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High Levels of Sugars and Fructan in Mature Seed of Sweet Wheat Lacking GBSSI and SSIIa Enzymes

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ABSTRACT: Sweet wheat (SW), which lacks functional granule-bound starch synthase I (GBSSI) and starch synthase IIa (SSIIa), accumulates high levels of free sugars in immature seeds. Here, we examined the effects of the lack of these two enzymes on mature kernel composition. Whole grain flour of SW had higher levels of sugars, particularly maltose, slightly higher ash and protein content, approximately two to three times higher lipid levels, and about twice as much total dietary fiber as parental or wild-type lines. Considerably higher levels of low-molecular-weight soluble dietary fiber (LMW-SDF), largely consisting of fructan, were also detected in SW. Although there were no differences in total amino acid levels, the free amino acid content of SW was approximately 4-fold higher than that of wild type, and the levels of certain free amino acids such as proline were particularly high. Thus, we were able to clearly demonstrate that the lack of GBSSI and SSIIa caused dramatic changes in mature seed composition in SW. These compositional changes suggest that SW flour may provide health benefits when used as a food ingredient.

KEYWORDS: Sweet wheat, sugars, fructan, free amino acids, granule-bound starch synthase I, starch synthase IIa

INTRODUCTION

Cereals such as rice, maize, and wheat store starch in endosperm tissue, and this starch represents an important energy source for humans. Starch consists of two types of homopolymers of glucose, namely, amylose and amylopectin. Amylose is an essentially linear molecule with glucosyl units linked via α-1,4 linkages, while amylopectin is a much larger molecule with branches produced by α-1,6 linkages. The endosperm starch of wheat generally contains about 20−30% amylose and 70−80% amylopectin. Many enzymes involved in starch synthesis in cereals have been identified and characterized, including ADP-glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), soluble starch synthase (SS), branching enzyme (BE), and debranching enzyme (DBE). In wheat, mutant lines lacking certain of these enzymes have been developed. Waxy (Wx) wheat, which accumulates amylose-free starch, was developed by the elimination of granule-bound starch synthase I (GBSSI),[1] which is responsible for amylose synthesis in endosperm tissue. Starch of Wx wheat showed a modified gelatinization curve, with a lower gelatinization onset temperature, higher peak viscosity, and lower setback than normal wheat, and also had higher retrogradation resistance.[2] Two classes of wheat that accumulate high amylose starch have also been developed. One of these classes was produced by suppressing both starch branching enzyme IIa (SBEIIa) and SBEIIb using an RNA interference method.[3] The amylose content in this line was greater than 70% and several indices of large-bowel function were improved in rats fed whole grain flour from this line compared to those fed standard flour. The second class of high amylose wheat was created via the elimination of starch synthase IIa (SSIIa; also called starch granule protein-1) by the stepwise crossing of three partial null lines. The amylose content in this line was approximately 1.3 times that of normal type starch.[4] Although flour from this line is not suitable in appearance for bread making,[5] it shows an increase in resistant starch levels,[6] which may have beneficial effects on intestinal function. Recently, a double mutant lacking both GBSSI and SSIIa was produced. On the basis of the high accumulation of sugars in immature endosperm tissue of this line, it was classified as sweet wheat (SW).[7]

The chemical and rheological properties of flour and/or starch from Wx and high amylose wheat lines have been analyzed to assess the potential of these new materials for the food industry.[8−13] We expect that SW flour will also serve as a new food ingredient able to give a specific texture or functionality to flour-based food products. However, to efficiently develop new applications, further information regarding the specific properties of SW flour is required. Therefore, in this study, the levels of components such as free sugar, ash, protein, lipid, starch, total dietary fiber, fructan, total amino acids, and free amino acids in SW flour were measured and compared with levels found in flour from wild-type cultivars and from the parental lines of SW.

