Anthocyanase$^1$ and Anthocyanins Occurring in Eggplant, *Solanum melongena* L.$^2$ (I).

By Sadao SAKAMURA and Yataro OBATA

Department of Agricultural Chemistry, Hokkaido University, Sapporo

Received May 22, 1961

Anthocyanins present in eggplant were decolorized by anthocyanase from flesh of eggplant. The anthocyanins consisted of at least three different anthocyanins containing delphinidin as common aglycone, and that a main component of those was nasunin, delphinidin-3-diglucoside acylated with p-coumaric acid.

Using the anthocyanin as substrate, the anthocyanase action was optimal at pH 6.0 and 35°C, and was inhibited by potassium cyanide, thiourea, and sodium chloride. The data obtained so far show that anthocyanase acts on the following anthocyanidin derivatives in order of increasing rate of decolorization; pelargonidin-=peonidin-<cyanidin-<delphinidin-<delphinidin-glucoside acylated with p-coumaric acid.

The fruit of eggplant (*Solanum melongena* L.) is a very popular and useful vegetable to Japanese, and its skin (epidermis) has a fine purple hue, so-called “Nasukon”, which comes from the anthocyanins$^3)$. Many manufacturers and housewives know that the addition of potassium alum to brine in preparing eggplant pickles, results in better retention of the bluish color and higher quality of the pickles. After storage, nevertheless, the pickles become dark in color by the loss of the pigments. On the other hand, a phenomenon, scald of the fresh fruit, is frequently observed, which may be caused by bruising and other pre-processing conditions.

The present authors attempted to demonstrate the mechanism of decolorization occurring in the fresh fruit and pickles on the basis of a characterization of the anthocyanins and the anthocyanase.

Previous investigations have established the presence of an enzyme capable of destroying anthocyanins in fungi$^4$) and in some higher plants$^5$-$^7$). Very recently, Wagenknecht$^7$) and coworkers have reported that the anthocyanase activity is possibly responsible for the scald in sour cherries. So far as optimum pH of the anthocyanase was investigated, a definite difference exists between the fungal and the higher plant ones, that is, the former has its optimum pH in the acid range, whereas the latter from neutral to alkaline range.

This report is, therefore, concerned with properties of the enzyme in eggplant capable of causing the destruction of anthocyanin, and the specific activity of the enzyme for the anthocyanin isolated from the skin of the fruits.

---

1) Previous investigators have proposed the name anthocyanase to an enzyme destroying the anthocyanin, so that the name is adopted here in that sense. Refer 4) and 7) cited.
EXPERIMENTAL

Preparation of the Pigments.
Ten kilograms of the fruits were purchased at a local market and were then destemmed and peeled. The procedure for the preparation of the anthocyanins was essentially that of Kuroda and Wada\(^3\). The collected skins were extracted at room temperature with a methanol solution containing 3% hydrochloric acid. The extract appeared red in color. To the extract was added a saturated lead acetate solution. The white precipitates of PbCl\(_2\) formed were filtered off, and further addition of lead acetate was continued until no more blue precipitate was produced. The resulting precipitates, collected on a Buchner funnel, were dissolved in a methanol solution containing 2% hydrochloric acid, and the solution was filtered again.

To the filtrate was added about ten times its volume of ether and brown precipitates formed were collected. Dissolving and re-precipitating as above, the final precipitates weighed 3.5 g as dry powder. This powder is described in terms of the crude anthocyanins in this report unless otherwise indicated.

Separation of the Crude Anthocyanins to their Components.
A preliminary analysis of the anthocyanins has revealed at least 3 components by ascending paper chromatography using n-butanol-2N hydrochloric acid (1:1, v/v) as solvent system. Subsequently, in order to obtain each component in a pure form, the crude anthocyanins were dissolved in 2% hydrochloric acid in methanol, placed on the top of a cellulose powder column (4.5 × 30 cm) and eluted with the n-butanol-2N hydrochloric acid solution. The optical density at 540 m\(\mu\) was measured with each 10 ml of the effluent and then plotted against the volume of the effluent. The effluents corresponding to one component were combined and concentrated in vacuo below 50°C. Each of the concentrates was subjected to re-chromatography as before. The effluent was concentrated to a small volume, dissolved in 2% hydrochloric acid in methanol, and kept at room temperature. After several days the precipitated pigment was recovered.

