VALUE ADDED WHEAT CRC
PROJECT REPORT

Amylose Content of
Triticosecale sp. and Triticum
timophevii germplasm

Summer Research Project, 2004 – 2005: PBI Cobbitty

Angela Dennett

Date: May 2005

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Amylose Content of *Triticosecale* sp. and *Triticum timophevii* germplasm

Summer Research Project, 2004-2005; PBI, Cobbity
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Abstract

Both Triticale (*X Triticosecale* Whittmack) and *Triticum timophevii* have undergone little selection relative to other feed grains for quality characters, including the amylose to amyllopectin ratio of starch in the grain. Using the differing swelling power (water absorption) and iodine binding properties of amylose and amyllopectin, purified samples of 247 lines of triticate and 20 lines of *T. timophevii* were screened for amylose content. A strong correlation ($R^2=0.8174$) was found between results of iodine binding (read in a spectrophotometer at 620nm and 535nm) and swelling power. The amylose content of *T. timophevii* lines ranged from 28.1% to 33.8%, which is a small range within values typically obtained for commercial wheat cultivars. Amylose content in triticate ranged from 12.8% to 35.1% - a much wider range reflecting the contribution of both the wheat and rye genomes. However as only maize standards were available, these results could not be confirmed. An SDS-page gel was run on the highest and lowest lines, revealing a large amount of polymorphisms between triticate lines, including the isoproteins of granule bound starch synthase.

Introduction

Triticale (*X Triticosecale* Whittmack, AABBRR genome) is a relatively new feed grain created by a rye and durum wheat cross. Despite exhibiting hybrid vigour for yield characteristics, triticale has relatively poor bread and noodle making qualities, owing partly to a short history of selection. Similarly, *Triticum timophevii* (AAGG genome, with GG being closely related to the BB of durum wheats) is a wild-type wheat and thus had little selection for grain quality. Relatively little is published on the genetics or analysis of starch content in either species, although general studies on metabolisable/digestible energy content of triticate have been performed (see review presented in van Barneveld and Cooper, 2002).

The amylose content of grain plays a major part in digestibility and breadmaking quality of the flour. A high amylose to amyllopectin ratio is preferred for human and ruminant consumption owing to slower digestion and absorption. Low amylose to amyllopectin ratio is preferred in the pig and poultry industries as the short amyllopectin chains are not only absorbed faster but increase hunger, leading to faster weight gain in monogastrics (Howes, 2004b).

Much research has been performed into the starch content of maize and wheat. Amylose content in Australian hexaploid wheat cultivars was found to range from 23.5% to 38.9%, with a similar range for exotic cultivars (17.6%-34.0%) in a study by Regina (2000). Similarly, a range of approximately 15%-45% amylose content in *Triticum durum* was observed by Watanabe et al (1998). A much wider range (0%-52%) was found in the F2 populations of rye studied by Mohammadhkani et al (1999), which were based on Australian and international parents with a range of amylose content from 8% to 27%. All these findings suggest a large variation of amylose content could be obtained in triticate. Sharma et al (2002) observed a range of approximately 20%-30% in triticales grown in Australia when comparing partially waxy to normal types.

Genetically, there appears to be a correlation between seed size and amylose content, with smaller seeds generally having higher amylose content than larger ones (Watanabe et al,
1998; Mohammadhkani et al., 1999). In addition to genetic factors, amylose content is influenced by the environment (particularly temperature and water stress at grain filling) and random effects within the head. The production of starch in the seed is complicated by the triploid nature of the endosperm, which contains two sets of maternal chromosomes and one set of paternal chromosomes (Marshall et al., 1999).

The complete mode of inheritance for high or low amylose content is unknown and most likely varies between species. The role of several major genes, including waxy endosperm (giving almost 0% amylose, gene symbols wx and wx²), sugary endosperm (high in water-soluble polysaccharides. symbols su₁, su₂ and su³⁰⁰), dull endosperm (high amylose, symbol du) and amylose extender (very high amylose, symbol ac) have been known for decades in Zea mays. More recently, most of these genes and some corresponding gene modifier complexes have been located on chromosomes and confirmed by several sources in other species, including Triticum durum, Triticum aestivum and Oryza sativa (Paschall and Whistler, 1965; Hayden, 1999; Regina, 2000; etc). Of these genes, the waxy gene is the most thoroughly documented. Encoding for granule bound starch synthase (GBSS), this gene controls amylose production in the endosperm. If the null allele is present at one or more of the three waxy loci (located on chromosomes 4A, 7A or 7D in wheat), little or no amylose is produced resulting in a high swelling power. Triticale contains GBSS at the 4A loci, 7A loci and one loci on the rye genome. A null allele at all three loci is undesirable in the grain industry as seeds are shrunken and contain little starch. Sharma et al (2002) documented average amylose contents in triticale between waxy (Null-4A) and normal genotypes as 21.5% to 26% respectively.

