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MALVIDIN 3-GALACTOSIDE AND DELPHINIDIN 7-GALACTOSIDE FROM *BLADHIA SIEBOLDII*

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Abstract—Two pigments have been isolated from the fruits of *Bladhia sieboldii* and their structures identified as malvidin 3-galactoside and a new pigment delphinidin 7-galactoside(bladhianin).

IN continuation of our work on natural anthocyanins[1] the pigments in the peel of *Bladhia sieboldii* have been investigated. About fifteen native species of *Bladhia* (Myrsinaceae) are found in Formosa. The fruits of *Bladhia sieboldii* change colour during ripening from pale yellow through pink, red and finally violet-black.

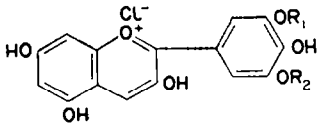
The crude pigment extract from ripe fruits showed, on paper chromatography, 3 pigment spots. Li and Wagenknecht[2] separated and purified two closely related anthocyanin pigments by metasilicic acid partition chromatography. Application of this method using silica gel finer than 200 mesh and various solvent systems did not effect separation of the pigments. On alumina column chromatography,[3] the alumina adsorbed the pigments so firmly that they were difficult to elute. In the present investigation a modified adsorption column chromatographic method involving silica has been devised for separation and purification of closely related anthocyanin pigments. Each of the three pigment fractions obtained by paper chromatography of the crude pigments, when further chromatographed on silica powder, gave four pigment zones indicating that there are no less than twelve kinds of anthocyanin pigments in the skins of *B. sieboldii.*

In order to obtain sufficient pure pigment to determine its structure, special methods were devised and two pigments termed "Anthocyanin A" and "bladhianin" were isolated.

Anthocyanin A does not contain an organic acid residue.[4] When hydrolysed with hydrochloric acid a violet-red anthocyanidin is produced. From the U.V. absorption spectra, qualitative tests[5] and paper chromatography the anthocyanidin was identified as malvidin chloride (I). The sugar in the hydrolysate of Anthocyanin A is galactose identified by comparison of the phenylosazone and paper chromatogram with authentic specimens. The quantitative hydrolysis shows that it is malvidin monogalactoside and the position of galactose was fixed at position 3 since it is not attacked by ferric chloride.[6] Thus Anthocyanin A is identified as malvidin 3-galactoside. The distribution number[7] is $9{\cdot}0 \pm 0{\cdot}8$ which agrees with the value of 8·6 for primulin (malvidin 3-galactoside)[8] as well as with those found for monoglycosides (9·8 to 11·0).[7]

[1] P. Y. Yeh, W. F. Ling and R. Takasaka, *Science* **128**, 312 (1958).
[2] K. C. Li and A. C. Wagenknecht, *J. Amer. Chem. Soc.* **78**, 979 (1956).
[3] P. Karrer and F. W. Strong, *Helv. Chim. Acta* **19**, 25 (1935).
[4] K. Hayashi, *Experimental Methods in Plant Pigments* (in Japanese). Nakayama-Shoten, Tokyo (1954).
[5] G. R. Robinson and R. Robinson, *Biochem. J.* **25**, 1687 (1931).
[6] A. Leon, A. Robertson, R. Robinson and T. Seshadri, *J. Chem. Soc.* 2672 (1931).
[7] R. Willstätter and E. H. Zollinger, *Liebigs Ann.* **412**, 208 (1916).
[8] R.Scott-Moncrieff, *Biochem. J.* **24**, 767 (1930); A. Bell and R. Robinson, *J. Chem. Soc.* 813 (1934).

Diglycosides usually gave distribution numbers below 2·0.[7] The colour reactions of Anthocyanin A are also in good agreement with those reported for primulin.[8]



I: $R_1 = R = Me$
II: $R_1 = R_2 = H$
III: $R_1 = H$, $R_2 = Me$

Bladhianin forms a scarlet red picrate and does not contain an organic acid residue. It distribution number is 13 ± 0·8, which is much higher than that (eight) reported for delphinidin 3-galactoside.[9] Quantitative hydrolysis yields one mole each of delphinidin chloride (II) and galactose. The ferric chloride test indicates that the galactose is not at position 3. Therefore, it is a new anthocyanin with galactose in the 5 or 7 position.

