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Physicochemical properties of amylose-free and high-amylose starches from transgenic sweetpotatoes modified by RNA interference

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Abstract

The transgenic sweetpotatoes having amylose-free and moderately high-amylose starches have been obtained by RNA interferences of granule-bound starch synthase I (GBSSI) and starch branching enzyme II [SBEII (class A)] genes, respectively. In this study, the physicochemical properties of such sweetpotato starches were investigated. From the amylose-free starches, structural changes in the starch molecules were found by suppressing GBSSI expression: lack of amylose, lack of long chains with more than DP 100 in amylopectin, and a slight decrease in chains with DP 6 and 7 in amylopectin. These results suggest that GBSSI participates in not only the synthesis of amylose molecules, but also the characterization of amylopectin molecules. In the case of the high-amylose starches, complicated structural changes were found by suppressing SBEII expression: increase in amylose, increase in phosphate content, alteration of crystalline structure, decrease in long chains with more than DP 100 in amylopectin, decrease in chains around DP 6–11, and increase in chains around DP 12–15 and DP 24–33 in amylopectin. As the results of the structural changes, the amylose-free sweetpotato starches showed a very slow retrogradation and high digestibility by pancreatin, whereas the high-amylose sweetpotato starches showed a higher pasting viscosity, more rapid retrogradation and lower digestibility compared to normal starch.

Keywords: Sweetpotato starch; Granule-bound starch synthase; Starch branching enzyme; RNA interference; Pasting; Retrogradation; Digestibility

1. Introduction

The sweetpotato (*Ipomoea batatas*) is one of the important starchy resources in Asia and Africa. In Japan, about one million tons of sweetpotatoes are produced in a year, and 20–30% of those are used for manufacturing starch. As for the application of sweetpotato starches in Japan, most are used in the saccharifying industry to make sugar products, and the rest are used for foodstuffs such as starchy noodles and traditional Japanese confectioneries. Out of the many botanical starches, there are few food uses for sweetpotato starches, and their poor diversity in starch traits limits the range of use. Based on this point of view, several sweetpotato starches having unique properties, such as low amylose content (Kitahara, Ueno, Suganuma, Ishiguro & Yamakawa, 1999) or low gelatinization temperature (Kitahara et al., 1999; Katayama et al., 2002; Katayama, Tamiya, & Ishiguro, 2004; Kitahara et al., 2005), were found in new sweetpotato lines, which were bred by a traditional breeding technique based on sexual hybridization. However, there are some inconvenient problems in sweetpotato breeding, such as sterility and cross-incompatibility (Otani, Shimada, Kimura, & Saito, 1998). Therefore, breeders must either take a longtime or be fortunately endowed to create a new line having certain

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desired characteristics. To overcome such problems, an alternative technology is genetic engineering.

Many starch-related genes and proteins have been identified in a range of plant species, and the outline of starch biosynthesis is fairly well understood, which makes it possible to create unique starches using gene manipulation (Morell & Myers, 2005). In the case of potatoes (*Solanum tuberosum*), for example, an amylose-free starch was produced by suppressing the expression of granule-bound starch synthase I (GBSSI) (Visser et al., 1991), and high-amylose starches were produced by suppressing the expression of one isoform of a starch branching enzyme, SBE A (Jobling et al., 1999), or by suppressing the expression of both SBE A and SBE B (Schwall et al., 2000; Blennow et al., 2005). Only one line of transgenic sweetpotato having amylose-free starch has been obtained by introducing full-length sense cDNA for sweetpotato GBSSI (Kimura et al., 2001). Although some properties of this sweetpotato amylose-free starch have been reported (Noda et al., 2002), structural information other than the apparent amylose content was limited to short unit chains (DP = 6–35) constituting the starch.

