

Biochemical Characterization of the Wheat Waxy A Protein and Its Effect on Starch Properties¹

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ABSTRACT

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Granule bound starch synthase1 (GBSS1) is a key enzyme in amylose biosynthesis and is encoded by the A, B and D GBSS1 *wx* loci in wheat. Wheat lines with mutations at the three GBSS1 loci have been identified. We have characterized and compared the grain starch of CDCW6 wheat line (null B and D for GBSS1) with PI235238 (null A and B for GBSS1), waxy (null A, B and D for GBSS1), and AC Reed (wild type wheat) grain starches. The grain starch of waxy, CDCW6, PI235238, and AC Reed lines contained =0, 12, 23, and 25% amylose (w/w), respectively. Waxy,

partially waxy, and wild wheat grain starches showed significant differences in onset and peak transition temperatures as determined by differential scanning calorimetric analysis. Grain starches extracted from waxy, CDCW6, and PI235238 also had higher enthalpy of gelatinization values than did wild wheat starch. X-ray diffraction analysis revealed the highest crystallinity for starch extracted from waxy wheat, followed by CDCW6. The starch produced from the CDCW6 line may find special food and industrial applications because of its relatively low amylose concentration.

Starch granules of plants contain two types of glucan polymers: a predominantly linear amylose and a highly branched amylopectin. The ratio of amylose to amylopectin determines the physicochemical properties of starch and, thereby, the end-use of the grain. Starch biosynthesis in plants is accomplished by the action of several enzymes that include adenosine glucose pyrophosphorylase, starch synthases, and branching and debranching enzymes (Preiss and Sivak 1998). One of the main starch biosynthetic enzymes is granule-bound starch synthase1 (GBSS1, EC 2.4.1.21), which is a key enzyme in amylose biosynthesis (Echt and Schwartz 1981). In wheat, GBSS1 is termed waxy protein (Wx) and is encoded by the waxy locus (*wx*) located on chromosomes 7AS, 4AL and 7DS (Chao et al 1989). Mutations at all three *wx* loci result in waxy wheat starch that is predominantly composed of amylopectin (Nakamura et al 1995). Chemically modified waxy wheat grain starch may find applications in the food industry similar to those identified for waxy maize starch (Reddy and Seib, *in press*). A mutation at the GBSS1 B locus results in a slightly reduced amylose concentration in endosperm starch and better noodle-making quality than is the case for wild wheat (Miura and Tanii 1994, Zhao et al 1998). There is also a strong correlation between the GBSS1 B mutation and flour swelling volume (Zhao et al 1998), and a reduction in amylose concentration also is associated with a higher amylogram peak viscosity (Zeng et al 1997).

Mutations at the *wx* loci in wheat have been reported by various groups (Yamamori et al 1994, Demeke et al 1997, Graybosch et al 1998, Zhao et al 1998). Over 200 accessions originating from Japan, Australia, and China, as well as 63 Canadian and U.S. wheat cultivars were screened by SDS-PAGE to identify null alleles at the GBSS1 locus (Demeke et al 1997). Ten percent of the Japanese accessions carried the null allele for the GBSS1 A isoprotein, and 14% of the Australian lines carried the null allele for GBSS1 B isoprotein. One double mutant for GBSS1 A and B isoprotein was also found among the Japanese accessions analyzed. Purification and separation of GBSS1 from more than 200 North American hexaploid wheats has resulted in the identification of

seven wheat lines with null alleles for GBSS1 A and 13 with null alleles for GBSS1 B, and one double mutant for GBSS1 A and B isoproteins (Graybosch et al 1998). However, none of the characterizations identified a wheat line carrying mutations at both B and D GBSS1 loci. The absence of individual GBSS1 isoproteins slightly reduces amylose concentration (Fujita et al 1998). The effect of different GBSS1 double mutants on amylose production and starch characteristics is not documented in the literature (Zhao et al 1998). In this study, we report on the identification of the wheat line CDCW6 with mutations at B and D GBSS1 loci. The starch characteristics of the CDCW6 wheat line were compared with those of PI235238 (null A and B for GBSS1), waxy, and wild (non-waxy or normal) wheat lines.