MATERIALS AND METHODS

Plant Materials. Sweet wheat (SW) carries null mutations at all *GBSSI* and *SSIIa* loci.[7] Selection for homozygous null alleles was performed using markers capable of distinguishing wild-type and null

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Table 1. Free Sugar Contents in Whole Wheat Flour from SW, Wild-Type, and Parental Lines[a]

sample[b]	fructose (mg/g dw)	glucose (mg/g dw)	sucrose (mg/g dw)	maltose (mg/g dw)	total sugars (mg/g dw)[c]
CS	0.22 a	0.18 a	12.29 a	0.48 a	13.17 a
Wx	0.44 b	0.31 a	14.76 a	0.67 a	16.18 a
HA	0.37 b	0.50 b	27.87 b	0.96 a	29.70 b
SW	0.66 c	1.59 c	49.07 c	5.72 b	57.04 c

[a] Values represented as the means (n = 4). Values followed by the same letter in the same column are not significantly different ($P < 0.05$); dw, dry weight basis. [b] CS, Chinese Spring (wild type); Wx, waxy wheat (lacking all functional *GBSSI* genes); HA, high amylose wheat (lacking all functional *SSIIa* genes); SW, sweet wheat (lacking all functional *GBSSI* and *SSIIa* genes). [c] Total amount of fructose, glucose, sucrose, and maltose of flour.

alleles of all six loci as described by Nakamura et al.,[7] and the absence of all GBSSI and SSIIa proteins was confirmed by SDS−PAGE. The SW line used in this study was a single seed descent line (F_6) from an F_4 plant selected on the basis of early maturity, plant height, and cold resistance. The SW parental lines waxy (Wx), which carries null mutations at all three *GBSSI* loci, and high amylose (HA), which carries null mutations at all three *SSIIa* loci, were used as controls, along with the common wheat line Chinese Spring (CS), in which all *GBSSI* and *SSIIa* genes are functional. To evaluate the accumulation of sugars and other components in mature SW grain under a controlled environment, SW and other lines were subjected to a 5 °C vernalization treatment at the three leaf stage, then grown in a greenhouse at 25/17 °C day/night temperature with a relative humidity of 55%. Seeds were harvested at 45 days after flowering and stored at 4 °C until analysis. Kernel weight was determined by taking the mean of five 100-kernel weight measurements. The seed moisture content was determined by heating at 135 °C for 2 h after grinding using a hand mill.

Preparation of Dry Ground Whole Grain Flour. Harvested seeds were ground using a ZM-200 ultracentrifugal mill (Retsch, Haan, Germany) with a 0.5 mm screen at a speed of 14,000 rpm, and the dry ground whole grain flour was used for analyses of seed components. The moisture content of the whole grain flour was determined using 5 g of whole wheat flour heated at 135 °C for 1 h.

Free Sugar Determination. After adding 40 μL of 80% ethanol per mg of whole wheat flour, samples were boiled for 20 min, and centrifuged at 17,800*g* for 5 min at 4 °C. A 400 μL aliquot of the supernatant was vacuum-dried, dissolved in 75 μL of water, and an equal volume of acetonitrile (Wako Pure Chemical Industries, Osaka, Japan) was added. The sample was filtered using a DISMIC-3_{JP} disposable syringe filter unit (PTFE, 0.5 μm) (ADVANTEC TOYO, Tokyo, Japan) and analyzed by HPLC. HPLC analysis was performed on an Alliance HPLC system with an e2695 Separation Module and a 2414 Refractive Index Detector using Empower 2 software (Nihon Waters, Tokyo, Japan). The column oven and detector temperatures were set at 30 °C. Separation was performed on NH2P 50G-4A and NH2P 50-4E columns (Shodex, Tokyo, Japan) using 75% acetonitrile as an eluent at a flow rate of 1.0 mL/min.

Determination of Protein, Ash, and Lipid Contents. Protein content (% N × 5.7) determination was performed in triplicate by combustion nitrogen analysis using a TruSpec N Nitrogen Determinator (LECO Japan, Tokyo, Japan), calibrated with EDTA, according to AACC Method 46-30.[14] Ash and lipid contents were determined according to AACC Method 08-02 and AACC Method 30-10, respectively.[14]

Determination of Starch Content. Starch content was determined using a TOTAL STARCH ASSAY kit (Megazyme, Brey, Ireland) according to the manufacturer's instructions, typically using 100 mg of whole grain flour. An ethanol pretreatment step for the removal of sugars and a gelatinization step in dimethylsulfoxide prior to digestion by thermostable α-amylase were included.

