Leuco-anthocyanin in Variable Flowers of *Camellia japonica**

by

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By the term leuco-anthocyanin a group of compounds is designated which yields anthocyanidin when heated with acid. On this account, some authors have postulated that transformation of leuco-anthocyanin *in vivo* into anthocyanin is possible, even though it is not known to occur usually in this way in plant tissues. According to Swain (1954) and Keppler (1956), leuco-anthocyanin might have a flavon-diol or -triol structure, which is closely related rather to catechin than to anthocyanidin.

There are many varieties of *Camellia japonica* cultivated in Japan, and their flower color types are roughly classified into three groups, *i. e.* red, white and variegated. Plants with variegated flowers often bring forth self-colored red flowers. Therefore, it is convenient to use the chimeral plants for the study of the biochemical effect of genes controlling anthocyanin biosynthesis, since both types of flowers have otherwise the same genetic background, including the mutable gene for flower color.

Bate-Smith (1953) suggests that leuco-anthocyanin is present in white flowers of *C. japonica*. The present author (1958) found that leuco-anthocyanin was contained together with anthocyanin in red flowers of some varieties of the species. If leuco-anthocyanin really is a precursor of anthocyanin, the red flowers of the chimeral plants should contain smaller quantities of it than the variegated flowers. In order to examine this, relative contents of both anthocyanin and leuco-anthocyanin were measured spectrophotometrically. On the other hand, the activity of copper enzyme partaking in polyphenol systems was also measured, because it was assumed that in both types of flowers different amounts of anthocyanin might be produced in parallel with a different enzyme activity. The present report includes some results of this investigation.

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Materials

More than forty horticultural varieties of *C. japonica* have been collected and maintained by Dr. Y. Takenaka. Among these, two varieties, Hasumishiro and Somekawa, were used in this experiment. Both plants have double flowers. Flowers of the former are white and those of the latter are variable, more than half of flowers being variegated.

The investigations were carried out chiefly with self-colored and variegated flowers of Somekawa. The white flowers of Hasumishiro were also examined for the sake of comparison with the data obtained from the flowers of Somekawa. This plant material was also proved to be an appropriate source of catechin-like substance including leuco-anthocyanin.

Description of flower color pattern: Flowers of Somekawa were observed towards the middle of April, 1957. Out of 173 flowers observed 66 were self-colored red, two were partially-self-colored, and the remaining were variegated. The flowers on the 2nd and 6th branches were uniformly self-colored. It suggests that the mutation from white to red had occurred early before the branching. One self-colored flower was found on the 8th branch, one partially-self-colored on the 7th and one on the 9th branch. In one of the partially-self-colored flowers, about four-fifth of the petals locating at inner circle were red. In another, petals in a half of the flower were red and the rest were variegated, as shown in Fig. 1.

Fig. 1. Photographs of self-colored, variegated and partially-self-colored flower which appeared in variety Somekawa.

Methods and Results

Preliminary tests: Acetone extract of white flowers of Hasumishiro was concentrated *in vacuo* at 30°C until brownish residue remained. The residue became brownish red, when heated several minutes in a boiling water-bath with propanol-hydrochloric

acid (Pigman *et al* 1953). The brownish red matter was partitioned into a small amount of amyl alcohol after addition of much water. Dark red alcoholic solution was paper-chromatographed with a solvent, 80% formic acid/concentrated hydrochloric acid/water (5:1:4, v/v) (Endo 1954). A red spot with a brownish trail appeared on the chromatogram, showing Rf value 0.20, which agreed with that of cyanidin co-chromatographed.

The red and variegated flowers of Somekawa contain a single anthocyanin which was proved paper-chromatographically to be cyanidinglycoside. The anthocyanin was removed from the extract prior to leuco-anthocyanin-test. Acetone extract from 30 g. of fresh red flowers was concentrated until red matter remained. The residue was dissolved in water and passed through a column (1.3×10 cm.) packed with Amberlite IRC-120 resin (H form). By this procedure, anthocyanin was quantitatively adsorbed on the resin column. Faintly orangish filtrate from the column was neutralized with dilute alkali, saturated with sodium chloride, and extracted with ethylacetate. The ethylacetate solution turned red, when heated with propanol-hydrochloric acid, whereas the aqueous layer reacted only very weakly. Extract from the variegated flowers gave the same results. Paper-chromatographic tests showed that both red and variegated flowers contained leuco-anthocyanin yielding cyanidin.

