). Other materials included protease (Bacterial Type XIV) (Sigma), BioRad AG-X8 mixed bed ion-exchange resin, methanol (Sigma-Aldrich), the debranching enzyme isoamylase (250 U/mg, 250 U/mL in 3.2 M ammonium sulfate, and 0.02% sodium azide; Megazyme, Wicklow, Ireland), 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) (Sigma-Aldrich), maltoheptaose, and sodium cyanoborohydride (Sigma-Aldrich). All reagents were analytical grade, and MilliQ (Millipore) water was used for all analyses.

To determine the contribution of the different components of rice flour to the processes of retrogradation, flour of each variety was divided into four samples. One sample was used directly, proteins were removed from the second sample, lipids were removed from the third sample, and both lipids and proteins were removed from the remaining sample. Proteins were removed using protease and a modified rapid visco analyzer (RVA) profile (*24*). Briefly, protease (5 U) was added to the standard RVA mixture of flour (3 g) and water (25 g), and the run was lengthened by adding 4 min to the beginning (at 50 °C). It has been shown that 5 U of protease completely digests the proteins within those 4 min and then is denatured when the temperature increases (*24*). Lipids were removed from flour by refluxing in 85% methanol for 20 h (*25*). For the fourth sample, defatted flour (3 g) was mixed with water (25 g) in a standard RVA canister, protease was added, and the extended profile, described above, was used.

Retrogradation was measured using gels made from the four subsamples of flour described above. Gels were prepared separately using the RVA with the standard heating and holding conditions for rice flour (AACC61-02) and with cooling to either 60, 40, or 20 °C or with the extended profile, described above, when protease was used. Retrogradation was measured as the firmness of the gels after storing and cooling, as compared with the firmness of freshly prepared gels. Firmness was measured as the force required to puncture the gel and penetrate 30 mm into the gel. The RVA canister, containing the gel, was placed on the platform of a Lloyd tensile tester. The probe was a round-ended, tempered plastic cylinder (17 mm diameter) attached to a 5 N load cell. The probe travelled at 100 mm/min to a distance of 65 mm (30 mm of gel). Nexygen software version 7.61 was used to control the probe and the load cell and to collect the data.

Retrogradation was induced by (i) rapidly cooling individual gels from 60 to either 40 or 20 °C, rewarming the gels immediately to 60 °C, and measuring the firmness at each temperature; (ii) storing samples

at either 20, 40, or 60 °C overnight and then measuring the firmness of the gels; (iii) storing gels at 4 °C overnight, then reheating individual samples to either 20, 40, or 60 °C, and then measuring the firmness of the gels; or (vi) storing the gels at 4 °C for 1 week, then reheating separate samples to 20, 40, or 60 °C, and then measuring the firmness of the gels. For gels intended for storage, the RVA canister was sealed immediately after the RVA run was completed. The sealed canisters were weighed immediately after being sealed and then again after storage to determine any loss of moisture. Each sample was prepared in triplicate.

Statistical analysis for determining the factors affecting firmness and retrogradation was carried out using balanced analysis of variance (ANOVA) using a completely randomized design and partitioning the treatment sum of squares using IRRIStat (version 5.0). Pairwise comparison of means was done using least significant difference (LSD) at both 5 and 1% significance levels. Significant differences between the difference in firmness between gels at 60 and 20 °C as a function of either cooling, storing, or warming were also analyzed using balanced ANOVA. LSD was likewise calculated at 5 and 1% significance levels.

The contribution of amylose to the firmness of gels was determined by using size exclusion chromatography (SEC) to measure the amount and the molecular weight distribution of hot water soluble amylose chains from the flours and from the gels after the temperature and storage treatments. The hot water soluble fraction (HWSF) was collected from the flour and defatted flour of both rices (*26*). The presence of proteins in the HWSF was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE). The molecular weight distribution of the chains in the HWSF was determined by debranching (*27*) the HWSF and analyzing it with calibrated SEC (*26*). Soluble starch was collected from freshly prepared gels, made from full flour of the Australian and Japanese samples, at either 60, 40, or 20 °C. Soluble starch was also collected from gels from full flour at the rewarming temperatures after they were stored for 24 h at 4 °C, and it was collected from gels of full flour stored overnight at the three temperatures. Soluble starch was collected by mixing a subsample of the gel (1 g) with water (2 mL) that had been previously warmed to either 20, 40, or 60 °C. The gel mixture was gently shaken (30 s) and then centrifuged (12500*g*, 10 min). The supernatant, containing starch that was able to be washed out of the gel, was debranched (*27*), and the molecular weight distribution of the chains was analyzed by calibrated SEC (*26*).

SEC was carried out using a Waters system comprising a pump (Alliance 2695) and a differential refractive index (DRI) detector. Empower software was used to control the pump and to acquire the data. The eluent was ammonium acetate (0.05 M, pH 4.75) flowing at 0.5 mL min^{-1} (*28*). The column was either an Ultrahydrogel 500 (UH 500) or 250 (UH 250) (Waters), and columns were held at 60 °C during each run. The UH 250 was used for the debranched HWSF from flour and defatted flour because it concentrates high molecular weight amylose chains into the void volume and separates lower molecular weight amylose chains. The UH 500 was used for the debranched starch washed from the gels because all amylose chains eluted in the separating phase (*26*). Aliquots (40 μL) of the HWSF and debranched HWSF were injected, and the run time was 35 min.