The complete genetic control of starch production is still unknown. Regina (2000) proposed at least one gene segregation with partial dominance for amylose was involved. However the results of Watanabe et al (1998) were consistent with either one major recessive gene for high amylose or 2-4 partially dominant/recessive genes. Marshall et al (1999) suggested different recessive genes were involved, and the same conclusion was reached using data produced by Hayden (1999). Marshall et al (1999) also concluded that additivity, dominance and interaction components were all important in determination of starch content, with a small cytoplasmic effect. Their data exhibited segregation ratios within wheats and ryes of either no segregation or ratios of 3:1, 1:2:1, 1:1 or 1:1:1:1.

The aim of this project is to identify lines of particularly high or low amylose content. with the view to be used in future selection programs for triticale, and to investigate the forms of GBSS in these lines. A further aim is to observe the range of swelling powers of a random selection of T. timopheevii lines and deduce the potential of T. timopheevii as a source of high or low amylose genetic material.

Method

The grain from 247 lines of triticale were sourced from CIMMYT by Jeremy Roake then grown simultaneously in the field. Seed from 20 T. timopheevii lines were sourced from a seedbank at Tamworth, NSW, then simultaneously grown in the field at the PBI in Cobbity.

Purification of starch for iodine binding and swelling power assay is performed as follows. 15-20 scs are manually cracked and soaked overnight at 4°C in approximately 2.5mL of 0.5M NaCl. Seeds are then manually mashed and the supernatant decanted into 2mL tubes. These are placed in a centrifuge at 2000rpm for 1 minute and the supernatant decanted. Following this, samples are 'washed' by adding 500μL of 100%w/v CsCl, placed in a vortex mixer at 1400rpm for 1 minute, then again into the centrifuge under the same settings. This washing is repeated with a further 500μL of 100%w/v CsCl, 1mL distilled water (twice), 1mL 2%w/v Sodium Dodecyl Sulfate, 1mL distilled water (twice), and 1mL 95%w/v ethanol. Following this 1mL of 85%w/v methanol is added and the samples are vortexed for 1 minute at 1400rpm before
being placed in a waterbath at 65°C for 20 minutes. Samples are again centrifuged for 1 minute at 2000rcf then placed in a desiccator after the supernatant is discarded.

Initial assay was by swelling power. 25mg of starch is weighed to the nearest 0.5mg in a pre-weighed 2mL Eppendorf tube. For the original assay of triticate and assay of T. timopheevii, 600μL of 0.1%w/v AgNO₃ is added and samples are mixed in a vortex mixer at 1300rpm for 1 minute. They are then placed in a waterbath for 30 minutes at 92.5°C, being manually mixed at 1 minute intervals for the first 5 minutes, in 2.5 minute intervals for the next 5 minutes then every 5 minutes for the remaining time. Following this they are placed in cold water at room temperature for 5 minutes then placed in a centrifuge for 5 minutes at 5000rcf. The supernatant is removed carefully using pipettes, then the tubes weighed. Swelling power is calculated by the following formula:

\[
\text{Swelling Power} = \frac{\text{Mass of swollen sample in tube} - \text{mass of tube}}{\text{Initial mass of sample (in our case, 0.025g)}}
\]

Maize standards are included in every assay to reveal experimenter error between batches of assay, however cannot be used reliably to determine the absolute amylose content of triticate as swelling power is determined by factors other that amylose content (including branching in amyllopectin, ratio of A and B granules and impurities).

To confirm the results of the highest and lowest lines, a confirmation swelling was performed (and repeated for some lines). The same method was followed except 1mL of 0.1%w/v AgNO₃ was added (in an attempt to increase the variabity in the lines of highest swelling power).

As a quick check for amylose content, iodine staining was performed on the freshly cut surface of grain endosperm. 1 – 2 drops of iodine solution (0.2g iodine, 2g potassium iodide and 250mL distilled water) were added to the surface. Blue seeds had high amylose, whilst reddish coloured seeds with poor staining were of high amyllopectin (waxy endosperm).

To determine the amount of amylose in lines with the 10 highest and 10 lowest swelling powers using iodine binding, two replicates of 5mg are weighed into 1.5mL screwtop tubes. Following this 75μL of 95%v/v ethanol and 450μL of 1M NaOH are added and tubes are vortexed then heated to 100°C in a waterbath for 30 minutes. After cooling, 50μL is extracted from each replicate and 500μL of 0.1M citric acid is added, then replicates are stained with 200μL of iodine solution made as above. Samples are diluted with 2250μL of distilled water then refrigerated for 40 minutes. The amount of iodine taken up is read off two 200μL subsamples of each preparation in a spectrophotometer at 620nm and 555nm. Following this the ratio of the absorbance from the two wavelengths is taken and the amount of amylose is read off a standard curve determined in GenStat version 7.0.

The isoforms of GBSS present in the highest and lowest amylose lines were investigated using gel electrophoresis. Triticate starch was prepared by crushing 3 grains and incubation in 500μl 4M urea for 30 minutes at room temperature with regular vortexing. The samples were then centrifuged at 16,000g for 10 minutes and the supernatants were stored at -20°C. The 4M urea extraction was repeated on the pellet, followed by extraction with 500μl 8M urea + 1% 2-mercaptoethanol for 2 hours with regular vortexing. All supernatants were stored at -20°C. Starch from a waxy wheat (TH waxy, null for GBSS 7A, 7D, 4A) and Triller (wheat containing all three GBSS isotypes) was also added.