MATERIALS AND METHODS

Plant Materials

Wheat (*Triticum aestivum* L.) seeds were collected from plants grown in a growth chamber maintained at 22 ± 2°C day/20 ± 2°C night, and 16-hr light/8-hr dark.

GBSS1 Protein Isolation, SDS-PAGE, and N-Terminal Protein Sequencing

Starch with the associated GBSS1 proteins was extracted from wheat kernels by the procedure of Zhao and Sharp (1996). GBSS1 proteins were extracted by dispersing 22–25 mg of starch in 475 µL of extraction buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, w/v, 5% 2-mercaptoethanol, 10% glycerol), followed by boiling for 10 min. The gelled starch solution was cooled on ice and centrifuged for 20 min at 15,000 × g. Bromophenol blue (0.005%, w/v) was added to the 450–500 µL of supernatant, and the proteins were separated on a preparative 15% acrylamide/bisacrylamide monomer (30:1) gel (ultrapure Protogel, National Diagnostics, Atlanta, GA). For N-terminal sequencing of the GBSS1 isoproteins, the separated GBSS1 polypeptides were transferred to a PVDF membrane by electroblotting (Millipore, Bedford, MA) and visualized by Coomassie blue staining. The pieces of immobilized blots carrying GBSS1 isoproteins were placed in the sample cartridge (Matsudaira 1987) of a Porton 2090E gas-phase protein sequencer (Beckman, Fullerton, CA) equipped with an on-line Hewlett-Packard 1090L HPLC for amino acid sequencing. The procedure for two-dimensional gel-electrophoresis was described earlier (Demeke et al 1997).

Determination of Amylose Concentration

Amylose concentration was determined according to the method of Gibson et al (1997) using the Megazyme assay kit (Megazyme International, Ireland). Wheat kernels were milled in a cyclone mill (Udy Corp., Fort Collins, CO) and sifted through a 0.5-mm

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sieve. A 25-mg sample of the flour was boiled in 1 mL of dimethyl sulfoxide (DMSO) for 15 min. Ethanol (95%) was added to the boiled solution to precipitate and defat the starch. The defatted starch pellet was redissolved in 1 mL of DMSO and diluted to 25 mL with 30% Concanavalin A (Con A) solvent. Concentrated Con A solvent contained 0.52M sodium acetate, 3.0M NaCl, 3.4 mM CaCl₂·2H₂O, 3.44 mM MgCl₂·6H₂O, and 3.54 mM MnCl₂·4H₂O, pH 6.4. A 0.5-mL aliquot taken from the 25 mL of solution was used to determine the total starch content by the amyloglucosidase/α-amylase method (Gibson et al 1997). Con A was added to a 1.0-mL aliquot taken from the 25 mL of solution, to precipitate amylopectin. The amylose in the supernatant was hydrolyzed to glucose by amyloglucosidase/α-amylase, and the amylose concentration was expressed as a percentage of the total starch content. Three replicates of each starch samples were used to calculate analysis of variance (ANOVA) using computer software (Minitab for Windows).

To determine the amylose concentration by high-performance size-exclusion chromatography (HP-SEC), a 5-mg starch sample was suspended in 5 mL of double-distilled water in a glass tube and incubated at 130°C for 30 min. To 1 mL of the vortexed starch solution, 55 μL of 1M sodium acetate, pH 4.0, was added. The solution was vigorously mixed, and four units of isoamylase (200 units/mL of stock solution, Megazyme) were added to debranch the starch. After 4 hr of incubation at 40°C, the reaction mixture was boiled for 20 min to inactivate the isoamylase, and the starch solution was freeze-dried. The debranched starch was dissolved in 200 μL of DMSO solution (99% DMSO and 1% nano pure water) and centrifuged at 15,000 × g for 10 min. Supernatant (40 μL) was injected into a PLgel 5 μM MiniMix-C guard column attached to a PLgel MiniMix 4.6-mm i.d. column (Polymer Laboratories, Inc., Amherst, MA) to separate amylose and amylopectin using an HPLC system (Waters 600 controller, Waters 610 fluid unit, Waters 717 plus autosampler, Waters 410 differential refractometer). The data were collected and analyzed using Millennium 2010 chromatography software. Starch samples, column, and detector were maintained at 40, 100, and 45°C, respectively. DMSO (99%) was used as an eluent at a flow rate of 0.2 mL/min. The amylose concentration of the starch samples was calculated by integration of the peak area corresponding to amylose to that of the peak area corresponding to both amylose and amylopectin.