Measurement of Dietary Fiber Content. The content of total dietary fiber, including insoluble dietary fiber (IDF), high-molecular-weight soluble dietary fiber (HMW-SDF), and low-molecular-weight resistant maltodextrin, which is referred to here as low-molecular-weight soluble dietary fiber (LMW-SDF),[15] was measured by Japan Food Research Laboratories (Tokyo, Japan) according to AACC method 32-41[14] with minor modifications in accordance with the Japanese official method.[16]

A portion of the ethanol-soluble fraction used for LMW-SDF determination was used to determine fructan content in this fraction. After the addition of glycerol, the sample was divided into three 10 mL aliquots; an equal volume of fructanase solution from a Fructan Assay kit (Megazyme) containing 100 units of exoinulinase and 1 unit of endoinulinase in 20 mM acetate buffer (pH 4.5) was added to the first aliquot, an equal volume of α-galactosidase solution (Megazyme), containing 200 units of α-galactosidase in 20 mM acetate buffer (pH 4.5), was added to the second aliquot, and an equal volume of 20 mM acetate buffer (pH 4.5) was added to the third aliquot, which served as a control. All samples were incubated for 4 h at 40 °C, following which samples were desalted, evaporated, subjected to volume adjustment, filtered, and analyzed by HPLC. Separation was performed on an Ultron PS 80-N column (i.d. 8.0 mm, 300 mm length) (Shinwa Chemical Industries, Kyoto, Japan) at 60 °C using distilled water as an eluent at a flow rate of 0.5 mL/min using the Alliance HPLC system described above. Peak areas corresponding to LMW-SDF were calculated after correcting for glycerol. The amount of fructan in LMW-SDF was determined according to the following calculation:

$$\text{fructan in LMW-SDF}\ (\%) = a \times [(b-c)/b - (b-d)/b] = a \times (d-c)/b \quad (1)$$

where a is the amount of LMW-SDF in whole wheat flour from Table 2, b is the peak area corresponding to the LMW-SDF peaks in the control, and c and d are the peak areas corresponding to the LMW-SDF peaks after the fructanase and α-galactosidase treatments, respectively.

Total Fructan Determination. Total fructan content was measured using a Fructan Assay kit (Megazyme) according to the manufacturer's instructions, with the inclusion of an α-galactosidase treatment.

Determination of Total Amino Acid Composition. Total amino acid composition was determined by the standard method for analysis of food nutrients at Japan Food Research Laboratories (Tokyo, Japan).[17]

Determination of Free Amino Acid Composition. Free amino acids were extracted from 100 mg of whole wheat flour in 5 mL of 75% ethanol by heating in boiling water for 5 min, then mixing by rotation at room temperature for 30 min. The resulting suspension was centrifuged at 800*g* for 10 min, and 1 mL of the supernatant was filtered sequentially through Millex-LH (0.45 μm) and Ultrafree-MC 30,000 NMWL (Nihon Millipore, Tokyo, Japan) filters. A 10 μL aliquot of filtered sample was mixed with 70 μL of AccQ Tag Fluor Borate Buffer and 20 μL of AccQ Tag Fluor reagent (Nihon Waters) to derivatize amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and the mixture was incubated for 20 min at 55 °C. Amino acids were separated using an HPLC system (Waters 717 plus Autosampler, Waters 600 controller, TOSOH CO-8000 column oven) on an AccQ Tag column (i.d. 3.9 mm, 150 mm length) (Nihon Waters) at 37.0 °C.

Table 2. General Analysis of Composition of Whole Wheat Flour from SW, Wild-Type, and Parental Lines[a]

					total dietary fiber		
sample	1000-kernel weight (g dw)[b]	ash (% dw)[c]	protein (% dw)[c]	lipid (% dw)[c]	(IDF)+(HMW-SDF) (% dw)[d]	(LMW-SDF) (% dw)[d]	starch (% dw)[c]
CS	30.9 a	2.4 a	20.6 a	3.1 a	13.5 a	2.0 a	52.3 a
Wx	34.2 b	2.2 b	15.8 b	2.8 a	14.0 b	2.6 b	56.9 b
HA	27.0 c	2.7 c	22.6 c	4.3 b	18.2 c	2.8 b	42.8 c
SW	22.0 d	3.0 d	23.0 d	8.0 c	28.7 d	8.5 c	17.8 d

[a] Values followed by the same letter in the same column are not significantly different ($P < 0.05$); dw, dry weight basis. [b] Values represented as the means ($n = 5$). [c] Values represented as the means ($n = 3$). [d] Values represented as the means ($n = 2$).

