

무당개구리의 복피 Carotenoid 색소에 관한 연구(제 4 보).
Lutein 과 3, 3'-Dihydroxy-3, 4, 3', 4'-Bisdehydro- β -
Carotene 의 분리 및 확인

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Studies on the Carotenoid Pigments in the Abdominal
Skin of *Bombina Orientalis*(IV). Occurrence of Lutein
and 3, 3'-Dihydroxy-3, 4, 3', 4'-bisdehydro- β -Carotene
in the Abdominal Skin of *Bombina Orientalis*

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요 약. 무당개구리의 복피 색소로부터 주 색소의 일부인 Lutein 과 3, 3'-dihydroxy-3, 4, 3', 4'-bisdehydro- β -carotene 을 분리하여 물리 화학적 성질, 가시광선 및 적외선 스펙트럼 특성, 그리고 크로마토그래피적 성질을 이용하여 그들의 구조를 확인 하였다.

ABSTRACT. Two more major pigments, lutein and 3, 3'-dihydroxy-3, 4, 3', 4'-bisdehydro- β -carotene, were isolated from the abdominal skin of *Bombina Orientalis* and their structures were established by physical and chemical properties, visible and IR spectral characteristics and chromatographic behaviors.

INTRODUCTION

The isolations and identifications of four major pigments; β -Carotene, α -Cryptoxanthin, 4'-Hydroxyechinenone, and 3-Hydroxycanthaxanthin from the abdominal skin of *Bombina Orientalis* were reported in this journal by these authors previously.¹⁻³ Two more major pigments were separated and identified as lutein and 3, 3'-dihydroxy-3, 4, 3', 4'-bisdehydro- β -carotene. Lutein

was originally isolated from green leaves by Willstätter and Mieg⁴ and later obtained from wide variety of plants⁵⁻⁹ and animals.¹⁰⁻¹⁴ However, 3, 3'-dihydroxy-3, 4, 3', 4'-bisdehydro- β -carotene is a new carotenoid not reported yet.

The pigments were separated on a silica gel PLC plate as the previous report with various solvents.¹⁻³ Lutein was obtained from a yellowish orange band G developed under the band F. This was further purified by repeated chro-

matography on a silica gel PLC plate with a mixed solvent; *n*-hexane: benzene: acetone(1 : 1 : 0.5).

Another pigment was separated from the moderately polar zone, the red colored band D. This was further purified by repeated chromatography on silica gel PLC plates with benzene. The purified pigment which was unable to crystallize was then saponified and the resulting product was again separated and purified by repeated chromatography on silica gel PLC plates with benzene containing 10 % of methanol.

The purified pigments were characterized with their physical and chemical properties and identified with their VIS-UV and IR spectra.

EXPERIMENTAL

Materials and Methods. The same materials and methods described in the previous papers¹⁻³ were used. The SiO₂ PLC plates were prepared using SiO₂ G for PLC Merk and activated at 110 °C for two hours in an oven.

Preliminary Partition of Pigment. The same partition was carried out as before³. The pigment G, the most polar portion of pigments on the SiO₂ PLC plate, formed a yellowish orange band. The pigment G zone was scraped off from the plate and the pigment was extracted with 5 % methanol in acetone. The pigments in acetone extracts were transferred to benzene layer by adding water. Benzene layer was washed with saturated NaCl solution and dried over anhydrous sodium sulfate, and evaporated. The residue was dissolved in a little volume of benzene and crystallized by adding hexane.

Pigment D, an broad red band on SiO₂ plate, was separated on SiO₂ PLC plate with benzene. Three zones, D₁ an orange band, D₂ a broad red band, and D₃ a narrow pink band, were separated. Each zone was scraped off from the plate, and the pigments were extracted with

acetone. The pigments were transferred to petroleum ether layer by adding water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure.

