Amylose content and starch properties in emmer and durum wheat lines with different waxy proteins composition

Running title: Starch properties in different waxy genotypes of tetraploid wheats

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Abstract

Background: Emmer wheat is a neglected crop that can be used in the breeding of modern durum wheat for quality. One important aspect of this quality is the starch composition which is related to the waxy proteins. A collection of emmer wheat was analysed previously for waxy protein composition, finding two new *Wx-B1* alleles by SDS-PAGE and DNA sequencing analysis. It is necessary to analyse the effect of these alleles in starch properties and compare to durum wheat ones.

Results: In the current study, emmer lines carrying three different *Wx-B1* alleles (*Wx-B1b,-B1g,-B1c**), including one with the null allele (*Wx-B1b)*, together with durum cvs. Langdon (*Wx-B1a*) and Mexicali (*Wx-B1c´*), were analysed for amylose content. Differences were detected between both species, and the line lacking Wx-B1 protein showed a remarkable low amylose content. In addition, data from blue value, swelling power and Rapid Visco Analyzer also suggested that there were differences in starch properties among the different *Wx-B1* alleles.

Conclusions: The present study suggests that the amylose content between emmer (*Wx-B1g*) and durum (*Wx-B1a)* standard materials is not the same; therefore some starch properties are different between the two species. The variation found could be used to enlarge the gene pool of durum wheat, and design new materials with desirable amylose content.

Keywords: amylose content, emmer wheat, starch properties, waxy proteins

INTRODUCTION

Starch is the major component of wheat flour and composed of two types of glucan polymers: the essentially linear amylose, and the highly branched amylopectin, in the range of 22-35% amylose/68-75% amylopectin. Some starch physico-chemical properties are very important for the end-use of the product as its gelatinization, pasting and gelation depend on the ratio amylose/amylopectin[1]. Starch with less amylose content is, for example, preferred for Japanese noodles[2]. For this reason several studies have been carried out during recent years in order to know which factors can affect to the ratio of both molecules[3]. Nowadays the genetic basis for amylose variation has been researched. The granule-bound starch synthase I or waxy protein is the enzyme responsible for amylose synthesis in cereal grain. Because the tetraploid (2n = 4× = 28, AABB) and hexaploid (2n = 6× = 42, AABBDD) condition of durum and common wheat respectively, these species carry two and three waxy proteins (Wx-A1 and Wx-B1 in durum wheat; Wx-A1, Wx-B1 and Wx-D1 in common wheat). Nakamura et al.[4] demonstrated the function of the waxy proteins and their exclusivity in the amylose synthesis in the wheat grain by crossing cv. Kanto 107 lacking Wx-A1 and Wx-B1 proteins with Chinese cv. Bai Huo lacking Wx-D1 protein to get a waxy (free of amylose) wheat. After, Yamamori and Quynh[5] showed that the lacking of Wx-B1 protein was more important than the lacking of Wx-D1 or Wx-A1 in common wheat, as its loss produces a greater effect in amylose content and starch properties. Similar conclusions were found by Araki et al.[6,7]. In addition to the null alleles, waxy proteins with different isoelectric points and molecular weight were discovered[8,9]. The effect of some of these alleles has been tested[10,11] finding that they can affect amylose content and starch properties, for example, *Wx-D1f* allele which carry internal mutation that

reduce the enzymatic activity.

Besides, the search of new variants of waxy proteins in wild species and ancient wheats was successful and new alleles were identified in different species[12,13], including null alleles and waxy proteins with different electrophoretic mobility. One of these ancient wheats, emmer wheat (*Triticum turgidum* ssp. *dicoccum* Schrank; 2n = 4× = 28, AABB), which cultivation was drastically reduced during the last century because its low yield compared to durum wheat, is recovering nowadays certain agronomic importance because the increasing interest in organic and traditional food. Another reason for its recovering is that emmer wheat based diets are known to digest slowly, or in other words, it could be considered as hypoglycemic, which is of therapeutic advantage[14]. Different authors have detected the polymorphism for the waxy proteins in this species[8,9]. However, no deeper studies have been done about how the polymorphism in the waxy proteins affect the amylose content and other starch properties in this species. Besides, little is known about the similarities and differences between durum wheat (*T. turgidum ssp. durum* Thell. em. Desf.) and emmer wheat starches, and this information would be very interesting to enlarge the genetic pool available for durum wheat breeders.

Recently, three *Wx-B1* alleles (*Wx-B1b*, *Wx-B1g* and *Wx-B1c**) were identified by SDS-PAGE and molecular characterization in a collection of Spanish emmer wheat[15]. Although the *Wx-B1g* and *Wx-B1c** alleles showed almost equal mobility in SDS-PAGE as the *Wx-B1a* and *Wx-B1c´* alleles, respectively; the analysis of their gene sequence showed the presence of several amino acid changes. The aim of the current study was to analyze the effect of these three *Wx-B1* alleles on amylose content and starch properties in emmer wheat, together with the comparison of these results with the ones obtained in durum wheat.

