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Genome-Specific Granule-Bound Starch Synthase I (GBSSI) Influences Starch Biochemical and Functional Characteristics in Near-Isogenic Wheat (*Triticum aestivum* L.) Lines

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ABSTRACT: Near-isogenic wheat (*Triticum aestivum* L.) lines differing at the *Waxy* locus were studied for the influence of genome-specific granule-bound starch synthase I (GBSSI/Waxy; Wx-A, Wx-B, Wx-D) on starch composition, structure, and in vitro starch enzymatic hydrolysis. Grain composition, amylose concentration, amylopectin unit-chain length distribution, and starch granule size distribution varied with the loss of functional GBSSI. Amylose concentration was more severely affected in genotypes with GBSSI missing from two genomes (double nulls) than from one genome (single nulls). Unit glucan chains (DP 6−8) of amylopectin were reduced with the complete loss of GBSSI as compared to wheat starch with a full complement of GBSSI. Wx-A and Wx-B had an additive effect toward short-chain phenotype of waxy amylopectin. Loss of Wx-D isoprotein alone significantly ($p < 0.05$) reduced the C-type starch granules. However, the absence of Wx-D in combination with Wx-A or Wx-B increased the B-type and C-type starch granules but decreased the volume of A-type starch granules. The rate of in vitro starch enzymatic hydrolysis was highest in completely waxy grain meal and purified starch. However, the presence of Wx-D reduced wheat starch hydrolysis as it increased the large A-type starch granule content (volume %) and reduced short chains (DP 6−8) in amylopectin. Factors such as small C-type starch granules, amylose concentration, and long chains of amylopectin (DP 23−45) also influenced wheat starch hydrolysis.

KEYWORDS: *near-isogenic wheat, waxy, amylopectin chain length distribution, starch hydrolysis*

INTRODUCTION

Endosperm of a wheat grain is occupied mostly (~70%) by starch, which is a glucan homopolymer, composed of one-fourth amylose (10^5−10^6 Da) and three-fourths amylopectin (10^7−10^9 Da). Amylose is a predominantly linear molecule made up of α-1,4-linked glucose residues with a degree of polymerization (DP) of ~800 (in wheat) and few branches.[1] Amylopectin, on the other hand, is a highly branched glucan polymer with 4−5% α-1,6 branches and DP of 10^5−10^7. The amylopectin branches form an organized structure and are categorized into short A-chains (DP 6−12), intermediate B-chains (DP 13−24 up to 50), and a long intercluster C-chain, with one free reducing end per amylopectin molecule.[2]

Amylose and amylopectin have different structural and physiological characteristics and hence exhibit different reactions within the body during digestion and subsequent release of glucose molecules for absorption.[3] Various studies have shown that the amylose to amylopectin ratio, DP, and branching of glucan polymers are important determinants of the extent of starch enzymatic hydrolysis and hence digestibility of food.[3−5] The rate of starch enzymatic hydrolysis is reduced in starches with higher amylose concentration.[4] The relationship between amylopectin chain length distribution and resistant starch has also been studied in different plant systems.[5] Changes in the amylopectin chain length distribution facilitate retrogradation to produce B- and V-type crystalline structures, leading to more resistant starch. It is generally believed that increased proportion of longer chains makes the starch more resistant to digestion.[6] A possible reason could be that longer chains form longer helices, which are further stabilized by hydrogen bonds, distributed over the entire crystalline region, hence decreasing digestibility.[6]

Starch, on the basis of its digestion behavior after consumption, can be divided into digestible and resistant starch.[7] The portion of starch that gets digested within 20−30 min of ingestion is called readily digestible starch (RDS), and that which gets digested within 120 min of ingestion is called slowly digestible starch (SDS). Resistant starch (RS) refers to that portion of starch which resists digestion after 120 min,[8] escapes digestion in the small intestine/upper gastrointestinal tract, and is fermented in the large intestine by gut microflora. RS offers many positive physiological effects on the human body such as reduction in glycemic index, production of short-chain fatty acids, particularly butyrate, which prevents colorectal cancer, and better absorption of minerals such as calcium and iron.[9]

Amylopectin synthesis is a complex process and involves an array of enzymes. These include ADP-glucose pyrophosphorylase (AGPase), starch synthases (SSI, SSII, SSIII, and SSIV), starch branching enzymes (SBEI, SBEII), and starch debranching enzymes (DBE).[10] Amylose synthesis and elongation, however, involve a single enzyme, granule-bound starch synthase I (GBSSI), also known as "waxy" protein.[10] Plants lacking the *waxy* gene, which encodes GBSSI, produce starch without amylose, known as waxy starch. GBSSI has also been

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reported to be involved in the elongation of amylopectin chains, particularly for very long branches.[11]

Wheat is an allohexaploid crop (*Triticum aestivum* L., $2n = 6x = 42$; BBAADD), consisting of three genomes derived from three diploid parents. Therefore, wheat endosperm has three isoforms of GBSSI encoded by the *Waxy* (*Wx*) loci, *Wx-A1*, *Wx-B1*, *Wx-D1*, located on chromosomes 7AS, 4AL (translocated from 7BS), and 7DS, respectively.[12] The absence of GBSSI in any of the three genomes would affect the concentration of amylose and amylopectin, starch structure, and functional properties. The objective of this work was to study the effect of GBSSI from the three wheat genomes on starch composition, structure, and in vitro starch hydrolysis. The terms "GBSSI" and "Waxy" have been used interchangeably.

MATERIALS AND METHODS

Materials. Plant material for this study included 32 Canadian western red spring (CWRS) near-isogenic waxy wheat (*T. aestivum* L.) lines, including four lines of each Wx-$A^+B^+D^+$, $A^-B^+D^+$, $A^+B^-D^+$, $A^+B^+D^-$, $A^-B^-D^+$, $A^-B^+D^-$, $A^+B^-D^-$, and $A^-B^-D^-$. These lines (CDC Teal*7/CDC Wx2) were derived by backcrossing CDC Wx2 (*Wx-AIb*, *Wx-BIb*, *Wx-DIb* null alleles; donor parent) to CDC Teal (complete complement of *Wx* alleles; recurrent parent) six times. Both parents were developed at the University of Saskatchewan.[13,14] Four replicates of each of the near-isogenic line were grown in a randomized complete block design in 2010 in Kernen (52° 09′ N, 106° 33′ W) and Goodale (52° 03′ N, 106° 29′ W) Crop Research Farms, University of Saskatchewan, Saskatoon, Canada. One thousand seeds from each genotype were weighed for thousand grain weight (TGW) determination. To prepare grain meal, 10 g of seeds was ground by using a UDY mill (UDY Corp., Fort Collins CO, USA) equipped with a 0.5 mm sieve. White bread (Wonder bread, George Weston Limited, Toronto ON, Canada) was used as control for in vitro hydrolysis study and calculation of hydrolysis index.

