Profile and characterization of anthocyanins extract from purple corn kernel during the ripening stage

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ABSTRACT

This study was performed to evaluate anthocyanins concentration and profiles in the kernel of purple corn Heukjinjuchal during the ripening stage because the compositions and concentrations of anthocyanins vary significantly depending on the kernel color. Purple corn presented a complex anthocyanins component on the absorbance wavelength of 520nm. As the day increases after silking stage, most of the peaks intensity were also increased. In order to isolate and identify anthocyanins, 11 standards of anthocyanins including cyanidin-3-β-D-glucoside were used. However, in this study, delphinidin-3-β-D-glucoside, peonidin-3-β-D-glucoside and malvidin-3-β-D-glucoside was separated and the other anthocyanin was not separated by UPLC under this research condition. Cyanidin-3-β-D-glucoside content ranged from 5.71 μg/100g of fresh weight (15th day after silking stage) to 40.97 μg/100g of fresh weight (31 day after silking stage). Pelargonidin-3-β-D-glucoside content ranged from 4.84 mg/100g of fresh weight (21th day after silking stage) to 13.59 μg/100g of fresh weight (31th day after silking stage). Malvidin-3-β-D-glucoside ranged from 2.11 μg/100g of fresh weight (21th day after silking stage) to 12.03 μg/100g of fresh weight (31th day after silking stage). From 21st day after silking stage, the content of cyanidin-3-β-D-glucoside rapidly increased. Pelargonidin-3-β-D-glucoside and malvidin-3-β-D-glucoside were not detected in the early development periods of purple corn kernel. The content of pelargonidin-3-β-D-glucoside and malvidin-3-β-D-glucoside was sharply increased from the 29th day after silking day. The relationship between the day after silking stage and individual anthocyanins had positive correlation with individual anthocyanins.

Key words: Purple corn, anthocyanins, ripening stage, profile.

INTRODUCTION

Purple corn is known as “maizmorado” and is mainly cultivated in Andes area in Latin America. Andes people have long been utilizing purple corn as color deserts and beverages. Recently, purple corn has been widely used in other continent including Asia and EU.

So far, several studies have shown that colored corn has various pigments including carotenoid, phenolic compounds and anthocyanins. Especially, the color of purple corn is due to anthocyanins. Anthocyanins from kernel pericarp were involved in the most percentage of total kernel anthocyanins content (Moreno et al., 2005). Also, Jing (2006) categorized the anthocyanin content containing various plant and food products.

The efficacy of anthocyanins has been reported to play an...
important role in scavenging free radical and preventing several chronic diseases. Anthocyanins pigments in purple corn show high antioxidant activities and have been reported to exhibit various biological activities such as anti-mutagenic, anticancer activities, and prevention of diabetes and obesity (Nagy, 2001; Tsuda et al., 2003; Smith et al., 2004; Wang and Stoner, 2008; Fukamachi et al., 2001; Sandler 2005; Olsson et al., 2004; Espin et al., 2000; Kähkönen and Heinonen, 2003; Stintzing et al., 2002).

The word anthocyaninss are derived from Greek terms, anthos, meaning flower, and kyanos, meaning blue (Kong et al., 2008). Anthocyanins are one of the largest group of natural compounds, water-soluble pigments known as flavonoid compounds which are widely distributed in plant.

Naturally occurring anthocyanins exhibit various colors depending on the pH, temperature, light and species of plants (Bridle and Timberlake, 1997; Mazza and Miniati, 1993; Moreno et al., 2005). Steyn et al. (2005) indicated that the degree of red color is determined by the content and composition of anthocyanins in the skin.

The basic carbon skeleton of a flavonoid contains 15 carbon in C_{6}-C_{3}-C_{6} arrangement with two aromatic rings connected by 3-carbon bridge. Studies conducted by many researchers reported more than 19 types anthocyanins based on skeleton (Strack and Wray, 1993; Nygaard et al., 1997; Pale et al., 1997; Kong et al., 2008). Anthocyanins are easily soluble under water and alcohol, but the other organic solvents cannot be dissolved easily. Also, the occurrence of anthocyanidins is seldom found in nature because of their poor stability.