The presence of protein in the HWSF was determined by SDS−PAGE (*27*). An aliquot of the HWSF (15 μL) was mixed with gel loading buffer (20 μL), and 10 μL was loaded onto the gel. SDS−PAGE was carried out with a Hoefer 10 cm × 8 cm Mighty Small, using a 10% gel as described in the operator manual. Gels were dried (Hoefer Easy Breeze) after staining with Coomassie Blue R-250. A molecular marker (Sigma-Alldrich) was used to determine the MW of the bands.

To determine the chain length distribution of the amylopectin molecules, the insoluble fraction (HWIF), remaining after extraction of the HWSF, was solubilized by mixing it with hot NaOH (1 mL, 0.25 M). After the HWIF was in solution, the weight of the mixture was adjusted to 4 g with milliQ water and then debranched (*26*). The debranched starch was labeled with APTS and analyzed by capillary electrophoresis (*29*). The area of each peak was converted to velocity area (*30*) to give $P(N)$ and then expressed as ln $P(N)$ for the determination of any differences in the MWD of amylopectin chains without normalization (*31*).

Table 1. Force (N) Required to Puncture Fresh and Stored Gels Made from Flour, from Flour with Proteins (P) Extracted, Flour with Lipids (L) Extracted, and Flour without L or P of Koshihikari Grown in Australia (AUS) or Japan (JAP)[a]

		flour		flour–P		flour–L		flour–P–L	
treatment	°C	AUS	JAP	AUS	JAP	AUS	JAP	AUST	JAP
fresh	20	1.125	0.671	0.910	0.447	3.157	3.192	3.205	2.424
	40	0.592	0.537	0.483	0.364	1.974	1.610	1.597	1.185
	60	0.350	0.362	0.206	0.223	0.251	0.259	0.294	0.257
ON at	20	1.342	0.720	1.029	0.529	2.997	2.272	3.380	2.151
	40	1.098	0.675	0.663	0.423	2.492	2.034	1.614	1.434
	60	0.752	0.589	0.421	0.390	0.615	0.578	1.018	0.869
ON at 4 °C	20	1.355	0.728	1.048	0.523	3.146	2.543	3.831	2.221
	40	1.159	0.624	0.902	0.460	2.451	1.764	3.249	2.034
	60	0.994	0.649	0.705	0.399	2.212	1.473	2.255	1.312
7 days at 4 °C	20	1.443	0.758	1.147	0.596	3.707	2.834	3.343	2.545
	40	1.084	0.646	0.866	0.479	2.563	2.214	3.090	1.671
	60	0.914	0.560	0.712	0.435	1.997	1.826	2.176	1.308

[a] ON, overnight. Means ($n = 3$) that are significantly different can be calculated using $LSD_{0.05} = 0.127$ and $LSD_{0.01} = 0.167$.

RESULTS

Content. The amylose content of the Koshihikari from Japan was 17.8%, and from Australia, it was 18.4%. The protein content of the Japanese rice was 5.6%, and for the Australian rice, the protein content was 5.5%.

Retrogradation of Gels. Statistical analyses showed no significant difference between replicates for each measurement. **Table 1** shows the firmness of the gels from each type of flour from Koshihikari grown in Australia and in Japan and indicates the LSD at two confidence intervals. For both growing locations, the removal of lipids significantly increases the firmness of the gels ($P < 0.01$), and the removal of proteins leads to significantly softer gels ($P < 0.05$). For almost every measurement, the gels from the rice grown in Japan are significantly softer than gels from the rice grown in Australia, but when lipids are removed, the firmness of fresh gels from Australian and Japanese flour is not significantly different.

Individual samples of fresh gels were cooled to either 60, 40, or 20 °C, and firmness was measured at each temperature. **Figure 1** shows that, within the range of 60−20 °C, the relationship between the temperature of the gel and the firmness is linear for the gels made from the rice grown in Japan, but for the gels made from rice grown in Australia, firmness increases exponentially between 40 and 20 °C. Partitioning the temperature sum of squares into linear and quadratic showed that the linear function was highly significant and that the quadratic function was not ($P < 0.01$), in almost every case. Thus, in the succeeding comparisons of the different types of retrogradation with the fresh gels, only the linear function of temperature was considered.

