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

Part II. Isolation and Identification of Chlorogenic Acid and Related Compounds from Eggplant

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From the eggplant fruits, chlorogenic acid, neochlorogenic acid and caffeic acid have been isolated and identified. Enzymic experiments show that their *O*-dihydroxyphenolic compounds can be the browning substrates in the fruit flesh, on the other hand chlorogenic acid accelerates the decoloration of nasunin (delphinidin-glycoside) and enables to decolorize pelargonidin-3-glucoside by the anthocyanase.

In the previous paper¹⁾, the anthocyanase in the eggplant fruits was demonstrated and its properties partially characterized, especially the specific activity for the anthocyanins and on inhibitors.

It has been found that the destruction of some cyanidin-glucosides is increased remarkably by addition of catechol in the enzyme system^{2,3)}.

On the other hand, the enzymic browning of the fresh fruits and vegetables has been recognized to arise from *o*-dihydroxyphenolic compounds such as chlorogenic acid⁴⁻⁷⁾, dopa⁸⁾, dopamine⁹⁾, and catechin¹⁰⁾ by the polyphenol oxidase.

Although many investigators^{11,12)} have reported on distribution of chlorogenic acid, its isomers and caffeic acid in number of plants, isolation and identification from eggplant have never been done except pointed out occurrence of caffeic acid derivative by means of spectrophotometrical method¹³⁾.

It seemed desirable to isolate and identify *o*-dihydroxyphenolic compounds in the fruits to learn if the compounds can be used as substrates of the enzymic browning and have any influence for the anthocyanase-substrate system. From such a point of view, this report was concerned with isolation and identification of chlorogenic, neochlorogenic and caffeic acids in the fruits flesh of eggplants. Subsequently, an evidence was presented that these compounds caused the enzymic browning by the action of the crude polyphenol oxidase preparation from eggplants, while chlorogenic acid had a specific action in the

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anthocyanase-substrate system, that is, addition of chlorogenic acid to the system accelerated markedly the decoloration of nasunin (delphinidin-glycoside) and enabled to decolorize pelargonididin-3-glucoside which was not affected by the anthocyanase. Thus, these facts may provide an interpretation about the decoloration and the followed browning of eggplant fruits caused by bruishing and other pre-processing conditions.

EXPERIMENTAL

Isolation of Chlorogenic Acid.

The peeled fresh fruit sliced by a stainless steel knife was placed in boiling water and left at boiling temperature for five minutes. After cooling to room temperature, the diffusion extract was filtered on Buchner's funnel. The filtrate gave green color with ferric chloride. The residue was discarded. By repeating the same procedure, the diffusion extract yielded 36 l from 35 kg of the peeled flesh. An aqueous solution saturated with lead acetate was added to the extract until no more yellow precipitate was produced. The resulting supernatant was discarded through a siphon and the precipitates were collected by centrifuging. To the aqueous suspension of the precipitates was added 5% H_2SO_4 to pH 3.0. The remained precipitates containing PbSO_4 were removed by centrifuging, and the supernatant was concentrated under reduced pressure to approximately 3.7 l. To this concentrates was added 7 l of absolute ethyl alcohol, the resulting precipitates were removed again. The supernatant was further extracted with ethyl acetate several times by mechanical shaking. The remaining aqueous solution (soln. A) is arranged for identification of neochlorogenic acid on the latter section. The ethyl acetate extract was combined, dried with anhydrous Na_2SO_4 , concentrated under reduced pressure and left in room temperature over night. The yellow crystalline solid was filtered and dried in a desiccator. The mother liquor (Soln. B) will be used to identify caffeic acid. The yield at this points was 23.2 g. This gave blue color with Folin's reagent in an alkaline range. Two crystallizations from hot water gave fine needles, m.p. $201\sim 3^\circ\text{C}$. Recrystallization 3 more times from hot water raised the melting point to $208\sim 9^\circ\text{C}$, yield 11.8 g, mixed melting point with authentic chlorogenic acid was

$208\sim 9^\circ\text{C}$, the chlorogenic acid had been isolated from coffee bean by the present authors according to the method described by Moores et al.¹⁴⁾ The crystals were dried for 3 hours over phosphorus pentoxide at 106°C . *Anal.* Found: C, 54.18; H, 4.96. *Calcd.* for $\text{C}_{16}\text{H}_{18}\text{O}_9$: C, 54.24; H, 5.12%.