Separation of Lutein. The solid G was dissolved in benzene and further purified on SiO₂ PLC plate with hexane: benzene: acetone (1 : 1 : 0.5). Two zones, G₁ a narrow pink band, G₂ a narrow orange band, were separated. The pigment G₂ was further chromatographed on SiO₂ column with 10 % acetone in benzene. The major orange zone was eluted out, and the eluted solution was washed with water, dried, and evaporated. The residue was examined by TLC on SiO₂ with 10 % acetone in benzene. No further separation was observed. The residue was crystallized from benzene and hexane, reddish orange plate crystals, m.p 192°C (Lit. 193 °). The VIS-UV absorption maxima in nm: 422, 446, 476, 476.5 in hexane; 420, 446, 476 in ethanol; 424, 450, 478 in benzene; 429, 456, 486 in CHCl₃; 446, 475, 505 in CS₂; 420, 445 in petroleum ether. IR(KBr-pellet); 3300 cm⁻¹ (-OH), 2850 cm⁻¹ (-CH₃), and 950cm⁻¹ (C-H in trans olefin). The R_f-value of G₂, determined on SiO₂ TLC plate with benzene: methanol (10 : 1) was 0.15; 4'-hydroxyechinenone, 0.65; 3-hydroxycanthaxanthin, 0.49; zeaxanthin, 0.15.

Separation of D₂'. The hexane solution of D₂ was saponified with 10 % KOH-methanol solution by allowing to stand at 25 °C for 76 hours under the N₂ atmosphere. The reaction mixture was transferred to a separatory funnel and added a small portion of benzene. It was washed with several portions of water and finally with saturated NaCl aqueous solution. The benzene layer was separated and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The residue was dissolved in a small portion of benzene and allowed to stand overnight in a refrigerator.

The solid was filtered and recrystallized from benzene and hexane. The purified pigment was examined by SiO₂ TLC plate with 10 % methanol in benzene. No further separation was observed. Violet plate with metallic luster (D₂'), m. p 147~9°C. The VIS-UV absorption maxima in nm: 486 in benzene; 472 in hexane; 470 in petroleum ether; 505~6 in CS₂; 486 in CHCl₃; 490 in pyridine. IR (KBr): 2.95 μ , 3.45 μ , 6.22 μ , 9.00 μ , and 10.4 μ . The VIS-UV absorption maxima of D₂, a broad round band, in nm: 465~468 in hexane; 478 in benzene; 463 in petroleum ether; 498 in CS₂; 480 in CHCl₃. R_f-value on SiO₂ plate with methanol-benzene(1:10): D₂', 0.14; D₂, 0.83; lutein, 0.15; 3-hydroxycanthaxanthin, 0.49; 4'-hydroxyechinenone, 0.65; zeaxanthin, 0.15; β -carotene, 0.96; and 3,4,4'-trihydroxy- β -carotene, 0.05 respectively.

Partition Test. The partition ratio of carotenoids between hexane and 95 % methanol were measured according to the method of Petracek and Zechmeister; 15 and found for D₂' hexane/95 % methanol, 13:87; for G₂, 12:88.

Epoxide Test. The epoxide tests for pigment D₂' and D₂ were carried out according to the method of Karrer¹⁶ as the following: 3 ml of carotenoid ethereal solution was placed in a test tube and 1 ml of concentrated HCl was added to it. No blue coloration observed in HCl layer after shaking the mixture.

RESULTS AND DISCUSSION

Two more major pigments, lutein and 3,3'-dihydroxy-3,4,3',4'-bisdehydro- β -carotene, were isolated from the abdominal skin of *Bombina Orientalis*. The shape of visible absorption curve of G₂, in hexane, has a similarity to α -carotene with a fine structure as shown in Fig. 1.

The visible absorption spectra of G₂ in various solvents, as shown in Table 1, were almost superimposable to that of lutein previously reported;^{17,18} 420, 447, 477 in hexane; 445, 475, 508 in CS₂; 428, 456, 487 in CHCl₃.