Retrogradation was induced by holding gels at either 20, 40, or 60 °C overnight and by storing gels at low temperatures overnight or for 1 week and then rewarming them to either 20, 40, or 60 °C. Between group and within group mean comparisons showed (i) that firmness of fresh gels was significantly different from all retrogradation treatments, (ii) that holding them overnight at a temperature was significantly different from holding them at 4 °C overnight and rewarming, and (iii) that the length of time of holding them at 4 °C had no significant effect on firmness after rewarming ($P < 0.01$). Canisters

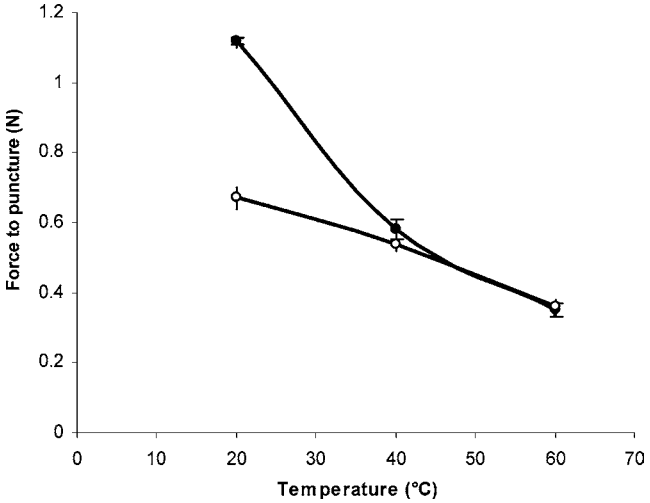

Figure 1. Relationship between temperature (°C) and force (N) required to puncture freshly cooling gels made from full flour of Koshihikari grown in Japan (○) and Australia (●). Gels were prepared and measured in triplicate, and mean values were presented ± standard deviation.

containing stored gels showed no difference in weight over the storage interval for any length of storage or any temperature of storage. Firmness could not be measured at 4 °C for any sample because the firmness of all gels at 4 °C exceeded the capacity of the load cell.

For each treatment, the difference in firmness between 60 and 20 °C was calculated from the means presented in **Table 1**. **Figure 2** shows these differences. The increase in firmness of fresh gels between 60 and 20 °C was significantly greater ($P < 0.01$) than the increase in softness of gels between 20 and 60 °C after storage at 4 °C (**Figure 2**).

Removal of proteins from the flour of both rices led to softer gels than those from the full flour for all retrogradation treatments (**Table 1**). However, within location, comparisons of the magnitudes of the changes in firmness for all retrogradation treatments without proteins were not significantly different ($P < 0.01$) from those of gels of flour (**Figure 2**).

When lipids are removed from the flour, cooling and storage lead to significantly firmer gels than when lipids are present ($P < 0.01$). **Table 1** shows that the firmness of all Australian and Japanese gels from lipid-free flours immediately at 60 °C is the same. Lipid-free gels stored overnight at 60 °C are significantly softer than lipid-free gels stored at 4 °C and rewarmed to 60 °C (**Table 1**). The magnitude of the change in firmness on cooling of lipid free gels is much greater than for gels that contain lipids (**Figure 2**). The difference is 10-fold for the gels made from rice grown in Japan (**Figure 2**). The increase in firmness as a function of cooling (fresh and overnight at temperature) is significantly greater than the decrease in firmness as a function of warming from 4 °C.

Gels made from full flour were cooled immediately from 60 to either 40 or 20 °C and then rewarmed immediately to 60 °C to examine the reversibility of immediate cooling and rewarming. **Table 2** shows that gels made from flour of Koshihikari grown in Japan, immediately rewarmed from 40 or 20 °C back to 60 °C, are not significantly firmer than fresh gels measured immediately at 60 °C ($P < 0.01$). **Table 2** also shows that gels from Koshihikari grown in Australia and measured immediately at 60 °C are softer (slightly significant) than gels rewarmed to 60 °C after cooling to 40 °C. However, rewarming from 20 °C back to 60 °C produces significantly firmer gels than those measured immediately at 60 °C ($P < 0.01$) (**Table 2**).

MWD of Starch Components. Figure 3 shows the SEC traces of the debranched HWSFs that were able to be washed from freshly made gels at each of the three temperatures. The debranched amylose chains from the HWSF of full flour are shown as a reference for molecular weight differences. **Figure 3a** shows that many more chains greater than DP 100, derived from amylose, could be washed from gels made from Australian flour than from gels made from Japanese flour (**Figure 3b**). **Figure 3** also shows that very few of the long chains could be washed from any of the gels. Slightly more amylose washed from the gels at 60 than at 40 °C and, again, slightly less washed from gels at 20 than at 40 °C (**Figure 3**). Furthermore, the average MW of chains that could be washed from gels decreased as the temperature of the gel decreased (**Figure 3**).

Figure 4 shows the debranched chains in the starch that were able to be washed from the gels after storage at 4 °C overnight and then rewarming to either 20, 40, or 60 °C before washing. The debranched amylose chains from the HWSF of full flour are again shown as a reference for molecular weight. **Figure 4** shows that the amount and the molecular weight of the chains that could be washed from these gels were both lower after storage at 4 °C and rewarming, as compared with immediate cooling shown in **Figure 3**. Very few amylose chains (greater than DP 100) washed from gels from either location rewarmed to 20 °C, a small amount of amylose chains washed from gels rewarmed to 40 °C, and more amylose chains could be washed from gels rewarmed to 60 °C (**Figure 4**). At both 40 and 60 °C, more amylose chains could be washed from the gels made from the Australian flour (**Figure 4a**) than from those made from the Japanese flour (**Figure 4b**).