HPLC analysis was performed according to the manufacturer's instructions using AccQ Tag eluent A (Nihon Waters), acetonitrile, and distilled water as eluents at a flow rate of 1.0 mL/min with the following gradient program: 100% eluent A initially, changing to 99% eluent A and 1% acetonitrile after 0.5 min, 95% eluent A and 5% acetonitrile after 18.0 min, 91% eluent A and 9% acetonitrile after 19.0 min, 83% eluent A and 17% acetonitrile after 29.5 min, 60% acetonitrile and 40% water after 33.0 min, and 100% eluent A after 36.0 min. Tryptophan (which shows autofluorescence) was detected with Waters 486 Tunable Absorbance Detector (Nihon Waters) at 254 nm, while the remaining 19 amino acids were detected using a TOSOH FS-8010 fluorescent detector (TOSOH, Tokyo, Japan) at 250 nm (excitation) and 395 nm (emission).

Statistical Analysis. Analysis of variance (ANOVA) and Scheffe's test at a 5% significance level were employed to detect significant differences.

RESULTS AND DISCUSSION

Sugar Levels in Whole Grain Flour. The distinguishing feature of SW is the high level of sugars present in immature seed.[7] Here, we compared the levels of sugars, including fructose, glucose, maltose, and sucrose, in whole wheat flour from mature SW seed to the levels in flour from the wild-type line CS and the SW parental lines Wx and HA (Table 1). For comparative purposes, seed was grown to maturity in a temperature-controlled greenhouse under the same conditions used to obtain immature seed in earlier experiments.[7] The glucose, fructose, and sucrose levels in CS and Wx were comparable to levels observed in two other wheat cultivars.[18] In comparison to CS, SW whole wheat flour contained higher levels of all sugars, particularly maltose. The maltose level in SW was 11.9 times that in wild-type, whereas glucose was 8.8 times the wild-type level. Sucrose was the predominant sugar in all lines, and the level in SW was 4.0-fold that in CS. The levels of sugars in SW were also higher than those in Wx and HA lines, although the differences were not always quite as pronounced (Table 1). The higher accumulation of maltose in SW corresponds to what was seen in immature SW seeds.[7] However, in whole grain flour from mature SW seed, sucrose was 8.6 times as high as maltose, whereas in immature seed, maltose was actually 1.3 times the amount of sucrose. Because of the increased sugar levels in mature SW seed, whole wheat flour from SW had a slightly sweet taste in comparison to that of other flours. Notably, glucose, sucrose, maltose, and total sugar levels were slightly higher in HA (which lacks SSIIa) than in Wx (which lacks GBSSI), suggesting that the SSIIa deficiency in the SW double mutant has a stronger effect on sugar levels than does the lack of GBSSI.

In maize, several lines with mutations in starch synthesis genes, including lines with mutations in two or more genes, are known to accumulate sucrose and other sugars.[19] Maize mutants lacking GBSSI and SSIIa are known as *waxy* (*wx*)[20] and *sugary2* (*su2*),[21] respectively. Interestingly, the maize double mutant *wx/su2* is not classified as sweet, and the sugar levels detected in this mutant were comparable to those of wild-type or parental lines.[19] However, elevated sugar levels were observed in the triple mutants *wx/su2/ae* and *wx/su2/du*.[19] The *ae* (*amylose extender*) mutation is known to result in a lack of branching enzyme IIb,[22] while *du* (*dull*) conditions a reduction in starch synthase III activity.[23,24]

Only the maize double mutant carrying *sugary enhancer* (*se*) and *sugary1* (*su1*) resembles SW in the accumulation of maltose in mature grains.[25] The gene involved in the *se* mutation has not yet been identified,[26] although *se* is known to be a recessive modifier of *su1*, causing an alteration in isoamylase-type debranching enzyme activity.[27] While the mechanism of maltose accumulation in SW and the maize double mutant may differ, in both cases the presence of maltose indicates the possible involvement of β-amylase,[25,28] which releases maltose from the nonreducing ends of starch molecules by the hydrolysis of α-1,4-linkages.[29] Although β-amylase is thought to be involved in the degradation of transient starch in chloroplasts,[30,31] neither the presence of this enzyme nor enzymatic activity has been reported in amyloplasts. Thus, it will be interesting to determine the exact role of β-amylase in the accumulation of maltose in SW.