The melting point of pigment G₂, reddish range plate, was 192°C (uncorr. evacuate

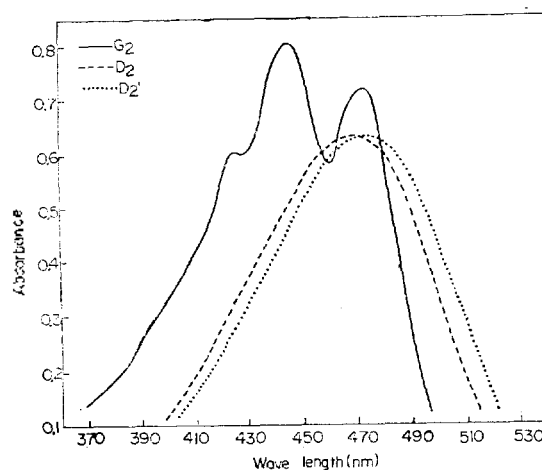


Fig. 1. Visible absorption curves of pigment G₂, D₂ and D₂'. Solvent: *n*-hexane.

Table 1. Visible Absorption Maxima of D₂, D₂' and G₂ in various solvents (in nm).

Solvent \ Pigment	Hexane	Benzene	CHCl ₃	CS ₂	Pet. ether	Ethanol
D ₂	465~468	478	480	498	463	
D ₂ '	472	486	486	505~6	470	
G ₂	422	424	429	446	420	420
	446	450	456	475	445	446
	476.5	478	486	507	476	476

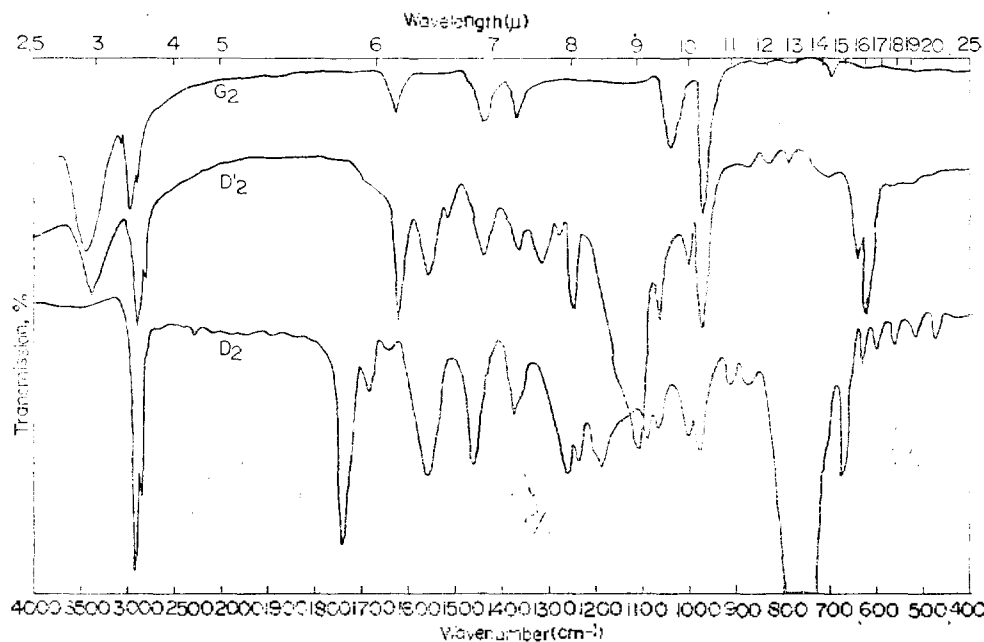


Fig. 2. IR spectra of pigment G_2 (KBr), D_2' (KBr) and D_2 (CCl_4).

capillary). The R_f -value of G_2 on a SiO_2 plate with benzen-methanol (10 : 1) was 0.15, identical with that of lutein isolated from egg yolk. The IR spectrum of G_2 showed a band at 3400 cm^{-1} , 2950 cm^{-1} , and 950 cm^{-1} , as shown in Fig. 2, which is in good agreement with that of lutein reported by Strain.¹⁹

On the mixed thin layer chromatography of pigment G_2 and an authentic sample of lutein from egg yolk, only one colored spot was observed. All these data strongly supported that pigment G_2 was undoubtedly lutein itself.