Figure 5 shows the debranched chains in the starch that were able to be washed from the gels after storage overnight at either 20, 40, or 60 °C. The debranched amylose chains from the HWSF of full flour are again shown as a reference for molecular weight. **Figure 5** shows that after storage at 60 °C overnight, almost no long amylose chains are able to be washed from the gel, but many of the shorter amylose chains can be washed. Storage overnight at 40 °C further decreases the MW of the chains that could be washed from gels, and storage at 20 °C overnight decreases both the amount and the molecular weight of extractable amylose. More chains could be washed from gels at each temperature after storage at those temperatures (**Figure 5**), as compared with those from gels stored overnight at 4 °C and then rewarmed to either 20, 40, or 60 °C (**Figure 4**).

Figure 6 shows the MWD of the debranched chains of amylose in the HWSF of flour from the Australian and Japanese Koshihikari on the UH 250 column; this column concentrates the peak for the long amylose chains. No amylose was retained in the hot water insoluble fraction of full flour (data not shown). **Figure 6** shows that significantly more chains greater than DP 100, and especially greater than DP 1000, were solubilized from the Australian flour than from the Japanese flour, despite the fact that both rices were of the same amylose content by iodine.

Figure 7 shows the MWD, on the UH 250, of chains in the HWSF from defatted flour. There is about a 3-fold increase in the number of large amylose chains from both varieties, relative to **Figure 6**, and the increase is much greater for the Japanese rice. Rice from Japan has slightly fewer long amylose chains than rice from Australian Koshihikari, consistent with the slightly lower amylose value obtained by iodine.

Proteins in the HWSF of flour from both rices were determined by SDS−PAGE of the HWSF. **Figure 8** shows two protein bands (60 and 22 kDa) in the HWSF of the Australian

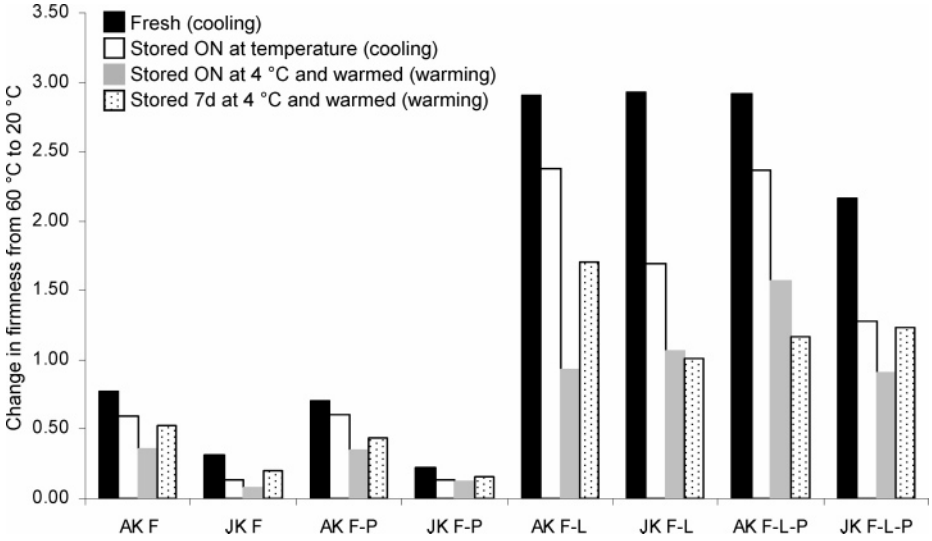

Figure 2. Change in firmness as a function of cooling from 60 to 20 °C or as a function of heating from 20 to 60 °C for each flour type and each form of retrogradation; ON, overnight.

Table 2. Force (N) Required to Puncture Freshly Prepared Gels from Full Flour of Koshihikari Grown in Either Japan or Australia[a]

	force (N) at 60 °C		
location	immediately	rewarmed from 40 °C	rewarmed from 20 °C
AUS	0.350	0.463*	0.616*
JAP	0.362	0.396	0.395

[a] Firmness was measured at 60 °C immediately and after rewarming the samples to 60 °C following cooling to either 40 or 20 °C; $n = 3$. The asterisk indicates significant differences between the three values for the Australian flour ($LSD_{0.05} = 0.046$).

sample and one protein band (22 kDa) in the HWSF of the Japanese sample.

Figure 9 shows the chain length distribution of the amylopectin of both rices expressed as ln $P(N)$, which allows comparison without normalization (*31*) and shows no difference between the chain length distribution of the two rices.

DISCUSSION

The amylose content is believed to make a large contribution to the firmness of freshly cooked and cooled and rapidly retrograded gels (*32*). The two rices used in this study are of very similar amylose and protein contents and are assumed to be genotypically identical. Retrogradation is measured as the increase in firmness of gels after different lengths and temperatures of storage. **Table 1** shows that the firmness of gels made from Koshihikari is significantly dependent on the geographical origin of the rice, and **Figure 2** shows that firmness of gels made from flour of Japanese Koshihikari changes little on cooling. Furthermore, when lipids are present in the gels, retrogradation of gels differs much more significantly with location, at a 1% level of confidence, than when lipids are removed (**Table 1** and **Figure 2**).