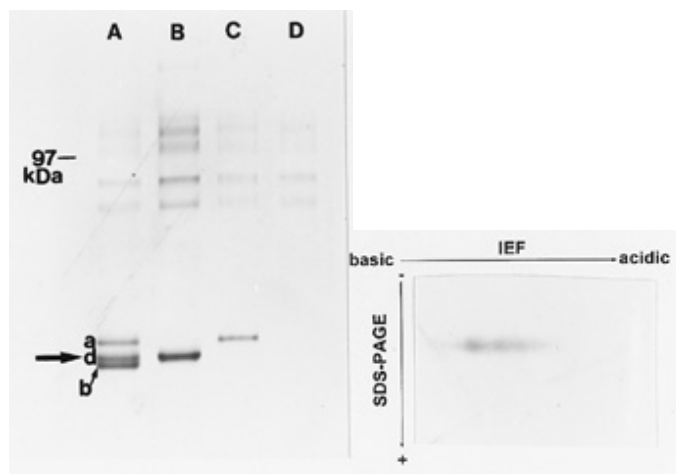


Fig. 1. Identification of proteins associated with starch granules extracted from the endosperm of wild, partially waxy, and waxy wheat lines. Left: SDS-PAGE. Lanes A–D: AC Reed (wild); PI235238 (null A and B); CDCW6 (null B and D); and waxy line. Arrow indicates location of the GBSS1 isoprotein (GBSS1A [a], GBSS1B [b], and GBSS1D [d]). Right: Isoelectric focusing followed by SDS-PAGE for CDCW6 GBSS1 isoprotein. Note that only a single protein with about three subunits is seen.

Analysis of Starch by Differential Scanning Calorimetry

The thermal characteristics of starch samples were measured by differential scanning calorimetry (DSC) (Mettler TA3000). A 4–5 mg starch sample was mixed with water in a DSC pan to give a water-volume fraction of 0.77, based on the total volume of starch (density 1.5). The sample was heated at 10°C min⁻¹ and the transition temperatures were recorded from a plot of heat flow versus temperature (35–120°C). The enthalpy of gelatinization (ΔH , J/g) was calculated by measuring the curve area. The onset (T_o), peak (T_p), and end of transition or conclusion (T_c) temperatures were taken from a plot of the relationship between differential (slope) and temperature. The differential was computed from a relationship between heat flow and temperature during starch gelatinization. Two determinations of three sets were analyzed for each wheat starch sample and the results are expressed as the mean of six determinations.

Analysis of Starch by X-ray Diffraction

Reagent-grade alumina powder (Anachemia, 200-mesh Al₂O₃) was used as an internal reference standard (reference peak at 38.50°, 2 θ) for measuring the relative intensity of starch diffraction peaks and providing an absolute diffraction angle (2 θ) reference position. A 1:10 mixture of alumina and starch (w/w) was densely packed into an aluminum sample holder. The X-ray diffraction pattern (diffractogram) of the starch sample was recorded on a Phillips model 42273 diffractometer with a Phillips PW-1965-60 proportional detector and an Enraf-Nonius FR590 2.0 kVA X-ray generator. The sample was scanned from 3° to 40° 2 θ at an equivalent angular velocity of 0.6° 2 θ /min, using a step interval of 0.02° 2 θ , a count time of 1.2 sec, and Cu K α radiation (1.54178Å) at 1.6 kVA. The d -spacing was calculated from 2 θ according to Bragg's equation ($n\lambda = 2d \sin\theta$; where d = intercrystalline spacing, $n = 1$, and $\lambda = 1.54178\text{\AA}$). The relative intensities of the major diffraction peaks of starch were calculated as the ratio between the absolute intensity of the starch peak and the reference peak.