Analysis of GBSSI Polypeptides. Starch was purified from wheat seeds (7−10) using a modified method involving cesium chloride density gradient centrifugation.[15] Proteins were extracted from the starch granules by suspending 10 mg of starch in 300 μL of denaturing electrophoresis buffer.[16] GBSSI was separated by polyacrylamide (10% v/v, 1 mm thick gel) electrophoresis (Protean II, Bio-Rad, Hercules, CA, USA) under denaturing conditions.[16] Separated polypeptides were visualized by silver staining.[17] The gel electrophoresis separated polypeptides were transferred on a nitrocellulose membrane at 10 V for 4 h.[18] Membrane with transferred polypeptides was processed and incubated with primary antibodies raised against wheat GBSSI.[19] The antigen−antibody complex was detected with goat anti-rabbit alkaline phosphate conjugate using BCIP/NBT immunoscreening color development kit (Bio-Rad).

Grain Constituents Concentration Determination. Total starch concentration was determined using the AACC approved method,[20] where ground meal samples (100 mg in duplicate) were hydrolyzed to dextrins and further D-glucose using α-amylase and amyloglucosidase, respectively. Total starch concentration was determined as free glucose by determining the absorbance at 510 nm[21] and calculated on a percent dry weight basis.[22] Protein concentration was determined by combustion method with the FP-528 Protein/Nitrogen Analyzer (LECO Corp., St. Joseph, MI, USA). Percent protein concentration was obtained by the formula $\%P = \%N \times C$, where C is 5.7 for wheat.[20] Crude lipid concentration was determined by an ANKOM^{XT15} extractor using petroleum ether as extraction solvent.[20] Percent lipid was expressed as weight of lipid per gram dry weight of the initial material used. β-Glucan concentration was determined using an enzymatic method.[20] Ground wheat meal (100 mg in duplicate) was digested with lichenase and β-glucosidase. Mixed linkage β-glucan concentration was calculated as free glucose by determining absorbance at 510 nm.[23]

Amylose Concentration Determination. High-performance size exclusion chromatography (HPSEC) was used to determine the amylose concentration.[19] Starch samples were debranched using isoamylase, freeze-dried, suspended in dimethyl sulfoxide (DMSO), and injected (40 μL) into a column (PL gel MiniMix 250 × 4.6 mm i.d. column, Polymer Laboratories Inc., Amherst, MA, USA). Amylose and amylopectin were separated using HP-SEC (Waters 600 controller, Waters 610 fluid unit, Waters 717 plus autosampler, Waters 410 differential refractometer; Waters Corp., Milford, MA, USA). Data were collected and analyzed using Empower 1154 chromatography software (Waters Corp.).

Amylopectin Chain Length Distribution Analysis. Glucan chain length distribution of amylopectin molecules was determined by fluorophore-assisted capillary electrophoresis (FACE)[24] using a Proteome Lab PA800 (Beckman Coulter, Fullerton CA, USA) equipped with a 488 mm laser module. A modified starch debranching protocol[25] was used to obtain unit amylopectin chains, which were labeled using 8-aminopyrene 1,2,6-trisulfonate (APTS) in the presence of sodium cyanoborohydride/tetrahydrofuran. The N-CHO (PVA) capillary with preburned window (50 μm i.d., 50.2 cm total length) was used to separate debranched samples.

Starch Granule Size Distribution Analysis. Granule size distribution (by volume) in starch slurries was determined using a laser diffraction particle size analyzer (Hydro 2000S, Malvern Instruments, Malvern WR, UK).[25] Volume percent particle size distribution values were obtained using Malvern Mastersizer 2000 software (Malvern Instruments).

In Vitro Kinetics of Enzymatic Starch Hydrolysis. Wheat grain meal and pure starch samples were enzymatically hydrolyzed in vitro for kinetic analysis, following a modified AACC approved method.[20] Meal and pure starch samples (100 mg) were mixed with pancreatic α-amylase (10 mg/mL) and amyloglucosidase (3 U/mL, Megazyme International Ltd., Wicklow, Ireland) for starch hydrolysis. Reaction mixtures were incubated for 0, 30, 60, 90, 120, and 240 min at 37 °C, during which starch was hydrolyzed to D-glucose. A 1 mL aliquot was taken from the reaction mixture after every incubation time, and reaction was terminated by adding an equal volume of 99% (v/v) ethanol and processed following the AACC approved method with modifications. Resistant starch and soluble starch concentrations were measured as free glucose by determining its absorbance at 510 nm.[22] Rate of starch hydrolyzed was expressed as percent of total starch at the end of each interval. Hydrolysis index was calculated using a nonlinear model described previously.[26,27]

Statistical Analyses. Analysis of variation (ANOVA) of the means, multiple means comparisons using Tukey's multiple-range tests at $p < 0.05$, and Pearson's bivariate correlations were performed with SPSS V. 19.0 software (SPSS Inc., Chicago, IL, USA). Dendrograms were obtained by Minitab V. 16.0 software (Minitab Inc., State College, PA, USA).

RESULTS

Screening of the Genotypes. Gel electrophoresis analysis of starch granule proteins showed that the most prominent polypeptide was GBSSI (molecular mass ∼ 60 kDa), which showed three bands, one originating from each genome (Figure 1). The waxy A, D, and B isoproteins have apparent molecular masses of 62.7, 58.7, and 56.7 kDa, respectively.[28] Although GBSSI-A was clearly distinct, GBSSI-B and -D were difficult to separate owing to their similar relative mobilities on denaturing polyacrylamide gel electrophoresis, which has also been observed previously.[16] Three resolved polypeptides were observed at 100−115 kDa, which could be SSIIa, as previously identified by immunoblotting antibodies against recombinant SSIIa-1.[18] In wheat, mature SBEI has been predicted to have a molecular mass of 87 kDa,[29] which was also observed in the present study. It has also been shown that *SBE1* gene produces alternatively spliced transcripts, which give an 87.4 kDa mature protein.[30] A larger variant of starch branching enzyme, SBEIc