Recently, RNA interference technique was applied to sweetpotatoes to obtain new transgenic lines with unique starch properties. One is sweetpotatoes suppressed the GBSSI expression (Shimada et al., 2005), and the other is sweetpotatoes suppressed the SBEII [class A branching enzyme (Burton et al., 1995)] expression (Shimada, Otani, Hamada, & Kim, 2006). Compared to the non-transgenic line, these starches were amylose-free and high-amylose, respectively. This was the first trial production of high amylose starch in sweetpotato. Since the primary starch structure is different according to plant species, sweetpotato starches with different amylose contents are expected to have, respectively, unique properties. In this paper, we report on the physicochemical properties of amylose-free and high-amylose starches from transgenic sweetpotatoes modified using RNA interference technique. In addition, the contribution of GBSSI or SBEII to the molecular structure of a sweetpotato starch is discussed by comparing the differences between transgenic and non-transgenic plants.

2. Materials and methods

2.1. Starches and reagents

Transgenic sweetpotatoes (cultivar, Kokei 14) modified by RNA interference of the construct encoding double-strand RNA of the first exon of sweetpotato GBSSI gene (GBSSI-RNAi) or a partial fragment of sweetpotato SBEII gene (SBEII-RNAi) lines have been obtained as described by Shimada et al. (2005) and Shimada et al. (2006), respectively. In this study, four lines (GR-1, GR-2, GR-4, GR-11) were used out of 38 GBSSI-RNAi lines, and three lines (ASGS-1, ASIS-1, ASIS-2) were used out of eight SBEII-RNAi lines. The peel and cambium tissues were removed from two or three tuberous roots of each line, and the starches were prepared from the inner tissues as described elsewhere (Kitahara et al., 2005). The starches were defatted by dissolving in dimethyl sulfoxide and precipitating with ethanol (Takeda, Hizukuri, & Juliano, 1986). The amylose and amylopectin of the defatted starches were separated by the butanol-complex method of Takeda et al. (1986), and the amylopectin was recovered from the supernatant fraction (Takeda, Hizukuri, & Juliano, 1987). The amylopectin samples obtained in this method were free from amylose, which was confirmed by the iodine-staining properties (absorbance at 680 nm and λ_{max}) of the eluates from a column of Toyopearl HW-75F (Tosoh, Tokyo, Japan) as described by Takeda et al. (1987).

All reagents and solvents, unless otherwise specified, were obtained from Wako Pure Chemical Industries, Osaka, Japan, and were of analytical grade.

2.2. General properties of starches

Defatted starch (50 mg, d.w.), obtained as described above, was dissolved by adding dimethyl sulphoxide (1 ml) and heating at 70 °C for 3 h, and then the solution was filled up to 100 ml with distilled water and allowed to stand for 1 h. To the defatted starch solution (8 ml) 4 ml of 0.2% iodine solution containing 2% potassium iodide was added and filled up to 50 ml. After standing for 1 h at 25 °C, the absorbance of the solution was measured at 680 nm (Abs_{680}, Spectrophotometer U-3310, Hitachi, Tokyo, Japan). Standard amylose and amylopectin were prepared as described above from a common sweetpotato starch (cv. Koganesengan). Under the same condition of the iodine coloration, the amylose and amylopectin gave the Abs_{680} of 2.973 and 0.445, respectively. The apparent amylose content was calculated from the Abs_{680} of the defatted starch with the following equation:

$$\begin{aligned}\text{Apparent amylose}(\%) = [&(\text{Abs}_{680}\text{ of defatted starch})\\ &-(\text{Abs}_{680}\text{ of amylopectin})]\\ &\times 100/[(\text{Abs}_{680}\text{ of amylose})\\ &-(\text{Abs}_{680}\text{ of amylopectin})]\end{aligned}$$

Phosphate groups bound to starch were determined as inorganic phosphate after ashing the defatted starch (Kitahara et al., 2005). An X-ray diffractogram was obtained using a Rigaku RU-200B X-ray diffractometer (Rigaku, Tokyo, Japan) under the following conditions: radiation CuKα, voltage 40 kV, current 100 mA, angle (2θ) 3–30° scan speed 1°/min.