RESULTS AND DISCUSSION

GBSS1 Isoprotein Profile and N-Terminal Protein Sequence of CDCW6 (Null B and D) Line

We previously had identified a GBSS1 null B and D wheat line while screening F2 seeds (K107 × Bai Huo) for waxy endosperm starch (Chibbar et al 1997). The seeds from this line (CDCW6) gave a reddish-brown color with blue spots in the center of the endosperm when stained with iodine. Comparison of the CDCW6 GBSS1

TABLE I
N-Terminal Amino Acid Sequences of GBSS1 A Isoproteins

GBSS1	Source	Protein Sequence
A	<i>Triticum monococcum</i>	A-T-G-S-G-G-M-N-L-V-F-V-G-A-G-M-A-P-V?
A	<i>T. urartu</i>	A-T-G-G-G-G-M-N-L-V-F-V-G-A-E-M-A-P ^a
A	CDCW6 (<i>T. aestivum</i>)	A-T-G-C-G-G-M-N-L-V-F-V-G-A-E-M-A

^a N-terminal protein sequence of *T. urartu* from Fujita et al (1996). Letters in bold indicate unique amino acids.

TABLE II
Amylose Concentration of Waxy (W2), Partially Waxy (CDCW6, PI235238) and Wild (AC Reed) Wheat Starches

Lines	Presence of GBSS1 Isoprotein	Amylose (%)	
		Megazyme	HP-SEC
CDCW6	A	12.4	12.7
Waxy 1	...	5.5	0.0
Waxy 2	...	5.5	0.0
PI235238	D	23.5	22.3
AC Reed	A, B, D	25.8	25.0
LSD $P < 0.05$		2.3	...
LSD $P < 0.01$		3.2	...

isoprotein profile with wild, PI235238, and waxy isoproteins indicated CDCW6 had only the GBSS1 A isoprotein (i.e., null B and D for GBSS1) (Fig. 1). Two-dimensional gel-electrophoresis showed the presence of a single GBSS1 isoprotein for CDCW6 (Fig. 1). The fully waxy type lacked all three GBSS1 isoproteins as expected, and PI235238 lacked the null A and B GBSS1 isoproteins.

N-terminal protein sequencing was performed to confirm whether the CDCW6 isoprotein was granule-bound starch synthase by comparison with the amino acid sequences of GBSS1A isoproteins from *Triticum monococcum* and *T. urartu* (Table I). Several earlier reports indicated that *T. monococcum* is the A genome donor for hexaploid wheat, but in a recent study, *T. urartu* was proposed as the A genome donor (Dovorak 1998). There was a high degree of amino acid sequence similarity among CDCW6, *T. monococcum*, and *T. urartu*, indicating the CDCW6 isoprotein to be GBSS1. However, there was a unique difference at position four, in that CDCW6, *T. urartu*, and *T. monococcum* had cysteine, glycine, and serine, respectively. *T. monococcum* also had a glycine instead of glutamic acid at position 15 in contrast to the amino acid sequence reported for *T. monococcum* by Taira et al (1995).

Amylose Concentration of CDCW6 vs. Other Lines

The amylose concentration of wheat endosperm starch reportedly varies depending on the method used in its determination. For example, the two wheat lines crossed to produce waxy wheat, K107 (null A and B for GBSS1) and Bai-Huo (null D for GBSS1) have reported amylose concentrations of 16–25% (Miura and Tanii 1994, Nakamura et al 1995, Hayakawa et al 1997, Kiribuchi-Otobe et al 1997) and 24–30% (Yamamori et al 1995, Hayakawa et al 1997), respectively. Some of the methods used to determine amylose concentration include: calorimetric determination of iodine complexed with amylose, also known as blue value (Knutson 1986, Knutson and Grove 1994, Yasui et al 1996), potentiometric iodine titration (Adkins and Greenwood 1966, Banks et al 1974),