The shape of visible absorption curve of D_2 and D_2' , saponified product of D_2 , was a broad and round curve in hexane, as shown in Fig. 1. The visible absorption maxima in various solvents are also presented in Table. 1. The visible spectral curves of carotenoids are characterized by the loss of fine structure in cases of conjugated carbonyl group on the β -ionon ring and conjugated double dehydrogenation on the β -ionon ring such as 3, 4, 3', 4'-bisdehydro- β -

carotene.²⁰⁻²³

D_2 and D_2' exhibit a round and broad absorption maxima in the visible spectra. IR spectrum of D_2 shows a strong absorption band due to the carbonyl stretching vibration at 1745 cm^{-1} in Fig. 2. This is regarded as the carbonyl stretching vibration of an ester group. Since no significant absorption band was observed in this region in the IR spectrum of D_2' , it is suggested that pigment D_2 and D_2' do not possess a conjugated carbonyl group on the terminal ring of the carotenoid. The shape of visible absorption curve of D_2' was almost identical with that of D_2 except a slightly bathochromic shift. This means that no significant skeletal change has been occurred during the saponification. The visible spectrum of D_2' was almost identical with those of 3, 4, 3', 4'-bisdehydro- β -carotene.²⁰⁻²³ The ester carbonyl group absorption in D_2 is disappeared in D_2' and a strong hydroxyl group absorption is observed at 3380 cm^{-1} in the IR spectrum. IR spectrum of D_2' shows a great

Table 2. R_f -value of related carotenoids on SiO₂ TLC. Solvent: benzene-methanol (10 : 1).

Pigment	R_f -value	Pigment	R_f -value
D ₂ '	0.83	Zeaxanthin	0.15
D ₂ '	0.14	3-Hydroxycanthaxanthin	0.49
β -Carotene	0.96	4-Hydroxyechinenone	0.65
Lutein	0.15	3, 4, 4'-Trihydroxy- β -carotene	0.05

similarity with that of zeaxanthin,^{24~27} however spectral curve of zeaxanthin is characterized by a fine structure in the visible region as λ_{max} , 425, 451, 479 nm in hexane. This results suggest that D₂' could not be zeaxanthin.

The R_f -value of D₂' determined on SiO₂ TLC plate with 10 % methanol in benzene was 0.14. The R_f -values of lutein isolated from egg yolk,¹³ 3-hydroxy canthaxanthin³ isolated from *Bombina Orientalis*, and 3, 4, 4'-trihydroxy- β -carotene, reduced product of 3-hydroxycanthaxanthin, were determined and are shown in Table 2.

The R_f -value of D₂' was smaller than that of 3-hydroxycanthaxanthin, and greater than that of 3, 4, 4'-trihydroxy- β -carotene, but almost the same to that of lutein which has two hydroxyl groups in its molecule. This result suggests the presence of two hydroxyl groups in its D₂' molecule. The IR spectrum of D₂' shows much difference from that of isozeaxanthin reported by Grob^{28,29} and Boder.³⁰ If D₂' passes two hydroxyl groups at 4, 4'-position on β -ionone ring, i. e. 3, 4, 3', 4'-bisdehydro compound of zeaxanthin, it would be expected a part of conjugated carbonyl group which is in equilibrium with enol form, and should have a similarity to IR spectrum of isozeaxanthin, and exhibits a conjugated carbonyl absorption in its IR spectrum. It means that the hydroxyl groups in D₂' could not be at 4' and 4 position. The partition ratio of D₂', determined by the method of Zechmeister,¹⁹ was 87:13. From all these data the pigment D₂' was characterized as 3, 3'-dihydroxy-3, 4, 3', 4'-bisdehydro- β -carotene.



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