Pigment Characterization in Grain Sorghum. II. White Varieties

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ABSTRACT

The location and properties of the pigments in six white seeded sorghum varieties and hybrids were investigated. Freezing microtome sections revealed an orange stilar area and yellow pigmentation in the epicarp and the endocarp of the grain examined. Where colored spots were visible on the surface of the seed, the epicarp, mesocarp, and endocarp exhibited orange pigmentation. A procedure was developed to separate the different pigments by paper chromatography. On the basis of spectral properties, color reactions, and Rf values, it is apparent that two forms of apigeninidin and two forms of luteolinidin were present in each of the six varieties or hybrids investigated.

Sorghum is a versatile plant which is grown for human food, animal feeds, and for industrial products. One of the main disadvantages of sorghum grain as a human food is the control of color during processing. A prime example is the production of off-colored starch during wet-milling. Anthocyanin-type pigments are considered the source of this discoloration (1).

The structure of the sorghum grain has been reviewed by Rooney and Clark (2). The pigments have been reported to be mainly located in the pericarp, the seed-tip (stilar area), and the subcoat. Red pigmentation in the epicarp and the endocarp of the pericarp and the stilar area in three red varieties has been reported recently (3). Cellular locations of pigments of sorghum grain with other pigmentation have not been investigated.

Nakayama (4) suggested the existence of a flavylium salt and compounds having anthocyanogen and dihydroxyphenol properties in sorghum grains. Blessin et al. (5) tentatively identified fisetinidin as one of the reaction products resulting from treatment of anthocyanogens with 12N hydrochloric acid at room temperature. Pelargonidin, and tentatively eriodictyol, were identified as products resulting from hydrolysis of the anthocyanogens isolated from the seed coat of commercial sorghum (6). Apigeninidin and luteolinidin were found in large quantities in the
glumes and to a much lesser extent in the tan pericarp and in the roots of the
Wheatland variety; only trace amounts of glycosides were detected. The presence of
large amounts of polymeric forms of luteolinidin was also suggested (7).
Apigeninidin, luteolinidin, 7-o-methyl luteolinidin, and its glycoside, along with a
polymeric pigment, have been identified from purple colored glumes (8).
Apigeninidin-5-glucoside and kaemferol-3-rutinoside-7-glucuronide have been
tentatively identified from three reddish-brown sorghum seed samples (3).
Recently, Bate-Smith and Rasper (9) reported that the principal tannin of sorghum
seed was a leucoanthocyanin yielding luteolinidin when treated with mineral
acids.

In summary, the cellular location and nature of sorghum grain pigments warrant
investigation. Anthocyanin-type pigments have been indicated to be present. It was
the purpose of this work to identify the location and investigate the physical and
chemical properties of pigments in several varieties of sorghum with white (ivory)
seeds.

EXPERIMENTAL PROCEDURE

Materials

Six varieties and hybrids (ATx607, Texioca54, ATx607 × Texioca54, BTx3197,
RTx2520, and BTx3197 × RTx2520) of sorghum with white seeds were used in
this investigation. These grains were ivory in color with brown-colored spots.

Location

Because the pigments in sorghum grains are water-soluble (10), the freezing
microtome technique was employed to avoid leaching of the pigments. The various
cell layers were identified by comparison with photomicrographs in the literature
(11).

Isolation

The seeds were air-cleaned to remove glumes and other foreign matter. One
hundred grams was then extracted with diethyl ether to remove the waxy coating
(5). The extraction was repeated again. The dewaxed seeds were then extracted
with 250 ml. of acidified methanol (HCl-MeOH, 1% v./v.) at room temperature in
darkness for 24 hr. The extract was collected and the extraction repeated again.
The extracts were combined and condensed to about 20 ml. in a flash evaporator at
40°C. The crude pigment concentrate was then applied to Whatman No. 3MM
chromatographic paper and descending chromatograms were developed.