Pasting property of the starches was measured using a rapid visco-analyzer, RVA-3D (Newport Scientific Pty. Ltd., NSW, Australia), at a 7% starch concentration. The temperature program of RVA was as follows: hold at 35 °C for 5 min, heat up to 93 °C at 3 °C/min, hold at 93 °C for 10 min and cool down to 35 °C at 3 °C/min. Ret-

rogradation of the 2% starch pastes during cold storage and digestibility of the starch granules by pancreatin (from hog pancreas) were evaluated by the same methods reported previously (Kitahara et al., 2005).

These experiments were performed in duplicate, and the differences between the replicate results were less than 2% of each mean value.

2.3. Unit-chain distributions of debranched starches and amylopectins

Defatted starch or amylopectin was debranched by isoamylase (Megazyme, Biocon Japan Ltd., Nagoya, Japan) as described previously (Kitahara et al., 2005). The unit-chain distribution of the debranched samples was determined by gel-permeation chromatography (CCPM-II pump and RI-8022 detector, Tosoh, Tokyo, Japan) using two linked columns of Superose 6 and Sephadex G25SF (1×30 cm each, Amersham Bioscience, Tokyo, Japan), which were eluted with 100 mM sodium phosphate buffer (pH 6.2) containing 0.02% sodium azide at 40 °C. The column system was calibrated using synthesized linear amyloses [peak degree of polymerization (DP) = 1953, 722, 438, 215; products once commercially available from Ajinoki, Aichi, Japan, but not available now] and maltoheptaose (Hayashibara Biochem. Lab. Inc., Okayama, Japan). Moreover, four standards were prepared in our laboratory by fractionating debranched waxy maize starch using a Sep-Pak Plus cartridge with C18 sorbent (Waters Corporation Japan, Tokyo, Japan). The debranched waxy maize starch (2.5 mg/2.5 ml) was passed through the cartridge and eluted with distilled water (5 ml) as a water fraction, and then eluted stepwise with 1% aqueous ethanol (5 ml), 2% aqueous ethanol (5 ml) and 3% aqueous ethanol (5 ml) according to the method reported previously (Kitahara & Copeland, 2004). The four fractions were lyophilized separately. The peak DP of the fractions was estimated to be 12 for the water fraction, 19 for the 1% ethanol fraction, 39 for the 2% ethanol fraction and 57 for the 3% ethanol fraction as determined by the fluorescent labeling method of Hanashiro and Takeda (1998). The debranched amylopectins were also examined by high-performance anion-exchange chromatography with pulsed amperometric detention (HPAEC-PAD) using a CarboPac PA-1 column and a guard column (Dionex DX-500, Nippon Dionex K.K., Osaka, Japan) (Kitahara, Imamura, Omae, & Suganuma, 1998). These chromatographic experiments were performed in duplicate, and good agreement between the replicate results was obtained.

3. Results and discussion

Table 1 shows the apparent amylose content and the phosphate content of the starches from the GBSSI-RNAi and SBEII-RNAi lines as well as a starch from a non-transgenic line. Since the values of absorbance at 680 nm (Abs_{680}) for the starch-iodine complexes of GR-1 ($Abs_{680} = 0.314$), GR-4 (0.328) and GR-11 (0.340) were lower than that of the standard amylopectin (0.445), the apparent amylose contents in these starches were calculated to be 0%. The GR-2 starch had a similar amylose content to the non-transgenic starch, suggesting the failure of gene silencing. The phosphate contents of the GR-1, 4 and 11 starches were slightly higher than those of the non-transgenic and GR-2 starches. This increment in phosphate content for the starches would be due to the increase in amylopectin content, because the phosphate groups of sweetpotato starch bind predominantly to the glucosyl residues of the amylopectin (Takeda, Tokunaga, Takeda, & Hizukuri, 1986). In the case of the SBEII-RNAi lines, the starches had apparent amylose contents of 23.6–25.1%, which was a moderate increase of about 10% compared to that of the non-transgenic starch. Since the apparent amylose content for Japanese sweetpotato varieties ranges from 10.3% to 24.3% (Kitahara, Ooi, Mizukami, Suganuma, & Nagahama, 1996; Noda et al., 1998; Kitahara et al., 1999), the SBEII-RNAi starches belong the highest class of amylose content. Interestingly the phosphate content of the SBEII-RNAi starches increased twofold compared to that of the non-transgenic starch. The similar increase in the phosphate content was previously found in transgenic potato lines where their starch branching enzyme expressions had been suppressed (Safford et al., 1998; Jobling et al., 1999; Schwall et al., 2000; Blennow et al., 2005). Blennow et al. (2005) suggests that this effect is caused by a specific action of α-glucan water dikinase, a starch phosphorylating enzyme, on the starch molecules, because this enzyme shows different specific activities for differing chain lengths (Mikkelsen, Baunsgaard, & Blennow, 2004). Fig. 1 shows X-ray diffractograms of the starches from the transgenic sweetpotato lines. Generally, sweetpotato starch shows a C-type diffractogram, as shown in that of the non-transgenic starch, which is a mixture of A- and B-crystalline structures (Imberty, Buléon, Tran, & Pérez, 1991). All GBSSI-RNAi

