Reexamination on the Structure of so-called "Hiviscin"*

by Mannen SHIBATA** and Motoko FURUKAWA**

Received March 28, 1969

Abstract

The major pigment, which was formerly isolated from the calyx and bract of roselle (*Hibiscus sabdariffa* L.) by Yamamoto *et al.* (1933), was reported to have the structure of delphinidin pentosido-glucoside. It was newly examined and found that it consists of each 1 mol of delphinidin (1 mol), glucose and xylose. By a series of analytical procedure its structure must be delphinidin 3-xylosido-glucoside. So it was compared with daphniphyllin (delphinidin 3-xylosido-glucoside), which was isolated from the berries of *Daphniphyllum macropodum* Miq. and it revealed their identity. Hence, the name "hiviscin", once given to this substance, must be eliminated from the literature.

As minor pigments, a small amount of delphinidin 3-monoglucoside, cyanidin 3-monoglucoside (chrysanthemin) and delphinidin were also isolated in crystalline state from dried calyx and bract.

The roselle (red sorrel or Jamaica sorrel) (*Hibiscus sabdariffa* L.), belongs to the family Malvaceae. It is an annual shrub, 1.5-2.5 m in height and its calyx and bract are thick and fleshy, deep red in colour. It was imported into Formosa from Malaya in 1910 by Mr. Fujine and grows now all over Formosa.

In 1932 Yamamoto and Oshima¹⁾ isolated an anthocyanin from the calyx and bract of roselle in crystalline state. They gave the name "hiviscin" (perhaps it may be due to a misprint of hibiscin) to this new anthocyanin and reported it to be a cyanidin monoglucoside.

By suggestion of R. Robinson, they reexamined it in next year and altered its structure to be delphinidin pentosido-glucoside²⁾.

According to the paperchromatographic test on crude extract of dried calyx of roselle, it was found that dark red colour of calyx is exhibited by four kinds of pigment (Rf value 0.56, 0.45 and 0.23, respectively in BuH*** and 0.59, 0.38, 0.26 and 0.09, respectively in AAH****).

The first pigment (S_1), Rf 0.59 in AAH, is the major component and amounts to about 7.5 parts of total pigment, while the second (S_2), Rf 0.38, to about 0.5 part, the third (S_3), Rf 0.26, to about 0.5 part and the forth (S_4), Rf 0.09, to about 1.5 part, respectively. The second pigment (S_2) corresponds to cyanidin 3-monoglucoside (chrysanthemin) and the third (S_3), to delphinidin 3-monoglucoside and the forth (S_4) to delphinidin. The forth pigment is supposed to be decomposition product of major pigment in

* This work was partly supported by a Grant in Aid of Scientific Research of Ministry of Education.

** Laboratory of Plant Physiology and Phytochemistry, Department of Biology, Faculty of Science, Kumamoto University, Kumamoto, Japan.

*** *n*-BuOH/HCl/H₂O, (7:2:5, v/v).

**** AcOH/HCl/H₂O, (3:1:8, v/v).

the course of drying of calyx and bract, although it is not known whether delphinidin is present in free state or not in fresh calyx.

The present investigation was undertaken to reexamine and to determine the chemical structure and precise nature of the major pigment contained in calyx and bract.

Experimental results and discussion

1. Extraction and purification of the pigment chloride

The dried calyx and bract of the roselle (600 g), imported from Formosa, were extracted with 1% methanolic hydrochloric acid (5 l) overnight. Re-extraction was made with the same solvent (5 l). The Rf value, proportion and colour shade on chromatogram of the extract were shown in Fig. 1 and Table 1.

The combined extract (9.5 l) was filtered through a thin layer of hyflosupercel and the filtrate was concentrated to 1/3 of its volume *in vacuo* below 35° and kept in a refrigerator overnight. The impurities precipitated were filtered off and to the concentrate lead subacetate was added under continuous agitation. The blue precipitate formed was filtered by suction and washed thoroughly with water and then methanol. The lead salt of anthocyanin was air-dried for a day in the room (1,050 g), pulverized in a mortar and converted into the chloride by treatment with 5% methanolic hydrochloric acid (1 l). Re-extraction of anthocyanin with 1% methanolic hydrochloric acid was repeated thrice and the extract (3 l) was concentrated to a half volume *in vacuo* below 35°. The concentrate was allowed to stand in a refrigerator for a day and the precipitate formed was filtered off. To the combined extract (2.5 l) fifteen times its volume of purified ether was added, whereby the pigment was deposited as syrupy mass. After decanting supernatant liquor, the pigment chloride was mixed with the same volume of methanol and an excess of ether was added to form amorphous precipitate. The precipitate was collected on a filter paper and dissolved in 1% methanolic hydrochloric acid. The pigment

Fig. 1. Paperchromatogram showing four kinds of anthocyanin contained in the extract of dried *Hibiscus*-calyx.

