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Effects of Homoeologous Wheat *Starch Synthase IIa* Genes on Starch Properties

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ABSTRACT: Near-isogenic lines (NILs) of the eight haplotypes of starch synthase IIa (SSIIa) were used to analyze the effects of *SSIIa* gene dosage on branch chain length, gelatinization, pasting, retrogradation, and enzymatic hydrolysis of starches. Compared to wild-type, the amylopectin of lines missing one or more active SSIIa enzymes had increases in the proportion of short branch chains (DP6−10) and decreases in midlength chains (DP11−24), and the size of these differences depended on the dosage of active SSIIa enzymes. Of the three loci, *SSIIa-A1* had the smallest contribution to amylopectin structure and *SSIIa-B1* the largest. The different effects of the three SSIIa enzymes on starch properties were also seen in gelatinization, retrogradation, pasting, and enzymatic hydrolysis properties. Such differences in starch properties might be useful in influencing the texture and shelf life of food products.

KEYWORDS: *wheat, SSIIa, starch properties, gelatinization, retrogradation, enzymatic hydrolysis*

INTRODUCTION

The two major components of starch are amylopectin, an α-1,4-linked glucan polymer that is highly branched via α-1,6 linkages, and amylose, an essentially linear molecule linked via α-1,4 linkages. The specific properties of starch, such as gelatinization, pasting, and retrogradation, are strongly related to the ratio of amylose to amylopectin and to the amylopectin chain length distribution. Therefore, modifying these characteristics can influence the suitability of starch for different uses and can influence the processing or textural properties of starch-based food products. Genetic modification of starch synthesis in plants can be accomplished by the engineering of starch-related enzymes via traditional breeding methods or genetic engineering techniques. For example, "waxy" or amylose-free starch is produced when granule-bound starch synthase I (GBSSI) activity is eliminated. The waxy phenotype was described and utilized in diploid plants such as maize and rice many years ago, whereas waxy wheat was first developed more recently.[1] Common wheat is a hexaploid plant; therefore, elimination of GBSSI activity required the identification of wheat lines with null alleles at each of the three *GBSSI* loci, followed by crossing of these lines and selecting plants that lacked all GBSSI enzymes. In addition to the fully null wheat GBSSI mutants, six "partial" waxy mutants have been produced that include various combinations of one or two active genes originating from the A, B, or D genome. These partial mutants show small, yet significant reductions in amylose levels, which are reflected in changes in starch properties such as gelatinization and pasting profiles.[2] For example, wheat lines lacking only GBSS-BI are known to be especially suitable for Asian noodle production because the slightly lower amylose content of starch from these lines imparts a specific viscoelastic texture to the noodles.[3,4] Thus, subtle modifications in starch can be achieved by taking advantage of the polyploid nature of common wheat, and starch that is naturally "fine-tuned" by this method is both useful and readily acceptable for food products.

Whereas GBSSI is the major enzyme involved in amylose synthesis, there are several enzymes employed in the synthesis of amylopectin. One such enzyme, starch synthase IIa (SSIIa), appears to have a particularly important role in amylopectin synthesis.[5−7] Genetic elimination of all SSIIa proteins (also known as starch granule protein-1 or SGP-1) by combining lines missing SSIIa originating from the A, B, and D genomes resulted in the production of wheat with a high amylose level.[8] Although there have been several studies on the properties and potential of the SSIIa null line,[9,10] information on the properties of partial SSIIa lines with one or two active enzymes is still limited. In a study comparing the starch properties of wild-type and single-, double-, and triple-null mutants selected from doubled haploid lines,[11] SSIIa protein dosage effects were observed for chain length distribution, pasting properties, swelling power, and gelatinization properties, but only the triple-null mutant showed significant changes in total starch content, granule morphology and crystallinity, granule size distribution, amylose content, and amylose−lipid complex dissociation.

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In this study, the starch properties of each of the eight homozygous SSIIa genotypes were compared using near-isogenic lines (NILs) generated from a cross between a Japanese cultivar and a fully null SSIIa line.