Table 1
Apparent amylose content and phosphate content of starches from transgenic sweetpotato lines

	Apparent amylose content (%)	Phosphate content (μmol/g starch)
Non-transgenic starch	15.2	6.18
GBSSI-RNAi starches		
GR-1	0[a]	6.89
GR-2	15.9	5.67
GR-4	0[a]	7.11
GR-11	0[a]	7.08
SBEII-RNAi starches		
ASGS-1	24.5	10.83
ASIS-1	25.1	10.42
ASIS-2	23.6	10.24

[a] The absorbance at 680 nm for the starch-iodine complexes were lower than that of the standard amylopectin.

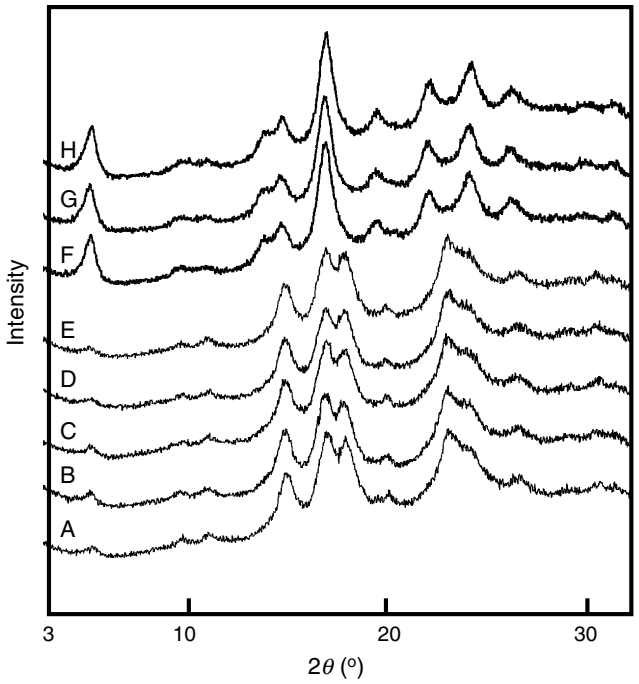

Fig. 1. X-ray diffractograms of starch granules from transgenic sweetpotato lines. (A) Non-transgenic, (B) GR-1, (C) GR-2, (D) GR-4, (E) GR-11, (F) ASGS-1, (G) ASIS-1, (H) ASIS-2.

starches showed the same difractograms as that of the normal starch, whereas the SBEII-RNAi starches showed a distinct diffractogram that was a B-type crystalline structure. These changes of the SBEII-RNAi starches in phosphate content and crystalline structure suggest some alterations for the branch structure of amylopectin.