Large format gels were prepared using a 16% polyacrylamide resolving gel (16% 30:0.1 bis-acrylamide, 375mM Tris-HCl pH 7.8, 0.1% w/v SDS, 0.1% w/v aluminium per sulfate, 0.06% v/v TEMED) with a 4% stacking gel (4% 30:1 bis-acrylamide, 126mM Tris-HCl pH 6.8, 0.1% w/v SDS, 0.1% w/v aluminium per sulfate, 0.1% v/v TEMED). 5μL and 20μL samples
from the 8M urea + 1% 2-ME wholemeal protein extracts were combined in a 1:1 ratio with SDS sample buffer (120mM Tris-HCl pH 6.8, 4% w/v SDS, 20% glycerol, 5% v/v 2-
mercaptoethanol, 0.0001% w/v bromophenol blue) and boiled for 5 minutes. Triticale lines 13, 9, 212 and 32 were omitted due to restrictions in lane numbers on the gel. The samples were loaded onto the gel with 10μL SeeBlue Plus2 protein standards (Invitrogen). The gels were run in Laemmli SDS running buffer overnight at 10mA.

For silver staining, the gels were fixed for 1 hour in fixing solution (50% v/v methanol, 12% v/v acetic acid, 0.05% 0.05% formaldehyde) then washed 3 times for 20min in 50% v/v ethanol. The gels were then preincubated for 1min in preincubation solution (0.02% w/v sodium thiosulfate) and rinsed 3 times for 20scc in milliQ water. The gels were then impregnated in silver nitrate solution (0.2% w/v silver nitrate, 0.075% v/v formaldehyde) for 20min then rinsed as before. The gels were placed in developer (6% w/v sodium carbonate, 0.0004% w/v sodium thiosulfate, 0.05% v/v formaldehyde) until protein bands were visualised then placed in 5% v/v acetic acid.

Results and Discussion

*T. Timopheevii*

The average swelling power of samples (which reflects the water absorbing capacity of starch and thus the proportion of short amylopectin branches) ranged from 13.7 to 16.7, correlating to amylose content ranging from 28.1% to 33.8% based on maize standards. This is a small range compared to the triticale however similar to common Australian wheat varieties (Howes, 2004b). Correlation between repeats (based on the same starch and performed only on the three highest and three lowest lines) was high (R²=0.9311).

Fig. 1 Swelling Power of selected T. timopheevii lines
Triticale

The average swelling power of triticale samples ranged from 12.5 to 23.6, with extremes of 12.2 and 24.5. This correlates to a range of 35.1% to 12.8% amylose based on maize standards.

The iodine binding ratio of absorbance at 620nm to 535nm ranged from 1.21 to 1.03 (see Appendix, Fig. A6). Based again on maize standards, low amylose lines had an amylose content ranging from 10.3% to 21.5%, however the exponential standard curve determined by the absorbance ratio of the standards had an asymptote at absorbance ratio of 1.13 (largely due to an unusually low absorbance of the 50% amylose standard), meaning not only is the curve likely to be inaccurate but also amylose content for high amylose lines could not be determined using iodine binding.

![Swelling Power of Triticosecale sp. lines](image)

**Fig. 2** Swelling Power of the range of triticale lines.

The correlation between raw results of the iodine binding and swelling power is quite high ($R^2=0.8174$). Therefore despite not being able to determine the amylose content of high amylose lines using iodine binding absorbance ratios, the high correlation infers that amylose percentages determined by swelling power are quite reliable compared to results obtained by iodine binding at all levels of amylose content. However, as only maize standards were available, the overall accuracy of the results is unknown. This is due to differences in structure of both the amylose and amylopectin molecules and their arrangement in granules between triticale and maize. Analysis by Lii and Lineback (1977) found triticale amylopectin to be highly branched relative to commercial wheat and rye varieties, which accounts for the high swelling observed in low amylose (but non-waxy) triticale relative to wheat and reinforces the inability to compare results between species.
Table 1. Amylose content of triticale grain as a proportion of total starch content (as determined by swelling power of the sample), and the corresponding swelling power and iodine absorbance ratios for the highest amylose lines. * = no starch available for iodine assay

<table>
<thead>
<tr>
<th>Line</th>
<th>% Amylose (Swelling)</th>
<th>Swelling Power</th>
<th>Iodine Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>35.1</td>
<td>12.5</td>
<td>1.21</td>
</tr>
<tr>
<td>12</td>
<td>33.0</td>
<td>13.5</td>
<td>1.20</td>
</tr>
<tr>
<td>29</td>
<td>32.4</td>
<td>13.9</td>
<td>1.19</td>
</tr>
<tr>
<td>152</td>
<td>32.3</td>
<td>13.9</td>
<td>1.17</td>
</tr>
<tr>
<td>247</td>
<td>32.1</td>
<td>14.0</td>
<td>1.19</td>
</tr>
<tr>
<td>13</td>
<td>32.1</td>
<td>14.0</td>
<td>*</td>
</tr>
<tr>
<td>21</td>
<td>31.6</td>
<td>14.2</td>
<td>1.14</td>
</tr>
<tr>
<td>201</td>
<td>31.2</td>
<td>14.5</td>
<td>1.20</td>
</tr>
<tr>
<td>158</td>
<td>31.2</td>
<td>14.5</td>
<td>1.19</td>
</tr>
<tr>
<td>8</td>
<td>31.1</td>
<td>14.5</td>
<td>1.16</td>
</tr>
<tr>
<td>9</td>
<td>30.9</td>
<td>14.6</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Table 2. Amylose content of triticale grain as a proportion of total starch content as determined by both iodine binding and swelling power, and the corresponding swelling power and iodine absorbance ratios for the lowest amylose lines. * = iodine ratio is greater than the asymptote in the fitted curve and thus cannot be determined.