MATERIALS AND METHODS

Plant Materials. The SSIIa genotypes of the NILs used in this study are listed in Table 1. The wild-type cultivar Shirogane-Komugi (SRGN) was used as a recurrent parent, and an SSIIa triple-null line, Wakei-RB3668-1, was used as a donor parent. Three codominant DNA marker sets were used to identify wild-type and null alleles,[12] and F2 plants heterozygous at the *SSIIa-A1*, *-B1*, and *-D1* loci were selected for backcrossing to SRGN. After five backcrosses, plants were self-pollinated, and the BC5F2 generation was used to select eight genotypes including wild-type (type1) and triple null (type 8) by marker-assisted selection. The selected BC5F3 seeds were planted in a field in November 2008 and harvested in June 2009.

Table 1. SSIIa Genotype, Starch Content, and Amylose Content of Plant Materials[a]

	SSIIa genotype				
line	*SSIIa-A1*	*SSIIa-B1*	*SSIIa-D1*	starch[b] (% dw)	amylose[c] (%)
type 1	+	+	+	62.8 a	23.0 b
type 2	−	+	+	58.7 a	22.6 b
type 3	+	−	+	63.2 a	21.9 b
type 4	+	+	−	59.7 a	21.9 b
type 5	+	−	−	61.9 a	22.4 b
type 6	−	+	−	61.1 a	22.0 b
type 7	−	−	+	63.9 a	22.7 b
type 8	−	−	−	46.8 b	31.4 a

[a]Values followed by the same letter in the same column are not significantly different ($P < 0.05$). [b]Starch content of whole wheat flour. [c]Amylose content of isolated starch.

Starch Isolation. Seed samples were ground using a ZM-200 ultracentrifugal mill (Retsch Japan, Tokyo, Japan) to obtain whole wheat flour. Starch isolation was conducted according to the method of Hayakawa et al.[13] The isolated starch was dried under vacuum, and moisture content was determined as the weight loss after heating for 1 h at 135 °C.

Starch Content Determination. The starch content of whole wheat flour was measured using a total starch assay kit (Megazyme International Ireland, Co. Wicklow, Ireland) according to the manufacturer's instructions.

Amylose Content Determination. Amylose contents were determined with an amylose/amylopectin assay kit (Megazyme International Ireland).

Chain Length Distribution Analysis. For debranching analysis, 2 mg of isolated starch was dispersed in 100 μL of dimethyl sulfoxide and boiled for 20 min with occasional mixing. After the mixture had cooled to room temperature, 2 μL of 0.5 M sodium acetate buffer (pH 4.0) was added to 20 μL of the boiled sample, the volume was adjusted to 100 μL with water, and 70 U of isoamylase (Megazyme International Ireland) was added. The mixture was incubated at 37 °C overnight and then heated in a boiling water bath for 5 min to inactivate the isoamylase. A 10 μL aliquot of the supernatant was vacuum-dried and used for analyzing chain length distribution by the FACE technique.[14] Samples were labeled with 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) and were analyzed using a Beckman PA800 capillary electrophoresis system (Beckman Coulter Japan, Tokyo, Japan) equipped with a fluorescence detector (488 nm Laser Module, Beckman Coulter Japan) and eCAP-NCHO capillary (Beckman Coulter Japan), controlled by 32 Karat version 8.0 software. The areas of peaks from DP6 to DP45 were summed, and the area percentage of each peak was calculated. For each DP, the differences in area percent between type 1 (wild-type) and other genotypes were determined.

Differential Scanning Calorimetery (DSC) Analysis. Gelatinization and amylose−lipid complex dissociation properties were measured using a differential scanning calorimeter (DSC-60A, Shimadzu, Kyoto, Japan). Isolated starch (5−7 mg) was placed in the DSC pan, 2× (volume/weight) distilled water was added, and the suspension was mixed using a needle. The pan was hermetically sealed, and the sample was stored overnight at room temperature. Alumina was used as the reference material. For measuring gelatinization and amylose−lipid complex dissociation properties, the sealed pan was heated from 30 to 120 °C at a rate of 5 °C/min, then held for 2 min at 120 °C, and cooled to 30 °C at the rate of 5 °C/min.