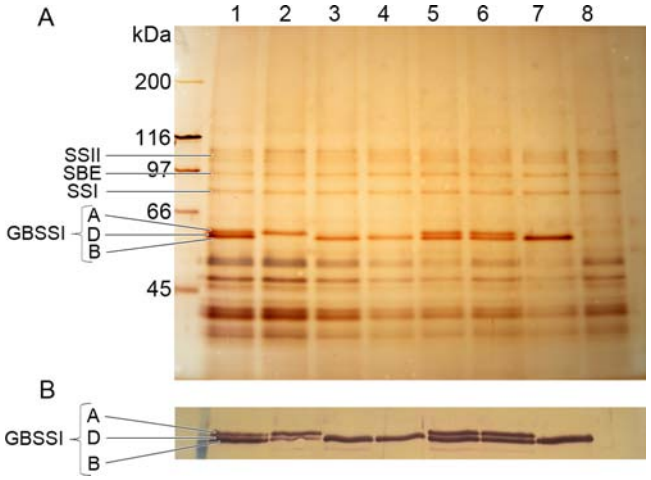

Figure 1. Analysis of GBSSI accumulation in wheat endosperm starch granules. (A) Denaturing polyacrylamide gel electrophoresis of starch granule-bound proteins. Lanes: 1, Wx-$A^+B^+D^+$; 2, Wx-$A^+B^-D^-$; 3, Wx-$A^-B^+D^-$; 4, Wx-$A^-B^-D^+$; 5, Wx-$A^+B^+D^-$; 6, Wx-$A^+B^-D^+$; 7, Wx-$A^-B^+D^+$; 8, Wx-$A^-B^-D^-$. Molecular mass of protein standards and migration of known starch granule-bound proteins are shown on the left. (B) Immunoreactive signal obtained with GBSSI-specific antibodies.

(152 kDa),[31] however, was not observed. At ~77 kDa, a polypeptide was observed in all genotypes, which could be starch synthase I (SSI).[32] In wheat the N-terminal sequence of 75 kDa polypeptide shows homology to rice soluble starch synthase.[33] In addition, various other lower molecular weight proteins such as puroindolines, 15 kDa,[34] were observed, which showed some differences between genotypes.

Carbohydrate Concentration. Thousand grain weight (TGW) in Wx-$A^+B^+D^+$ genotype was 40.1 g, whereas it varied significantly ($p < 0.05$) between 39.3 and 43.6 g for the other waxy null genotypes (Table 1). Lowest TGW (39.3 g) was observed for Wx-$A^-B^-D^-$ and Wx-A^-D^- genotypes, whereas highest TGW was observed for Wx-D^- genotype (43.6 g).

The total starch concentration in Wx-$A^+B^+D^+$ was 63.1%, whereas it ranged from 57.7 to 64.8% in the partial and completely waxy genotypes (Table 1). Wx-$A^-B^-D^-$ showed a significantly ($p < 0.05$) lower amount of starch (58.1%). However, double-null genotypes showed a higher starch concentration (62.9−64.8%) than single-null genotypes (57.7−60.5%). Because starch content is positively correlated with grain yield,[21] increase in TGW should be related to higher total starch concentration. However, Wx-D^- genotype with higher TGW had lower starch concentration (57.7%).

Amylose concentration varied significantly ($p < 0.05$) among the different waxy null genotypes. The Wx-$A^+B^+D^+$ genotype showed a higher amylose concentration of 28.7%, whereas the Wx-$A^-B^-D^-$ genotype showed nondetectable to very low (2.7%) concentration (Table 1). Among the partial waxy genotypes, amylose concentration varied from 21.5 to 23.1% in double nulls, whereas it varied from 26.8 to 28.3% in single-null genotypes, concurring with a previous report that double nulls are more affected than single nulls.[19] This suggests that genome-specific GBSSI has a substantial dosage effect on amylose concentration. However, because the amylose concentration was not completely reduced in single- and double-null genotypes, it can be suggested that genome-specific GBSSI activity is not limiting for amylose synthesis and shows a compensatory role of Wx proteins from the different genomes.

Protein Concentration. Protein concentrations in Wx-$A^+B^+D^+$ and Wx-$A^-B^-D^-$ were 15.9 and 15.6%, respectively, whereas it ranged from 13.0 to 18.8% in the partial waxy genotypes (Table 1). Similar to the trend observed for amylose concentration, a higher protein concentration was recorded for single-null genotypes (15.5−18.8%) than double-null genotypes (13.0−14.7%). Wx-D^- had significantly high ($p < 0.05$, 18.8%), whereas Wx-A^-D^- had significantly low ($p < 0.05$, 13.0%), protein concentration.

Lipid Concentration. Wx-$A^+B^+D^+$ genotype showed 2.3% lipid concentration, whereas it was highest (2.8%) in Wx-$A^-B^-D^-$ genotype (Table 1), agreeing with a previous report in barley.[25] In addition, a negative correlation was observed between lipid and amylose ($r = -0.61$, $p < 0.01$) (Table 2), represented by higher lipid concentration in the Wx-$A^-B^-D^-$ line.

β-Glucan Concentration. β-Glucan concentrations in the partial and completely waxy genotypes varied from 0.6 to 0.9%, being 0.7% in Wx-$A^+B^+D^+$ genotype (Table 1). Different cereal grains were analyzed for their whole grain and endosperm β-glucan contents, and very low (0.23−0.36%) concentration for wheat endosperm was reported.[35] Wx-$A^-B^-D^-$ genotype recorded significantly ($p < 0.05$) higher concentration of β-glucan (1.1%) than the partial waxy genotypes. A similar pattern was observed in a previous study in barley, where a waxy cultivar CDC Fibar with low starch and amylose concentrations had higher β-glucan concentration.[25]

Amylopectin Chain Length Distribution. A typical chain length distribution curve can be divided into seven regions, DP

Table 1. Proximate Analysis[a] of Wheat Grains with Different Combinations of Waxy (GBSSI) Isoproteins

		concentration (%)				
genome composition	thousand grain weight (g)	total starch	amylose	protein	lipid	β-glucan
$A^+B^+D^+$	40.1 ± 0.4 ab[b]	63.1 ± 0.5 bc	28.7 ± 0.5 c	15.9 ± 0.1 cd	2.3 ± 0.0 ab	0.7 ± 0.0 a
$A^-B^+D^+$	40.9 ± 0.7 abc	59.6 ± 0.5 ab	26.8 ± 0.4 c	15.5 ± 0.2 cd	2.3 ± 0.0 ab	0.7 ± 0.0 a
$A^+B^-D^+$	42.2 ± 0.1 bc	60.5 ± 0.5 ab	28.3 ± 0.6 c	16.7 ± 0.8 d	2.4 ± 0.1 ab	0.7 ± 0.0 a
$A^+B^+D^-$	43.6 ± 0.5 c	57.7 ± 0.1 a	27.0 ± 0.4 c	18.8 ± 0.0 e	2.3 ± 0.0 ab	0.6 ± 0.0 a
$A^-B^-D^+$	41.0 ± 0.9 abc	62.9 ± 1.1 bc	23.1 ± 0.4 b	14.7 ± 0.2 bc	2.3 ± 0.0 ab	0.9 ± 0.0 b
$A^-B^+D^-$	39.3 ± 0.4 a	63.2 ± 0.6 bc	22.5 ± 0.2 b	13.0 ± 0.0 a	2.3 ± 0.0 a	0.9 ± 0.0 b
$A^+B^-D^-$	40.8 ± 0.6 ab	64.8 ± 0.9 c	21.5 ± 0.4 b	13.7 ± 0.2 ab	2.5 ± 0.1 b	0.9 ± 0.0 b
$A^-B^-D^-$	39.3 ± 0.3 a	58.1 ± 0.3 a	2.7 ± 0.2 a	15.6 ± 0.0 cd	2.8 ± 0.1 c	1.1 ± 0.0 c

