Journal of Research in Biology

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Characterization of carotenoid pigments in amphibian, *Rhacophorous bipunctatus*

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ABSTRACT:

Pigmentation has attracted human beings from time immemorial. Various pigmentations and their orientation are a matter of curiosity regarding their occurrence, metabolism as well as functional properties. Several issues has been discussed and referred as cited by earlier workers on the diversity of piscian and amphibian species showing magnificent colouration, their chemistry, physiology as well as metabolic transformation and more, especially on the existence of different retinoid molecules. Animals use different types of pigments to acquire their colourful ornaments. Knowing the types of pigments that generate animal colours often provide valuable information about the costs of developing bright coloration as well as the benefits of using these signals in social or sexual contexts. Within the various classes of natural pigments, the carotenoids are the most widespread and structurally diverse pigmenting agents. Many animals use carotenoid pigments to colour their integument red, orange, or yellow (Fox, 1976). Carotenoids are a family of over 600 natural lipid-soluble pigments that are produced within microalgae, phytoplankton, and higher plants. Thus to study the carotenoid profile, *R.bipunctatus,* a small brilliantly coloured tree frog, was selected and collected. The carotenoids profile from the entire skin was analysed through HPLC. Carotenoids such as astaxanthin, β-carotene, cryptoxanthin, lutein, zeaxanthin were present in the amphibian species of the present study.

Keywords:

Carotenoids, pigmentation, ß carotene, lutein.

Article Citation:
Pinky Baruah and Goswai UC.
Characterization of carotenoid pigments in amphibian, *Rhacophorous bipunctatus*.
Journal of research in Biology (2012) 2: 114-118

Dates:
Received: 31 Jan 2012 /**Accepted:** 06 Feb 2012 /**Published:** 20 Feb 2012

Journal of Research in Biology

INTRODUCTION

Many animals use carotenoid pigments to colour their integument red, orange, or yellow (Fox, 1976). These carotenoid-based colours are often more pronounced in males and serve as important sexually selected traits that signal mate quality (Olson and Owens, 1998; Hill 1999; Møller, *et al*., 2000; McGraw, Hill, Stradi, Parker, 2001, 2002). Evolutionary biologists have expressed recent interest in identifying the carotenoid-derived nature of animal colours, as knowing the specific type of ornamental colour is important for determining the signalling information contained within showy sexual displays (Owens and Hartley, 1999; McGraw and Hill, 2000, 2001; Grether, *et al*., 2001).

Carotenoids are pigments that are found in plants and micro-organisms but are not synthesized in animals. They are synthesized through the isoprenoid pathway which also produces such diverse compounds as essential fatty acids, steroids, sterols, vitamins A, D, E, and K. Within the various classes of natural pigments, the carotenoids are the most widespread and structurally diverse pigmenting agents. They are responsible, in combination with proteins, for many of the brilliant yellow to red colours in plants and the wide range of blue, green, purple, brown and reddish colours of fish and crustaceans.

Thus, biochemical tests are needed to unequivocally identify pigments as carotenoids in animal tissues.

MATERIALS AND METHODS

Collection of specimen:

Rhacophorus bipunctatus were collected from Mizoram. The specimens were brought live in glass bottles with perforated lids.

Solvents and chemicals :

Light petroleum ether (b.p. 40-60 °C), Diethyl ether, Chloroform, Absolute ethanol, Anhydrous sodium sulphate, Aluminium oxide active neutral LR for chromatographic absorption analysis, were procured from BDH, Laboratory Chemicals Division, Glaxo Laboratories (India) Pvt. Ltd. Alumina was deactivated, prior to use, by adding the requisite amount of water (usually 5-8 % v/w) in a glass mortar. It was then mixed thoroughly. Different authentic retinoids samples, such as retinol, dehydroretinol, retinyl propionate β-carotene, lutein, cryptoxanthin, astaxanthin, β-apocarotenals and CAEE samples were supplied by F.Hoffman La Roche, Basel, Switzerland.

UV-vis spectrum:

The UV-visible spectra were recorded in a Beckman DK-2 spectrophotometer. β-carotene, lutein, anhydrolutein and 3-dehydroretinol were assayed on the basis of the following $E^{1\%/}{}_{1cm}$ values: 2500, 2200, 2031 and 1455 respectively (all at the point of maximum absorption).