Table 1. Colour shade, proportion and Rf value on chromatogram (one-way ascending procedure, Tôyô No. 52 filter paper, $28 \pm 1^\circ$) of four kinds of anthocyanin.

Sample	Rf value in		Proportion (parts)	Colour shade
	AAH	BuH		
S ₁	0.59	0.38	7.5	red purple
S ₂	0.38	0.45	0.5	purplish red
S ₃	0.26	0.23	0.5	red purple
S ₄	0.09	0.45	1.5	red purple

chloride was precipitated again on addition of three times its volume of ether, collected and dissolved in 1% methanolic hydrochloric acid. To the filtrate of pigment chloride a half volume of 5% methanolic hydrochloric acid was added and allowed to stand for a few days. The crystals commenced to separate on the surface of the solution and then deposited on the bottom of vessels. The crystals were filtered by suction. The second crop was also obtained on allowing the mother liquor to stand for a few days more. The recrystallization of the pigment was best achieved by dissolution in 1% methanolic hydrochloric acid and addition of a half volume of 8% methanolic hydrochloric acid. The yield was about 0.75 g, that is, 0.125% of the dried material used. As the crop was found to be the mixture of four kinds of pigment, they were separated mass paper or column chromatographically from each other and eluted. Each eluate was concentrated, crystallized and used for experiments.

2. The properties of pigment chloride

The pigment was obtained in brownish red long prism, but it seems greyish violet under microscope (Fig. 2). When collected, it exhibits deep brownish red colour showing brilliant golden reflex. The air-dried specimens decomposed at 212.7° (uncorr.) to form a blackish mass. It contained $2\frac{1}{2}$ mol water of crystallization. The crystals are moderately soluble in cold water, ethanol and 1% methanolic hydrochloric acid, sparingly soluble in warm 7% hydrochloric acid, and almost insoluble in cold 10% hydrochloric acid. The colour of dilute ethanolic solution of the pigment turns blue upon addition of aqueous sodium carbonate, which fades away into yellow brown, transient blue with aqueous sodium hydroxide, blue green which discoloured with aqueous ferric chloride, and violet with aqueous sodium acetate. Upon addition of aqueous lead acetate it gives blue precipitate and of picric acid no precipitate.

Fig. 2. Microphotograph of pigment chloride.

3. Anthocyanidin chloride (aglycon)

A. Complete hydrolysis

Air-dried crystals of the pigment chloride (30 mg) were dissolved in a small quantity of warm water and to the solution was added an equal amount of 35% hydrochloric acid. The solution was boiled for 3 min. over direct flame, chilled in ice and then allowed to stand for a few hours in a refrigerator.

The yield of aglycon liberated was about 14 mg, that is 46.6% of the pigment chloride used. Recrystallization of aglycon was carried out by dissolution in 1% methanolic hydrochloric acid and addition of a half volume of 8% methanolic hydrochloric acid.

Fig. 3. Crystals of aglycon prepared from the pigment chloride.

The paperchromatographic and spectrophotometric data also proved the identity of the aglycon with authentic delphinidin chloride, which was prepared from daphniphyllin, an anthocyanin contained in the pericarp of berries of *Daphniphyllum macro-podum* Miq.³⁾ (Figs. 3 and 4).

B. Determination of the bound organic acid and sugar

After collection of the aglycon liberated by hydrolysis, the acidic hydrolysate was shaken with a small amount of ether several times and the combined ethereal solution was evaporated to dryness. The residue obtained was dissolved in methanol and paperchromatographed, whereupon authentic organic acids were also co-chromatographed as standard. The result showed the absence of bound organic acid in the molecule of the pigment chloride.