<table>
<thead>
<tr>
<th>Line</th>
<th>% Amylose (Iodine)</th>
<th>% Amylose (Swelling)</th>
<th>Swelling Power</th>
<th>Iodine Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>10.3</td>
<td>12.8</td>
<td>23.6</td>
<td>1.03</td>
</tr>
<tr>
<td>180</td>
<td>11.2</td>
<td>12.9</td>
<td>23.6</td>
<td>1.04</td>
</tr>
<tr>
<td>144</td>
<td>14.7</td>
<td>13.1</td>
<td>23.5</td>
<td>1.08</td>
</tr>
<tr>
<td>43</td>
<td>10.8</td>
<td>13.3</td>
<td>23.4</td>
<td>1.04</td>
</tr>
<tr>
<td>10</td>
<td>13.3</td>
<td>14.2</td>
<td>22.9</td>
<td>1.07</td>
</tr>
<tr>
<td>25</td>
<td>14.1</td>
<td>14.8</td>
<td>22.6</td>
<td>1.07</td>
</tr>
<tr>
<td>192</td>
<td>21.5</td>
<td>15.1</td>
<td>22.5</td>
<td>1.12</td>
</tr>
<tr>
<td>213</td>
<td>13.7</td>
<td>15.2</td>
<td>22.4</td>
<td>1.07</td>
</tr>
<tr>
<td>50</td>
<td>11.5</td>
<td>15.3</td>
<td>22.4</td>
<td>1.05</td>
</tr>
<tr>
<td>212</td>
<td>*</td>
<td>15.8</td>
<td>22.1</td>
<td>1.16</td>
</tr>
</tbody>
</table>

As a method, swelling power has several disadvantages. Swelling power does not measure amylose content directly; it measures the ability of starch granules to absorb water irreversibly when heated in an aqueous solution (termed 'gelatinisation'). Only branched sections of amylpectin and amylose molecules irreversibly absorb water, meaning the swelling depends not just on the relative amount of amylpectin but also on it's structure (including distribution of molecular weight, branching and length of outer amylpectin branches – Paschall and Whistler, 1965). In addition, smaller starch granules hydrate and swell more efficiently due to a high surface area to unit mass ratio (Regina, 2000). This means not only that comparisons cannot be made between species, but also that the gene/s giving a line it's characteristic swelling power may act in a different way to other lines of similar swelling. For example, low swelling can be due to both high amylose or a higher proportion of long amylpectin branches, possibly produced by starch debranching enzyme (Regina, 2000).

Several factors which may affect swelling power were examined in swollen samples. They were:-

- tube colour;
- visible impurities (bran);
- number of days between sample preparation and assay for swelling power;
- Presence of layers;
- Correlation between amount of visible impurities and colour; and
- Effect of position in a batch a sample was assayed.

As shown by graphs of these factors (see Appendix, Fig. A1-A5 and Fig. A7), the only significant interactions appear to be the number of layers and correlation between colour and bran. The presence of at least 2 layers probably indicates inadequate shaking of samples which restricts access of water to the base of the tube and reduces swelling power. Although there was an apparent trend linking bran and colour to each other, both the colour and bran individually were consistently spread across all swelling powers. Also, although a significant interaction was found for both bran and colour with swelling power (P=0.006 for both), very high leverage was recorded for such a large proportion of the data that the analysis was discarded. Also, data collected on these factors are subjective and thus a more thorough study is required before objective conclusions can be drawn.
The absorbance of 50% amylose maize standards used in iodine binding yielded some unusual results, which created a fairly ‘flat’ standard curve. This meant that the fitted curve had a horizontal asymptote at 1.13, below the absorbance ratio of the higher amylose lines. The fitted exponential equation ($R^2 = 0.989$, $P < 0.001$, see Appendix, Table A3) was given as:

\[ \text{Absorbance ratio} = 1.1367 - 0.4926 \times 0.8629^{\%\text{amylose}} \]

Fig. 4 Standard curve for absorbance ratio.

Regression for swollen standards revealed a linear trend ($P < 0.001$, $R^2 = 0.989$), with equation:

\[ \text{Swelling Power} = -0.4978 \times \%\text{amylose} + 29.984 \]

This equation was used to determine amylose content based on the maize standards to which 1mL was added. It is worth noting however that when only 600μL of 0.1%w/v AgNO₃ was added, the 0% amylose standards had much lower swelling powers (around 17, compared to swelling power of 30 when 1mL was added). If the swelling power of earlier standards were used to create a standard curve (where 600μL was added), the fitted model is also exponential.