General Composition of Whole Grain Flour. The levels of ash, protein, lipids, total dietary fiber, and starch for each sample, as well as kernel weights, are given in Table 2. The highest kernel weight was seen in Wx, while SW had the lowest weight, as was previously observed.[7] Ash and protein percentages were 1.3 and 1.1 times higher in SW than in CS, respectively, while lipids and total dietary fiber percentages in SW were 2.6- and 2.4-fold higher. Similar trends were observed when comparing SW flour to Wx or HA whole wheat flour.

The amount of starch in SW whole grain flour was much lower than the levels in other lines (Table 2), with flour from CS containing almost three times as much starch as SW flour. Starch levels were determined using the Total Starch Assay kit, which involves an ethanol pretreatment that eliminates free sugars; therefore, measurements were not influenced by the high levels of maltose and glucose in SW. The low starch content in SW likely contributes to the relatively high proportions of ash, protein, lipids, and total dietary fiber observed in this line.

Since SW is a double mutant lacking the starch synthesis enzymes GBSSI and SSIIa, it is not surprising that starch synthesis is reduced compared to that of other lines. Relatively low starch levels in immature kernels were also seen in a number of high sugar maize lines carrying mutations related to starch synthesis.[19]

LMW-SDF Composition and Total Fructan Content. SW flour showed markedly higher LMW-SDF content than CS, Wx,

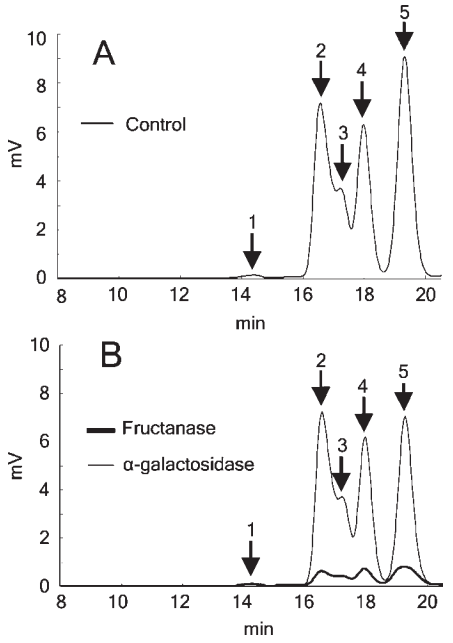

Figure 1. Chromatograms of the LMW-SDF fraction from SW. Separation was performed on an ULTRON PS 80-N column (i.d. 8.0 mm, 300 mm length) (Shinwa Chemical Industries). (A) Chromatogram of the undigested LMW-SDF fraction; (B) Chromatogram of the LMW-SDF fraction digested by fructanase or α-galactosidase. Peaks denoted by arrows correspond to LMW-SDF. The numbers indicate peaks with the same retention times.

Table 3. Amount of Fructan in LMW-SDF and Total Fructan[a]

sample	fructan in LMW-SDF (% dw)[b]	total fructan (% dw)[c]
CS	1.0 a	1.1 a
Wx	1.7 b	2.0 b
HA	1.8 b	2.0 b
SW	6.8 c	7.2 c

[a] Values are represented as the mean (n = 3). Values followed by the same letter in the same column are not significantly different ($P < 0.05$); dw, dry weight basis. [b] Fructan content in LMW-SDF fraction of whole wheat flour, as determined by the digestion of LMW-SDF fraction with fructanase and α-galactosidase (see Materials and Methods). [c] Fructan content in whole wheat flour was obtained using a FRUCTAN ASSAY kit.