In Forestal* solvent

Fig. 4. Paperchromatographic comparison of *Hibiscus*-aglycon with authentic anthocyanidins.

S₁: *Hibiscus*-aglycon C: Cyanidin
P: Pelargonidin D: Delphinidin

Fig. 5. Paperchromatogram showing the sugar of *Hibiscus*-anthocyanin and authentic specimens (Tôyô No. 52 filter paper, $28 \pm 1^\circ$, one-way ascending procedure, using BuPyr** for irrigation).

G: Glucose S₁: *Hibiscus*-sugar X: Xylose A: Arabinose

* AcOH/HCl/H₂O, (30:3:10, v/v)

** *n*-BuOH/Pyridin/H₂O, (6:3:1, v/v)

After saponification of the pigment chloride, there was observed no discordance of R_f value in BuH. Therefore, it could be safely concluded that there is no bound organic acid in the pigment chloride.

After determination of organic acid, the acidic hydrolysate was shaken with *iso*amyl alcohol to remove the remaining aglycon and then with ether to remove the remaining trace of *iso*amyl alcohol. The hydrolysate thus treated was neutralized with 10% methanolic sodium hydroxide, evaporated *in vacuo* to dryness. The residue obtained was dissolved in newly distilled methanol and chromatographed with authentic sugars. Two spots were clearly observed on chromatogram by silver nitrate reagent. Fig. 5 shows that the sugar moieties attached to the aglycon of the pigment chloride are glucose and xylose.

4. Partial hydrolysis of the pigment chloride

According to the method described by Abe and Hayashi⁴⁾, partial hydrolysis of the pigment chloride was carried out in order to determine the sequence of the bound sugar.

A few milligrams of recrystallized pigment chloride were dissolved in 1% methanolic hydrochloric acid (5 ml) and mixed with an equal volume of 20% hydrochloric

Fig. 6. Paperchromatogram showing the products of partial hydrolysis of the pigment chloride (Tôyô No. 52 filter paper, $28 \pm 1^\circ$, one-way ascending procedure).

D₂: Daphniphyllin S₁, 1: Sample ("hiviscin") D, 3: Delphinidin
D₂, 2: Delphinidin 3-monoglucoside

acid. The solution was heated on a water bath at 70° for 140 min. During hydrolysis a small amount of the reaction mixture was pipetted out at regular intervals and was spotted in duplicate on a large sheet of chromatographic paper, using the the pigments of known constitution as the standard, that is, delphinidin 3-monoglucoside and daphniphyllin (delphinidin 3-xylosido-glucoside). Then the chromatographic papers were developed with BuH and AAH. As shown in Fig. 6 the results revealed that the pigment is diglycoside of delphinidin, but its R_f value differs from delphin and also discloses the absence of 5-glucoside and the presence of 3-glucoside in the course of partial hydrolysis. It is suspected to be 3-xylosido-glucoside of delphinidin.

Fig. 7. Absorption spectra
— daphniphyllin
--- the pigment "hiviscin"

Visible and ultraviolet absorption spectra of the pigment chloride was estimated and compared with those of daphniphyllin chloride. Absorption maxima were 281 mμ, 355-357 mμ and 548-549 mμ for daphniphyllin and almost similar with those of the pigment as shown in Fig. 7.

IR spectra of the pigment and daphniphyllin were also estimated and compared each other as shown in Fig. 8.

According to the experimental results, as mentioned above, the anthocyanin obtained from the calyx and bract of *Hibiscus sabdariffa* L. must be delphinidin 3-xylosido-glucoside.

Fig. 8. IR spectra of the pigment and daphniphyllin (KBr disc method).

Hiv: the pigment Da: Daphniphyllin

The authors wish to express their cordial thanks to Mr. N. Takahashi of the Institute of Agricultural Science in Kumamoto city for the supply of dried calyx and bract of roselle.

References

- 1) Yamamoto, R., and Oshima, Y., Sci. Pap. Inst. Phys.-chem. Res. **19**: 134 (1932).
- 2) ———, and Kato, A., Nettai Nogaku Kaishi **5**: 273 (1933).
- 3) Shibata, M., and Ishikura, N., Bot. Mag. Tokyo **77**: 277 (1964).
- 4) Abe, Y., and Hayashi, K., ibid. **69**: 577 (1956).