Neutral equivalent, using as indicator the yellow-green color formed on neutralization by base, 355, *calcd.*, 354. Its ultraviolet absorption curve was identical with the authentic specimen (Fig. 1).

Pentaacetyl Chlorogenic Acid.

A mixture of 0.3 g chlorogenic acid and 1.5 ml of acetic anhydride was reacted in a boiling water bath for 2 hours. The acetate was precipitated by routine procedure. Two recrystallizations from ethyl alcohol gave melting point $178\sim 180^\circ\text{C}$, yield 0.19 g, bitter taste. An admixture with authentic pentaacetyl chlorogenic acid did not depress the melting point.

Saponification of Chlorogenic Acid.

Two grams of chlorogenic acid were dissolved with 10 ml of hot water, cooled in ice-water, and then mixed with 10 ml of 2 N sodium hydroxide. After having been bubbled with N_2 gas, the mixture in a test tube was sealed with a stopper. After storage at room temperature for 16 hours, addition of hydrochloric acid caused the immediate precipitation of yellow crystals. The crystals were filtered and washed with water. Recrystallization twice from hot water yielded 160 mg of crystals, m.p. 220°C (decomp.). *Anal.* Found: C, 59.95; H, 4.36. *Calcd.* for $\text{C}_6\text{H}_8\text{O}_4$: C, 60.00; H, 4.44%.

Identification of Caffeic Acid.

The mother liquor (Sol. B) of the crystalline chlorogenic acid was subjected to paper chromatography using *n*-butyl alcohol-acetic acid- H_2O (4:1:5, v/v/v) as developer. As shown in Table I, the result indicates an existence of caffeic acid besides chlorogenic acid. Therefore, the mother liquor was diluted with water, shaken with ethyl ether, and the ether layer was evaporated. Then, it was diluted again with a small portion of water. When it was left in a refrigerator for 2 weeks, the yellow precipitates were formed. The precipitates were collected by filtration, washed with water, and they weighed 35 mg. After recrystallization from hot water, it melted at $219\sim 220^\circ\text{C}$ (decomp.).

An admixture with the authentic caffeic acid (de-

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FIG. 1. Ultraviolet Absorption Spectra of Chlorogenic Acid and Neochlorogenic Acid.

- I. Chlorogenic acid from coffee bean, 0.95 mg/100 ml H₂O.
 II. Chlorogenic acid from eggplant, 1.04 mg/100 ml H₂O.
 III. An isomer. neochlorogenic acid from eggplant, measurement of which was carried out with the syrup resulting from the F-III fractions noted on Fig. II.

TABLE I. THE RESULTS OF PAPER CHROMATOGRAPHY

	<i>R_F</i> Value and Color								
	<i>R_F</i>	I	II	<i>R_F</i>	I	II	<i>R_F</i>	I	II
Soln. A*				0.61	dBl	YB	0.49	dBl	YB
Soln. B*	0.84	dBl	YB	0.61	dBl	YB			
Caffeic acid from eggplant	0.83	dBl	YB						
Caffeic acid from chlorogenic acid by saponification	0.83	dBl	YB						
Chlorogenic acid from eggplant				0.61	dBl	YB			
Chlorogenic acid from coffee				0.61	dBl	YB			
Neochlorogenic acid from eggplant							0.49	dBl	YB
Authentic neochlorogenic acid							0.49	dBl	YB

* See text on the details.

Solvent: *n*-butyl alcohol-acetic acid-water (4:1:5 v/v/v.).