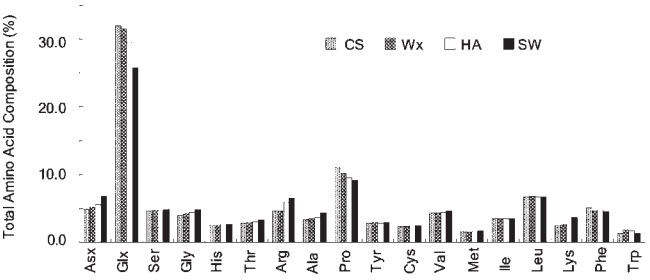

Figure 2. Total amino acid composition of whole wheat flour from CS, Wx, HA, and SW. Asx includes asparagine and aspartic acid, and Glx includes glutamine and glutamic acid. Each value was determined in duplicate.

and HA, with levels 4.3-, 3.3-, and 3.0-fold as high, respectively (Table 2). The LMW-SDF fraction represents low-molecular-weight soluble dietary fiber that is soluble in 78% ethanol. Since wheat seed has been shown to contain raffinose-series oligosaccharides and fructooligosaccharide (fructan),[32,33] the LMW-SDF fraction was digested by fructanase and α-galactosidase to detect these components.

The chromatograms of digested and nondigested (control) LMW-SDF fractions are shown in Figure 1. The area of the peaks corresponding to LMW-SDF was decreased by fructanase digestion to 12.3% of the control, while the peak area after α-galactosidase treatment remained at 92.1% of the control. Therefore, the digestion ratios for fructanase and α-galactosidase were approximately 87.7 and 7.9%, respectively. α-Galactosidase digests raffinose-series oligosaccharides (including raffinose, stachyose, and verbascose) but does not digest fructan, although fructanase is also able to digest raffinose-series oligosaccharides.[34] Thus, the net reduction of LMW-SDF resulting from the digestion of fructan was approximately 79.8%, and this fructan makes up approximately 6.8% of SW whole wheat flour (Table 3). The chromatograms also indicated that the LMW-SDF fraction included oligosaccharides with varying degrees of polymerization. Since all lines had LMW-SDF peaks with the same retention times (data not shown), the higher level of fructan in SW represents increases in existing species of fructan rather than the presence of novel oligosaccharides.

To determine if fructan was present in other fractions besides LMW-SDF, the total fructan content in flour from mature seeds of all four wheat lines was measured (Table 3). The total fructan content in CS was somewhat lower than that in Wx and HA, but the levels in all of these lines were comparable to the previously reported values for wheat.[34,35] However, the total fructan level in SW was 6.5 times the level observed in CS, and 3.6 times that in Wx and HA. The data also clearly established that the majority of the fructan was in the LMW-SDF fraction (Table 3).

It was interesting to note that the lack of two enzymes involved in starch synthesis resulted in the accumulation of fructan, which belongs to a different class of polysaccharides. The observed increase in fructan in SW may be due to the presence of a higher amount of sucrose, which is a substrate for fructan synthesis.[36] Fructan is also thought to play a key role in stress-induced metabolic processes in plants and is elevated in wheat exposed to stress conditions such as low temperature, drought, or salinity.[37,38] The SW plants used here were produced in a greenhouse under controlled temperature and humidity, and therefore were not subjected to stress during growth. In barley, the mutant line M292, which lacks SSIIa, was reported to accumulate higher levels of sugars and fructan than the control variety Himalaya, and stress-related genes were up-regulated during the maturation of M292 seeds grown in a controlled environment.[39] Higher sugar levels might result in abnormal conditions in the maturing grain, such as alterations in osmolarity, which could cause the induction of stress response mechanisms.

Fructans act as prebiotics which can selectively stimulate the growth of beneficial bacteria such as bifidobacteria in the colon.[36,40] The high level of fructans in SW, together with the fact that total dietary fiber constitutes 37.2% of SW flour (Table 2), suggests that whole wheat flour from SW might provide significant health benefits. Dietary fructans can also lead to an increase in mineral absorption, and the molecular weight and structure of fructans are thought to affect the efficiency of this absorption.[41] While it is known that wheat plants accumulate