Fig. 2 shows the unit-chain distributions of the whole starches and their isolated amylopectins. The distributions were divided into three fractions, Fr.1, Fr.2 and Fr.3. The three fractions from the debranched whole starch correspond roughly to an amylose fraction and two unit-chain fractions from amylopectin in order of elution (Ikawa, Glover, Sugimoto, & Fuwa, 1978). Table 2 summarizes the proportions of each fraction and the ratio of Fr.3 to Fr.2 as an index of the amylopectin structure. The non-transgenic starch showed 18.4% for Fr.1 and 1.9 for Fr.3/Fr.2. Carbohydrates for the GR-1, GR-4 and GR-11 starches were not detectable in Fr.1, which demonstrates that the starches are practically free from amylose. It was reported that sweetpotato amylopectins had more amylose-like long chains than the other plant amylopectins (Hanashiro, Tagawa, Shibahara, Iwata, & Takeda, 2002). As also shown in Fig. 2, the unit-chain distributions of GBSSI-RNAi amylopectins revealed that the non-transgenic and GR-2 amylopectins had the long chains with more than DP 100 eluting at Fr.1, whereas the amylopectins from the amylose-free starches had no such long chains. The lack of long chains in the amylopectins from the amylose-free starches would be responsible for their lower Abs_{680} of the starch-iodine complexes than that of the standard amylopectin. These results suggest that the GBSSI contributes to not only the creation of amylose, but also the formation of long chains of amylopectin. The synthesis of long chains of amylopectin by GBSSI

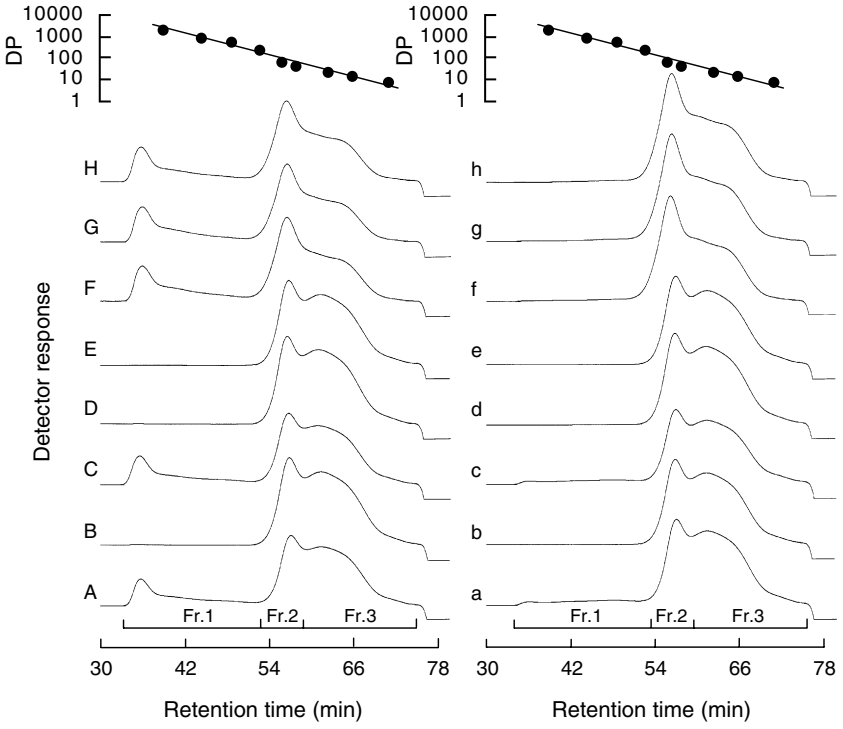

Fig. 2. Unit-chain distributions of starches and amylopectins. Capital letters indicate the whole starches, and small letters indicate the amylopectins. A and a, Non-transgenic; B and b, GR-1; C and c, GR-2; D and d, GR-4; E and e, GR-11; F and f, ASGS-1; G and g, ASIS-1; H and h, ASIS-2.