When the three highest and three lowest swollen lines were stained with iodine, all absorbed iodine and stained a dark purple colour. No waxy lines were present, even for lines with exceptionally high swelling power (lines 43, 144 and 180, which exhibited average swellings of 23.4, 23.5 and 23.6 respectively). This supports data from the iodine binding which found the lowest amylose content to be 10.3%. No lines exhibited appreciable differences between the shrivelled and plump seeds chosen for each staining.

Based on a SDS-page gel, two of the GBSS isoforms observed in hexaploid wheat (7A and 4A) are present in triticale, in addition to the isoform in rye (Fig. 6). This is confirmed by Sharma et al. 2002. The faster running (lower) 4A band is differentiated from the upper band representing the 7A and rye GBSS isoproteins. Lines 180, 10, 25, 192, 200, 12, 152, 247 and 158 appear to be null-4A, however further investigation is required before conclusions can be drawn. A high degree of polymorphism is present not just in GBSS, but other proteins resolved in the gel, which also needs further investigation.
Experimental conditions must be identical for each purification and assay of starch. Fig. 7 shows the correlation between results obtained by two different operators for the same lines ($R^2=0.4963$). One possible explanation is the difference in time and force of shaking during swelling varies between experimenters. Data from samples assayed on the 30/11/2004 were ignored in the overall summary due to less vigorous shaking, with the average swelling power for samples on that day being 15.3, compared to averages between 18 and 20 for assays performed (albeit of other samples) on different days. Therefore it is possible different people shake tubes with different force during swelling, yielding different results. This is evidenced in some tubes by a layer of different colour near the bottom of the tube. Other factors which may lower the swelling are overshaking (breaks up starch granules) and shaking for a longer time, (temperature drops during the time samples are out of the waterbath). Thus standardization of experimental conditions can create significant problems for comparison of results.
One unexpected observation is that the confirmation repeats of high swelling lines actually have lower swelling power than originally, despite addition 400µL more of AgNO₃. However the overall ranking was similar, again with the exception of line 32.

It is interesting to note however that the purified maize standards purchased from Penfords have almost identical swelling powers between replicates in both the same day and different days of assay. This may be due to increased refinement of maize standards, making them more pure and giving granules a more consistent surface area. This allows them to absorb water more evenly. As shown by Fig. 8, correlation between repeated lines is good ($R^2=0.8682$, excluding line 32 – see case study below). Typically the correlation between higher swelling lines was lower than that of low swelling lines, due to low swelling lines absorbing more consistent amounts of water, and being less affected by irregular shaking.

**Case study – line 32**

<table>
<thead>
<tr>
<th>Date of Assay</th>
<th>Starch Preparation</th>
<th>Swelling Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>30/11/2004</td>
<td>Original</td>
<td>16.9</td>
</tr>
<tr>
<td>5/1/2005</td>
<td>Original</td>
<td>12.9</td>
</tr>
<tr>
<td>14/1/2005</td>
<td>New</td>
<td>23.3</td>
</tr>
<tr>
<td>18/1/2005</td>
<td>New</td>
<td>23.6</td>
</tr>
<tr>
<td>18/1/2005</td>
<td>Original</td>
<td>24.1</td>
</tr>
</tbody>
</table>

Table 4. Comparison of swelling powers from line 32 produced in different batches of assay using different starch preparations.

In total, five swellings of line 32 were performed, yielding a variance of 25.1. Although confirmation swellings using both original and a new starch yielded consistently high swelling power (within a typical range of variation), a very large difference is observed between both the confirmation results and the two original results (Table 4). Iodine binding yielded a low absorbance ratio, consistent with the high swelling of latter repeats and indicating low amylopectin (See table 2). Iodine staining confirmed this line is not waxy.

Inspection of the assay tubes offers some possible explanations (see Fig. 9). The first tube, from the 30/11/2004, exhibits three layers—a small darker layer on the bottom sits below a thin whitish strip, which is under the bulk of the swollen starch similar in colour to the bottom layer. This means that water has not been in excess throughout the tube during swelling and this tube has not swollen to its full potential. The second tube is visually less swollen. One explanation is weighing error, or possibly that part of the starch was blown or fell out of the tube whilst it was being removed from the balance. The swelling for 32IB is exceptionally low—one of the lowest recorded for a triticale and certainly the lowest recorded for that batch of assayed tubes (which had a higher average swelling than all other batches), further supporting experimental error. The next two tubes (where new starch was used) are both relatively clear, and are swollen to roughly the same height as the final tube, which is of yellow colour. All three tubes containing the original starch are a yellowy colour and have large amounts of impurities e.g. bran visible in the tubes. However the similar swelling for the
new (pure) and original (impure) samples indicates that visible impurities in other tubes may not play a significant role in swelling power (see Appendix, Fig. A3).

Conclusions

Based on the above data, I recommend lines 200, 12 and 28 for use in triticale breeding for high amylose, and lines 32, 180 and 144 in programs breeding for low amylose. Use of a waxy wheat variety backcrossed to lines 32, 180 and 144 may also be of benefit.