Preparation of the Enzyme Solution.
Eggplant anthocyanase was partially purified according to the method used by Wagenknecht\(^7\) in studying cherries. Three hundred grams of the peeled fruits were mixed in a homogenizer with acetone, chilling with salted ice. The filtered residues (acetone powder) were extracted with de-ionized water in a refrigerator for three hours. Ammonium sulfate was added to the extract to 60% saturation and allowed to stand for several hours in a refrigerator. The mixture was centrifuged at 2000 r.p.m., and the supernatant liquid was decanted and discarded. The precipitates were dissolved in de-ionized water and the insoluble matter, if any, was centrifuged off and the supernatant was used as the enzyme solution.

Determination of the Anthocyanase Activity.
The reaction mixture consisted of 2 ml of anthocyanin solution, 1 ml of the enzyme solution, and 1 ml of McIlvaine buffer solution (pH 6.0). For a control, the enzyme solution was replaced by an enzyme solution inactivated by heating in boiling water bath for three minutes. After 10 minutes incubation at 35°C, the mixtures were added with 4 ml of 2% hydrochloric acid in methanol, and an aliquot was then diluted with an equal volume of 1% hydrochloric acid in methanol.

The optical density at 540 m\(\mu\) was determined for each sample using a Beckman spectrophotometer. The enzyme activity was expressed by difference in the optical densities between the reaction mixture and the control.

For the purpose of comparing the substrate specificity of the enzyme the following anthocyanins were also employed in addition to the nasunin isolated from the crude anthocyanins; pelargonidin-3-glucoside from strawberry, peonin from petals of peony, synthesized cyanidin\(^8\), peonidin-3-galactoside\(^9\), cyanidin-3-galactoside\(^9\) from American cranberry, a delphinidin-glucoside (not combined with \(p\)-coumaric acid) from calyx of Hydrangea macrophylla SERING. These compounds except for cyanidin were prepared by the present authors. When these compounds were used as substrate, the enzyme activity was compared by measuring optical density at the individual peak.

RESULTS AND DISCUSSION

Preliminary Decolorization Experiment.
When the skins of eggplant were extracted with a boiling potassium alum solution, the extract retained the fine blue color for a considerably long period. On the other hand,
when the press juice from the fruit flesh was added to the extract, the mixture became dark in color losing the bluish tint and did not appear reddish in color even after making it strongly acidic by adding hydrochloric acid and subsequent boiling. Thus, the decolorization can occur independently of pseudobase formation\(^{10}\) of anthocyanin and may be caused by an enzymic action.

Demonstration of the decolorization by the anthocyanase was carried out on the reaction mixture shown in Table I, and the results are shown in Fig. 1. It was shown that by heating in a boiling water bath for three minutes the destruction of the pigment of anthocyanin was suppressed almost completely, while the enzyme solution caused a remarkable pigment destruction leading to an increase in the optical density of the reaction mixture in a region of shorter wave length and decrease at 540 m\(\mu\) in the acidified solution.

**Separation and Partial Characterization of the Crude Anthocyanins.**

The crude anthocyanins were treated to give pure anthocyanin as aforementioned. As shown in Fig. 2 each anthocyanin was fractionated to Fraction I, II, III, and IV. Each fraction was re-chromatographed separately. After concentration under reduced pressure, Fraction I was recovered as a crystalline mass from 2% hydrochloric acid in ethanol. Its absorption spectrum is shown in Fig. 3.

For the purpose of identifying the aglucone of each component, hydrolysis was carried out with 20% hydrochloric acid in a boiling water bath for thirty minutes, followed by extraction with isoamyl alcohol. To the alcohol phase was added petroleum ether, and a lower aqueous phase containing nearly all of the pigment was separated. After concentration, an ali-

---

**TABLE I. REACTION MIXTURE.**

<table>
<thead>
<tr>
<th></th>
<th>Enzyme Soln.</th>
<th>Pigment Soln.</th>
<th>Enzyme Soln. Inactivated by Heating</th>
<th>De-ionized Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2 ml</td>
<td>2 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2 ml</td>
<td>2 ml</td>
<td></td>
<td>2 ml</td>
</tr>
<tr>
<td>III</td>
<td>2 ml</td>
<td></td>
<td></td>
<td>2 ml</td>
</tr>
<tr>
<td>IV</td>
<td>2 ml</td>
<td></td>
<td></td>
<td>2 ml</td>
</tr>
</tbody>
</table>

---

**Fig. 1. A Spectrophotometric Demonstration of the Pigment Decolorization by Enzymic Action.**

After incubation at 30°C for 20 minutes, a 1 ml aliquot of the mixture was diluted with de-ionized water to make 8 ml in total volume (left); another 1 ml aliquot was diluted with 2% HCl in methanol, followed by adding 1% HCl in methanol to a total volume of 8 ml (right).