Bladhianin was exhaustively methylated with dimethyl sulphate in aqueous alkaline solution with the formation of a chalcone. Since anthocyanidin methyl ether is not formed on acid hydrolysis of the chalcone,[6,10] the sugar residue is not attached to the 5 position and therefore the structure of bladhianin is delphinidin 7-galactoside. Although 3,7-diglucoside and 5,7-diglucoside of cyanidin chloride[11] and pelargonidin 7-β-glucoside chloride[6] were synthesized, bladhianin is the first natural anthocyanin bearing a sugar group on the 7-position.

It is of interest that malvidin 3-galactoside is found in the highest zone of the silica chromatogram obtained from the highest spot on the paper chromatogram of the crude pigments, and delphinidin 7-galactoside is found in the lowest zone of the silica chromatogram of the lowest spot on the paper chromatogram. As mono- or di-glucosides of malvidin (I), petunidin (III) and delphinidin(II) are found together in grape,[12] it is reasonable to presume the presence of galactosides of petunidin as well as other glycosides of malvidin, petunidin, and delphinidin in the skins of the fruits of *Bradhia sieboldii*.

EXPERIMENTAL

Silica powder and silica gel (granular) used were C.P. grades, Wako Chem. Co., Japan. Silica and silica gel, ground and screened with a 110 mesh sieve, were fractionated by sedimentation in water to remove the finer particles which retarded the flow of solvent in chromatography. The Spaeth and Rosenblatt's method[13] was modified as follows: Silica (or silica gel) powder (100 g) was stirred vigorously with distilled water (1300 ml) for ½ hr and kept undisturbed for 5 min. The supernatant mixture was decanted and was allowed to settle for 30 min. The first and second sediments were dried at 100–110° for 8 hr, the former was screened with a 200 mesh sieve and combined with the latter to use in chromatography.

The fruits (1 kg) were washed with water immediately after the collection and dried at room temp. The fruits were extracted 3 times, over a period of 9 days with ethanol containing 1% HCl in a refrigerator, to give 1 l. of combined extracts. The extracts gave, when chromatographed on Toyo filter paper No. 2, three spots, having R_f values 0·29 (the highest spot), 0·21 (middle spot) and 0·16 (the lowest spot), when developed with 4:1:1 BuOH–AcOH–H_2O and 0·59, 0·51, and 0·41 in 5:1:4 80% HCOOH–36% HCl–H_2O.

[9] K. Hayashi, G. Suzushino and K. Ouchi, *Proc. Japan. Acad* **27**, 430 (1951); *Chem. Abstr.* **46**, 7567g (1952).
[10] P. Karrer, *Helv. Chim. Acta* **10**, 750 (1927); **11**, 837 (1928); **12**, 292 (1929); C. G. Nordström, *Acta Chem. Scand.* **10**, 1491 (1956).
[11] R. H. MacDowell, R. Robinson and A. R. Todd, *J. Chem. Soc.* 806 (1934).
[12] E. C. Spaeth and D. H. Rosenblatt, *Analyt. Chem.* **22**, 1321 (1950).

A mixture of lead acetate (5 g) and yellow lead oxide (3 g) in water (6 ml) was shaken at room temp until white in colour. The mixture was kept at room temp overnight and the supernatant liquid, contained lead subacetate,[13] was diluted with an equal volume of ethanol. To 1 l. of the pigment extract, 50 ml of twice diluted lead subacetate solution was added, and the precipitate separated and dried (21 g). The paper chromatogram of the pigments obtained by decomposing the lead salts showed 3 spots, while that of the pigments in the mother liquor of the lead salts showed mainly one spot, the highest spot. The mother liquor was reserved for the isolation of Anthocyanin A.

Powdered lead salts (10 g) was mixed with absolute ethanol containing 5% hydrogen chloride (30 ml). The red anthocyanin solution was separated by centrifuge. The residue was further decomposed 5 times with 5 portions of 5% ethanolic HCl (20 ml). This gives a better yield of the crude pigments than treatment of the lead salts with all the ethanolic HCl at one time. Anhydrous ether (4 vols) was added to the combined red pigment solution, the mixture kept in a refrigerator overnight, yielding crude pigments (0·996 g) from which bladhianin was isolated.