After much experimentation, a sequence of solvents for separation and
purification of the pigments evolved (Table I). After the first development, the
chromatograms were air-dried in a hood at room temperature. The different bands
were cut out and eluted with about 100 ml. of HCl-MeOH (1% v./v.) for at least 12
hr. and the eluates were condensed to about 3 ml. in a flash evaporator at 40°C.
The condensed and partly purified extracts were rechromatographed several times
with different solvent systems until the pigments appeared to be chromatographically pure (Table I).

Characterization

The purified pigments were spotted on Whatman No. 1 chromatographic paper
TABLE I. SOLVENT SYSTEMS USED IN THE ISOLATION AND CHARACTERIZATION OF SORGHUM PIGMENTS\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Code</th>
<th>Chemicals</th>
<th>Proportions</th>
<th>Layer Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAIW</td>
<td>1-Butanol:acetic acid:water</td>
<td>4:1:5</td>
<td>Top</td>
</tr>
<tr>
<td>BAIW-HCl</td>
<td>1-Butanol:acetic acid:water:conc. HCl</td>
<td>60:10:20:1</td>
<td>Miscible</td>
</tr>
<tr>
<td>BuOH-HCl</td>
<td>1-Butanol:2N HCl</td>
<td>1:1</td>
<td>Top</td>
</tr>
<tr>
<td>Formic</td>
<td>Formic acid:conc. HCl:water</td>
<td>5:2:3</td>
<td>Miscible</td>
</tr>
<tr>
<td>Forestal</td>
<td>Acetic acid:conc. HCl:water</td>
<td>30:3:10</td>
<td>Miscible</td>
</tr>
<tr>
<td>10% A-HCl</td>
<td>Acetic acid:conc. HCl:water</td>
<td>87:3:10</td>
<td>Miscible</td>
</tr>
<tr>
<td>HAc-HCl-1</td>
<td>Acetic acid:conc. HCl:water</td>
<td>15:3:82</td>
<td>Miscible</td>
</tr>
<tr>
<td>HAc-HCl-2</td>
<td>Acetic acid:conc. HCl:water</td>
<td>10:3:87</td>
<td>Miscible</td>
</tr>
<tr>
<td>1% HCl</td>
<td>Conc. HCl:water</td>
<td>3:97</td>
<td>Miscible</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All solvent systems used were freshly prepared.  
\textsuperscript{b} See refs. 7, 15.

and developed in several solvent systems (Table I). Pelargonidin chloride obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., was used as a marker to standardize the environment. \( R_f \) values of the purified pigments were calculated. Colors of the purified pigments on paper under visible and ultraviolet lights, and with or without ammonia fuming, were recorded (12,13,14). For spectral measurements, acidified methanol (HCl-MeOH, 0.01% v/v) was used to elute the pigments from the chromatogram (12,15). Each spectrum was measured with a Beckman DB spectrophotometer equipped with a Beckman potentiometric recorder. Each pigment eluate was measured against a blank eluted from an appropriate area of the chromatogram prepared at the same time as the corresponding pigment eluate. Bathochromic shifts upon addition of aluminum chloride and sodium hydroxide were observed. \( R_f \) values, color reactions, and spectral measurements of the purified pigments were compared with published data for tentative identification.

RESULTS AND DISCUSSION

Location

Microscopic examination of the freezing microtome sections revealed that the stylar areas of the six samples investigated were orange in color. The epicarp and the endocarp of the pericarp were yellow. The mesocarp of the pericarp was colorless. However, the pericarp was orange in areas where brown or orange spots occurred on the surface of the kernel. The yellow pigmentation in the epicarp and the endocarp is in contrast to the orange-red pigmentation of the red varieties and hybrids (3).