autoanalyzer (Nakamura et al 1995), gel-filtration chromatography (Denyer et al 1995), and HP-SEC (Bradbury and Bello 1993). Each method is limited in its ability to accurately determine amylose content. Recently, Gibson et al (1997) reported a procedure where Con A is used to complex and selectively precipitate amylopectin, thereby allowing the determination of amylose. Using this method, we found significant differences in the amylose concentrations of waxy, CDCW6, PI235238, and AC Reed (Table II). The CDCW6 and PI235238 lines contained ≈12 and 23% amylose, respectively (Table II). The 23% amylose concentration of PI235238 starch was similar to that of K107 starch (null A and B) as reported by Nakamura et al (1995). These results suggest that mutations at the GBSS1 A and B loci did not substantially reduce the amylose concentration in wheat starch. It has also been reported that mutation at the B locus reduces amylose concentration by >3%, while mutation at either the A or D locus reduces amylose concentration by <2% (Miura and Sugawara 1996), as compared with wild type wheat starch. Our results showed that a combination of null B and D GBSS1 alleles significantly reduced the amylose concentration as compared with the null A and B GBSS1 alleles. However, studies incorporating different lines of all three GBSS1 double mutants could further reveal effects of waxy A, B, and D isoproteins on amylose concentration. The waxy lines contained ≈5% amylose, which was higher than the 0.5 to 2% reported by others (Nakamura et al 1995, Yasui et al 1996).

HP-SEC was used to confirm the amylose concentration data obtained by precipitation with Con A (Fig. 2). In this procedure, grain starch was debranched by isoamylase and fractionated by size-exclusion on an HPLC system. Under the experimental conditions, amylose and debranched amylopectin had retention times of ≈11.0 and 16.0 min, respectively. The analysis showed no amylose for waxy wheat, 12.7% amylose for CDCW6, 22% amylose for PI235238, and 25% amylose for AC Reed (Fig. 2). Thus, the results were similar to those obtained with the Con A method of Gibson et al (1997), with the exception of waxy wheat starch (Table II). In the case of waxy wheat starch, Con A might not have precipitated all of the amylopectin, thus giving a relatively high amylose concentration. It is also possible that amylopectin in

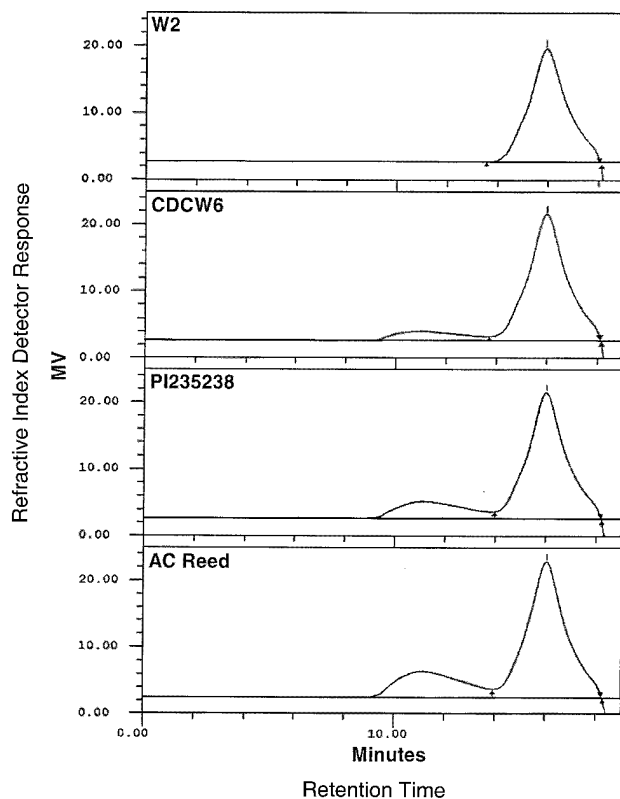


Fig. 2. High-performance size-exclusion chromatography (HP-SEC) analysis of debranched starch isolated from waxy, partially waxy, and nonwaxy wheat lines. Waxy, CDCW6, PI235238, and AC Reed contained 0, 12.7, 22.3, and 25% amylose, respectively.