Anthocyanase and Anthocyanins Occurring in Eggplant, *Solanum melongena* L.  

FIG. 2. Optical Density at 540 m\(\mu\) of Eggplant Anthocyanins Eluted from a Cellulose Column.

The crude anthocyanins, 94 mg; cellulose powder 100–200 mesh; column size, 4.3×30 cm; influent, n-butanol-2N HCl (1:1, v/v).

Figure 2 shows the optical density at 540 m\(\mu\) of eggplant anthocyanins eluted from a cellulose column. The crude anthocyanins, 94 mg, were dissolved in cellulose powder (100–200 mesh) and passed through a column with an influent of n-butanol-2N HCl (1:1, v/v).

The resulting crystalline precipitates were paper-chromatographed together with samples from Fraction III, IV and authentic anthocyanidins. Fraction II was too small in amount to be subjected to the chromatography. The results of paper chromatography are shown in Table II. All of the fractions gave delphinidin as aglucone, one of which obtained from Fraction I showed close agreement with that of the authentic delphinidin given by K. Hayashi on absorption spectrum (See Fig. 3).

**Sugar Moiety.**

The aqueous solution remaining after the extraction of the hydrolysed anthocyanin from Fraction I with isoamyl alcohol was desalted by passing successively through the columns of Amberlite IRA 402 and Amberlite IR 120. The extract was concentrated under reduced pressure and a small aliquot was subjected to paper chromatography in n-butanol-acetic acid-water (4/1/5, v/v/v). Spraying the chromatogram with aniline hydrogen phtha-

![Figure 3](image-url)  
**FIG. 3.** Spectra of the Anthocyanin from Fraction I and Delphinidin in 1% HCl in Methanol.  

- Authentic delphinidin, 1.02 mg %.  
- Delphinidin from the anthocyanin, 0.9 mg %.  
- Delphinidin-glucoside (Nasunin), 1.45 mg %.
TABLE II. RF VALUES OF EGGPLANT ANTHOCYANINS AND THEIR ANTHOCYANIDINS

<table>
<thead>
<tr>
<th>Pigment</th>
<th>n-But.-HCl&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AcOH-HCl&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>0.49</td>
<td>0.74</td>
</tr>
<tr>
<td>III</td>
<td>0.11</td>
<td>0.66</td>
</tr>
<tr>
<td>IV</td>
<td>0.06</td>
<td>0.51</td>
</tr>
<tr>
<td>Hydrolysate I</td>
<td>0.42</td>
<td>0.30</td>
</tr>
<tr>
<td>III</td>
<td>0.42</td>
<td>0.30</td>
</tr>
<tr>
<td>IV</td>
<td>0.42</td>
<td>0.30</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>0.42</td>
<td>0.30</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>-</td>
<td>0.50</td>
</tr>
<tr>
<td>Peonidin</td>
<td>-</td>
<td>0.67</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>-</td>
<td>0.79</td>
</tr>
</tbody>
</table>

<sup>a</sup> n-butanol, 2 N hydrochloric acid (1:1 v/v).
<sup>b</sup> water, acetic acid, conc. hydrochloric acid (10:30:3 v/v/v).

This suggested that the sugar was either glucose or galactose. The sugar was identified as glucose through its osazone, m.p. 203°C, prepared from the de-ionized hydrolysate. The authentic osazone prepared from pure glucose showed m.p. 205°C.

**Spectrum of the Anthocyanin.**

The absorption spectrum of the anthocyanin from Fraction I was recorded after dissolving in 1% hydrochloric acid in metha-

![FIG. 4. Effect of pH on the Enzyme Activity.](image)

![FIG. 5. Effect of Temperature on the Enzyme Activity.](image)

Anthocyanase and Anthocyanins Occurring in Eggplant, *Solanum melongena* L.