Isolation

A procedure was developed to separate the different pigments extracted from the various sorghum varieties and hybrids. The following sequence of solvent systems for descending chromatography was found to be most effective: 1) formic, 2) HAc-HCl-1, 3) BuOH-HCl, 4) formic, and 5) HAc-HCl-1. The procedure suggested by Stafford (7) was not effective because of excessive tailing of the pigments in those solvents. Four pigments, two yellow (Y-1 and Y-2) and two orange (O-1 and O-2), were isolated from each white variety or hybrid investigated.
Other yellow pigments were also observed in ATx607, Texioca54, and ATx607 × Texioca54 which did not separate in the solvent system investigated. However, similar orange pigments were found in BTx3197, RTx2520, and BTx3197 × RTx2520. These varietal differences in pigments were unexpected.

Characterization

The yellow pigments Y-1 and Y-2 turned purple upon treatment with ammonia fumes which indicated anthocyanin properties. The pigments did not change color under UV light, with and without exposure to ammonia. Spectrophotometric measurements in HCl-MeOH (0.01% v./v.), with or without addition of concentrated sodium hydroxide (Figs. 1 and 2), also suggested anthocyanins. Addition of 5% alcoholic solution of aluminum chloride did not induce spectral shifts. The absorption maxima of these two pigments in the visible spectrum indicated derivatives of the 3-deoxyanthocyanidin apigeninidin (12). However, comparison of R_f values in several solvent systems with those published in the literature (Tables II and III) did not provide sufficient evidence for identification. It would appear that Y-1 and Y-2 were two unidentified forms of apigeninidin (Figs. 1 and 2; Tables II and III).

The orange pigments, O-1 and O-2, turned blue upon fuming with ammonia. These pigments did not change color under UV light, with or without exposure to ammonia. This suggested anthocyanins. The absorption spectra of these two pigments in HCl-MeOH (0.01%, v./v.), with or without addition of concentrated sodium hydroxide (Figs. 3 and 4), further suggested that they were derivatives of the 3-deoxyanthocyanidin, luteolinidin (12). Addition of 5% alcoholic solution of aluminum chloride did not induce a spectral shift which indicated that the pigments were not identical to luteolinidin. In addition, comparison of R_f values in various solvent systems with those reported in the literature indicated the two pigments did not resemble the luteolinidin derivative reported earlier (Tables II and III). It is
Fig. 2. Spectra of sorghum pigment, Y-2, in 0.01% HCl-MeOH before and after treatment with sodium hydroxide.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Rf Values (× 100)</th>
<th>Rf Values (× 100)</th>
<th>Rf Values (× 100)</th>
<th>Rf Values (× 100)</th>
<th>Rf Values (× 100)</th>
<th>Rf Values (× 100)</th>
<th>Rf Values (× 100)</th>
<th>Rf Values (× 100)</th>
<th>Rf Values (× 100)</th>
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<tr>
<td>Y-1</td>
<td>75</td>
<td>58</td>
<td>73</td>
<td>74</td>
<td>4</td>
<td>20</td>
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<td>Y-2</td>
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<td>72</td>
<td>80</td>
<td>4</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>O-1</td>
<td>73</td>
<td>45</td>
<td>49</td>
<td>47</td>
<td>3</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-2</td>
<td>61</td>
<td>35</td>
<td>48</td>
<td>48</td>
<td>2</td>
<td>12</td>
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<tr>
<td>PeCl^b</td>
<td>75</td>
<td>84</td>
<td>26</td>
<td>12</td>
<td>22</td>
<td>46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ap^c</td>
<td>75</td>
<td>44</td>
<td>74</td>
<td>...^d</td>
<td>...^d</td>
<td>...^d</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ap-5-glu^c</td>
<td>...^d</td>
<td>...^d</td>
<td>41</td>
<td>38^d</td>
<td>22^d</td>
<td>55^d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lu^c</td>
<td>61</td>
<td>35</td>
<td>56</td>
<td>...</td>
<td>...</td>
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<td></td>
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<tr>
<td>Pe^c</td>
<td>...^d</td>
<td>...^d</td>
<td>31</td>
<td>14</td>
<td>23</td>
<td>45</td>
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</table>

^aAll the solvents were freshly prepared.