Acknowledgements

I would like to thank Nilufa Sultana for her preliminary work on these triticale lines, and patient explanations and support. Similarly I would like to thank my supervisor Dr Neil Howes for his patience in answering all my queries and ideas for further exploration.

Thanks to James Chin, Peter and Wheat CRC staff at EMAL for their assistance and equipment in running the SDS-page.

Thanks also to Omid Ansari his help in the lab, and Tamiko Jinhazse and Jeremy Roake for providing the T. timopheevii and triticale seed respectively. Dr Peter Sharp, Dr Robert (Bob) McIntosh and Dr Norman Darvey have also given gratefully received advice. Finally I would like to acknowledge the Value Added Wheat CRC for provision of funds for this project.

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Howes (2004a), High Amylose Wheats. Summary document produced for the Value Added Wheat CRC.

Howes (2004b), personal communication.


Appendix

Swelling Power of Samples Prepared varying numbers of Days Before Measurement

![Swelling Power Chart](image)

Fig. A1 Range of swelling powers recorded with differing amounts of time between purification of a sample and assay for swelling power.

Affect of Colour on Swelling Power

![Affect of Colour Chart](image)

Fig. A2 Range of swelling powers recorded over different colour scores.

Table A1. Description of colour scores, recorded in tubes within a day of swelling.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Clear</td>
</tr>
<tr>
<td>1.0</td>
<td>Limited Yellowing</td>
</tr>
<tr>
<td>2.0</td>
<td>Yellow</td>
</tr>
<tr>
<td>3.0</td>
<td>yellow-brown</td>
</tr>
<tr>
<td>4.0</td>
<td>Milky White</td>
</tr>
</tbody>
</table>
Fig. A3 Range of swelling powers recorded on samples with different levels of visible impurities e.g. bran.

Table A2. Bran scores and descriptions, recorded within a day of swelling

<table>
<thead>
<tr>
<th>Bran No</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>No Bran</td>
</tr>
<tr>
<td>2</td>
<td>Some Bran</td>
</tr>
<tr>
<td>3</td>
<td>Lots of Bran</td>
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</table>

Fig. A4 Correlation between visible impurities (bran) and colour of tubcs.
Effect of Presence of Layers

Fig. A5 Swelling power of samples which exhibited at least two layers and those with no visible layering.

Absorbance Ratios of high and low amylose lines

Fig. A6 Results of the iodine absorbance assay.
Fig. A7 a) Effect of position analysed on swelling power within a batch of 48 samples.

b) Effect of position analysed on swelling power within a sub-batch of 24 samples.

48 samples could be placed in the waterbath at any time, however reagents were added to 24 samples at a time before that half of the batch (sub-batch) was placed in the vortex mixer while reagents were added to the other sub-batch. This longer time exposed to water may have affected swelling power. Similarly, the supernatant was removed in the same order the reagent was added, however at a much slower rate, thus samples at the end of both each batch and sub-batch were exposed to water for a longer period of time (however all were exposed to heat for 30 minutes).
Table A3 GenStat output for formation of standard curves

***** Regression Analysis *****

Response variate: Maize_STD_Swelling_Power
  Fitted terms: Constant, Maize_STD_Amylose

*** Summary of analysis ***

<table>
<thead>
<tr>
<th></th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
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<td>424.900</td>
<td>424.900</td>
<td>342.23</td>
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<tr>
<td>Residual</td>
<td>3</td>
<td>3.725</td>
<td>1.242</td>
<td>6.12</td>
<td>&lt;.001</td>
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<tr>
<td>Total</td>
<td>4</td>
<td>428.624</td>
<td>107.156</td>
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Percentage variance accounted for 98.8
Standard error of observations is estimated to be 1.11

*** Estimates of parameters ***

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<th>t pr.</th>
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<tr>
<td>Constant</td>
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<td>0.967</td>
<td>31.01</td>
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<tr>
<td>Maize_STD_Amylose</td>
<td>-0.4976</td>
<td>0.0269</td>
<td>-18.50</td>
<td>&lt;.001</td>
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</table>

***** Nonlinear regression analysis *****

Response variate: Absorbance_Ratio
  Explanatory: R_Amylose
  Fitted Curve: A + B*(R**X)
  Constraints: R < 1

*** Summary of analysis ***

<table>
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<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
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<th>F pr.</th>
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<tr>
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<td>0.3199768</td>
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<tr>
<td>Residual</td>
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<td>0.005770</td>
<td>0.0006411</td>
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<tr>
<td>Total</td>
<td>11</td>
<td>0.645724</td>
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</table>

Percentage variance accounted for 98.9
Standard error of observations is estimated to be 0.0253

*** Estimates of parameters ***

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<tr>
<td>B</td>
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<td>0.0185</td>
</tr>
<tr>
<td>A</td>
<td>1.1367</td>
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Appendix to VAW-CRC student project report by Angela Dennett:

Analysis of GBSS expression patterns in high and low % amylose Triticale lines using a panel of GBSS-specific MAb’s

Peter Schofield, VAW-CRC Project 1.1.2 Antibody Based Diagnostics

Introduction

A follow-up analysis was performed on the high and low % amylose Triticale lines identified from the swelling power survey of 250 lines. The aim of the study was to determine the GBSS expression patterns of Triticale lines with varying amylose:amyllopectin ratios using a panel of MAb’s specific to different GBSS isoforms. Another aim of the study was to validate the potential of GBSS as a surrogate marker of starch quality in Triticale lines by correlation of GBSS ELISA results with % amylose as determined by swelling power.