Color development:

I, sprayed with Folin's reagent and exposed to ammonia vapor 7.)

II, sprayed with the crude polyphenol oxidase (see text). dBl=deep blue, YB=Yellow-brown.

rived from chlorogenic acid) did not depress the melting point.

Identification of Neochlorogenic Acid.

The remaining aqueous solution (Soln. A) extracted with ethyl acetate to separate chlorogenic acid was

subjected to the paper chromatography. This gave mainly 2 spots (Table I) one of which had an *R_F* value 0.61 corresponding to that of chlorogenic acid, and another of which had an *R_F* value 0.49. The latter showed entirely the same behavior as chloro-

genic acid displayed to the color reactions and to the fluorescence under UV-light. In order to isolate this compound, the solution was shaken several times with *n*-butyl alcohol. The alcohol layers were combined, and concentrated to syrup under reduced pressure. This was subjected to silicic acid chromatography.

The silicic acid column technique used here was an adaptation of the method described by Sondheimer^{12,15}. The column consists of 120 g silicic acid (Mallinckrodt, 100 mesh) mixed with 82 ml of 0.5 N H₂SO₄. Approximately 5 g of the syrup mixture in 10 g of silicic acid was placed on the top of the column. Development was carried out with 15%, 25% and 35% *n*-butyl alcohol-chloroform saturated with 0.5 N H₂SO₄. Fractions of 50 ml were collected and used for the absorption measurements at 330 m μ . The composition of the blanks used in the spectrophotometer was changed as the solvent composition

at 330 m μ , it was not considered probable that caffeic acid derivative was present. F-I, F-II and F-III fractions showing the peak at 330 m μ were combined respectively and the solvent was evaporated under reduced pressure, yielding the syrups, but F-II was too small quantity for further work. After being dissolved in a small portion of ethyl alcohol, they were precipitated as solids on addition of chloroform. By the paper chromatography using *n*-butyl alcohol-acetic acid-water (4:1:5 v/v/v) the solids from F-I gave a single spot corresponding to chlorogenic acid, while that from F-III showed also a single spot of lower *R_F* value, 0.48, appearing to be identical to neochlorogenic acid (see Photograph 1). When compared to the authentic neochlorogenic acid^{15,16}, which was kindly supplied by Corse, there was no difference between the *R_F* values on the chromatogram, (see Photograph 1).

FIG. 2. Chromatographic Separation of Chlorogenic Acid and Neochlorogenic Acid on Silicic Acid Column.

The column was developed with 15%, 25% and 35% *n*-butylalcohol in chloroform as noted on the figure. Other details of the chromatographic separation are given in text.

altered. The result obtained is plotted in Fig. 2. Since F-0 fractions did not show the absorption peak

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PHOTOGRAPH 1. Results of the Paper Chromatography.

Soilvent, *n*-butyl alcohol-acetic acid-water (4:1:5, v/v/v). Spray reagent, Folin's reagent.⁶⁾

I, Soln. A. II, chlorogenic acid. III, neochlorogenic acid obtained from the column chromatographic separation. IV, authentic neochlorogenic acid.

Enzymic Browning of Chlorogenic Acid and Related Compounds.

The *o*-dihydroxyphenolic compounds from the eggplant flesh were subjected to paper chromatography. The chromatograms dried in room temperature were sprayed with the crude polyphenol oxidase in M/15 phosphate buffer (pH 6.0), which was prepared from

the eggplant flesh by the similar way as the potato polyphenol oxidase was done previously⁹⁾.

After incubation at 30°C for ten minutes, the chromatogram gave the yellow-brown spots. The results are shown in Table I. Thus, it is found that chlorogenic, neochlorogenic and caffeic acids are oxidized to brown products, melanin-like compounds by the

polyphenol oxidase.

Function of Chlorogenic Acid in the Anthocyanase-Substrate System.

Enzymic experiments used here were essentially the same as previous report¹³.