In a study conducted by Abdel-Aal et al. (2006) and Aoki et al. (2002), it was shown that the anthocyanin types of purple corn include cyanidin-3-O-β-D-glucoside, pelargonidin-3-O-β-D-glucoside, peonidin-3-O-β-D-glucoside, cyanidin-3-O-β-D-(g-malonyl-glucoside), pelargonidin-3-O-β-D-(g-malonyl-glucoside), and peonidin-3-O-β-D-(g-malonyl-glucoside).

In the aforementioned studies, the concentration and profiles of anthocyanins in the kernel of purple corn Heukjinjuchal during the ripening stage were evaluated because the compositions and concentrations of anthocyanins vary significantly depending on the kernel colors. In addition, the relationship between anthocyanins and the other factor, such as physicochemical characteristics and antioxidant assay, was analyzed.

**MATERIALS AND METHODS**

**Sample preparation of purple corn**

The corn variety using purple corn kernel was a single crosshybrid Heukjinjuchal which was developed by a purple color waxy corn at National Institute of Crop Science (NICS) (Junget al., 2009). The corn kernel was harvested from 15th to 31th days after silking stage. Harvested corn kernels were immediately, to block the enzymatic activities, stored at −72°C. Prior to the experiments, all sample were lyophilized and ground into a flour used with liquid nitrogen (N2).

**Chemical and reagents**

3',4',5,7-Tetrahydroxy-3',5'-dimethoxyflavlyium chloride (Malvidin chloride), 3,3',4,5,7-Pentahydroxyflavlyium chloride (cyanidin chloride), 3,3',4',5,5',7-Hexahydroxyflavlyium chloride (delphinidin chloride), 3,4',5,7-Tetrahydroxy-3'-methoxyflvium chloride (peonidin chloride), 3,3',4',5,7-Pentahydroxy-5'-methoxyflavlyium chloride (petunidin chloride), 3,5-Bis(glucosyl)-4',7-dihydroxyflavlyium chloride (pelargonidin chloride), cyanidin-3-O-glucoside chloride (kuromanin chloride), peonidin-3-O-glucoside chloride, delphinidin-3-β-O-glucoside chloride (myrtillin chloride), malvidin-3-β-O-glucoside chloride (oenin chloride) and pelargonidin-3-O-glucoside chloride (callistephin chloride) were purchased from Extrasynthese Co. (France). All of the chemicals and regents used in the experiments were of analytical grade.

**Sample extraction**

The ground kernel of purple corn with liquid nitrogen was extracted according to the applied method described by Aoki et al. (2002). Approximately, 2g of each purple corn powder were added to a flask containing 20 mL of 0.1% hydrochloric acid (HCl) aqueous solution. This flask was shaken on a laboratory platform shaker at 150 rpm and 37°C for 24 h. The extracts of each sample were filtered through Whatman No. 42 filter paper. The extracted solution was stored in refrigerator at 4°C and under dark condition.

**Qualitative and quantitative analysis of anthocyanins using UPLC Mass spectrometer**

Qualitative and quantitative analysis of anthocyanins involving the kernel of purple corn was obtained by ultra performance liquid chromatography (UPLC, ACQUITY, MA, USA). Solvents, such as high performance liquid chromatography-grade water and acetonitrile (ACN) for UPLC analysis were purchased from J. T. Baker (NJ, USA). The analysis column was applied by Waters ACQUITY BEH C18 column (particle size 1.7 µm, 2.1×100 mm, Waters). For analysis of purple corn extract, the detection was observed with Photodiode Array Detector (PDA) at 530 nm. The sample extracts were filtered through a 0.2 µm membrane syringe filter before injection. The mobile phase A was 0.1%formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile (ACN). For analysis, the condition of the mobile phase is stated in Table 1. The condition of MS
Table 1: The solvent ratio for quantitative analysis of anthocyanins.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (ml/min)</th>
<th>Solvent B(^1)</th>
<th>Solvent B(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
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<td>100</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.35</td>
<td>90</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>0.35</td>
<td>85</td>
<td>30</td>
</tr>
<tr>
<td>20</td>
<td>0.35</td>
<td>75</td>
<td>15</td>
</tr>
<tr>
<td>22</td>
<td>0.35</td>
<td>70</td>
<td>15</td>
</tr>
<tr>
<td>24</td>
<td>0.35</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

1) Solvent A: 0.1% formic acid in water  
2) Solvent B: 0.1% formic acid in acetonitrile

Figure 1: Peak change of purple corn five day intervals from 15th to 30th day after silking stage.
*: No. means day after silking stage.

method was TQD (Tetraquadrople detector, equipped with an electro-spray ionization-tandem mass/mass, Waters) in the positive ion mode, Con voltage: 30 V, capillary 4 kV, drying gas \(\text{N}_2\), 800 L/h.