[a]Values were calculated on dry weight basis (except amylose, which is percent of total starch) and represent the means of three biological replications and two independent observations for each replicate ± standard error. [b]Mean values within a column followed by different letters are significantly different ($p < 0.05$), with "a" representing the smallest value.

Table 2. Correlation Analysis[a] between Grain Constituents and Starch Enzymatic Hydrolysis Parameters

	TGW	starch	amylose	protein	lipid	β-glucan	RDS M	SDS M	RS M	HI M
TGW	1	−0.25	0.48*	0.65**	−0.28	−0.65**	−0.36	−0.41*	0.49*	−0.37
starch		1	−0.05	−0.74**	−0.13	0.35**	−0.24	0.18	0.01	−0.19
amylose			1	0.31*	−0.61**	−0.85**	−0.61**	−0.18	0.49**	−0.77**
protein				1	−0.01	−0.63**	−0.13	−0.32*	0.32*	−0.22
lipid					1	0.46**	0.30*	−0.15	−0.06	0.41**
β-glucan						1	0.58**	0.28	−0.55**	0.69**
RDS M							1	0.12	−0.68**	0.92**
SDS M								1	−0.81**	0.05
RS M									1	−0.59**
HI M										1

[a]*, significant at $p < 0.05$; **, significant at $p < 0.01$

6−8 (R-I), DP 9−11 (R-II), DP 12−14 (R-III), DP 15−18 (R-IV), DP 19−22 (R-V), DP 23−36 (R-VI), and DP 37−45 (R-VII), depending on its changing nodes (Figure 2). Differences

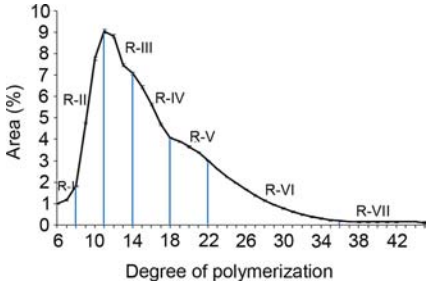

Figure 2. Amylopectin chain length distribution for starch extracted from nonwaxy wheat genotype. The amylopectin unit chains were divided into seven regions, R-I (DP 6−8), R-II (DP 9−11), R-III (DP 12−14), R-IV (DP 15−18), R-V (DP 19−22), R-VI (DP 23−36), R-VII (DP 37−45), on the basis of changing slopes of the curve.

were observed in R-I, which had comparatively more chains of DP 6−8 in wheat starch with complete GBSSI complement than the partial and completely waxy starches (Table 3). Similarly, subtractive graphs between Wx-$A^+B^+D^+$ and other waxy null starches showed that nonwaxy wheat had higher content of short chains of DP 6−10 compared to GBSSI-deficient starches (Figure 3). Chains of DP 6−10 occupied ∼15% more area in the wheat starches with a complete complement of GBSSI proteins (Wx-$A^+B^+D^+$) compared to all other partial and completely waxy starches (Figure 3). Among the waxy null starches studied, Wx-D^- showed ∼10% more area for DP 6−10 than Wx-A^- and Wx-B^- (Figure 3). For other

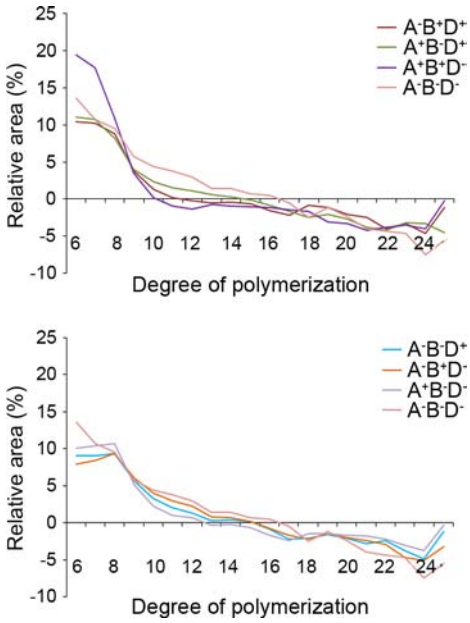

Figure 3. Amylopectin chain length distribution profile in partial and completely waxy wheat starch and nonwaxy wheat starch. Percent relative chains were obtained by subtracting the normalized area percent occupied by chains of waxy null starch from that of Wx-$A^+B^+D^+$ starch, with the upper graph showing single nulls and the lower graph showing double nulls.

regions of chain length distribution, Tukey's test, however, revealed no clear differences among various waxy null combination genotypes (Table 3). No significant differences were observed for average DP as well.