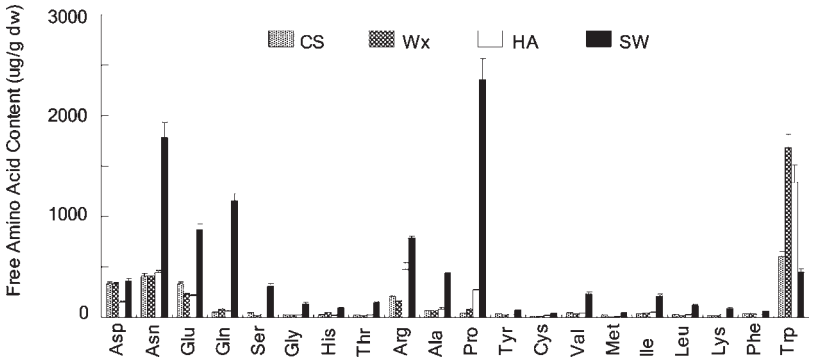

Figure 3. Free amino acid levels in whole wheat flour from CS, Wx, HA, and SW. Each value was determined in triplicate. Error bars represent the standard deviation of the mean.

graminan-type fructans in mature blades,[42] and some fructooligosaccharides such as 6-kestose and neokestose have been identified in grain,[32,43] to our knowledge, detailed information on the composition of mature wheat grain fructan is not available. It will therefore be necessary to evaluate the health effects of the specific fructans found in SW to develop functional food-related uses for SW flour.

Significant genotypic variation for fructan content occurs in wheat, and fructan contents ranging from 0.7% to 2.9% were detected among 62 wheat lines,[35] with QTLs having major effects on fructan concentration identified on chromosomes 6D and 7A.[44] Thus, it may be possible to further increase the level of fructans in wheat by crossing SW with other high-fructan lines. Interestingly, *GBSS-A1* and *SSIIa-A1* are also located on chromosome 7A.[45−47]

Total Amino Acid Composition and Free Amino Acids. Differences in total amino acid compositions were not seen in the four lines used here (Figure 2), and the ratios among the amino acid levels were consistent with results from other wheat lines.[48,49] However, a notable characteristic of SW was the high content of free amino acids in whole wheat flour (Figure 3). The total free amino acid content in CS, Wx, HA, and SW flour was 2.4, 3.4, 3.4, and 9.8 mg/g, respectively. Levels of individual amino acids are shown in Figure 3, and for CS, the levels of individual free amino acids were consistent with those obtained in mature grain by Labanauskas et al.[48] Eighteen of the 20 amino acids were present at higher levels in SW than in other lines. The major free amino acid seen in SW was proline, although in other lines, tryptophan was at the highest level. Proline accounted for 24.1% of the free amino acids in SW, and was 57.8-, 29.2-, and 8.7-fold higher than in CS, Wx, and HA, respectively. Glutamine, the amino acid present at the third highest level in SW, was present at 23.3, 14.1, and 19.2 times the levels in CS, Wx, and HA, respectively. Only two free amino acids (aspartic acid and tryptophan) were not found at higher levels in SW. The amount of tryptophan in SW was slightly lower than that in CS and more than 3-fold lower than the amounts found in Wx and HA, while the aspartic acid level in SW was comparable with that of CS and Wx.

The free amino acid content in wheat grain can be affected by soil temperature,[48] and salt stress also leads to an accumulation of free amino acids in plant tissues[50] including wheat seeds.[51] The increase of free amino acids in grain exposed to high salinity is thought to be due to a decrease in the rate of incorporation of free amino acids into proteins.[51] Similarly, the high level of free amino acids in SW grain may result from the inhibition of free amino acid incorporation into proteins by stress conditions such as abnormal osmolarity due to high sugar levels. However, the possibility that osmotic stress causes increased proteolysis of SW endosperm storage proteins cannot be ruled out. The substantial increase in free proline in SW is particularly notable since proline is known to accumulate in stressed plants[52,53] including wheat.[54,55] The accumulation of three well-known stress-induced metabolites in SW grain (sucrose, fructan, and proline) provides a strong indication that some form of stress is likely occurring during grain development.

In conclusion, our data clearly showed that the lack of GBSSI and SSIIa enzymes caused pleiotropic effects on the composition of whole wheat flour from mature grain and had a particularly dramatic effect on the levels of sugars, fructan, free amino acids, and starch present in mature seed. The specific compositional changes that occurred in SW seed suggest that SW flour may provide health benefits when used as a food ingredient. The high accumulation of sucrose, fructan, and proline also suggested that some form of stress was occurring in SW grain during seed maturation.

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