For determining percentage of retrogradation, the cooled pan was stored at 4 °C for 7 or 14 days. After storage, the pan was equilibrated to room temperature for 30 min and subjected to the same thermal program described above. Percentage of retrogradation (%R) was determined according to the following calculation:

$$\%R = (\text{enthalpy change of stored sample}) / (\text{enthalpy change of gelatinization}) \times 100$$

Rapid Visco Analyzer (RVA) Measurements. The pasting profile was established using an RVA according to AACC method 76-21 using profile STD3.[15]

Turbidimetric Analysis. Sixty milligrams of starch (dry weight) was suspended in 20 mL of distilled water and heated in a boiling water bath for 30 min with occasionally mixing. After cooling to room temperature, the turbidity was determined by measuring absorbance at 640 nm (0 days). Samples were stored at 4 °C for 1, 7, and 14 days. Prior to measuring turbidity, samples were kept at room temperature for 30 min and mixed.

Enzymatic Hydrolysis of Raw Starch and Cooked Starch. Enzymatic hydrolysis of raw wheat starch was carried out following the method of Setiawan et al.[16] with some modifications. Starch (1%, w/v) was hydrolyzed into soluble sugars by incubation with porcine pancreatic α-amylase (PPA, Sigma Chemical Co., St. Louis, MO, USA) (500 units/g starch, dry weight) at 35 °C with shaking (100 rpm). The soluble sugars in the supernatant were collected after centrifugation (5200*g*, 5 min) and further digested into glucose using glucoamylase. The concentration of glucose released was quantified using a D-glucose assay kit (Megazyme International Ireland). The percentage of starch hydrolysis was calculated as

$$\text{starch hydrolysis (\%)} = 100 \times \text{total mass of glucose released/initial dry mass of starch} \times (162/180)$$

To measure the enzymatic hydrolysis of cooked starch, wheat starch (200 mg, dry weight) was first cooked by heating in 19 mL of deionized water in a boiling water bath for 15 min. After cooling to 37 °C in a water bath, 1 mL of phosphate buffer solution (100 mM, pH 6.9, 5 mM calcium chloride) containing 32 units of PPA was added to the cooked starch sample. The hydrolysis was conducted at 37 °C with shaking (100 rpm). At time intervals of 30, 60, 90, and 120 min, an aliquot of 0.4 mL was removed and mixed with 0.6 mL of 100% ethanol. The soluble sugars were collected and hydrolyzed as described above.

Statistical Analysis. Experiments were performed in at least duplicate for individual lines. A one-way analysis of variance was performed, and differences were assessed by a Scheffe test.

RESULTS

Starch Content and Amylose Content. For both starch and amylose contents, no significant differences were detected among wild-type, single-null, and double-null lines (Table 1). However, type 8 had the lowest starch and highest amylose contents (Table 1), suggesting SSIIa enzyme elimination results

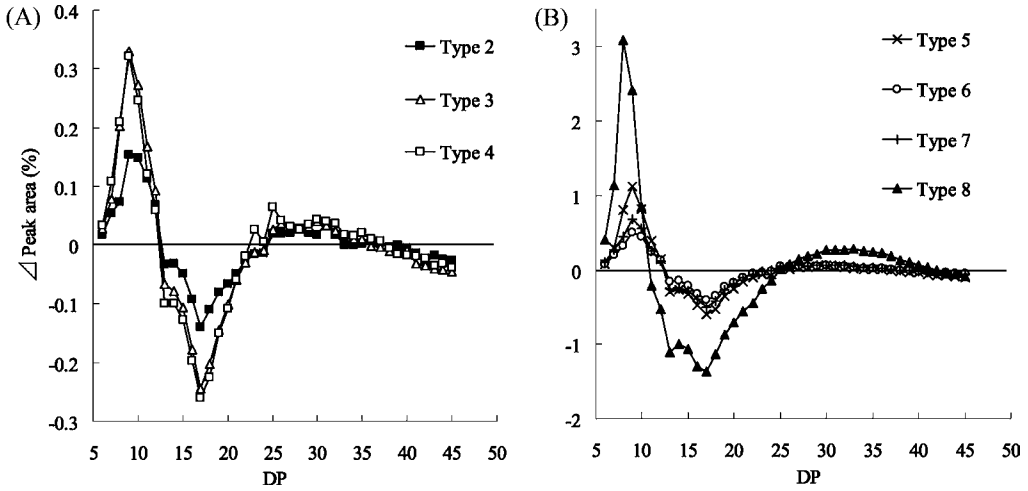

Figure 1. Differences in chain length distribution between type 1 and other NILs: (A) differences between type 1 and types 2−4; (B) differences between type 1 and types 5−8. Data points are the means of at least three measurements.