Retrogradation describes the hardening of cooked rice upon cooling or storage. At least two processes could lead to a rapid increase in the firmness of rice gels upon storage. First, the increase in firmness with decreasing temperature could be purely a function of viscosity of the starchy paste, which relates directly to the mobility of the molecules. Second, it could be molecular interactions that occur between the less mobile molecules that

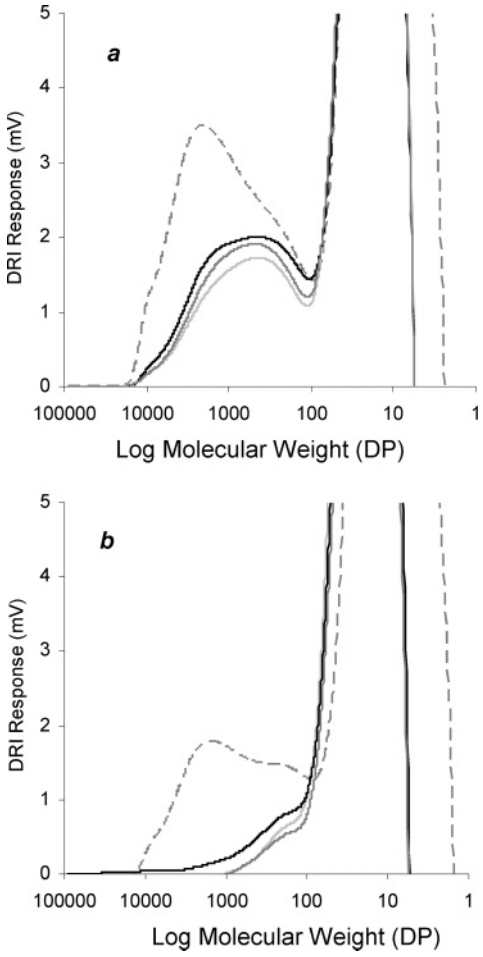

Figure 3. MWD of amylose chains from HWSF of flour (dashed line) from Australian (**a**) and Japanese (**b**) grown Koshihikari and of chains washed from gels immediately as they cooled to 60, 40, and 20 °C. Gels were washed at the same concentration, but the HWSF of flour is not the same concentration as the gels. HWSF is presented as a reference for the complete MWD of chains in the flour.

increase the resistance to puncture by probe or teeth. **Figure 1** shows the increase in firmness of gels made from full flour and cooled immediately to either 60, 40, or 20 °C. **Figure 1** shows a linear relationship between firmness and temperature for the

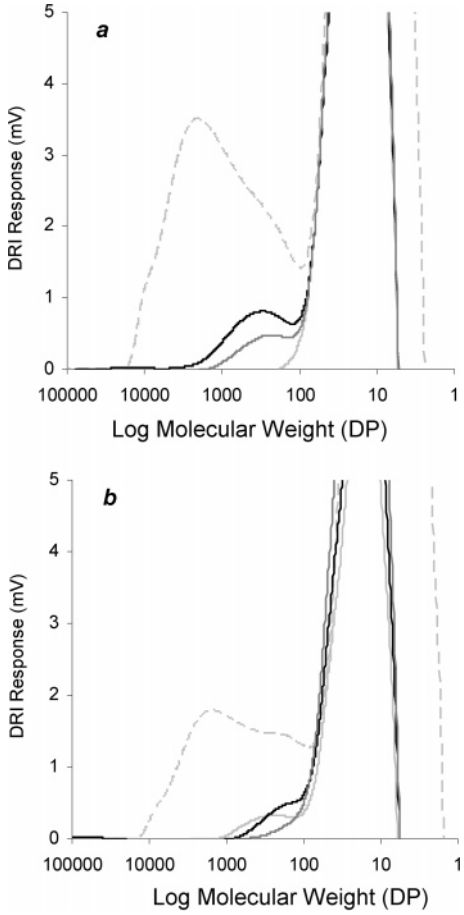

Figure 4. MWD of amylose chains from HWSF of flour (dashed line) from Australian (**a**) and Japanese (**b**) grown Koshihikari and of chains washed from gels after storing them overnight at 4 °C and rewarming them to either 20, 40, or 60 °C. Gels were washed at the rewarmed temperatures. All gels were washed at the same concentration, but the HWSF of flour is not the same concentration as the gels. HWSF is presented as a reference for the complete MWD of chains in the flour.