Isolation of Anthocyanin A. Lead subacetate solution (diluted 5 times) was added in 2 portions (79 ml and 315 ml) to the mother liquor (3·5 l.) from the preparation of the lead salts. The first crop of lead salts, containing mainly Anthocyanin A, was separated before the second portion of the diluted subacetate solution was added. The second crop, containing almost exclusively Anthocyanin A, was decomposed with 0·1% aqueous HCl (700 ml) to give aqueous pigment solution.

The silica powder (40 g) and 0·1% aqueous HCl (35 ml) were transferred to a column. Then, under 30 mm vacuum, the aqueous pigment solution (70 ml) was introduced and eluted with 0·01% ethanolic HCl. After the first narrow band had been removed, 0·02% ethanolic HCl was introduced to elute the main zone. The remainder of the mother liquor of the lead salts was treated in the same way. The eluates were combined (2 l.) and concentrated to 40 ml below 30° under reduced press. Dark red-brown crystals (90 mg) with metallic luster separated and recrystallized from aqueous ethanol HCl to give 60 mg pure Anthocyanin A. (Found: C, 50·47; H, 4·84; Cl, 6·30; OMe, 11·20; H_2O, 3·11. Calc. for $C_{23}H_{25}O_{12}Cl.H_2O$: C, 50·51; H, 5·00; Cl, 6·48; OMe, 11·35; H_2O, 3·29.)

The colour reactions of Anthocyanin A are the same as those given by Scott-Moncrieff for primulin.[8]

Isolation of bladhianin. The crude pigments (3 g) were dissolved successively in 4 portions water (15 ml) and the solutions combined. Fractional precipitation of the picrates by addition of 3 portions of 1% aqueous picric acid (22 ml) kept 20 min at room temp gave successive fractions termed "Picrate I" (585 mg), "Picrate II" (342 mg), "Picrate III" (73 mg) and "Mother liquor A".

Paper chromatography of the pigments obtained by decomposing Picrates I, II and III revealed that the compositions of the picrates, measured by colorimetric method and expressed by approximate ratio of the highest spot:the middle spot:the lowest spot, were Picrate I, 1:0·5:0·05, Picrate II, 1:1:0·5, and Picrate III, 0·1:0·5:1.

Picrate II (684 mg) was ground twice in water (20 ml each) for 10 min. and centrifuged. The combined supernatant liquid was cooled in an ice-salt bath overnight to give precipitate and "Mother liquor B". The compositions checked by paper chromatography and expressed in the approximate ratio of the 3 spots were insoluble picrate (480 mg), 1·00:1·00:0·40: the precipitate (38 mg), 0·00: 0·05:1·00, and Mother liquor B, 0·00:0·001:1·00. Similarly, Picrate III (145 mg) was treated with water to give insoluble picrate (79 mg), precipitate (46 mg) and mother liquor. Each of these 3 parts has the same composition as that of the corresponding part from Picrate II.

Mother liquor A was cooled in an ice-salt bath to give 112 mg of picrates the composition of which was the same as that of Mother liquor B. The picrate was decomposed with 5% absolute ethanolic HCl and reprecipitated with dry ether to give crude anthocyanin chloride (100 mg) which was dissolved in hot water (0·4 ml, 75°) and centrifuged. An equal volume of 2·5% ethanolic HCl was added to the hot aqueous solution. The precipitate (65 mg) was separated after the mixture had been cooled in ice-water and recrystallized 4 times to give crude bladhianin (35 mg). paper chromatography of which showed only the lowest spot. It was further chromatographed on silica powder (100 g). No zone was eluted with aqueous 0·1% HCl or ethanol–water (3:7). When the column was eluted with 0·01% ethanolic HCl, however, 3 zones were seen. The lowest red zone, the middle blue zone, and the highest red zone were eluted successively with 0·01%, 0·02%, and 0·1% ethanolic HCl. After the elution, there was one red zone (4th zone) at the top of the column. The lowest zone was the main fraction, from which bladhianin (10 mg) was obtained.

[13] R. Shindler, *Chem. Zentr.* 779 (1845).

Bladhianin, forms dark violet microcrystals, is easily soluble in water, methanol, and ethanol to form a red solution, sparingly soluble in cold acetone to form a violet solution and insoluble in ether, benzene, and ethyl acetate.