in a trade-off between starch content and amylose content. These data were in general agreement with previously reported results.[8,11]

Branch Chain Length Distribution of Starch. The branch chain length distribution (CLD) profile of type 1 (wild-type) starch was compared with that of starch originating from the *SSIIa* null allele lines (types 2−8). As shown in Figure 1, types 2−8 showed increases in the proportion of short chains (DP6−10) and decreases in DP11−24 chains compared with type 1. These differences were largest in type 8 (carrying all null alleles), followed by the double-null lines (types 5−7) (Figure 1B and Table 2). Although increases in short chains and decreases in medium chains were also detected in single-null lines (Figure 1A), these changes were very small compared to the double-null lines. Type 8 had a higher proportion of DP8 chains than types 1−7, which is a specific property of this type (Figure 1). These trends were consistent with previous data.[11]

Table 2. Branch Chain Length Distribution[a]

line	DP6−10 (%)	DP11−24 (%)	DP25−45 (%)
type 1	13.6 a	64.7 a	21.8 a
type 2	14.0 ab	64.1 ab	21.8 a
type 3	14.5 bc	63.7 abc	21.8 a
type 4	14.5 bc	63.5 abc	22.0 a
type 5	16.7 e	61.7 d	21.6 a
type 6	15.1 cd	62.8 bcd	22.0 a
type 7	15.6 d	62.3 cd	22.1 a
type 8	21.5 f	54.0 e	24.6 b

[a]Values represent means of at least three determinations. Values followed by the same letter in the same column are not significantly different ($P < 0.05$).

When a detailed analysis of CLD was conducted, differences were observed between single-null lines (Table 2). Although there was no difference between types 1 (wild-type) and 2 (*SSIIa-A1* null) in the amount of short chains (DP6−10), significant differences occurred between type 1 and both types 3 and 4, which carry *SSIIa-B* and *D1* null alleles, respectively. Among the three genotypes carrying two null alleles, significant differences were detected in the level of DP6−10 chains. Types 6 and 7, which carry null alleles at the A locus, showed smaller changes than type 5, which is missing SSIIa enzymes from the B and D genomes. These results indicated that, in terms of CLD, the SSIIa enzyme derived from the A genome has a smaller contribution than the other two enzymes. Although differences between genotypes carrying null alleles from the B and D genomes were less pronounced, it appeared that SSIIa-B1 had a stronger effect on CLD than the SSIIa-D1 enzyme.

DSC Profile. Starch gelatinization onset temperatures (T_o) differed significantly with SSIIa dosage (Table 3). Compared with wild-type, T_o was 1.4−1.8 °C lower in lines with single-null alleles and 2.9−4.2 °C lower in lines with two null alleles. As previously reported,[11] type 8 showed a large decrease in T_o, with an onset temperature 9.7 °C lower than that of wild-type. Peak temperature (T_p), which was also lower in mutant than in wild-type lines, was more useful in distinguishing the effects of specific alleles from the A, B, and D genomes. Type 2 (null *SSIIa-A1*) had a significantly higher T_p than types 3 (null *SSIIa-B1*) and 4 (null *SSIIa-D1*). Among lines carrying two null alleles, type 6 (*SSIIa-A1* and *SSIIa-D1* null) had the highest T_p, whereas type 5 (*SSIIa-B1* and *-D1* null) had the lowest peak temperature.

A clear dosage effect on enthalpy change was also detected, with enthalpy change decreasing according to the number of active SSIIa enzymes. In the lines with a single-null gene (types

Table 3. Gelatinization Properties of Isolated Starch Determined by DSC[a]

	gelatinization peak			amylose−lipid dissociation peak		
line	T_o (°C)	T_p (°C)	enthalpy change (J/g)	T_o (°C)	T_p (°C)	enthalpy change (J/g)
type 1	54.2 a	59.5 a	10.3 a	96.5 a	102.0 a	0.7 a
type 2	52.8 b	57.8 b	9.4 b	97.6 a	102.0 a	0.7 a
type 3	52.5 b	57.0 c	9.4 b	97.1 a	101.9 a	0.6 a
type 4	52.4 b	57.2 c	9.3 b	97.1 a	102.4 a	0.7 a
type 5	50.0 d	53.5 f	7.8 d	96.9 a	101.7 a	0.7 a
type 6	51.3 c	55.6 d	8.8 bc	96.9 a	101.7 a	0.7 a
type 7	50.4 d	54.6 e	8.3 cd	96.3 a	102.3 a	0.8 a
type 8	44.5 e	50.1 g	2.1 f	92.8 b	99.5 b	1.5 b

[a]Values represent means of at least three determinations. Values followed by the same letter in the same column are not significantly different ($P < 0.05$). T_o, onset temperature; T_p, peak temperature.