Materials and Methods

Monoclonal and Polyclonal Antibodies

Wheat GBSS-specific antibodies were used for immunoblotting and ELISA. MAb’s 1F2 (GBSS-7A specific), F32C1C1 (GBSS-4A specific), F32A4E10 (GBSS-4A7D specific) and F32C1G6 (pan-GBSS) were produced by project 1.1.2 of the Value Added Wheat-CRC (VAW-CRC) at Elizabeth McArthur Agricultural Institute (EMAI). A pan-GBSS PAb was also produced by project 1.1.2. A second pan-GBSS MAb (91484) was provided by Goodman Fielder.

Triticale lines

Grain samples from the nine highest and ten lowest % amylose triticale lines identified by the experimentation of Angela Dennett (VAW-CRC summer studentship, 2005) were supplied (PBI, Cobbity). The high amylose lines (T200, T12, T28, T152, T247, T21, T150, T13, and T9) had % amylose between 30.9 and 35.1% as determined by swelling power compared to maize standards. The % amylose of the low amylose lines (T180, T144, T43, T10, T25, T192, T213, T50, T212, and T32) ranged between 12.8 and 15.8%. A white rye sample was also provided (PBI, Cobbity).

Starch Granule Preparation

Starch granules (SG) were prepared from the white rye line and the three highest (T200, T12, T28) and three lowest (T180, T144, T32) % amylose lines. 5g grain samples were crushed using a coffee grinder and soaked in 30ml 4% radiant detergent overnight. The soaked grains were homogenized using a mortar and pestle and filtered through a 65μm mesh. The filtrate containing SG was centrifuged at 3,500rpm for 3min. the SG pellet was washed twice with 40mL milliQ H2O and then once with 40mL absolute ethanol (centrifugation for each wash as above).
SG Sequential Urea Extraction

0.4g of each SG preparation was placed into 50ml tubes and extracted with 0.6ml 1M urea for 30min at room temperature. The tubes were centrifuged at 3,500rpm for 3 min and the supernatant stored at -20°C. The extraction was repeated on the pellet with 0.6ml 2M urea, 0.6ml 4M urea and finally 6ml 8M urea, 5% 2-mercaptoethanol. The centrifugation was increased to 20min for the final extraction.

16% SDS PAGE

Large format gels were run in a Hoefer 600 SE Ruby system (Amersham Biosciences). The resolving gel was 16% acrylamide (30:0.1), 375mM Tris-HCl (pH 7.8), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.06% (v/v) TEMED. The stacking gel contained 4% acrylamide (29:1), 125mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1% (w/v) SDS, 0.1% (v/v) TEMED. Samples were combined 1:1 with loading buffer (5% w/v SDS, 25% v/v glycerol, 0.01% bromophenol blue, 0.15M Tris-HCl pH 6.8) and boiled for 5min. Gels were run overnight at 7mA/gel then silver stained or immunoblotted.

Silver Staining

Gels were placed in fixing solution (50% v/v methanol, 12% v/v glacial acetic acid, 0.05% v/v formaldehyde). The gels were washed in 50% ethanol three times for 20min then placed in pretreatment solution (0.02% w/v sodium thiosulfate) for 1min. The gels were rinsed in milliQ water three times for 20sec then impregnated with silver nitrate (0.2% w/v silver nitrate, 0.075% v/v formaldehyde) for 20min. The rinse step was repeated followed by developer (2% pretreatment solution, 6% w/v sodium carbonate, 0.05% w/v formaldehyde). Development was stopped at sufficient visualization by placing the gel in 5% acetic acid.

Immunoblotting

16% SDS-PAGE was performed using the triticale extracts as described earlier. The gels were soaked in CAPS buffer (10mM CAPS, 10% methanol, 0.01% SDS, pH 11.0) and blotted onto Hybond P VDF membrane (Amorsham) with a current of 0.8mA/cm² for 2hr using a Hoefer semi-dry transfer unit (Amersham). Membranes were washed in PBST for 5min 3 times then blocked in 3% skim milk/PBST for 2hr. The washing step was repeated followed by incubation in various dilutions of the GBSS-specific Mab’s in 3% skim milk/PBST. The washing step was repeated followed by incubation in a 1:3000 dilution of anti-mouse HRP in 3% skim milk/PBST and a final wash step. Binding of the second antibody to the membrane was visualised by incubation with TMB substrate. The substrate reaction was stopped by placing the blot in milliQ H₂O.