Statistical analysis

These experiments were done intricipates for all measurements. All data obtained from the analysis were statistically analyzed using SAS statistic program ver. 9.3 for Windows (Statistical Analysis Systems Institute Inc., NC, USA).

RESULTS AND DISCUSSION

Profile of anthocyanins of purple corn at individual ripening stage

Anthocyanins are glycosilatedpolyhydroxy or polymethoxyderivatives of 2-phenylbenzopyrillum. According to functional groups, Strack and Wray (1993) classified naturally occurring anthocyanidins into five groups such as common basic structures, common methylated structures, 3-deoxy structures, rare hydroxylated structures and rare methylated structures.

As point of view in chemistry and biochemistry, anthocyanins are easily soluble under water and alcohol, but the other organic solvents cannot easily dissolve. Also, the occurrence of anthocyanidins is seldom found in nature because of their poor stability. Ignat et al. (2011) reported that the analysis of anthocyanins is a complicated process due to their capacity to undergo structural transformation and multiplex reaction. Also, a variety of chemical and physical condition such as oxygen, high temperature, and pH values have an important influence on the stability of anthocyanin compound (Cavalcanti et al., 2011). The various anthocyanin compounds have similar chemical structures and there often exist complex mixtures, which make them exceedingly difficult to isolate and identify (Andersen and Jordheim, 2010). As a result, the possibility of anthocyanins analysis was evaluated.

To determine the profile of anthocyanins, each standard was purchased by Extrasynthese Co. (Genay, France). As the day after silking stage increases, many peaks in the Ultra Performance Liquid Chromatography (UPLC) were found (Figure 1). In Purple corn, it was detected, complex
anthocyanins components on the absorbance wavelength of 520 nm. As the days after silking stage increases in purple corn Heukjinjuchal kernel, the number and absorbance intensity of peaks were also found to increase. Some researchers indicated that content and types of anthocyanins among each of the different plants, plant part and cultivars in the same plant varies and were affected by internal factors such as genes, environmental factor such as light and temperature, and agronomic factors such as growing conditions and maturity (Stein et al., 2002; Jing 2006; Amarowicz et al., 2009). When each anthocyanins compounds were analyzed at five day intervals from the 15th to 30th day after silking stage, the retention time, which ranged from 4 to 12 min showed a drastic difference (Figure 1).

To isolate and identify anthocyanins, 11 standards of anthocyanins including cyanidin-3-β-O-glucosidewere used. However, in this study, cyanidin-3-β-O-glucoside, peonidin-3-β-O-glucoside and malvidin-3-β-O-glucosidewere well separated and had high content. But the other anthocyanins were not separated by UPLC under this research condition. The chromatogram analysis showed many unknown peaks. This means that an extract of purple corn has a complicated mixture of other chemical compounds. Thus, it is necessary further to use mass spectrometer (MS) and proton nuclear magnetic resonance (NMR) for more accurate identification of unknown peaks.

Therefore, to identify the three types of standard, mass spectrometer was used. This result showed that the dominant anthocyanins of purple corn kernel was cyanidin-3-β-O-glucoside, pelargonidin-3-β-O-glucoside and malvidin-3-β-O-glucoside among the anthocyanins compound standards.

Anthocyanins content of purple corn at individual ripening date

Anthocyanins are one of the important flavonoid group and have been studied for long (Brouillard, 1982; Aoki et al., 2002; Espin et al., 2000; Mazza and Miniati, 1993; Anderson and Jordheim, 2010; Bridle and Timberlake, 1997). Generally, anthocyanins are known for being mostly in the form of glycosides than the existence of aglycon forms. Anthocyanins usually contain a single glucoside unit form. In some cases, many anthocyanins combine multiple positions of the basic carbon skeleton with two, three or more sugars.