The extracts from these flowers gave negative reaction in reduction test (magnesium and hydrochloric acid), but became green with ferric ion and produced voluminous white precipitates with a modified Steasny reagent (concentrated hydrochloric acid/28% formalin, 1:2, v/v). Therefore, the flowers of the plant contain a considerable amount of catechin-like substance without flavone.

Paper-chromatography of catechin-like substance: The acetone extracts obtained above were examined by two-way paper-chromatography, using distilled water in one direction and phenol saturated with water in the other. Polyphenol substances were detected by spraying with aqueous methanolic solution saturated with diazotized sulfanilic acid. As shown in Fig. 2, five spots and one trail with brownish yellow color could

Fig. 2. Paper chromatograms of polyphenol substances extracted from red, variegated (Somekawa) and white flowers (Hasumishiro). The chromatograms were run at first upwards with water and then right to left with phenol.

be detected on the chromatograms of the three types of flowers. It appears that each flower contains almost the same components. R_f value of one of the spots (0.50/0.30: water/phenol) agreed with that of *d*-catechin (0.48/0.31), obtained in treatment with the same solvent, but this agreement may not necessarily mean identification of the spot. It is likely that some of the spots are involved with leuco-anthocyanin.

Preparation of catechin-like substance: White flowers of Hasumishiro (550 g. in fresh weight) were macerated in a blender with total three liter of methanol and filtered. The filtrate was concentrated *in vacuo* up to 400 cc. and refiltered through Celite. The aqueous filtrate was saturated with sodium chloride and was extracted five times with 100 cc. portion of butanol. Combined butanolic solution was dried with sodium sulfate and concentrated *in vacuo* up to about 100 cc. at 60°C. On addition of 300 cc. benzene, catechin-like substance was separated as yellowish brown precipitate. Collected precipitate was dissolved in acetone and filtered. The filtrate was dried up and brownish material (754 mg.) was obtained. This material, which showed strongly leuco-anthocyanin-reaction, was used as substrate for copper enzyme occurring in flowers of this plant.

Spectrophotometric analysis: The above catechin-like substance was compared with *d*-catechin spectrophotometrically in aqueous solution by the Shimadzu QB-50 Spectrophotometer. Both samples were found to be very similar as regards two absorption maxima, *i. e.* 218-224 and 278-280 $m\mu$, as depicted in Fig. 3 (a). However, a remarkable difference appeared in the spectra, when they were heated for ten minutes at 68°C in 7% ethanolic hydrochloric acid. The *d*-catechin produced a large amount of brownish precipitate, and the supernatant showed an absorption maximum at 462 $m\mu$. Spectra of treated catechin-like substance showed newly two maxima at 457 and 546 $m\mu$, of which the former was less prominent than the latter. It appears that the two absorption maxima of treated catechin-like substance correspond to 462 $m\mu$ of treated *d*-catechin and 542 $m\mu$ of cyanidin respectively, as shown in Fig. 3 (b).

Relative concentrations of the catechin-like substance and of the anthocyanin were estimated. For the estimation of catechin-like substance, five grams of fresh petals were macerated in a blender and extracted twice with 100 cc. portion of acetone. Combined extract was filtered and concentrated *in vacuo* up to a syrup. The syrup was dissolved in 50 cc. of water and passed through Amberlite IRC-120 (H) resin column (1.3×10 cm.). The filtrate from the column was adjusted to 100 cc. with water. Ten cc. was again diluted to 100 cc. with water and measured. Absorption spectra of the filtrates are shown in Fig. 4. Optical densities at 280 $m\mu$ were 0.650 (for red flower), 0.428 (for variegated) and 0.820 (for white), respectively.

For the estimation of the anthocyanin, five grams of fresh petals were extracted thrice with 30 cc. portion of 1% aqueous hydrochloric acid and filtered on a sintered glass funnel. Combined extract was filled up to 100 cc. with the extractant. Absorption maximum was found at 520 $m\mu$ with the extract of red flowers. Then, optical densities at 520 $m\mu$ were 1.46 and 1.36 (for red flower), 0.16 and 0.20 (for variegated) and 0.00 and 0.05 (for white). Accordingly, it was found that the red flowers contained about nine to seven times as much more anthocyanin as the variegated flowers.