Table 2
Fractions of debranched starches and amylopectins

	Fr.1[a] (%)	Fr.2[a] (%)	Fr.3[a] (%)	Fr.3/Fr.2[b]	Δ Fr.1[c] (%)
Non-transgenic					
Starch	18.4	27.7	53.9	1.9	11.9
Amylopectin	6.5	33.3	60.2	1.8	–
GBSSI-RNAi					
Starches					
GR-1	n.d.[d]	34.9	65.1	1.9	–
GR-2	18.9	29.6	51.5	1.7	12.2
GR-4	n.d.[d]	34.0	66.0	1.9	–
GR-11	n.d.[d]	34.5	65.5	1.9	–
Amylopectins					
GR-1	n.d.[d]	35.4	64.6	1.8	–
GR-2	6.7	31.7	61.6	1.9	–
GR-4	n.d.[d]	35.1	64.9	1.8	–
GR-11	n.d.[d]	34.1	65.9	1.9	–
SBEII-RNAi					
Starches					
ASGS-1	23.3	37.7	39.0	1.0	20.3
ASIS-1	24.1	37.6	38.3	1.0	20.9
ASIS-2	22.7	38.0	39.3	1.0	20.2
Amylopectins					
ASGS-1	3.0	49.2	47.8	1.0	–
ASIS-1	3.2	47.6	49.2	1.0	–
ASIS-2	2.5	47.5	50.5	1.1	–

[a] Fractionation of each fraction is shown in Fig. 2.
[b] Ratio of Fr.3 to Fr.2.
[c] Difference of the starch and amylopectin in Fr.1.
[d] Not detected.

has been shown in starches from *Chlamydomonas* (Maddelein et al., 1994), wheat (Yoo & Jane, 2002) and rice (Inouchi et al., 2005). The Fr.3/Fr.2 comparison is similar among the GBSSI-RNAi amylopectins including the non-transgenic amylopectin, indicating that these amylopectins have a similar branch structure. In the case of the SBEII-RNAi lines, it was found that the proportions of the transgenic starches in Fr.1 (22.7–24.1%) were larger and the proportions of their amylopectins in Fr.1 (2.5–3.2%) were smaller than those (18.4% and 6.5%, respectively) of the non-transgenic line. Therefore, the true amylose fraction of starches was corrected as a difference of the starch and amylopectin proportions in Fr.1. The differential amounts in Fr.1 (ΔFr.1) were estimated to be 11.9% for the non-transgenic starch and 20.2–20.9% for the SBEII-RNAi starches, which was confirmed to be due to an increase in true amylose molecules. There was a clear difference in the elution profiles of Fr.2 and Fr.3 comparing the SBEII-RNAi and non-transgenic amylopectins as well as the GBSSI-RNAi amylopectins. The Fr.3/Fr.2 ratio changed from 1.8 for the non-transgenic amylopectin to 1.0 or 1.1 for the SBEII-RNAi amylopectins, indicating a large decrease in the proportion of chains in Fr.3 of SBEII-RNAi amylopectins. There was also an overall increase in the chain length in Fr.2 and Fr.3.

Furthermore, the distribution of unit chains between DP 6–36 of the amylopectins was analyzed by HPAEC-PAD. As shown in Fig. 3, the distributions are expressed as a difference in the chain distributions between the transgenic and non-transgenic amylopectins. All amylopectins from the amylose-free starches showed similar distributions to that of the non-transgenic amylopectin, except for the amount of chains with DP 6 and 7. This minor structural change in the amylopectin of the amylose-free starches was consistent with a previous result reported for amylose-free

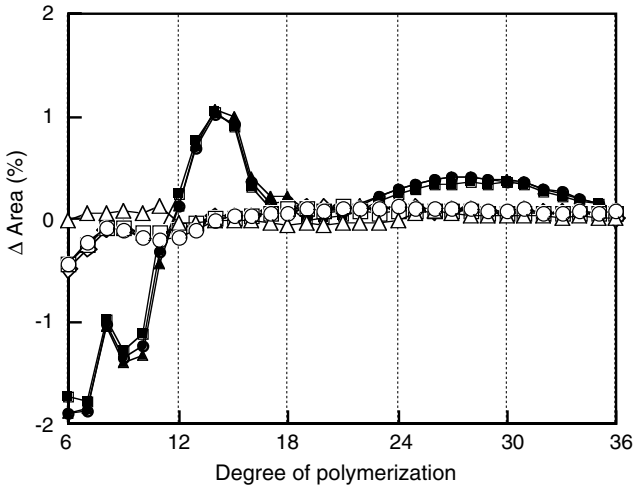

Fig. 3. Differences in short chain distributions of transgenic amylopectins compared to the non-transgenic amylopectin. ○, GR-1; △, GR-2; □, GR-4; ◇, GR-11; ●, ASGS-1; ▲, ASIS-1; ■, ASIS-2.