Table 3. Amylopectin Chain Length Distributions[a] in Wheat Starch

	distribution (%)							
genome composition	R-I (DP 6−8)	R-II (DP 9−11)	R-III (DP 12−14)	R-IV (DP 15−18)	R-V (DP 19−22)	R-VI (DP 23−36)	R-VII (DP 37−45)	av DP
$A^+B^+D^+$	4.2 ± 0.0 b[b]	22.0 ± 0.3 a	23.4 ± 0.2 a	20.4 ± 0.1 a	14.0 ± 0.1 a	15.0 ± 0.4 a	1.1 ± 0.2 a	16.2 ± 0.1 a
$A^-B^+D^+$	3.8 ± 0.0 ab	21.7 ± 0.2 a	23.5 ± 0.2 a	20.7 ± 0.1 a	14.2 ± 0.1 a	15.2 ± 0.4 a	1.0 ± 0.1 a	16.2 ± 0.1 a
$A^+B^-D^+$	3.8 ± 0.0 ab	21.5 ± 0.1 a	23.2 ± 0.1 a	20.7 ± 0.1 a	14.3 ± 0.2 a	15.3 ± 0.2 a	1.2 ± 0.2 a	16.3 ± 0.1 a
$A^+B^+D^-$	3.6 ± 0.1 a	22.0 ± 0.2 a	23.7 ± 0.3 a	20.7 ± 0.1 a	14.4 ± 0.1 a	14.8 ± 0.4 a	0.9 ± 0.1 a	16.2 ± 0.1 a
$A^-B^-D^+$	3.8 ± 0.1 ab	21.2 ± 0.2 a	23.1 ± 0.1 a	20.5 ± 0.1 a	14.2 ± 0.1 a	15.9 ± 0.3 a	1.3 ± 0.1 a	16.5 ± 0.1 a
$A^-B^+D^-$	3.8 ± 0.1 ab	21.1 ± 0.2 a	23.1 ± 0.2 a	20.6 ± 0.1 a	14.2 ± 0.0 a	15.9 ± 0.3 a	1.3 ± 0.2 a	16.5 ± 0.1 a
$A^+B^-D^-$	3.9 ± 0.1 ab	21.5 ± 0.1 a	23.3 ± 0.2 a	20.6 ± 0.1 a	14.0 ± 0.1 a	15.5 ± 0.2 a	1.3 ± 0.1 a	16.4 ± 0.0 a
$A^-B^-D^-$	3.7 ± 0.1 a	21.0 ± 0.4 a	22.9 ± 0.3 a	20.5 ± 0.3 a	14.3 ± 0.1 a	16.2 ± 0.7 a	1.4 ± 0.4 a	16.5 ± 0.2 a

[a]Values are based on average of three replications ± standard error. [b]Mean values within a column followed by different letters are significantly different ($p < 0.05$), with "a" representing the smallest value.

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Starch Granule Size Distribution. Starch granule size showed bimodal distribution in Wx-$A^+B^+D^+$ and the waxy null starches. Starch granule diameter ranged from 1.5 to 52.5 μm, with maximum volume occupied by granules of 17.4 μm diameter (Figure 4A,B). On the basis of diameter, starch granules can be divided into C-granules (<5 μm), B-granules (5−15 μm), and A-granules (>15 μm).[36] Wx-$A^-B^-D^-$ starch showed the highest volume percentage of C-granules and the lowest volume percentage of A-granules among nonwaxy and waxy null starches (Figure 4C). Wx-A^-B^- and Wx-D^- starches showed similar results, that is, reduced and increased volume percentage of C-granules and A-granules, respectively. This suggests a comparable role of Wx-D individually, and Wx-A, Wx-B together, in influencing the granule size distribution. The presence of Wx-D, either individually or in combination with Wx-A and/or Wx-B, showed an increase in the volume percentage of A-granules (Figure 4C). However, the absence of Wx-D along with Wx-A or Wx-B increased the B- and C-granule volume (%) and reduced the A-granule volume (%) (Figure 4C). Normal distribution of number percent of starch granules can be divided into three portions: (i) d(0.1), 10% of granules smaller than diameter set point; (ii) d(0.5), 50% of granules smaller than diameter set point; and (iii) d(0.9), 90% of granules smaller than diameter set point. Values for d(0.1) in Wx-$A^-B^-D^-$ starch were significantly higher than those for Wx-$A^+B^+D^+$ (data not shown), suggesting the influence of waxy allele on starch granule size distribution.

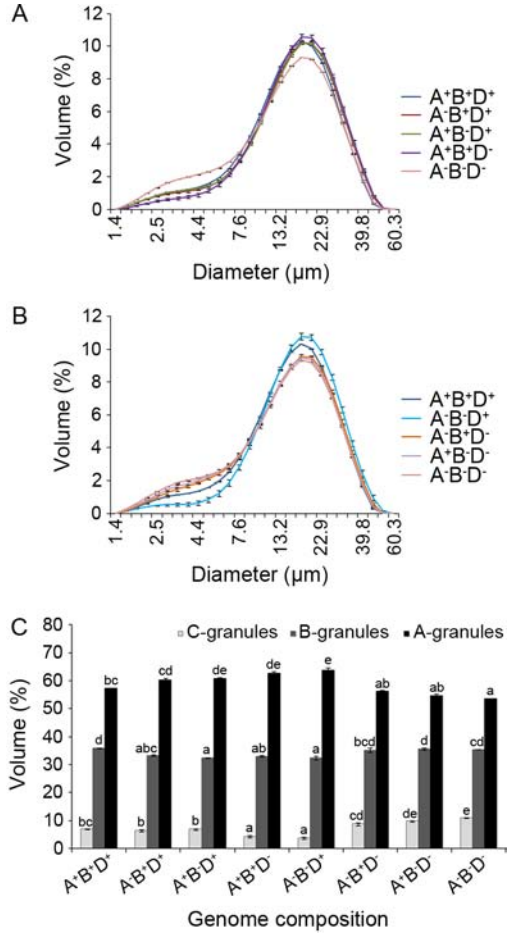

Figure 4. Starch granule size distribution in wheat endosperm starch. Line graphs show the volume occupied by starch granules of different diameters in (A) single waxy-null genotypes and (B) double waxy-null genotypes. (C) Effect of the absence of genome-specific GBSSI on C-type (<5 μm), B-type (5−15 μm), and A-type (>15 μm) diameter range of starch granules. Different letters on the same colored columns indicate they are significantly different ($p < 0.05$), with "a" representing the smallest value.

In Vitro Starch Hydrolysis. The kinetics of hydrolytic studies in meal and pure starch showed a rapid increase in starch hydrolysis until 30 min, which is considered as RDS. From 30 to 120 min, there was a slow increase in the rate of starch hydrolysis, which is considered as SDS. After 120 min, starch hydrolysis in pure starch reached saturation and was completely digested by 240 min. This portion of starch is known as RS.[8] In meal samples, however, the rate of starch hydrolysis was slower than in purified starch (Figure 5).

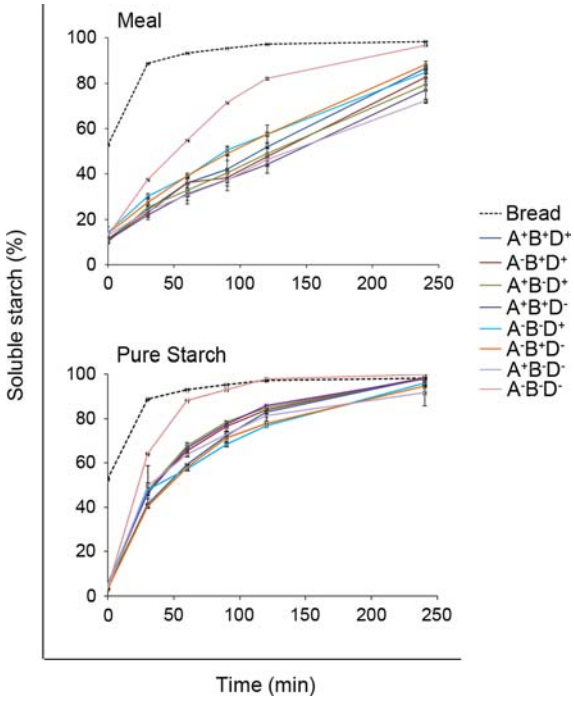

Figure 5. Rate of starch enzymatic hydrolysis curve showing the amount of starch hydrolyzed at selected time intervals in meal and pure starch samples from nonwaxy, partial waxy, and completely waxy genotypes.