Colour reactions of bladhianin. In aqueous solution: Sodium carbonate, pale green → green-yellow → pale orange; sodium acetate, violet → blue; lead acetate, blue-violet precipitate.

In ethanol solution: Sodium carbonate, blue-violet → dark brown precipitate; sodium acetate, blue; lead acetate, blue-violet precipitate. (Found: C, 45·18; H, 4·82; Cl, 6·26; H_2O 11·68. Calc. for $C_{21}H_{21}O_{12}Cl.3H_2O$: C, 45·45; H, 4·91; Cl, 6·39; H_2O, 11·54%).

Saponification of Anthocyanin A (for any ester linkage).[4] To a red solution of Anthocyanin A (1 mg) in anhydrous 1% ethanolic HCl (2 ml) anhydrous 10% ethanolic KOH (2 ml) was added to give a blue solution. The air above the was replaced by hydrogen and the solution kept at room temp for 1 hr. To this blue solution anhydrous ethanolic HCl was added until the solution turned red. Then 3 volumes of anhydrous ether was added and the precipitate formed was separated by centrifuging and again dissolved in 1% ethanolic HCl to give a red solution. The paper chromatography of this solution developed with 4:1:1 BuOH–AcOH–H_2O at about 20° on a Toyo No. 2 paper showed the same R_f value as that of Anthocyanin A (0·30) indicating no ester linkage is involved in the latter compound.

Hydrolysis of the glycosidic linkage in Anthocyanin A.[4] A solution of Anthocyanin A (5 mg) 20% HCl (1 ml) was boiled for 3 min to give needle crystals of the aglycone which separated while cooling. The filtrate was reserved for the identification of sugar. The aglycone from Anthocyanin A, violet-red needles, gave a positive qualitative tests for malvidin chloride.[5]

The oxidation test. A solution (1 ml) of the aglycone in 5% aqueous HCl was mixed with 10% NaoH (0·5 ml) and shaken for 5 sec. On adding 5 drops of 36% HCl to the above blue solution, the colour turned to reddish, and was extracted with isoamyl alcohol, showing that the aglycone is not decomposed by oxidation.

The R_f values using Toyo No. 2 paper and developed at 28° were 0·39 in 5:1:5 AcOH–36% HCl–H_2O and 0·28 in 5:2:3 80% HCOOH–36% HCl–H_2O. The co-chromatography with an authentic malvidin chloride also showed the same R_f values.

The U.V. spectra of the aglycone was the same as that of authentic malvidin chloride.

Identification of the sugar in the hydrolysate of Anthocyanin A *and in that of bladhianin.* The brown crude sugar solution obtained from the hydrolysis of Anthocyanin A (or bladhianin) was extracted with isoamyl alcohol until both layers became colourless. The aqueous layer was further extracted with benzene 3 times to remove the isoamyl alcohol. The colourless aqueous solution in a watch glass was dried overnight in a evacuated desiccator over sodium hydroxide and calcium chloride, The solid residue was again dissolved in water (0·5 ml) and dried. The unknown sugar in the residue showed on paper chromatography only single spots with several developing solvents, and the R_f values were the same as authentic galatose, which has been used individually and co-chromatographically. The sugar was also identified by conversion to the phenylosazone m.p. 179–181°.

Quantitative hydrolysis of Anthocyanin A. A solution of Anthocyanin A (7·644 mg) in 1·5 ml water was hydrolysed as described above and the reaction product cooled overnight. The crystals of the aglycone separated by centrifuging were washed with 3 drops water. The washings were combined with the mother liquor for the determination of sugar. The aglycone was dried over phosphorous pentoxide at 100–105° under 5 mm vacuum. It weighed 4·925 mg, 64·43% of the anthocyanin. The calculated value for the aglycone in malvidin galactoside monohydrate, $C_{23}H_{25}O_{13}Cl.H_2O$, was 67·06%.