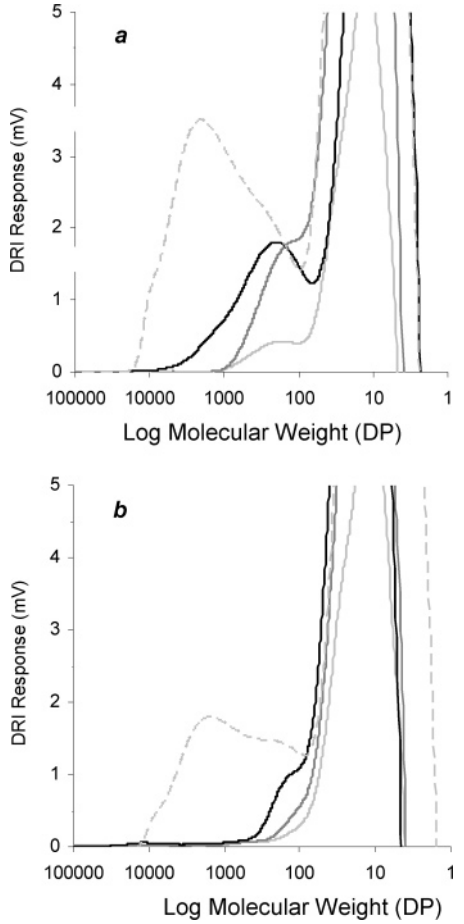

Figure 5. MWD of amylose chains from HWSF of flour (dashed line) from Australian (**a**) and Japanese (**b**) grown Koshihikari and of chains washed from gels after storing them overnight at either 20, 40, or 60 °C. All gels were washed at the same concentration, but the HWSF of flour is not the same concentration as the gels. HWSF is presented as a reference for the complete MWD of chains in the flour.

gels made from the rice grown in Japan, but for the gels made from rice grown in Australia, firmness increases exponentially between 40 and 20 °C, and the firmness at 20 °C is significantly different between the two locations. **Table 2** shows that the firmness of the gels made from the Japanese flour after immediate cooling and rewarming was the same as when freshly measured at 60 °C indicating (i) that no, or minimal, irreversible molecular interactions occurred in those gels and (ii) that the trend seen in **Figure 1** is most likely due to temperature-dependent decrease in mobility of the molecules. However, for the gel made from Australian-grown flour, the increase in the slope between 40 and 20 °C in **Figure 1** and the significant increase in firmness after cooling to 20 °C and immediately rewarming to 60 °C (**Table 2**) suggest that irreversible molecular interactions occurred very quickly between 40 and 20 °C, which increased the firmness of gels made from Australian flour.

The hot water soluble components of rice flour play a large role in the kinetics and molecular dynamics of retrogradation (*10*). The HWSF of rice flour contains most, if not all, of the amylose, some amylopectin (*26*), and some protein (**Figure 8**). The only proteins in rice endosperm that are soluble in water are albumins, which account for about 2% of the total protein in rice (*33*) and are about 20−22 kDa (*34*), and some soluble enzymes. The protein band in both HWSFs (**Figure 8**) is about 20 kDa, so it is likely to be an albumin. The band at about 60 kDa in the Australian HWSF is likely to be a soluble enzyme of starch synthesis. The primary component of the HWSF is starch, and analyses of the MWD of the hot water soluble starch of flour (**Figure 6**) indicate one explanation for the differences in firmness between the retrograded and the fresh gels from the two locations. The average chain length of amylose in the HWSF of the flour from the Japanese rice is about DP 200, but it is greater than DP 1000 for the Australian rice. Furthermore, there are many more chains above DP 1000 that are soluble in the Australian rice. In aqueous systems, at concentrations of the order used here, the chain length of amylose is strongly related to (i) aggregation rates, (ii) the likelihood of precipitation or crystallization, and (iii) the likelihood of forming a cross-linked gel (*35*). Short chains of amylose, around DP 100, tend to precipitate, whereas longer chains of amylose form cross-linked gels on cooling (*35*). The extent of cross-linking is greater for longer molecules of amylose (*4*). Precipitated or crystallized amylose is not expected to affect the firmness of the gel, but cross-linked gels of amylose are. The Australian flour contains many more long chains of amylose than the Japanese flour (**Figure 6**), which would enable the formation of a much more extensively cross-linked gel at cooler temperatures than would form in a gel from the Japanese flour.

Figure 3 shows that in progressively cooler gels, the amylose chains that are not involved in the structure of the gel, and that can be washed from the gel, are shorter chains. Furthermore, as the temperature decreases, the average chain length distribu-

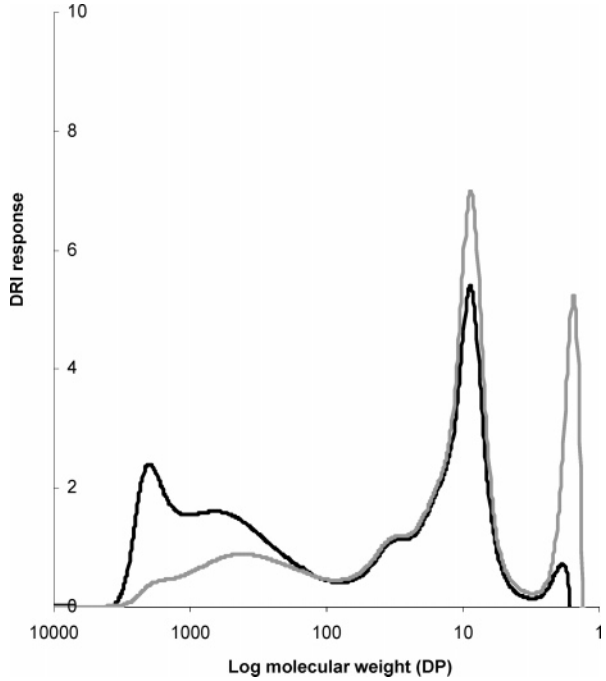

Figure 6. MWD of debranched starch in the HWSF of flour of Koshihikari grown in Australia (black) and Japan (gray).