Percent soluble starch in white bread reached ~90% within 30 min as compared to nonwaxy and partial waxy genotypes, which reached ~25% (meal) and 40−45% (pure starch) in the same time period (Figure 5). Completely waxy genotype showed a significantly higher rate of starch hydrolysis than all partial waxy genotypes, reaching ~40 and ~60% in meal and pure starch samples, respectively, within 30 min of incubation with hydrolytic enzymes. For meal samples of partial waxy genotypes, percent soluble starch increased linearly throughout the experiment duration, however, reaching saturation at 120 min in the completely waxy genotype. For pure starch samples, the rate of hydrolysis was significantly higher than that of meal. In the completely waxy, pure starch samples reached saturation in 90 min compared to 120 min in meal samples (Figure 5).

In wheat grain meal, endogenous components such as protein, lipid, and β-glucan are present in addition to starch. Completely waxy genotype showed highest rate of hydrolysis,

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reaching a point of 50% hydrolysis in ~45−50 min, whereas other genotypes reached a similar stage after ~120 min. RDS in completely waxy genotype was significantly ($p < 0.05$) higher (24.8%) as compared with nonwaxy (12.9%) and partial waxy genotypes (11.0−16.0%) (Figure 6). RS in completely waxy

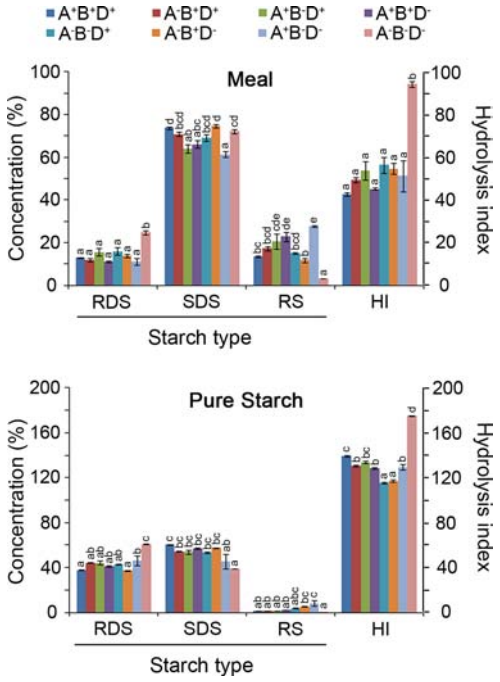

Figure 6. Enzymatic starch hydrolysis parameters of meal and pure starch from nonwaxy, partial waxy, and completely waxy genotypes. RDS, SDS, and RS are percent values. HI is an absolute value.

genotype was significantly ($p < 0.05$) lower (3.1%) than nonwaxy (13.4%) and partial waxy genotypes (11.7−27.6%). SDS values in different genotypes ranged from 61.4 to 74.5%, with no specific pattern. Wx-B$^-$D$^-$ genotypes showed lowest concentration of RDS and highest concentration of RS.

To investigate the influence of GBSSI on starch hydrolysis, purified starch was used to remove the confounding effects of other endogenous grain constituents. The hydrolysis curve indicated that 50% of pure starch was hydrolyzed between 30 and 60 min in all genotypes. Similar to meal, completely waxy genotype showed the highest amount of RDS (60.9%), followed by nonwaxy and partial waxy genotypes (Figure 6). RS concentration was lowest in completely waxy genotype (0.2%). Consistent with the pattern of hydrolysis in meal, highest RS concentration was also found in Wx-B$^-$D$^-$ genotypes in pure starch hydrolysis assay.

Hydrolysis index (HI) for meal was highest in completely waxy genotype (94.3). Nonwaxy genotype showed HI of 42.7. HI (pure starch) was also highest in completely waxy genotype (175.0), whereas it ranged from 117.1 to 139.3 in nonwaxy and partial waxy genotypes (Figure 6).

DISCUSSION

GBSSI in Relation with Grain Components Accumulation. Wheat flour composition influences its properties and end use. Amylose concentration and amylopectin structure are integral parts of starch functionality. In bread baking, substitution of nonwaxy wheat flour with ~10% waxy wheat flour results in higher loaf volume, whereas >30% substitution can lead to loss of granule rigidity and fusion of starch granules.[37] In the present study, GBSSI double-null genotypes showed significantly higher total starch concentration (62.9−64.8%) than single-null genotypes (57.7−60.5%) (Table 1) without significantly affecting TGW. In a recent study in sorghum, *GBSSI* gene associated single nucleotide polymorphic (SNP) markers affected starch content, independent of amylose concentration.[38] In addition to starch, dose-dependent effect of Wx protein on amylose concentration was observed (Table 1), concurring with a previous study.[19] The gradual reduction in amylose concentration from wild type to two *Wx* gene null genotypes can be explained by the ability of the remaining functional gene to partially compensate for the missing *Wx* allele, as suggested previously.[39] However, overexpression of *Wx* genes does not affect amylose content, despite the increase in the amount of Wx protein.[40] Thus, this suggests evolutionary limitation of amylose threshold, above which it becomes inert to *Wx* gene dosage. However, considering previous and present results, it can be suggested that, in wheat, genome-specific GBSSI activity is compensatory for amylose synthesis.

On the basis of GBSSI catalytic potential for amylose synthesis, Wx-A1a was lowest, whereas Wx-B1a and Wx-D1a had similar activities.[41] Wx-A1 and Wx-B1 proteins have been reported to be additive in action, whereas Wx-D1 is independent and strongest among all of the GBSSI isoproteins in wheat endosperm.[42] Various reports have suggested a nonadditive behavior of Wx proteins, with interaction being the dominating character influencing amylose concentration.[43] Although order of contribution toward amylose synthesis is reported to be Wx-B1 ≥ Wx-D1 > Wx- A1[41] and/or Wx-D1 > Wx-B1 > Wx-A1,[42] no such pattern was observed in the present study (Table 1). Variants of *Wx* alleles have been studied in the past for their amylose synthesis ability, and the postulated order is Wx-A1b (null) = Wx-A1e < Wx-A1c < Wx-A1a < Wx-B1c = Wx-B1d ≤ Wx-D1a < Wx-D1c ≤ Wx-B1e.[44] These variations in amylose-synthesizing capacity of genome-specific Wx protein support the idea of regulatory mechanisms between genomes. However, this trait has also been reported to be influenced by growing environment and genetic background,[45] which could be the reason for different results reported in the literature.