EXPERIMENTAL

Plant materials

Six lines of Spanish emmer wheat, along with durum wheat cultivars Langdon (*Wx-B1a* allele) and Mexicali (*Wx-B1c'* allele), were grown during 2008-2009 inside a greenhouse in the IFAPA in Cordoba (Ambient A) and in Rabanales Campus in Cordoba (Ambient B), using standard agronomic practice for the region. The *waxy* allelic composition of the emmer lines for the *Wx-B1* gene is showed in Table 1. All materials showed the *Wx-A1a* allele.

After harvest, the grain was tempered to 16% of moisture and milled on an experimental Brabender mill.

Starch isolation and properties

Flours were homogenized in a solution consisting of 20 g kg^{-1} sodium dodecyl sulfate (SDS) and 100 ml L^{-1} glycerol. Homogenates were passed through a 100 µm nylon mesh and centrifuged. A yellowish layer was removed with a spatula, and then mixed with the SDS solution. This was repeated twice, and then the pellet was washed with distilled water twice, and twice with acetone.

Twenty milligrams of the resulting starch was used for measuring in triplicate amylose content by Megazyme Amylose/Amylopectin kit. This content was also measured by an Autoanalyzer (Bran+Luebbe) using 40 mg of starch. Four measures were done for each sample.

The Blue value (BV) and maximum absorbance (λ_{max}) were measured as described by Yamamori et al.[16] Absorption curves of gelatinized starch-iodine complexes were measured at 500-700 nm to determine the absorbance at 680 nm

(apparent BV) and λ_{max} of the iodine-starch complex. The values were determined in triplicate.

Water-washed starch was also prepared. A dough ball was made with flour and NaCl 20 g kg^{-1}, which was kept in cold distilled water for 1 hr. The dough ball was then kneaded in water until all starch granules were extracted. The resultant starch was filtered through a 100 µm nylon mesh and centrifuged. The starch was washed three times with water and a yellowish layer was removed with spatula in each of the washing.

After drying, 160 mg of water-washed starch were weighed in a 10 ml test tube, and then 5 ml of 1 g kg^{-1} $AgNO_3$ was added. Capped test tubes were incubated at 70°C in a water bath for 10 min with shaking, and then incubated in boiling water for 10 min. After cooling, tubes were centrifuged at 1.700 *g* for 4 min and the supernatant was removed. Swelling power (SP) was measured as sediment weight divided by dry sample weight (g/g). Four measures were done for each sample.

To examine the starch pasting properties, 3 g of water-washed starch in 25ml water was subjected to Rapid Visco Analyzer (RVA). The suspension was first retained at 34ºC for 2 min, then heated from 34 to 94ºC at a rate of 5ºC /min and held at 94ºC for 5 min, then cooled to 34ºC at a rate of 5ºC /min, and held at 34ºC for 4 min. The mixture was stirred at 160 rpm and viscosity values were expressed as RVA units (RVU). Peak viscosity (PV), holding strength (HS), final viscosity (FV), breakdown (B) and setback (S) were recorded. Samples were analysed in triplicate.

Statistical analysis

All data were analysed by ANOVA, and means were compared using least significant difference (LSD) test.

Secondary structure prediction

The Garnier Emboss tool[17] was used to predict the secondary structure motifs of the amino acid sequences described previously[15].

RESULTS AND DISCUSSION

Amylose content and Blue Value

Amylose content of all the lines analysed are shown in Table 1. Differences in amylose content were significant between some of the lines, specially the ones carrying *Wx-B1a* and *Wx-B1b* alleles. The emmer-52 line, with the *Wx-B1 null* allele showed the lowest mean value (205 g kg^{-1} and 210 g kg^{-1}) using Megazyme Amylose/Amylopectin kit. However, the decrease in amylose content in this line was not as high as it was expected (only 5% respect to other lines), because *Wx-B1* protein is supposed to be 35%-25% more abundant than *Wx-A1* protein[8] and its lack should cause a drastic effect on amylose content. In other studies in common wheat, the lack of only one protein does not cause an important fall in amylose content[5,7]. These results indicate that amylose content is not linearly proportional to the number of *waxy* genes, suggesting that they may act in an epistatic manner as Lafiandra et al.[18] suggested. Unfortunately no other works about the effects of *null* alleles in durum or emmer wheat have been done before so comparison is not possible. Cv. Langdon, carrying the standard *Wx-B1a* allele showed the highest amylose content, which differed significantly from emmer samples with *Wx-B1g* allele. These differences could be explained by a different activity of both *Wx-B1* alleles due to their internal differences we reported previously[15]. However, durum cv. Mexicali (*Wx-B1c´)* showed a lower value, similar to emmer lines. This data support our hypothesis, because the *Wx-B1* DNA sequence of this cultivar had 99.0 % homology compared to emmer ones, while cv. Langdon showed 91.2 %