Sandwich ELISA

ELISA plates were coated with 100µl GBSS PAb diluted 1:4000 in carbonate buffer (0.035M NaHCO₃, 0.015M Na₂CO₃ pH 9.6) overnight at 4°C. The wells were washed 3 times with phosphate buffered saline pH 7.4 containing 0.05% tween (PBST) and then blocked for 1hr with 200µl 3% skim milk in PBST. The washing step was repeated and the wells were incubated with various dilutions (generally 1:50 or 1:200) of the triticale SG extracts in 0.1M Tris-HCl (pH 7.8). The wells were then washed and incubated with
100µL of a 40:1 combination of TMB substrate solutions A (205mM potassium dihydrogen citrate, 0.015% w/v urea hydrogen peroxide pH 4.0) and B (8mM tetraethylammonium borohydride, 40mM 3,3',5,5' tetramethyl benzidine in N,N dimethylacetamide) for 20 min. The reaction was stopped with 1M phosphoric acid and the O.D. at 450nm was measured.

Results and Discussion

16% SDS-PAGE and Immunoblotting of SG extracts

16% SDS-PAGE of the triticale 8M urea SG extracts revealed two bands (GBSS bands 1 and 2) in the waxy protein region (Fig 1). The expression of GBSS band 2 differed between the 6 triticale lines. The band could only be visualized clearly in the high % amylose lines T200, T28, and T12. However the band was faint or absent in the low % amylose lines T32, T144 and T180.

Previous studies claimed that band 1 was a mixture of rye GBSS and wheat GBSS-7A, with band 2 containing wheat GBSS 4A (Sharma et al 2002). The identity of the bands was confirmed in this study by immunoblotting with monoclonal antibodies specific for wheat GBSS (Fig 2). Immunoblotting with pan-wheat GBSS MAb F32C1G6 showed recognition of both GBSS bands 1 and 2 (Fig 2A). Recognition of band 1 in the rye extract demonstrated the cross reactivity of MAb F32C1G6 with rye GBSS. Immunoblotting with GBSS-4A specific MAb F33C1C1 showed strong reaction with band 2, present in lines T200, T28 and T12 (Fig 2B). Immunoblotting with wheat GBSS-7A specific MAb 1F2 showed reaction with band 1 in extracts from T200 and T28 only (Fig 2C).

ELISA analysis of SG extracts

Expression of the three GBSS isoforms was quantified by ELISA analysis of the SG extracts with GBSS-specific MAb. Sandwich ELISA using pan-GBSS MAb F32C1G6 showed a marked difference in overall GBSS expression between high and low % amylose triticale lines (Fig 3A). The range of O.D. 450nm for the high amylose lines was 1.778-2.431 for the pan-GBSS MAb whereas the range for the low amylose lines was 0.482-0.912. Analysis with the MAb F33C1C1 also showed a clear difference in expression of GBSS-4A between high and low amylose lines, with O.D. 450nm readings ranging from 0.572-0.663 and 0.081-0.145 respectively (Fig 3B). ELISA analysis using MAb 1F2 reflected the immunoblotting results, with apparent expression of GBSS-7A in high amylose lines T200 (0.648) and T28 (0.618) but not in the remaining lines (0.059-0.112) (Fig 3C).

The sandwich ELISA results using MAbs F33C1C1 and F32C1G6 was correlated to the apparent % amylose (determined by swelling power, see report by Angela Dennet) for triticale lines 200, 28, 12, 32, 144 and 180 (fig 4A and 4B respectively). The correlation was highly significant in both cases (p < 0.005).
Conclusion

Immunoblotting of SG protein extracts from high and low % amylose triticale lines has illustrated a differential expression of GBSS-4A. There is higher expression of GBSS-4A in high % amylose lines. Further complexity is added by the differential expression of GBSS-7A. GBSS-7A was only expressed in two of the three high amylose lines. These differences in GBSS expression were quantified by sandwich ELISA. The ELISA readings correlate with high significance to amylose:amylopectin ratio of triticale lines. This indicates that GBSS expression may be a suitable surrogate marker for % amylose in unknown samples.

Further ELISA analysis of extracts from triticale lines with a more complete spectrum of % amylose is required. The analysis should also be extended to high/low % amylose wheat breeding lines.
Fig 1. Silver stained 16% SDS-PAGE of 8M urea starch granule extracts. Lanes 1-3 contain high amyllose triticate lines T200, T28 and T12. Lanes 4-6 contain low amyllose lines T32, T144 and T180. Lane 7 contains white rye.
Fig 2. Immunoblots of triticale 8M urea SG extracts against A) 1:2 pan-GBSS MAb (F32C1G6) B) 1:100 GBSS-4A (F33C1C1) C) 1:3000 GBSS-7A MAb (1F2). Lanes 1-7 contain extracts as in Fig 1.
Fig 3. Sandwich ELISA of A) 1:3000 pan-GBSS MAb (91484), B) 1:20 GBSS-4A MAb (F33C1C1) and C) 1:1000 GBSS-7A MAb (1F2) against 8M urea SG extracts from three HAT’s (shown in black) three LAT’s (white) and Rye.
Fig 4. Correlation of % Amylose (determined by swelling power, see report by Angela Donnott) with SG sandwich ELISA readings (O.D. 450nm) described in figure 3A and 3B.