Manometric estimation of copper enzyme activity: Twenty grams of fresh flowers were macerated in a blender together with 100 cc. water and 60 g. of ammonium sulfate and were filtered by suction. From the residue, enzyme was extracted successively thrice with 15 cc. portion of citrate-phosphate buffer at pH 5.0. Combined extract was

Fig. 4. Absorption spectra of catechin-like substance extracted from 1. white flowers of Hasumishiro, 2. red flowers and 3. variegated flowers of Somekawa.

adjusted to 50 cc. with the same buffer solution. The activity of copper enzyme was measured by means of oxygen uptake in Warburg apparatus at 30°C, whereby 4 cc. of the crude enzyme solution and definite amount of substrate solution were applied. Catechol, *p*-cresol, *p*-phenylenediamine and ascorbic acid were used as substrate. The oxygen uptake of the enzyme solution is shown in Table 1. Enzymes participating in oxidation of the substrates are inferred by a review of Dawson and Tarply (1951).

Table 1. Copper enzyme activity of red and variegated flowers of Somekawa and of white flowers of Hasumishiro (oxygen uptake, μ l/hr. Temp. 30°C)

Substrate	Concentration	Participating enzyme	Enzyme activity of flowers of		
			red	variegated	white
<i>p</i> -Cresol	$8 \times 10^{-3}M$	Polyphenolase	7.8	15.7	3.4
Catechol	$2 \times 10^{-3}M$	Polyphenolase and Laccase	20.9	20.2	27.8
<i>p</i> -Phenylenediamine	$6 \times 10^{-3}M$	Laccase	15.7	19.0	39.2
Ascorbic acid	$6 \times 10^{-3}M$	Ascorbase and Laccase	75.0	68.0	106.0

The extracted catechin-like substance was not affected by potato tyrosinase at

Fig. 5. Oxygen uptake curve of enzymatic oxidation of catechin-like substance (μ l/hr., Temp. 30°C). Intact enzyme activity of 1. variegated flowers (Somekawa), 2. red flowers (Somekawa) and 3. white flowers (Hasumishiro). Blocked enzyme activity of 4. variegated flowers, 5. red flowers and 6. white flowers.

all, but has partially by the above enzyme solution. The activity of the enzyme was estimated in Warburg apparatus, taking 1 cc. of the enzyme solution, 6 mg. of the catechin-like substance and 4 cc. of demineralized water or aqueous solution of phenylthiourea as inhibitor. The results are shown in Fig. 5. It was found that the activities of each enzyme solution were inhibited as much as about half by $6.8 \times 10^{-4}M$ solution of phenylthiourea. On the other hand, when 4 cc. of the enzyme solution extracted from the red flowers and 6 mg. of the catechin-like substance were mixed in a vessel of the apparatus, oxygen uptake rapidly increased to its highest level within ten minutes, but considerable amount of leuco-anthocyanin was found to remain in the reactant solution.

From these results, it may be concluded that no significant difference exists between activities of copper enzymes extracted from the red and the variegated flowers of Somekawa, and that the leuco-anthocyanin is not transformed into any coloring matter with the enzyme obtained above.

Discussion

Though the data obtained here are tentative, it is very probable that leuco-anthocyanin is not an immediate precursor of anthocyanin. It appears more plausible that both compounds are formed in parallel, since leuco-anthocyanin accumulates in the flowers irrespective of anthocyanin formation. This opinion has been put forward from other kinds of experiments by several authors, as reviewed by Blank (1947), Geissman and Hinreiner (1952) and Paech (1955).

As regards to the role of copper enzyme, the present experiments have not shown any conclusive evidence in relation to anthocyanin biosynthesis. Robinson (1936) suggested that oxidation rather than reduction was surely in harmony with the whole of the circumstances of anthocyanin formation. It may be expected from this view point that cyanic flowers have a more active or higher level of oxidation system than acyanic ones. On the other hand, Edmondson and Thimann (1950) have reported that in *Spirodela* there is evidence for the mediation of copper in anthocyanin biosynthesis. Accordingly, it seems likely that a certain copper enzyme participates at least temporarily in the course of anthocyanin formation, although estimation of copper enzyme activity showed no significant difference among the examined flowers of this plant.

Summary

Flowers of *Camellia* variety, Somekawa, are variegated. Frequently self-colored red flowers occur, probably owing to mutation of a mutable recessive gene. Co-existence of anthocyanin and leuco-anthocyanin was found in both types of flowers. Estimations of anthocyanin and catechin-like substance including leuco-anthocyanin were carried out spectrophotometrically. The results have shown that leuco-anthocyanin accumulates in the flowers irrespective of anthocyanin formation. Copper enzyme

activities in red and variegated flowers compared manometrically, but there was no significant difference between them.

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