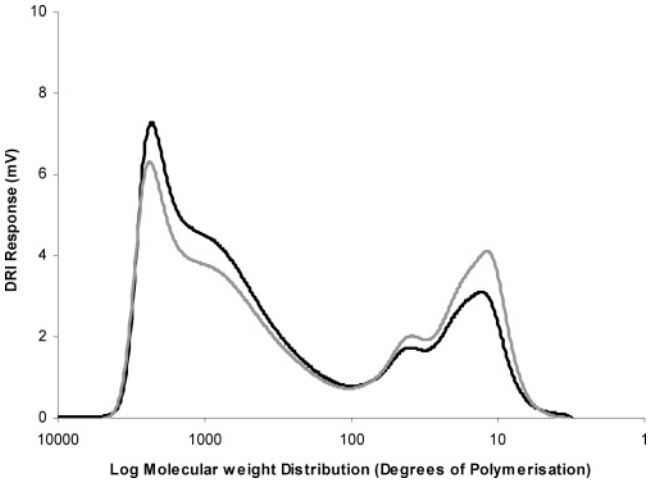

Figure 7. MWD of debranched starch in the HWSF of defatted flour of Koshihikari grown in Australia (black) and Japan (gray).

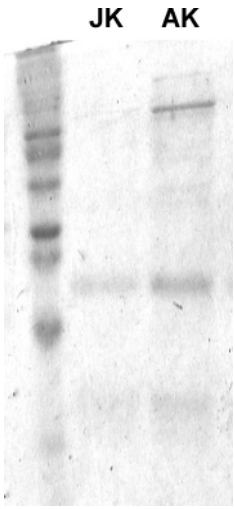

Figure 8. SDS–PAGE of proteins in the HWSF of flour from Koshihikari grown in Japan (JK) and Australian (AK). The first lane is the molecular weight marker.

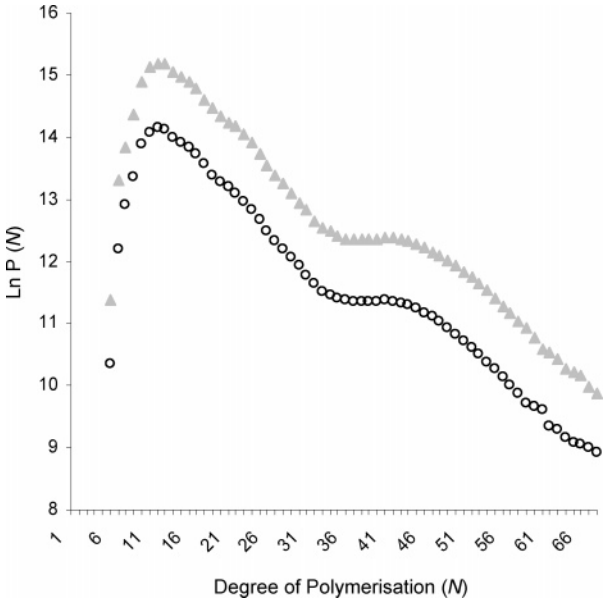

Figure 9. Ln *P*(*N*) plot of the MWD of debranched amylopectin chains of Koshihikari grown in Australian (○) and Japan (▲). The sample from Japan has been offset by 1 unit on the *y*-axis to allow comparison.

tion of the amylose washed from the gels decreases, suggesting that the longer chains that are present in the Australian rice (**Figure 6**) are preferentially involved in aggregating or cross-linking as the temperature decreases. As molecular interactions increase, longer chains are likely to interact at multiple locations along the length of the chain (*4*), thus linking the large amylose molecules of the Australian rice more permanently into the gel structure. The magnitude of the change in firmness on cooling immediately (full flour) as compared with the change in firmness on warming (**Figure 2**) is consistent with interactions, like cross-linking of long amylose chains, which are not reversible in the temperature range used here.

Figure 4 shows that significantly fewer chains of amylose could be washed from gels after storage at 4 °C and rewarming. Rewarming to 40 or 60 °C results in progressively more amylose chains leaching from the gel (**Figure 4**), suggesting that not all of the amylose chains are irreversibly locked into a gel. Reversibility of this form of retrogradation appears strongly related to chain length since only amylose chains of intermediate length could be washed from freshly cooled gels (**Figure 3**), but only short chains could be solubilized from gels rewarmed after cooling to 4 °C (**Figure 4**). The irreversible entanglement of the intermediate chains after storage overnight at 4 °C explains the firmer gel obtained after warming to 60 °C as compared with storage at 60 °C (**Table 1**) for the Australian rice. The same comparison of firmness for the Japanese rice was not significant (**Table 1**), but neither were the differences in amylose chain lengths that washed from the gels (**Figures 3**−**5**). Aside from amylose, protein, lipids, and amylopectin are all components of rice grains, and interactions between these significantly affect the firmness of the gels.