Substrate.

Nasunin isolated and purified from the eggplant skin¹³.

Pelargonidin-3-glucoside (crystals) from the strawberry¹⁷.

Anthocyanase.

The final stage of the preparation of the anthocyanase was altered¹³. The acetone powder (10 g) was extracted with de-ionized water (100 ml) in a refrigerator, filtered off and followed by 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ in the extract. The precipitates formed were dissolved in M/15 phosphate buffer pH 6.0 (100 ml) and after centrifugation, the supernatant was used as the enzyme solution. Measurement of the decolorization by anthocyanase.

The anthocyanase experiment was carried out as shown in Table II. After reaction, the mixtures were immediately made to total volume 8 ml with 2% HCl in methanol. Optical density was measured at an individual maximum at the visible light ($520\text{ m}\mu$ for pelargonidin-3-glucoside, $540\text{ m}\mu$ for nasunin). Blank of the spectrophotometrical measurement consisted of 2 ml of the inactivated enzyme solution by heating, 1 ml of the chlorogenic acid solution (20 mg%) and 1 ml of water, and followed by making the total volume to 8 ml with 2% HCl in methanol.

When chlorogenic acid alone was added to the anthocyanase, the reaction mixture became pale brown in color, but it showed no optical density at the ab-

sorption peaks of the anthocyanins under the determination conditions. Therefore, it is recognized that the presence of chlorogenic acid in the enzyme experiments does not interfere with determination of the anthocyanins.

Subsequently, destruction rate (%) is defined as follows:

Destruction rate

$$(\%) = \frac{\text{O.D. of control} - \text{O.D. of sample}}{\text{O.D. of control}} \times 100$$

As shown in Table II, the results prove that the presence of chlorogenic acid affects in a great deal to decolorization of the both anthocyanins by the anthocyanase.

A probable assumption concerning the function of chlorogenic acid is made tentatively.

The anthocyanase preparation is considered either to be the polyphenol oxidase itself or to be contaminated by the polyphenol oxidase based on the preparation procedure. Thus, the discoloration of pelargonidin-3-glucoside in the presence of chlorogenic acid by the anthocyanase is due to a coupled reaction, in which the polyphenol oxidase participates as follows:

On the other hand, the destruction of nasunin in the absence of chlorogenic acid may be understandable from data on the substrate-specificity of the anthocyanase in the previous report¹³.

Evidence on the above assumption is under study.

TABLE II. DECOLORATION OF THE ANTHOCYANINS BY THE ANTHOCYANASE

No.	Reaction mixture				*Destruction rate (%)	
	The enzyme solution	Anthocyanin (20 mg %/H ₂ O)	H ₂ O	Chlorogenic acid (20 mg %/H ₂ O)	Pelargonidin-3-glucoside	Nasunin (Delphinidin-glycoside)
1	2 ml	1 ml	1 ml		0	53
2	2 ml	1 ml		1 ml	92	94
Control	Inactivated by heating for 3 minutes.	1 ml		1 ml	0	0

Reaction temperature, 35°C; Reaction time, 10 minutes.

* See text on the details.

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CONCLUSION

1) From 35 kg of the eggplant flesh, 11.8 g pure crystalline chlorogenic acid was isolated and identified.

2) Caffeic acid was also obtained in a state of crystals and identified, but its quantity was extremely little.

3) Of the some isomers of chlorogenic acid, neochlorogenic acid was separated by column chromatography using silicic acid and identified by comparison with authentic specimen by paper chromatography.

4) Their *o*-dihydroxyphenolic compounds are substrates of the crude polyphenol oxidase in eggplants and converted to yellow-brown

substances.

5) The anthocyanase preparation does not substantially act on pelargonidin-glucoside, but the addition of chlorogenic acid make possible the decoloration of the pigment.

6) The anthocyanase preparation which is able to decolorize nasunin markedly increase the decolorizing activity by the addition of chlorogenic acid.

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