^bPeCl = Pelargonidin-3, 5-diglucoside chloride from Aldrich Chemical Co., Milwaukee, Wis.

^cAp = Apigeninidin; Ap-5-glu = Apigeninidin-5-glucoside; Lu = Luteolinidin; Pe = Pelargonidin-3, 5-diglucoside (15).

^dNot available.

suggested that these pigments are two unidentified forms of luteolinidin (Figs. 3 and 4).

The yellow pigments isolated from sorghum varieties with reddish-brown seeds (3) were not isolated from these white varieties and hybrids. The absence of these pigments in the white varieties and hybrids is indicative of varietal differences in kinds and number of pigments. It is necessary to investigate other sorghum varieties of different pigmentation in order to understand the pigmentation patterns of various colored sorghum seed.
TABLE III. \( R_f \) VALUES (X 100) OF THE DIFFERENT PURIFIED
PIGMENTS ISOLATED FROM SORGHUM SEED IN VARIOUS
SOLVENT SYSTEMS USING ASCENDING CHROMATOGRAPHY\(^a\)

<table>
<thead>
<tr>
<th>Pigment</th>
<th>BAW-HCl</th>
<th>10% A-HCl</th>
<th>Formic</th>
<th>Forestal</th>
<th>HAc-HCl-2</th>
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</thead>
<tbody>
<tr>
<td>Y-1</td>
<td>67</td>
<td>91</td>
<td>59</td>
<td>79</td>
<td>14</td>
</tr>
<tr>
<td>Y-2</td>
<td>68</td>
<td>80</td>
<td>59</td>
<td>80</td>
<td>14</td>
</tr>
<tr>
<td>O-1</td>
<td>51</td>
<td>82</td>
<td>53</td>
<td>81</td>
<td>13</td>
</tr>
<tr>
<td>O-2</td>
<td>49</td>
<td>68</td>
<td>40</td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>PeCl(^b)</td>
<td>16</td>
<td>69</td>
<td>85</td>
<td>78</td>
<td>39</td>
</tr>
<tr>
<td>Ap(^c)</td>
<td>60</td>
<td>20</td>
<td>68</td>
<td>85(^d)</td>
<td>\ldots(^d)</td>
</tr>
<tr>
<td>Ap-5-glu(^c)</td>
<td>25</td>
<td>45</td>
<td>90</td>
<td>\ldots(^d)</td>
<td>\ldots(^d)</td>
</tr>
<tr>
<td>Ap-3(^c)</td>
<td>60</td>
<td>28</td>
<td>79</td>
<td>94</td>
<td>\ldots(^d)</td>
</tr>
<tr>
<td>Lu(^c)</td>
<td>42</td>
<td>10</td>
<td>53</td>
<td>70</td>
<td>\ldots(^d)</td>
</tr>
<tr>
<td>Lu-5-glu(^c)</td>
<td>21</td>
<td>35</td>
<td>70</td>
<td>\ldots(^d)</td>
<td>\ldots(^d)</td>
</tr>
<tr>
<td>Lu-3(^c)</td>
<td>42</td>
<td>15</td>
<td>65</td>
<td>85</td>
<td>\ldots(^d)</td>
</tr>
</tbody>
</table>

\(^a\)All the solvents were freshly prepared.

\(^b\)PeCl = Pelargonidin-3, 5-diglucoside chloride from Aldrich Chemical Co., Milwaukee, Wis.

\(^c\)Ap = Apigeninidin; Ap-5-glu = Apigeninidin-5-glucoside; Ap-3 = unidentified apigeninidin;

Lu = Luteolinidin; Lu-3 = unidentified luteolinidin (7).

\(^d\)Not available.

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**Fig. 3 (left).** Spectra of sorghum pigment, O-1, in 0.01% HCl-MeOH before and after treatment with sodium hydroxide.

**Fig. 4 (right).** Spectra of sorghum pigment, O-2, in 0.01% HCl-MeOH before and after treatment with sodium hydroxide.
Literature Cited


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