In the absence of protein, the gels are all softer than when made from full flour (**Table 1**). Proteins, although not accounting for much of the grain—both rices are around 5% protein—can account for up to 40% of the added water (*31*). Most proteins in rice (up to 95%) hydrate rather than solubilize (*33*), and they bind water a few degrees below the temperature at which starch begins to bind water (*36*, *37*), leaving less water

available for the starch. When proteins are absent, all of the water is available for the starch, and the gel and starch solution is much more dilute than when proteins are present (*24*). Although it is clear that proteins affect firmness (**Table 1**), they do not affect retrogradation (**Figure 2**). The difference in firmness of gels between 60 and 20 °C, as a function of cooling or warming, is the same, within location, for gels from flour and from gels from which only proteins have been removed (**Figure 2**). Therefore, proteins play a direct role in relative firmness but not in temperature-induced changes in firmness within this temperature range.

Table 1 shows that when lipids are removed from the flour, the freshly cooked and retrograded gels are much firmer than when lipids are present, and gels from flour from both locations are the same. **Figures 6** and **7** show that when lipids are removed from the flour, the HWSF contains about three times more amylose than when lipids are present, especially the HWSF from the Japanese flour. Furthermore, removal of lipids releases the long chains of amylose (**Figures 6** and **7**), and removal of lipids shows that both rices contain approximately the same amount of amylose, in agreement with the iodine values and consistent with identical values for firmness.

The difference between the amylose seen by SEC of HWSFs of the flours (**Figure 6**) as compared with the similarities shown by iodine is that the iodine method includes a step to saponify lipids so that all of the lipid−amylose complexes are dissolved (*38*). Thus, all of the amylose is able to complex with iodine and contribute to the reading. If some of the amylose, in this case **Figures 6** and **7** indicate about 60%, is locked in amylose−lipid complexes, then the iodine value is not an adequate predictor of functional amylose—that which contributes to sensory traits. Amylose molecules form helices, and the lipids lodge in the cavity of the helix (*3*). Many amylose−lipid complexes do not melt until temperatures above 100 °C are reached (*39*). Thus, amylose bound in lipid complexes has no functional influence in rice cooked or processed at or below the boiling point of water.

When the long chains of amylose are available to contribute to the gel, after being released from amylose−lipid complexes, gels at 40 and 20 °C are significantly firmer than gels at 60 °C (**Table 1**) and the difference in firmness between gels at 20 and 60 °C is also significantly greater. In the time that it takes to cool a freshly prepared gel to 20 °C, the long chains of amylose molecules are already able to form a gel that requires a significantly greater amount of force to puncture (JK F-L) than when almost no long chains are present (JK F) (**Table 1** and **Figures 2**, **6**, and **7**). Storage of the lipid-free gels at 40 °C overnight in a sealed container allows the amylose network to strengthen, indicating the relationship with time, but storage at 60 °C overnight is obviously warm enough to inhibit the extensive cross-linking that takes place at the cooler temperatures (**Table 1**). Storage of the lipid-free gels at 4 °C overnight and rewarming to 60 °C show very clearly that the molecular entangling and cross-linking of long amylose chains that occurs at the cooler temperatures are not reversible, at least by rewarming to 60 °C (**Table 1** and **Figure 2**). Thus, the main factor separating the retrogradation parameters of these two rices appears to be the proportion of amylose that is complexed in lipids and prevented from contributing to the formation of networks.

It has been shown that rewarming retrograded gels to temperatures higher than the gelatinization temperature of the rice can reverse the effects of retrogradation (*6*), but this is the melting of recrystallized amylopectin molecules (*6*). Storing gels for 1 week promotes retrogradation of amylopectin (*11*), but **Table 1** and **Figure 2** show that warming of gels stored for 1 week did not fully reverse the changes in firmness. However, for the treatment without lipids and proteins, where the only component remaining is starch, the magnitude of the change in firmness of gels rewarmed to the different temperatures after a week at 4 °C does not differ between locations (**Figure 2**). This is consistent with the finding that the MWD of chains of both amylose (**Figure 7**) and amylopectin (**Figure 9**) is the same for both rices. In all other treatments, the magnitude of the change in firmness of gels warmed after a week at 4 °C differs between location (**Figure 2**). This indicates that proteins, lipids, and starch (perhaps at an organizational level higher than the MWD of chains) interactively affect the reversibility of long-term storage of rice gels.

The difference between the retrogradation parameters of the two rices seems to be solely the proportion of long chains of amylose that is complexed with lipids and the proportion of long chains of amylose that is available to form an irreversibly tangled and cross-linked gel. It is unknown whether the amylose−lipid complexes form during the preparation of the gel or during grain filling and grain development. Very little is known about the processes of lipid synthesis in cereals, let alone how environment affects lipid synthesis, but these two rices clearly demonstrate that environmental conditions do affect lipid synthesis in rice, which, in this study, is the factor that underlies the environmental effect on retrogradation, which is a key trait of rice quality.

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