homology[15]. This was traduced in only three amino acid changes in cv. Mexicali respect to *Wx-B1g* but fifteen in cv. Langdon. To asses the possible effect of these amino acid changes on the structure and thus on the transport lead by the transit peptide and the activity of the proteins, the Garnier Emboss tool was used to predict the secondary structure motifs in each of the sequences (Supporting Material 1). As it is showed, cv. Langdon sequence had some remarkable changes respect to emmer and cv. Mexicali ones as the presence of a small α-helix in the transit peptide (residues 35-40) or the appearance of a β-strand and two turns between residues 355-370 and residues 550-560. These changes in the structure of the Wx-B1 proteins could be the reason of a different activity and thus of the different amylose content obtained. Other possible hypothesis to explain this difference in the amylose content is that the genetic background of the two species will differ, although the data from cv. Mexicali does not support this idea. Anyway, the results between the two species agree with the ones found by Rodríguez-Quijano et al.[19] who reported 40.7 % amylose in durum wheat and 37.2 % in emmer wheat in a study focused on waxy proteins of tetraploid wheats. To support the results mentioned above, amylose content was measured again using an Autoanalyzer (Table 1). The result was the presence of three groups: first with cv. Langdon (*Wx-B1a)*, second with *Wx-B1g, Wx-B1c`* and *Wx-B1c** lines, and third with emmer *Wx-B1* null line. Although the Autoanalyzer values were higher than the ones from Megazyme kit, the trend of the values is the same with both methods. The technique of the Autoanalyzer is based on a colorimetric measurement of the iodine-starch complex, and sometimes the formation of amylopectin-iodine complexes leads to an overestimation of amylose content, while the Megazyme kit is based on the specific precipitation of the amylopectin with concanavalin A and a posterior digestion of the amylose in order to measure the free glucose. Obtaining the same conclusions with both methods supports

the idea that amylose content is different between lines with *Wx-B1a*, *Wx-B1g/Wx-B1c´/Wx-B1c** and *Wx-B1b* alleles.

To confirm amylose content results, the absorbance at 680 nm (Blue Value) and maximum absorbance (λ_{max}) of the iodine-starch complex were determined (Table1). Both parameters were related positively with amylose content. Again emmer-52 line and cv. Langdon showed the lowest and highest values in both parameters, supporting the data obtained in amylose content and suggesting that emmer-52 line has less amylose than the rest of the lines and cv. Langdon has higher content, although in Langdon the differences were not significant compared with other samples. The rest of the samples showed similar values due to their differences in amylose content are not remarkable.

Starch pasting properties by swelling power and Rapid Visco Analyzer

The swelling power is the capacity of starch to increase its volume when it is heated with excess of water. All our samples were analysed for swelling power (Table 1) and a negative correlation with amylose content was found. The emmer-52 line with *Wx-B1* null allele showed the highest value and cv. Langdon the lowest one. Swelling power was studied in this work because it was reported to be related with amylose content; swelling power is inhibited by amylose and lipids[20]. The same researchers suggested that swelling power is a property of the amylopectin, and that amylose acts as a diluent. So, swelling power is supposed to be negatively related with amylose content and our data supports their suggestion. In several studies swelling power has been measured as an alternative to amylose content because its simplicity and reproducibility to confirm the data from amylose content analysis [5,7,21]. In all those studies a negative relationship between the two parameters was found, as in our case. Emmer-52 line

showed a very different value from the rest, demonstrating a prominent effect of the lack of the *Wx-B1* protein. Other lines showed values a little different from the rest (emmer-66 and emmer-88 in ambient A), although they showed medium amylose content. As lipids can also influence this property, maybe they could be the reason of these values. However, in this study the effect of lipid was not examined. The swelling power of cv. Langdon showed again a remarkable difference between durum and emmer starches.

At last, Rapid Visco Analyzer was used to analyse other pasting properties. Some of the parameters (Table 2) showed the expected values and relation to amylose content. Peak viscosity (PV) was negatively correlated with amylose content: emmer-52 line presented the highest value and cv. Langdon the lowest one. Emmer-39 showed an unexpected high value. This line also showed abnormal values in other parameters as Hold Strength, Final Viscosity and Setback. The reason to explain these values are unknown. In other studies[7,22] the relationship was the same between PV and amylose content. Besides, Araki et al.[7] supports the theory of Zhao et al.[23] that the high PV values found in lines carrying *Wx-B1* null allele are not only the consequence of a quantitative decrease in amylose but also could be explained because a qualitative change in amylose due to the lack of *Wx-B1* protein.