2−4) the decrease in enthalpy change was 0.9−1.0 J/g, compared with 1.5−2.5 J/g in double-null lines (types 5−7). Although lines missing a single SSIIa enzyme (types 2−4) had similar gelatinization enthalpy changes, differences were observed between the lines missing two SSIIa enzymes, with the lowest enthalpy change value observed in type 5 and the next lowest in type 7. A significant difference was observed between types 5 and 6.

Overall, the *SSIIa-A1* locus had the smallest effect on gelatinization properties and *SSIIa-B1* the largest. Type 5 showed the largest decreases in T_o, T_p, and enthalpy change, and type 7 showed larger decreases in these three parameters than type 6. Among single-null lines, the effect of a null mutation at the A locus (type 2) on T_o and T_p was smaller compared to effects from the other two loci. However, differences between *SSIIa-B1* and *-D1* were not clearly detectable in single-null lines.

In the DSC parameters for dissociation of the amylose−lipid complex, only type 8 showed significant differences from other genotypes (Table 3), which is reasonable given the greater amylose content of type 8 (Table 1).

Starch Retrogradation. Because current information in the literature regarding retrogradation properties of SSIIa mutant starches is limited, both calorimetric and turbidimetric assays were used to investigate retrogradation properties. For calorimetric assays using DSC, the retrogradation profile was determined using gelatinized starch stored at 4 °C for 7 and 14 days (Table 4), with percentage of retrogradation calculated as

Table 4. Thermal Properties of Retrograded Starch[a]

	7 days			14 days		
line	T_o (°C)	T_p (°C)	%R	T_o (°C)	T_p (°C)	%R
type 1	37.4 a	46.4 a	41.5 a	36.4 b	45.2 a	58.3 a
type 2	37.0 a	45.7 a	40.2 ab	36.7 b	44.8 a	53.2 ab
type 3	38.1 a	46.2 a	37.7 ab	37.6 ab	44.6 a	47.2 ab
type 4	38.3 a	46.5 a	38.9 ab	37.1 ab	45.1 a	52.3 ab
type 5	37.7 a	45.1 a	34.1 b	37.3 ab	44.4 a	42.1 b
type 6	38.2 a	45.6 a	34.4 b	37.2 ab	44.9 a	47.2 ab
type 7	38.2 a	45.5 a	34.2 b	36.8 b	44.4 a	44.8 b
type 8	ND	ND	0.0 c	39.4 a	42.9 a	16.3 c

[a]Values represent means of at least three determinations. Values followed by the same letter in the same column are not significantly different ($P < 0.05$). T_o, onset temperature; T_p, peak temperature; %R, percentage of retrogradation.

the ratio of enthalpy change after storage to gelatinization enthalpy change.[17] In types 1−7, the onset temperatures of stored starch samples ranged from 36.4 to 38.3 °C and peak temperatures ranged from 44.4 to 46.5 °C, but a corresponding peak was not seen in type 8 after 7 days of storage. In starch samples stored for 7 days, a reduction in gene dosage was associated with reduced starch retrogradation, and significant differences in percentage of retrogradation were observed between the wild-type line and lines lacking two *SSIIa* genes. After 14 days of storage, types 5 and 7 showed significant differences in percentage retrogradation compared to type 1. Among the double-null lines, the lowest retrogradation was seen in type 5 and the highest value in type 6, suggesting that the relative contribution of each loci followed the same trend seen in chain length distribution and gelatinization properties.