The carotenoids extracted were estimated using HPLC technique. The HPLC system includes (water) with column 300 mm × 3.9 mm Nova - Pak C18 (4mm) and a Guard - Pak precolumn module (water 5) were used. Standard retinoids samples (5.0 mg) were dissolved in 100 ml tolune : methanol. Standard retinoids samples (5.0 mg) were dissolved in 100 ml tolune : methanol (1:1) containing 500 mg BHT (butylated hydroxytolune) / litre for producing 50 m/ml standards. These standard stock solutions could be preserved at -20° C for 4 months. HPLC grade solvents, acetonitrile : dichloromethane : methanol : water : propionic acid (71:22:4:2:1, v/v) were used as mobile phase with the flow rate of 1.0 ml/minute in the first 10 minute run, detection of carotenoid pigments was performed at 450 nm and retinol in 352 and 326 nm.

RESULTS

R.bipunctatus is a small tree frog with a pointed snout and body length of about 37-60 mm when adult, females being larger than males. Its back is intensely green to violet-brown in living animals. The arms and legs have very faint darker bands. The sides, belly and toes are brilliant yellow. Behind the arms, there is almost always a conspicuous large black spot on the flanks; towards the hind legs there may be another one or two such spots, but very rarely the flank spots are entirely absent. The well-developed webbing of the toes is bright orange-red and unspotted. The eyes are dull green, sometimes with a yellow rim.

Extraction and estimation of carotenoids from *Rhacophorous bipunctatus*

The carotenoids pigments are extracted from the dorsal and ventral skin separately. The pigments containing the lipids were extracted from freshly killed specimen. The skins were grounded with anhydrous sodium sulphate separately. Lipid solvents such as acetone, light petroleum, benzene, diethyl ether, etc. were used for the extraction of various pigments. The extractions containing the carotenoids were pooled together and evaporated and were estimated from their UV-visible absorption maxima and from the $E^{1\%}/_{1cm}$ value of the respective compounds. The esters were saponified with methanolic KOH (10%) under refluxed conditions and were later extracted with light petroleum (40-60^0 C). Alkali was removed after 5-7 washings with distilled water. It was again extracted with light petroleum 40-60^{0}C and dried over anhydrous Na_2SO_4 and measured for alcoholic form. The different carotenoids and vitamin A analogues were estimated.

The authentic carotenoids samples were prepared and a standard HPLC chromatogram for each carotenoid was standardized as shown in the **Figure 1.** The details of the solvents used and the elution profiles have been described earlier.

Carotenoids profiles in adult *Rhacophorous bipunctatus:*

The magnificent multicolored skin of *Rhacophorous bipunctatus* is of great attraction for naturalists. The green coloured dorsal skins and yellow ventral skins give rise to several questions on the mode

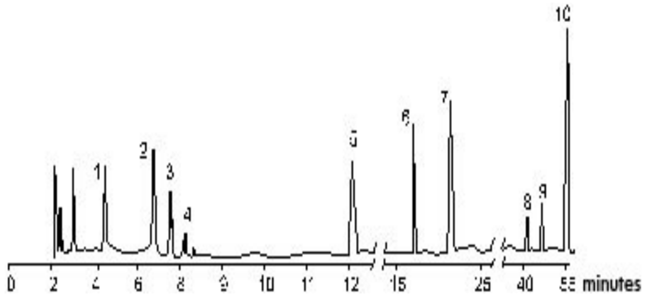

Fig. 1: Chromatogram of the standard carotenoids ($2.0\ \mu g.ml^{-1}$).
List of carotenoids from investigated specimens :
(1) Astaxanthin; (2) Isozeaxanthin; (3) Phoenicoxanthin; (4) Zeaxanthin; (5)CAEE; (6)Cryptoxanthin; Lutein; (8) Echinenone; (9) α-carotene; (10) β-carotene.

of deposition of carotenoids as well as its metabolism. In the present studies 2 samples (25, 28 g wt., males) were collected from Mizoram and both dorsal as well as ventral regions were dissected out and their lipid contents were extracted as described. The carotenoids profile and the chromatograms have been shown the **Table 1,** and in figures **(Fig. 2 and 3).**

DISCUSSIONS

In amphibians, carotenoids (including both β-carotene and lutein) are widely distributed in the body. The amphibian species can be studied through the life cycle, as embryos, as tadpoles and as immature and mature frogs; there is a marked seasonal cycle in both

Table 1 : Carotenoids profiles (µg/ 100 g) of skins of *Rhacophorous bipunctatus*. The values are the mean of 2 samples are given in the table.