For Holding Strenght (HS) and Break Down a negatively relation with amylose content was also found. Again, the emmer-52 line and cv. Langdon showed the highest and lowest values, with the exception of emmer-39 line witch presented the highest value for HS, although not significant different respect to emmer-52. Setback showed a not strong positive relation with amylose content. Emmer-52 line showed the lowest value due to its low amylose content. Final viscosity (FV) did not show any relation with amylose content and the values were very heterogeneous. Other authors have

found a positive correlation between FV and amylose content[7,24] or a negative correlation[22]. And finally, Setback showed a weak positive correlation with amylose content. Again emmer-52 line showed a remarkable difference with the rest showing the important difference of its starch respect to the rest of lines.

CONCLUSIONS

The present study suggests that the amylose content between lines with *Wx-B1a*, *Wx-B1g/Wx-B1c´/Wx-B1c** and *Wx-B1b* alleles is not the same. As *Wx-B1a* and *Wx-B1g* alleles are the most frequent in durum and emmer wheat respectively, some starch properties are different between the two species. In our opinion this difference is likely due to differences in the activity of the *Wx-B1* protein whose internal gene structure was demonstrated to differ in both species. These data could be very useful for durum breeders to enlarge the genetic pool and get the desirable amylose content in each species.

Besides, the effects of a *Wx-B1* null allele on starch in emmer wheat confirmed its utility to develop materials with less amylose content and different starch properties in this crop. This could be important for enlarging the genetic pool of emmer wheat, because this crop is recovering importance in the last years for the increasing interest in traditional products and sustainable agriculture.

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Table 1: amylose content, blue value, λ_{max} and swelling power of wheat starches.

Specie	Line	Wx-B1 allele	Amylose g kg^{-1} Megazymes		Amylose g kg^{-1} Autoanalyzer		Blue Value		λ_{max}		Swelling power	
			Amb. A	Amb. B	Amb. A	Amb. B	Amb. A	Amb. B	Amb. A	Amb. B	Amb. A	Amb. B
Emmer	Emmer-39	*Wx-B1g*	254bc	266bc	366cd	377cd	0.351c	0.390c	602cd	602c	16.1d	19.5c
wheat	Emmer-66	*Wx-B1g*	248b	261b	349b	367b	0.333b	0.358b	600bcd	596b	17.6b	19.7c
	Emmer-71	*Wx-B1g*	254bc	262b	359c	373c	0.352c	0.362b	601bcd	596b	16.2d	20.8b
	Emmer-88	*Wx-B1g*	253bc	266bc	365cd	379d	0.332b	0.351b	599b	596b	17.5b	19.7c
	Emmer-49	*Wx-B1c**	259c	270c	371d	392e	0.344bc	0.387c	600bcd	601c	16.9c	19.1c
	Emmer-52	*Wx-B1b*	205a	211a	310a	319a	0.274a	0.305a	591a	586a	20.1a	25.3a
Durum	cv. Langdon	*Wx-B1a*	272d	-	383e	-	0.361c	-	602d	-	14.9e	-
wheat	cv. Mexicali	*Wx-B1c´*	253bc	261b	362c	372c	0.355c	0.382c	600bc	601c	16.0d	19.2c

Means with the same letter are not significant different at 95%.

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Table 2: Starch-pasting properties by RPV

Line	Peak viscosity		Hold strenght		Final viscosity		Breakdown		Setback	
	Amb. A	Amb. B	Amb. A	Amb. B	Amb. A	Amb. B	Amb. A	Amb. B	Amb. A	Amb. B
Emmer-39 (*Wx-B1g*)	221b	258b	168a	200a	518a	575b	53c	58c	298a	306b
Emmer-66 (*Wx-B1g*)	206c	254c	146b	185b	461b	564a	60b	70b	260c	324a
Emmer-71 *(Wx-B1g*)	189d	216d	143bc	169c	447c	498c	47d	46e	257c	282d
Emmer-88 *(Wx-B1g*)	183e	216d	142c	169c	406d	483d	41e	46e	222d	268e
Emmer-49 (*Wx-B1c**)	179f	203e	131e	151e	444c	472e	48d	52d	265b	269e
Emmer-52 (*Wx-B1b*)	242a	278a	165a	202a	406d	454f	76a	76a	166f	176f
cv. Langdon (*Wx-B1a*)	129h	-	111f	-	342e	-	18g	-	212e	-
cv. Mexicali (*Wx-B1c´*)	166g	204e	137d	163d	450c	501c	28f	43f	285b	298c

Means with the same letter are not significant different at 95%.

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