As shown in Figures 2 and 3, three types of anthocyanins were detected by UPLC. In several studies, anthocyanins content also tended to increase when increasing day after silking stage. Previous report of Aoki et al. (2002) showed that cyanidin, pelargonidin and their monoloylated derivatives are present in purple corn seeds. Another study evaluated the red and pink colored corn sand found cyanidin3-glucoside and pelargonidin 3-glucoside as the major anthocyanin components, respectively (Abdel-Aal et al., 2006).

These studies showed that cyanidin-3-β-O-glucoside was a major anthocyanin constituent and also play important role as anthocyanins content of purple corn during ripening stage. Cyanidin-3-β-O-glucoside content ranged from 5.71 µg/100g of fresh weight (15th day after silking stage) to 40.97 µg/100g of fresh weight (30th day after silking stage). Pelargonidin-3-β-O-glucoside content ranged from 4.84 mg/100g of fresh weight (21st day after silking stage) to 13.59 µg/100g of fresh weight (30th day after silking stage). While Malvidin-3-β-O-glucoside ranged from 2.11 µg/100g of fresh weight (21st day after silking stage) to 12.03 µg/100g of fresh weight (30th day after silking stage). Pelargonidin-3-β-O-glucoside and malvidin-3-β-O-glucosidewere detected 21 days after silking stage. In statistical analysis, significant quantitative differences of all detected anthocyanins were found in day after silking stage.

Moreno et al. (2005) found cyanidin in colored corn as a major and important anthocyanin component. This study showed that cyanidin-3-β-O-glucoside had the highest content in the whole ripening stage. Lopez-Martinez et al. (2009) pointed out that the total anthocyanins of Mexican maize strains ranged from 1.54 to 850.9 mg cyanidin-glucoside equivalents/100g of whole grain flour; and purple-colored strains were most enriched with anthocyanins. Urias-Lugo et al. (2015) indicated that the anthocyanins content ranged from 646 to 1,052 mg cyanidin-3-β-O-glucoside/kg of maize and the elite blue maize hybrids could be an important source of antioxidant compounds with potential for either food or nutraceutical industries. Therefore, it is possible to obtain food with high content levels of health beneficial bioactive compounds and with antioxidant ability as purple corn.

Table 2 shows the change of major anthocyanins content in purple corn. The content of cyanidin-3-β-O-glucoside was slowly increased before 21st day after silking stage. However, from the 21st day after silking stage, the content of cyanidin-3-β-O-glucoside rapidly increased. Pelargonidin-3-β-O-glucoside and malvidin-3-β-O-glucosidewere not detected in the early development periods of purple corn. Heukjinjuchal kernel.

The content of pelargonidin-3-β-O-glucoside and malvidin-3-β-O-glucoside did not show significant statistical difference and slowly increased from 21st to 28th days after silking stage. But the content of pelargonidin-3-β-O-glucoside and malvidin-3-β-O-glucosidewere sharply increased from 29th day after silking stage. It may be inferred that the ripening stage have a significant influence on anthocyanins accumulation in purple corns.

The change in content between each anthocyanins and day after silking stage is shown in Table 3. Regression analysis between cyanidin-3-β-O-glucoside and days after silking stage was y = 2.3958x + 35.976 and R² = 0.9024. The regression analysis between days and the change of content between pelargonidin-3-β-O-glucoside and malvidin-3-β-O-
Figure 2: Mass scan chromatogram from 15th day after silking stage to 30th day after silking stage.

1) C3G: Cyanidin-3-β-O-glucoside.
2) P3G: Pelargonidin-3-β-O-glucoside.
3) M3G: Malvidin-3-β-O-glucoside.

glucoside was \( y=0.9269x-15.847 \) (\( R^2=0.9168 \)) and 
\( y=0.7703x-13.795 \) (\( R^2=0.8918 \)), respectively. Hu and Xu (2011) estimated that the anthocyanin and phenol content of black corn increased during maturation but the levels of anthocyanin and phenolics decreased for white and yellow corns.