Lipid concentration was highest in completely waxy genotype as compared to nonwaxy and partial or completely waxy genotypes (Table 1). The presence of amylose−lipid inclusion complexes is known in wheat.[46] Crystallinity studies[47] in waxy wheat did not report the formation of such complexes, due to the absence of amylose.[48] Because nonwaxy wheat varieties have lipid integrated with amylose, in the present study a lower concentration of free lipids was observed in nonwaxy and partial waxy genotypes. Waxy wheat genotypes devoid of amylose−lipid complex are suitable candidates for bioethanol production.[49]

GBSSI in Relation with Amylopectin Fine Structure. The role of Wx protein in starch synthesis has been extensively studied in different plant systems.[1,10,11] Previous studies have suggested an indispensible role of functional *Wx* allele either for synthesis of intermediates in amylose production or for synthesis of long-chain fraction of amylopectin.[11] On the other hand, a study in wheat showed that starch synthase and starch branching enzymes are not regulated by the *Wx* null alleles,[42] suggesting that amylopectin synthesis pathway is not affected by the lack of one, two, or three *Wx* alleles. In addition, it has been shown that amylopectin structure is identical in waxy and their respective parents in near-isogenic wheat

Table 4. Correlation Analysis[a] between Starch Characteristics and Starch Enzymatic Hydrolysis Parameters

	starch	amylose	C-granules	B-granules	A-granules	RDS PS	SDS PS	RS PS	HI PS	R-I	R-II	R-III	R-IV	R-V	R-VI	R-VII	av DP
starch	1	−0.05	0.01	0.05	−0.03	−0.25	0.07	0.33*	−0.40**	0.25	−0.20	−0.14	−0.23	−0.37*	0.20	0.35*	0.24
amylose		1	−0.54**	−0.33*	0.49**	−0.63**	0.53**	0.00	−0.61**	0.06	0.35*	0.26	0.15	0.01	−0.33*	−0.27	−0.33*
C-granules			1	0.70**	−0.95**	0.35*	−0.36*	0.17	0.50**	0.16	−0.30	−0.30	−0.03	0.07	0.23	0.23	0.24
B-granules				1	−0.89**	0.11	−0.18	0.22	0.32	0.24	−0.17	−0.20	−0.15	−0.15	0.17	0.23	0.19
A-granules					1	−0.27	0.31	−0.21	−0.46**	−0.21	0.27	0.28	0.09	0.02	−0.23	−0.25	−0.24
RDS PS						1	−0.92**	0.19	0.76**	−0.35*	−0.12	−0.08	−0.16	0.05	0.21	0.09	0.18
SDS PS							1	−0.57**	−0.53**	0.40**	0.15	0.08	0.17	0.04	−0.26	−0.11	−0.22
RS PS								1	−0.26	−0.27	−0.10	−0.03	−0.08	−0.20	0.20	0.09	0.17
HI PS									1	−0.04	0.03	−0.03	−0.09	0.08	0.01	0.00	0.00
R-I										1	0.09	−0.15	−0.05	−0.24	−0.17	0.09	−0.13
R-II											1	0.91**	0.38**	−0.42**	−0.89**	−0.75**	−0.93**
R-III												1	0.49**	−0.33*	−0.87**	−0.79**	−0.90**
R-IV													1	0.34*	−0.68**	−0.65**	−0.65**
R-V														1	0.11	−0.06	0.15
R-VI															1	0.75**	0.96**
R-VII																1	0.89**
av DP																	1

[a]*, significant at $p < 0.05$; **, significant at $p < 0.01$.

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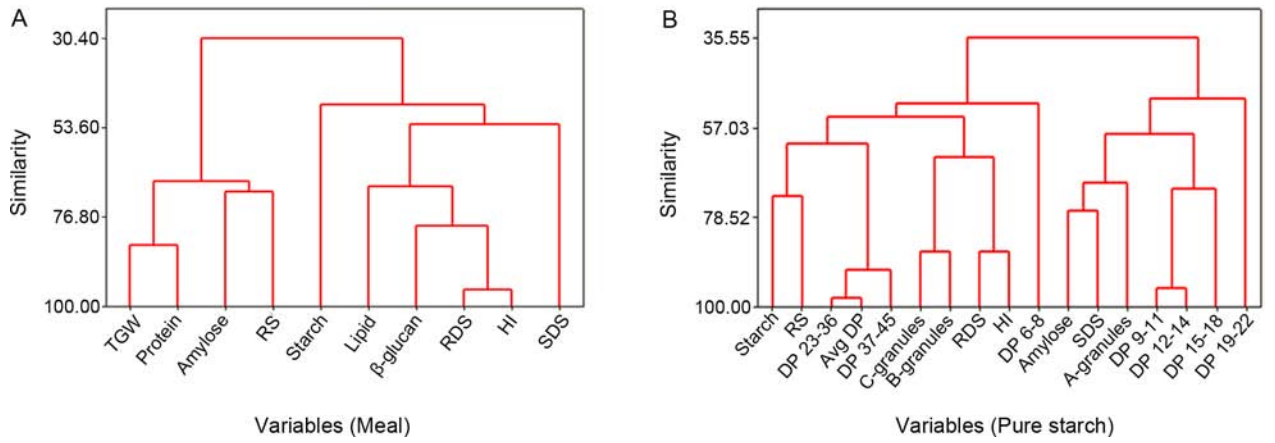

Figure 7. Average linkage dendrogram depicting relationship between different components of wheat grain related to starch enzymatic hydrolysis: (A) meal characteristics; (B) starch characteristics.

lines.[41,46] However, in the present study, minor but significant ($p < 0.05$) differences were observed in the very short chain fraction, with Wx-$A^+B^+D^+$ showing a higher concentration of DP 6−8 chains than Wx-$A^-B^-D^-$ (Table 3). Among the partial waxy starches, Wx-$A^+B^+D^-$ showed relatively higher concentration of short chains of DP 6−8 (Figure 3). However, Wx-A or Wx-B individually did not show such phenotype, thus suggesting an additive contribution of Wx-A + Wx-B toward the short-chain phenotype of waxy amylopectin. Although absence of Wx-D singly (Wx-$A^+B^+D^-$) showed a relative increase in DP 6−8 chains content, the presence of Wx-D with Wx-A or Wx-B did not influence the short-chain fraction (Figure 3), thereby suggesting a suppressive role of Wx-D toward the formation of short chains of waxy starch amylopectin. Significant ($p < 0.05$) differences were not observed in medium- or long-chain regions between nonwaxy and partial or completely waxy genotypes (Table 3). Hence the results suggest that reduced GBSSI dosage subtly affects short glucan chain synthesis in amylopectin, however it is not a limiting factor for the development of amylopectin structure. In addition, the deficiency in short amylopectin chains of DP 6−8 could be because of pleiotropic effects of other starch synthesizing enzymes,[10] such as SSI or SSIIa, which are responsible for synthesis of this fraction of chains.