Retrogradation profiles were also determined using a turbidimetric assay (Figure 2). Low concentrations of boiled

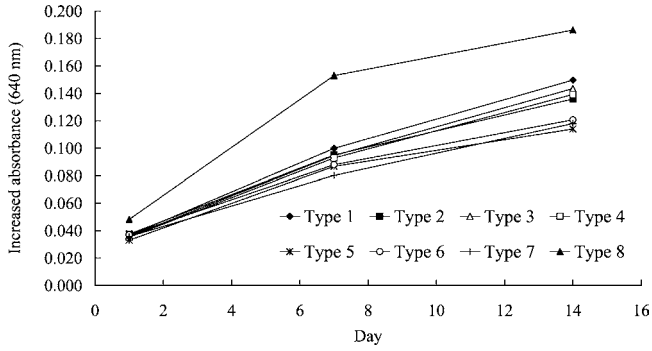

Figure 2. Changes in turbidity during storage of gelatinized starch. Turbidity was measured as absorbance at 640 nm. Data points are the means of at least three measurements.

starch placed at 4 °C undergo slow retrogradation, or recrystallization, resulting in turbidity development over time. Changes in turbidity were measured over a 14 day period (Figure 2). Type 8 gave the largest increase in turbidity throughout the experiment. With the exception of type 8, development of turbidity decreased with a reduction in active SSIIa enzymes. Type 1, having three active SSIIa enzymes, showed larger increases in turbidity than the partial null lines, and the lines lacking two SSIIa showed a slower increase in turbidity than lines with two or three active SSIIa enzymes. However, the relative effects of individual loci were not distinguishable.

Pasting Profiles. SSIIa activity has been shown to affect specific pasting properties of starch in a number of species and, correspondingly, each of the eight wheat SSIIa genotypes showed a distinctive RVA pasting profile (Table 5 and Figure

Table 5. Pasting Profile of Starch[a]

line	peak viscosity (cP)	breakdown viscosity (cP)	final viscosity (cP)
type 1	1819 a	449	3366 a
type 2	1497 ab	228	3151 a
type 3	1545 ab	224	3120 ab
type 4	1327 abc	130	2925 abc
type 5	874 cd[b]	0	2182 d
type 6	1144 bc	39	2577 bcd
type 7	1071 bc	10	2421 cd
type 8	393 d[b]	0	809 e

[a]Values are means of duplicate determinations. Values followed by the same letter in the same column are not significantly different ($P < 0.05$). [b]Viscosity upon initially reaching 95 °C.

3). Notably, types 8 and 5 did not produce a clear pasting peak; therefore, in these lines the maximum peak viscosity was taken as the viscosity measured when the temperature initially reached 95 °C, and the breakdown value was taken as zero. Type 1 had the highest peak, breakdown, and final viscosity levels among the eight genotypes (Table 5 and Figure 3). Among the lines carrying a single active SSIIa, the highest values for all parameters were found in type 6, which carries an active enzyme from the B genome, whereas the lowest values were found in the line with an active enzyme from the A genome (type 5). The overall RVA profile of type 8 differed substantially from other lines, with no real curve detected. Similar changes in RVA parameters due to elimination of all

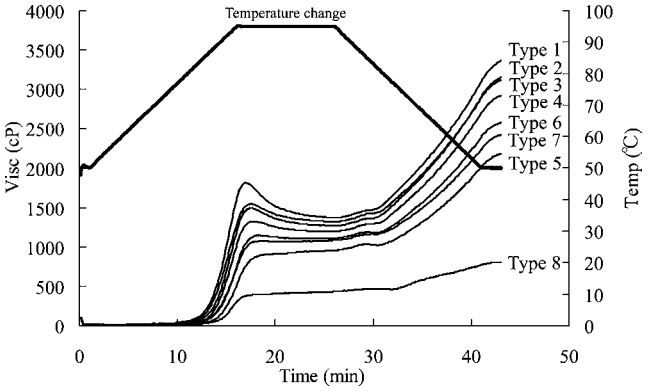

Figure 3. Comparison of pasting profiles of starch from eight *SSIIa* NILs as measured using a Rapid Visco Analyzer (RVA).

SSIIa enzymes were observed by Yamamori et al.[18] and Konik-Rose et al.[11]

Hydrolysis of Raw and Cooked Starch by Porcine Pancreatic α-Amylase. For all samples, nearly 80% of raw starch was hydrolyzed by porcine pancreatic α-amylase within 24 h (Figure 4A). Raw starch from type 1 wheat was hydrolyzed at the lowest rate among all samples throughout the reaction. Hydrolysis of type 8 starch seemed to reach a plateau phase within 3 h. Among the single-null genotypes, types 2 and 4 were hydrolyzed at almost the same rates, whereas type 3 starch appeared to have lower resistance to the amylase. Among the double-null genotypes, type 5 showed the highest and type 6 the lowest digestibility.