The spectrum as compared with that of delphinidin is shown in Fig. 3. The two peaks in the ultraviolet range at 280 and 310 m\(\mu\), due to the superimposition of the absorption of \(p\)-coumaric acid (310 m\(\mu\)) upon that of the pigment, indicated the presence of \(p\)-coumaric acid in the anthocyanin according to the data reported by Harborne\(^{12}\). The anthocyanin is, therefore, identical with nasunin, delphinidin-3-diglucoside acylated with \(p\)-coumaric acid, the molecular structure of which has been proposed by Kuroda and Wada\(^{3}\).

**Properties of Anthocyanase.**

**pH Optimum.**

As shown in Fig. 4, the optimum pH of the anthocyanase was determined to be 6.0. The anthocyanin used was decolorized significantly in the absence of active enzyme, particularly in alkaline range. Therefore, the anthocyanase activity, was derived by subtracting the optical density values of the control from those of the enzyme system at each pH. Bayer showed that the optimum pH for coleus cyanoxidase was 7.0 to 7.5\(^{6}\). This is considerably alkaline in range as compared with our result, but the reason may be due to the fact that no attention was paid to autocatalytic decolorization of the anthocyanin.

**Temperature Optimum.**

As shown in Fig. 5, the optimum temperature for anthocyanase activity was about 35°C, so that all of the subsequent experiments were carried out at this temperature. The time course of the reaction under the optimum conditions is shown in Fig. 6. The fact that highly salted pickles of eggplant do not readily lose their blue color led the present authors to examine the inhibiting action of some compounds related to oxidase inhibitor. As shown in Table III, potassium cyanide inhibited the anthocyanase activity most effectively and thiourea next. The fact that sodium chloride was inhibitory to a considerable degree is of interest in relation to the practical use.

Specificity of the Enzyme.

Following the confirmation that the anthocyanin (nasunin) could serve as the substrate for the enzyme (anthocyanase) in eggplant, the specificity of this enzyme was investigated. The results obtained with various substrates are recorded in Table IV.

Bayer\textsuperscript{6} had noted that the coleus enzyme needed addition of catechol to destroy cyanin. Wagenknecht and coworkers\textsuperscript{7} have also reported that catechol addition in the enzyme system enhanced the decolorization of antirrhinin as a substrate, while little enzymic action took place without catechol. The compounds used by these workers were limited to cyanidin derivatives.

In this report, other anthocyanidin derivatives were also examined, as substrate and cyanidin derivatives were, in fact, not particularly reactive and peonidin and its derivatives were still less reactive. Only nasunin, an anthocyanin, was destroyed at the highest rate and a delphinidin derivative not containing p-coumaric acid was the next.

In general, structural features which appear to govern the substrate-specificity of anthocyanase are the number of hydroxy groups substituted at the 3', 4', 5' positions of 2-phenyl-benzopyrylium nucleus and the presence of p-coumaric acid in anthocyanin molecule.

**Acknowledgement.** The authors wish to express their thanks to Prof. K. Hayashi of Botanical Institute, Faculty of Science, Tokyo University of Education, for his kind supply of delphinidin, and also to Dr. W. B. Esselen, Head of Department of Food Technology, University of Massachusetts, for his encouragement and his interest in this work.

---

**Table IV. Substrate-Specificity of the Enzyme for Various Anthocyanins**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aglycone</th>
<th>Activity Δ O. D × 100 (mₘₒ, measured at)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin-3-glucoside</td>
<td>R-OH-OH-OH</td>
<td>2 (520)</td>
</tr>
<tr>
<td>Peonidin-3-galactoside</td>
<td>R-OH-OH</td>
<td>2 (530)</td>
</tr>
<tr>
<td>Peonin</td>
<td>R-OH-OH</td>
<td>2 (520)</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>R-OH-OH</td>
<td>8 (540)</td>
</tr>
<tr>
<td>Cyanidin-3-galactoside</td>
<td>R-OH-OH</td>
<td>8 (530)</td>
</tr>
<tr>
<td>Delphinidin-glycoside</td>
<td>R-OH-OH</td>
<td>19.6 (540)</td>
</tr>
<tr>
<td>Nasunin</td>
<td>R-OH-OH</td>
<td>57.8 (540)</td>
</tr>
</tbody>
</table>

Each anthocyanin concentration was adjusted so that controls had certain O.D. values near 0.70 at their maximum absorption wavelengths when measured as described in text after incubation. Temperature, 35°C; the buffer solution, pH 6.0; reaction time, 10 minutes; controls, the enzyme solution inactivated by heat treatment.