TABLE III
Thermal Transition Temperatures (T_o , T_p , T_c)^a and Enthalpies of Waxy (W2), Partially Waxy (CDCW6, PI235238), and Wild (AC Reed) Wheat Starches

Starch	Endothermic Transition Temperatures (°C)				
	T_o	T_p	T_c	$T_c - T_o$	ΔH (J/g)
W2	55.6	63.5	73.5	17.9	10.3
CDCW6	56.0	66.3	75.8	19.8	11.0
PI235238	56.4	64.0	72.2	15.8	10.4
AC Reed	51.9	60.8	71.4	19.5	9.2
LSD 0.05	0.54	0.49	ns ^b	ns	0.99
LSD 0.01	0.89	0.81	ns	ns	ns

^a Onset (T_o), peak (T_p), conclusion (T_c), range ($T_c - T_o$), and enthalpy of gelatinization (ΔH , J/g).

^b Not significant.

TABLE IV
Percent Relative Intensity^a of Major Peaks in the X-ray Diffraction Patterns of Waxy (W2), Partially Waxy (CDCW6, PI235238), and Wild (AC Reed) Wheat Starches

Starch	X-ray Diffraction Angle (2 θ) ^b			
	15.2 (5.8)	17.2 (5.2)	18.0 (4.9)	23.2 (3.8)
W2	27.8	36.7	36.7	36.7
CDCW6	25.8	30.8	30.8	30.0
PI235238	19.3	28.9	29.8	28.9
AC Reed	16.9	20.2	20.8	20.2

^a Starch peak intensity/standard peak intensity \times 100.

^b Numbers in parentheses are starch crystal d -spacings (Å).

waxy wheat might be less branched and contains lower molecular weight polymers that are not precipitated with Con A. However, this assumption needs to be confirmed by detailed analysis of starch structure. Our results suggest that HP-SEC of debranched starch gives a more accurate estimation of amylose and amylopectin concentrations in grain starch than the Con A method.

Thermal Transition Temperature of CDCW6 vs. Other Lines

The data for transition temperatures (T_o , T_p , and T_c) and enthalpy (ΔH) is summarized in Table III. Grain starches extracted from waxy, CDCW6, and PI235238 wheat lines exhibited relatively high thermal transition temperatures. There were significant ($P = 0.05$) differences among the T_o and T_p temperatures, as well as in the ΔH . The T_o and T_p temperatures for waxy, CDCW6, and PI235238 were higher than corresponding values for AC Reed (wild type). Starch extracted from waxy, CDCW6, and PI235238 also had higher ΔH than did AC-Reed. Waxy wheat starch requires a large amount of energy for gelatinization (Fujita et al 1998) and thus has a high ΔH , as confirmed by our study. Hayakawa et al (1997) also have reported higher transition temperatures and ΔH for waxy wheat starch than for nonwaxy wheat starch. Significant variations in the T_o were also observed in starches of partially waxy and nonwaxy wheat cultivars (Zeng et al 1997). Information on molecular weight, degree of polymerization and branching of waxy, partially waxy, and wild wheat starch would further elucidate the differences in thermal and pasting characteristics.

X-ray Diffraction Analysis of CDCW6 vs. Other Lines

The four starch samples were all determined to be that of the A-type polymorph, which is common for cereal starches (data not shown). The relative peak intensities in X-ray diffraction data indicate the crystallinity of starch granules (Abdel-Aal et al 1997). Waxy starch had the highest crystallinity, followed by CDCW6, and wild wheat starch had the lowest crystallinity (Table IV). The high crystallinity of waxy wheat starch results from its high amylopectin content (Hayakawa et al 1997, Fujita et al 1998). In addition, the X-ray data was negatively associated with amylose content as determined by HP-SEC (Table II), in that the lower the amylose content, the higher the crystallinity, and vice versa.

CONCLUSIONS

We have characterized a CDCW6 wheat line with mutations at B and D GBSS1 loci that had a lower amylose concentration (12%) in endosperm starch as compared with PI235238, a null A and B line containing $\approx 23\%$ amylose. Starch from waxy and CDCW6 wheat lines had higher crystallinity, ΔH , and transition temperatures than did wild wheat starch. The uniqueness of the CDCW6 line in terms of its starch properties will be of interest for waxy protein studies, and its starch, having a reduced amylose concentration, may find special applications in food and industrial uses.

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