For cooked starch, a lower level of digestion was observed only in type 8, whereas the other seven lines showed similar levels of hydrolysis (Figure 4B). This was consistent with observations made by Chanvrier et al.,[19] who showed that enzymatic digestion levels of extruded products made with wild type (type 1 here), single-null (type 2), and double-null (type 6) lines were higher than for triple-null lines (type 8). The higher resistance of type 8 might be due to the formation of resistant starch.[18,19]

DISCUSSION

In this study, we investigated the relative effects of the three homoeologous *starch synthase IIa* (*SSIIa*) genes on starch properties, such as structure, gelatinization, pasting, retrogradation, and enzymatic hydrolysis properties. A similar study by Konik-Rose et al.[11] observed clear SSIIa dosage effects on starch properties such as amylopectin branch chain length distribution, gelatinization properties, and pasting properties. In their study, the eight possible homozygous *SSIIa* genotypes were selected from a doubled haploid population derived from progeny of a cross between an SSIIa null wheat line and an Australian cultivar. In our experiments, NILs obtained from a cross between an SSIIa null line and a Japanese cultivar were used to adjust for genetic background. Our results were largely consistent with those of Konik-Rose et al.,[11] confirming the significant dosage effect of SSIIa on starch properties, even in lines originating from different parents.

Additionally, we noted a dosage effect on the thermal profile of retrogradation (Table 4). Interestingly, in studies of retrograded starch, amylopectin from type 8 had extremely low enthalpy changes (Table 4). Similar trends have been noted in the maize *sugary-2* (*su2*) mutant,[20] which is also lacking SSIIa activity. The thermal properties of retrograded starch are mainly affected by the melting characteristics of recrystallized amylopectin, and outer branches with a length ranging from DP14 to 18 are involved in the crystallization process of amylopectin.[21] Type 8 showed an especially large decrease in chains of this range compared to wild-type (type 1), but all lines missing one or more SSIIa enzymes also showed decreases in these chains (Figure 1). This reduction in DP14−18 branches was likely to slow the retrogradation process.

Results obtained from the turbidimetric analysis were not as straightforward. Lines missing a single SSIIa enzyme showed slower development in turbidity than the wild-type line, and lines with two null alleles grouped below the single-null allele lines (Figure 2). Inconsistent with this trend, however, type 8 showed the largest increase in turbidity over time. The slower development of turbidity in the partial null lines was not unexpected, because the clarity of paste from short-chain amylopectin is thought to be more stable than that from normal amylopectin.[22] However, turbidity is also influenced by amylose,[23] and only type 8 had a significantly larger amylose content than wild-type (Table 1). Additionally, amylose with DP80−100 has a higher tendency toward retrogradation than longer chains,[23] and the amylose from type 8 has a relatively lower molecular weight than wild-type amylose.[24] Thus, the

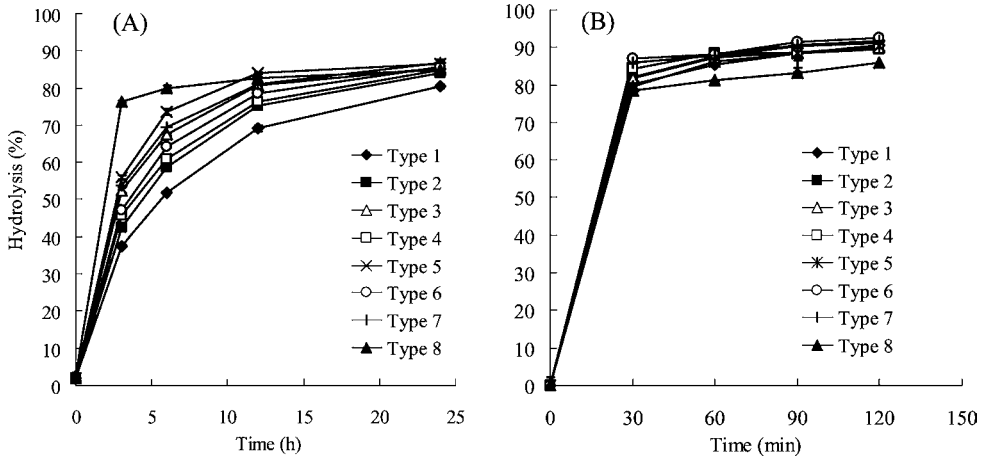

Figure 4. Enzymatic hydrolysis of raw starch (A) and cooked starch (B) by porcine pancreatic α-amylase (PPA). Data points represent the mean of duplicate analyses. Error bars represent standard deviation.