In the correlation between the day after silking stage and individual anthocyanins (Table 4), day after silking stage
Table 2: The content of individual anthocyanins in kernel of the purple corn at different ripening stage.

<table>
<thead>
<tr>
<th>DASS$^{1)}$</th>
<th>Anthocyanins</th>
<th>C3G$^{2)}$</th>
<th>P3G$^{3)}$</th>
<th>M3G$^{4)}$</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µg/100g fresh weight)</td>
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<tr>
<td>15</td>
<td>nd$^{5)}$</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5.71</td>
<td>nd</td>
<td>nd</td>
<td></td>
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<tr>
<td>17</td>
<td>6.55</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>8.11</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>8.93</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>10.19</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>14.34</td>
<td>4.84</td>
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<td></td>
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<td>22</td>
<td>14.6</td>
<td>5.48</td>
<td>2.09</td>
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<td>23</td>
<td>15.5</td>
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<td>2.1</td>
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<td>24</td>
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<td>25</td>
<td>25.33</td>
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<td>8.02</td>
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<td>28</td>
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<td>6.73</td>
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<td>29</td>
<td>40.87</td>
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<td>12.37</td>
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<td>31</td>
<td>40.91</td>
<td>13.59</td>
<td>12.03</td>
<td></td>
</tr>
<tr>
<td>LSD$_{(0.05)}$$^{6)}$</td>
<td>5.67</td>
<td>1.8</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

$^{1)}$ DASS: Day after silking stage. $^{2)}$ C3G: Cyanidin-3-β-O-glucoside. $^{3)}$ P3G: Pelargonidin-3-β-O-glucoside. $^{4)}$ M3G: Malvidin-3-β-O-glucoside. $^{5)}$ nd: not detected. $^{6)}$ LSD$_{(0.05)}$: Least Significant Difference (LSD) at the 5% probability.

Figure 3: Major anthocyanins compound mass spectra of purple corn Heukjinjuchal.

1) C3G: Cyanidin-3-β-O-glucoside.
2) P3G: Pelargonidin-3-β-O-glucoside.
3) M3G: Malvidin-3-β-O-glucoside.
had positive correlation with individual anthocyanins (r=0.94** in cyanidin-3-β-O-glucoside, r=0.95** in pelargonidin-3-β-O-glucoside, r=0.92** in malvidin-3-β-O-glucoside).

The ripening stage associated with purple kernel corn is very important and as such, should be considered in making decision on harvesting period and whether purple kernel corn serves as green corn or industrial goods. The results of the present study may provide corn breeder with basic information on component breeding with anthocyanins.

REFERENCES


Table 3: Regression among day after silking stage and individual anthocyanins.

<table>
<thead>
<tr>
<th></th>
<th>Regression</th>
<th>R²</th>
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<tr>
<td>C3G</td>
<td>y=2.3958x-35.976</td>
<td>0.9024</td>
</tr>
<tr>
<td>P3G</td>
<td>y=0.9269x-15.847</td>
<td>0.9168</td>
</tr>
<tr>
<td>M3G</td>
<td>y=0.7703x-13.795</td>
<td>0.8918</td>
</tr>
</tbody>
</table>

1) DASS: Day after silking stage.
2) C3G: Cyanidin-3-β-O-glucoside.
3) P3G: Pelargonidin-3-β-O-glucoside.
4) M3G: Malvidin-3-β-O-glucoside.
* ** represent significance at the 5% and 1%.

Table 4: Correlation coefficient (r) among day after silking stage and individual anthocyanins.

<table>
<thead>
<tr>
<th></th>
<th>DASS</th>
<th>C3G</th>
<th>P3G</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3G</td>
<td>0.94**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3G</td>
<td></td>
<td>0.96**</td>
<td></td>
</tr>
<tr>
<td>M3G</td>
<td>0.92**</td>
<td>0.95**</td>
<td>0.95**</td>
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</table>

1) DASS: Day after silking stage.
2) C3G: Cyanidin-3-β-O-glucoside.
3) P3G: Pelargonidin-3-β-O-glucoside.
4) M3G: Malvidin-3-β-O-glucoside.
* ** represent significance.


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