sweetpotato starch (Noda et al., 2002). On the other hand, the SBEII-RNAi amylopectins showed a large difference compared to the distributions from the non-transgenic amylopectin: fewer chains with DP 6–11 and more chains with DP 12–15 and DP 24–33. The increase in chains with DP 12–15 was contrary to the results from transformed potatoes as the result of suppressing the expression of SBE A (Jobling et al., 1999) or by suppressing the expression of both SBE A and B (Schwall et al., 2000). Thus, the structural properties of the sweetpotato amylose-free and high-amylose starches were characterized.

The pasting and retrogradation properties and enzyme digestibility of starches are important evaluations for the starch utility. The pasting profiles of 7% starches by RVA are shown in Fig. 4. All of the amylose-free starches showed similar profiles and had characteristics of lower peak viscosity during heating, and sharper breakdown and slower rising curve during cooling than those of the non-transgenic and GR-2 starches. These changes in pasting properties were similar to a previous report on amylose-free sweetpotato starch (Noda et al., 2002). In the case of the high-amylose starches, all starches showed an overall increase in viscosity in comparison with the non-transgenic starch. No marked change in pasting temperature was observed among the transgenic starches. Fig. 5 shows the turbidity developments of 2% starch pastes during storage at 4 °C for 7 days as an index of starch retrogradation. There was a clear difference in the retrogradation rates of starches between the respective transgenic lines. The pastes of the non-transgenic and GR-2 starches showed an intermediate turbidity development between those of the amylose-free and high-amylose starches. The amylose-free starches were made into transparent pastes and the clear pastes were maintained for 7 days. In addition, the clear pastes of the amylose-free starches did not change during further storage for 1 week (data not shown). Interestingly, the high-amylose starches were also made into transparent pastes just after gelatinization and turbidity developed rapidly. Finally, the digestibilities of the starch granules by pancreatin, which consisted of digestive enzymes including α-amylase from hog pancreas, were observed. The results are shown in Fig. 6. It can clearly be seen that all amylose-free starches showed higher digestibility and all high-amylose starches showed lower digestibility than the non-transgenic and GR-2 starches. A higher degree of hydrolysis for amylose-free sweetpotato starch was also observed in the case of digestion by crystalline glucoamylase from *Rhizopus niveus* (Noda et al., 2002).

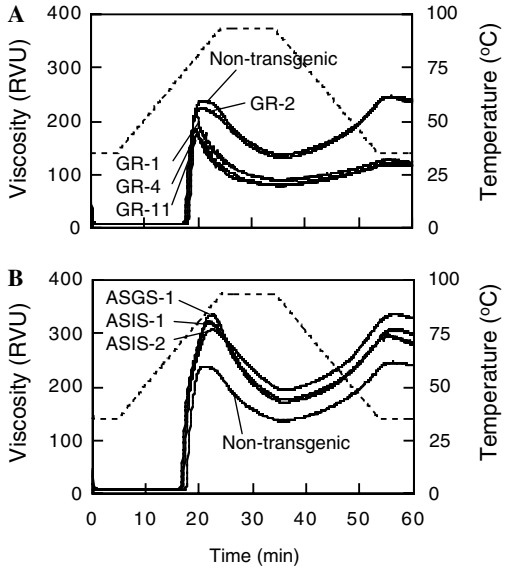

Fig. 4. RVA viscograms of GBSSI-RNAi (A) and SBEII-RNAi (B) starches. Solid line, viscosity; Dotted line, temperature.