molecular weight and quantity of amylose found in type 8 starch might account for the greater increases in turbidity observed in this genotype. In good agreement with these results, type 8 had the lowest enzymatic hydrolysis rate among all of the cooked starches (Figure 4B).

The levels of enzymatic hydrolysis of raw starch generally decreased according to the dosage of active SSIIa, although the enzymatic susceptibility of type 3 starch surpassed that of type 6 (Figure 4A). Perera et al.[20] reported that starch granules with larger proportions of short branch chains have less perfect crystalline structures and therefore are more susceptible to enzymatic hydrolysis, which agrees well with the results shown here.

In cooked starch, however, only type 8 showed a lower degree of enzymatic hydrolysis (Figure 4B). This might be partially due to the higher amount of amylose−lipid complex formation in type 8 (Table 3). Yamamori et al.[18] also reported that heat−moisture-treated high-amylose starch (type 8) contained >10% more enzyme-resistant starch (RS) than wild-type. A similar tendency toward resistance to hydrolysis was seen in the high-amylose starch of the maize *SSIIa* mutant, *su2*.[20,25]

In addition to dosage effects, or effects attributable to the total number of SSIIa enzymes, our results demonstrated the differences in the contributions of individual SSIIa enzymes to starch structure and properties. The use of BC5 NILs in this study was advantageous in distinguishing these differences, and lines with a single active enzyme (types 5, 6, and 7) appeared particularly useful for this purpose. In these double-null lines, an SSIIa effect on chain length distribution appeared in the number of short branch chains with DP6−10 (Table 2). This is reasonable because SSIIa enzymes elongate short branch chains of amylopectin.[8,26] The relative number of short chains is significantly higher in type 5, which has the wild-type *SSIIa-A1* gene, and lower in type 6, with the wild-type *SSIIa-B1*. Considering that there are no significant differences in amylose contents among types 1−7 (Table 1), we can conclude that SSIIa-A1 has the lowest contribution toward amylopectin synthesis and SSIIa-B1 the highest, whereas the contribution of SSIIa-D1 appears to be between those of these two enzymes. This difference was reflected in most starch properties studies here. Next to type 8, type 5 showed the largest changes in comparison to wild-type in almost all analyses.

It is interesting that the order of *SSIIa* gene contribution to starch properties observed here corresponds to that of GBSSI enzymes on amylose content, with GBSSI-B1 having the highest contribution followed by GBSSI-D1.[2] Further studies on activity or expression levels among the homoeologous sets of starch synthesis enzymes may help elucidate the mechanisms behind these differences.

Relatively small modifications in the amounts or activity levels of starch synthesis enzymes can have important effects on starch characteristics. The Japonica type rice cultivar Nipponbare, for example, is known to have reduced SSIIa activity compared with Indica type rice cultivars, leading to starch with increased levels of shorter chains.[7,27] However, Nipponbare retains a small but detectable level of SSIIa protein, and starch from this cultivar does not demonstrate the typical changes in granule morphology and starch content[27,28] observed in SSIIa mutants of wheat,[8] barley,[5] and pea.[6] The differences in amylopectin structure between Nipponbare and Indica-type rice cultivars more closely resemble the differences between type 1 and the partial SSIIa null wheat lines, particularly the double-null lines, rather than those between types 1 and 8. The reduced SSIIa level in Nipponbare appears to contribute to a lower gelatinization temperature, which is related to cooked rice texture, and a lower degree of retrogradation, which can improve the eating quality of rice that has been cooked and stored.[28] Thus, from a practical point of view, combining null alleles for SSIIa enzymes might result in modified starches that are able to impart slight but significant textural changes to flour products, particularly during storage.

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Notes

The authors declare no competing financial interest.

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