GBSSI in Relation with Starch Granule Size Distribution. Granule size distribution of wheat starch is influenced by genotype[50] and environment.[51] Granule size is an important characteristic that influences the composition of starch and its functionality. Studies on starch granule sizes from various plant sources suggest their correlation with physicochemical properties such as thermal, viscoelastic properties, gelatinization behaviors, pasting properties, swelling power, and water-binding capacity, in both wild type and waxy genotypes.[48]

In the present study starch granule size showed bimodal distribution in nonwaxy, partial waxy, and completely waxy starch (Figure 4A,B). However, in a previous study waxy maize starch showed altered starch granule size distribution compared to nonwaxy maize starch.[52] The completely waxy starch in this study showed highest volume percentage of small C-type starch granules and lowest volume percentage of large A-type starch granules, compared to nonwaxy and partial waxy starch (Figure 4C). In previous studies as well, the volume percentage of small starch granules was shown to be higher in waxy starch compared to the nonwaxy starch in wheat[53] and barley.[27] Additionally, a decrease in large A-type starch granule content with increase in waxy character has been reported.[39] It has been suggested to be a secondary effect of Wx protein dosage, which could occur by either alteration of glucan chains, thereby reducing starch debranching enzyme activity, or a pleiotropic effect of *Wx* gene on other starch biosynthetic enzymes, hence altering large and small starch granule properties.[39] In the present study, significant ($p < 0.05$) differences in starch granule size distribution between nonwaxy and completely waxy starch were observed, concurring with a previous study in waxy wheat.[39] Contrastingly, in a few other studies, differences in starch granule size distribution between waxy and nonwaxy wheat varieties have not been observed.[47]

GBSSI in Relation with Starch Hydrolysis. Grain and starch characteristics affect the starch digestibility of cereal grains.[25] Amylose is a negative regulator of starch digestibility.[4] The reason for such characteristic of amylose lies in its linear hydrogen bond stabilized double-helical structure, which is resistant to enzymatic hydrolysis. Recently, production of barley amylose-only starch granules resulted in 65% RS for gelatinized starch, which was 2.2-fold higher than the nonwaxy starch.[54] In the present study, Pearson's bivariate correlation analysis showed a negative correlation of amylose with hydrolysis index ($r = -0.77$, $p < 0.01$) and RDS ($r = -0.61$, $p < 0.01$) and a positive correlation with RS ($r = 0.49$, $p < 0.01$) (Table 2). This is also reflected in completely waxy genotype, with low amylose concentration and highest hydrolysis index, as compared to nonwaxy and partial waxy starch.

Correlation between starch characteristics showed that amylose was positively correlated with large A-type starch granules ($r = 0.49$, $p < 0.01$) and negatively correlated with small C-type starch granules ($r = -0.54$, $p < 0.01$) (Table 4), which is in agreement with a previous paper[36] but differs from a recent study in barley.[25] In addition, large A-type starch granules were negatively correlated with hydrolysis index ($r = -0.46$, $p < 0.01$), and small C-type starch granules were positively correlated with hydrolysis index ($r = 0.50$, $p < 0.01$) and negatively correlated with SDS ($r = -0.36$, $p < 0.05$) (Table 4). Larger size, lenticular shape, higher amylose,[15] and long chains of amylopectin[55] in A-type starch granules could be the reasons for their reduced digestibility. The reason for the higher rate of hydrolysis in C-type starch granules could be attributed to more surface area or more free ends of glucan chains for amylases to act on.

To determine characteristics associated with starch-specific and meal parameters, cluster dendrograms with average linkage correlation were constructed (Figure 7). For wheat grain meal, amylose and RS are parts of the same clade, suggesting a close association between the two components. For pure starch, RDS and small granules were present on branches of the same clade, suggesting a positive correlation between these variables. SDS was present close to amylose and large A-type starch granules. RS was located near longer chains of amylopectin DP 23−45. This further supports that A-type starch granules, amylose, and longer chains of amylopectin play substantial roles in starch hydrolysis potential.

Genome-specific GBSSI showed variations in starch characteristics and relationship with starch hydrolysis. The absence of Wx-D showed an increase in short chains of amylopectin (Figure 3), whereas the presence of Wx-D, either singly or in combination, showed reduced short chains of DP 6−8. This suggests a suppressive role of Wx-D in the formation of the short-chain fraction of amylopectin. In addition, the presence of Wx-D, either singly or in combination, led to increased volume percentage of large A-type starch granules (Figure 4C). Moreover, individual Wx-D showed a significantly ($p < 0.05$) lower hydrolysis index, whereas the rate of hydrolysis was higher when Wx-D was present with Wx-A or Wx-B (Figure 6). Therefore, it can be suggested that among the Wx isoproteins, Wx-D might be the major contributor influencing starch hydrolysis in wheat.

In conclusion, this study indicates dosage-dependent amylose synthesis, where the absence of one Wx protein reduces the amylose concentration by 1.3% and, further, the absence of two Wx proteins reduces it to 22.4 from 28.7% in nonwaxy wheat starch. Results also show that GBSSI is not limiting for amylopectin synthesis, because only subtle differences in amylopectin chain length distribution were observed in nonwaxy, partial, and completely waxy starches. The complete absence of Wx protein affects starch granule size distribution, with a 37% increase in C-type starch granules in completely waxy relative to nonwaxy starch. The present study suggests that the absence of GBSSI affects starch granule growth by inhibiting glucan chain elongation. The rate of starch hydrolysis is significantly influenced by granule size, amylose concentration, and the long-chain fraction of amylopectin. The above-mentioned starch properties could be utilized to develop specialty starches for food, feed, and industrial applications.

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Notes

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ABBREVIATIONS USED

AGPase, ADP-glucose pyrophosphorylase; DP, degree of polymerization; GBSSI, granule-bound starch synthase I; HI, hydrolysis index; M, grain meal; PS, purified starch; RDS, readily digestible starch; RS, resistant starch; SBE, starch branching enzyme; SDS, slowly digestible starch; SS, starch synthase; TGW, thousand grain weight; Wx, waxy

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