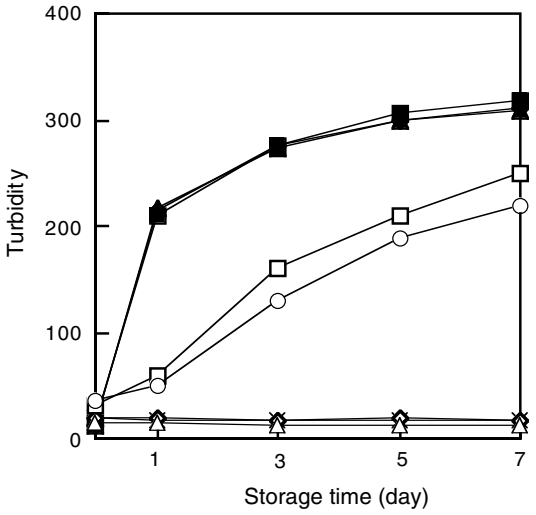

Fig. 5. Turbidity developments of 2% starch pastes during storage at 4 °C. ○, Non-transgenic; △, GR-1; □, GR-2; ◇, GR-4; ∗, GR-11; ▲, ASGS-1; ■, ASIS-1; ◆, ASIS-2.

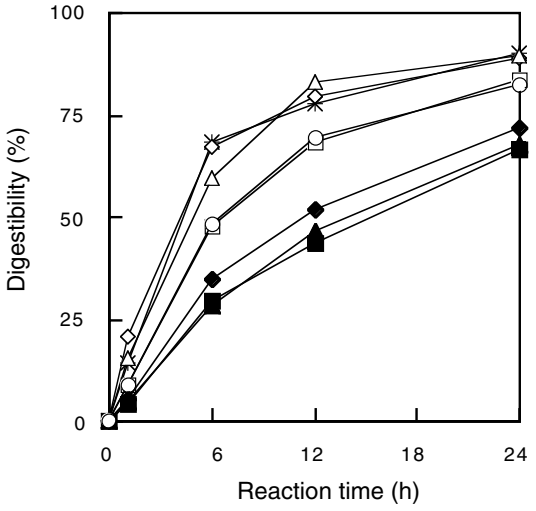

Fig. 6. Digestibility of starch granules by pancreatin. ○, Non-transgenic; △, GR-1; □, GR-2; ◇, GR-4; ∗, GR-11; ▲, ASGS-1; ■, ASIS-1; ◆, ASIS-2.

4. Conclusions

In this study, the physicochemical properties of amylose-free and moderately high-amylose starches from transgenic sweetpotatoes were investigated. The amylose-free starches from three GBSSI-RNAi lines and high-amylose starches from three SBEII-RNAi lines showed consistent results among the respective lines. From the amylose-free starches, some structural changes in the starch molecules were found by suppressing GBSSI expression: lack of amylose, lack of long chains with more than DP 100 in amylopectin, and a slight decrease in chains with DP 6 and 7 in amylopectin. These results strongly suggest that GBSSI participates in not only the synthesis of amylose molecules, but also the characterization of amylopectin molecules. Consequently, the amylose-free sweetpotato starches showed very slow retrogradation and high digestibility by pancreatin as compared to normal sweetpotato starch. For the high-amylose starches, complicated structural changes in the starch molecules were found by suppressing SBEII expression: increase in amylose, increase in phosphate content, alteration of crystalline structure, decrease in long chains with more than DP 100 in amylopectin, decrease in chains around DP 6–11, and increase in chains around DP 12–15 and DP 24–33 in amylopectin. As a result of the complicated structural changes, the high-amylose sweetpotato starches showed higher pasting viscosity, more rapid retrogradation and lower digestibility than those of normal starch. Although there has been some pioneering work in the area of genetically engineered starches such as potatoes with amylose-free (Visser et al., 1991) and high-amylose (Jobling et al., 1999; Schwall et al., 2000; Blennow et al., 2005) starches, we expect unique applications for amylose-free and high-amylose sweetpotato starches because the basic starch structures are different from those of other botanical sources.

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