The sugar solution was first diluted with water to 3 ml, then extracted with isoamyl alcohol until the latter became colorless. The isoamyl alcohol extracts were combined and extracted with water (1 ml). The aqueous extract was combined with the sugar solution and again washed with benzene (1 ml) to remove isoamyl alcohol. The benzene washing was extracted with water (1 ml) and the aqueous extract was combined with the sugar solution. The sugar solution was dried in a vacuum desiccator with sodium hydroxide and calcium chloride. The residue was dissolved in water and again evaporated *in vacuo* to dryness. In order to remove HCl which may interfere with the colouration by anthrone, the above procedure (dissolve in water and evaporation) was repeated 5 times. The final residue, a colourless solid mass, was dissolved in water (8 ml). The solution (pH 6·12) gave no precipitate with silver nitrate solution. The amount of galactose in this solution was determined as

2·375 mg. (31·07% of the anthocyanin) by the anthrone method using authentic galactose solution as control.[14] The calculated value for monogalactoside was 32·94%.

Determination of the position of the sugar.[6] An equal volume of aqueous 0·25% ferric chloride was was mixed with 0·004% pigment solution (in aqueous 1% HCl solution). The resulting red colour did not change during a period of 3 days. This shows that the position 3 is not free. The same experiment was carried out with the aglycone from Anthocyanin A for control. The solution became colourless within 3 min, showing that the 3-position is free.

Saponification of bladhianin (for any ester linkage). The similar treatment of bladhianin as that of Anthocyanin A gave unreacted material as shown from the following R_f values when co-chromatographed with bladhianin (Toyo No. 2 paper): 4:1:1 BuOH–AcOH–H_2O, 0·16; 5:1:4 80% HCOOH–36% HCl–H_2O, 0·41.

Hydrolysis of the glycosidic linkage in bladhianin. A solution of bladhianin (5 mg) in 20% HCl (1 ml) was boiled for 3 hr and extracted with isoamyl alcohol. The aglycone obtained was soluble in water. It gave the Robinson's colour reactions for delphinidin.

In contrast to the aglycone of Anthocyanin A, that of bladhianin was decomposed in the oxidation test by alkalinizing its solution followed by shaking for 5 sec.

The aglycone showed the same R_f values as those of authentic delphinidin isolated from the hydrolysate of the pigment in the skins of the fruits of *Solanum melongena*[15] on co-chromatography on Toyo No. 2 paper at about 28° as follows: 5:1:5 AcOH–36% HCl–H_2O, 0·20; 5:2:3 80% HCOOH–36% HCl–H_2O, 0·14.

The U.V. spectra of the aglycone from bladhianin was the same as that of delphinidin.

The sugar from bladhianin as that in Anthocyanin A is galactose.

The quantitative hydrolysis of bladhianin (9·463 mg) with 20% HCl gave sugar (2·896 mg, 30·60% of bladhianin). The calculated figure for delphinidin monogalactoside trihydrate, $C_{21}H_{21}O_{12}Cl.3H_2O$, is 32·47% sugar. The amount of dried delphinidin chloride obtained from the isoamyl alcohol extract of the hydrolysate was 5·480 mg, 57·91% of bladhianin (Calc. for anhydrous aglycone, 59·84%).

The position of the sugar substituent was determined by the oxidation test with ferric chloride. On adding ferric chloride solution, the red solution of bladhianin decolourized within 30 min, proving the 3-position free. The red colour of a solution of the aglycone from bladhianin decolourized after 3 min. With the ferric chloride test, decolourization is complete in 35 min if position 3 is free. On the other hand, the colour is unchanged for 3 days if 3-position is not free.[6]

Exhaustive methylation and hydrolysis of bladhianin. Bladhianin (2 mg) was dissolved in 3 drops of 1 N NaOH. The colour of the solution changed instantly from red to blue. With vigorous stirring, 0·5 ml freshly distilled dimethyl sulphate and 30% aqueous NaOH (2 ml) was added dropwise during 1·5 hr in nitrogen atm and stirring was continued for another hour. The orange-yellow mixture was neutralized with 2 N HCl and made about 4 N with 36% HCl. After heating at 100° for 30 min the colour (orange-yellow) of the mixture remain unchanged, proving the 5-position to be free.

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[14] J. R. Helbert and K. D. Brown, *Analyt. Chem.* **27**, 1791 (1955); D. D. Morris, *Science* **107**, 254 (1948).
[15] C. Kuroda and M. Wada, *Proc. Imp. Acad* **9**, 51 (1933).