Carotenoids	Dorsal skins	Ventral skins
	Amount (µg/ 100 g)	Amount (µg/ 100 g)
Astaxanthin	35 ± 2.5	25 ± 1.5
β-carotene	60 ± 1.5	40 ± 0.5
Cryptoxanthin	65 ± 3.5	70 ± 0.5
Lutein	75 ± 5.5	78 ± 0.5
Zeaxanthin	55 ± 2.5	30 ± 1.5
Others	110 ± 10.5	85 ± 0.5
CAEE	97 ± 1.5	94 ± 1.5

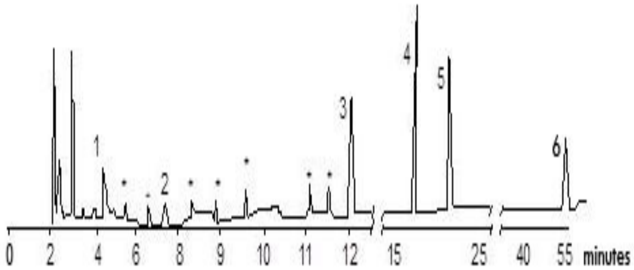

Fig 2 : Chromatogram of *Rhacophorous bipunctatus.* Dorsal skin : 1. Astaxanthin; 2. Zeaxanthin; 3. CAEE; 4. Cryptoxanthin; 5. Lutein; 6. β-carotene; * not identified.

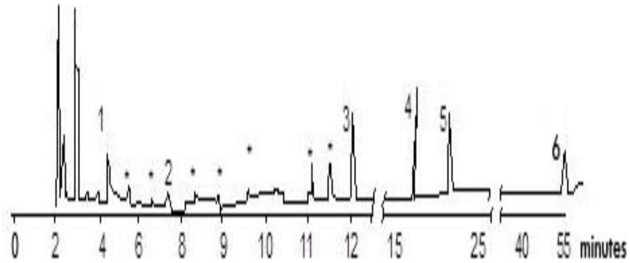

Fig. 3 : Chromatogram of *Rhacophorous bipunctatus.* Ventral skin : 1. Astaxanthin; 2. Zeaxanthin; 3. CAEE; 4. Cryptoxanthin; 5. Lutein; 6. β-carotene; * not identified.

sexes and the reproductive phase entails a heavy drain on body reserves, especially in the female. Moreover, as an amphibian, the frog provides a link between work on mammals and fishes. There are, therefore, numerous factors potentially capable of correlation with data on the distribution of carotenoids so as to lead to hypotheses concerning the functions of these substances.

Rhacophorous bipunctatus is an interesting amphibian, where two different patches of coloration showing variation of pigmentation in both dorsal and ventral region of the skin are found. In the present study, several carotenoids molecules were detected and showed the occurrence of principal forms of pro-vitamin A. It is interesting to note that astaxanthin is present throughout their life. It has been discussed and referred that astaxanthin molecules are present stating from several species of invertebrates and continuing its occurrence up to the human species (Goodwin, 1984). Astaxanthin is metabolized into dehydroretinol and retinol. The perfect metabolic stage is not known, where the maximum amount of astaxanthin is utilized for its transformation

Rhacophorous bipanctatus

into either dehydroretinol or retinol. Further it has been shown (Goswami, 1984; Goswami and Sharma, 2005; Goswami, 2007) that the metabolic transformation of dehydroretinol provided several other metabolites such as 3-hydroxyretinol, 3-hydroxyanhydroretinol etc. Several studies on the metabolism of astaxanthin have been perused in several species of fish and salmonids in particular.

β-carotene is present in low to moderate concentration, which provides low to medium intensities of yellowish-greenish colouration. Lutein and cryptoxanthin are interesting pigments. Cryptoxanthin is converted into both retinol and dehydroretinol (Goswami, 1984) and it has been an effective precursor in higher animals (Goswami and Sharma, 2005). It is interesting to note that lutein is not an effective precursor of retinol, whereas it could be converted into dehydroretinol through 3-hydroxyretinol, 3-hydroxyanhydroretinol (Goswami, 2006; 2007; 2011).

Yellowish skin showed more lutein and cryptoxanthin, and less astaxanthin. The food habit of the animal is an indicator of such coloration. It is definite that certain animals consume more such food particles which are absorbed and assimilated in their body. Lutein and cryptoxanthin accumulation might provide more synthesis of dehydrotetinol as well as more porphyropsin or its corresponding aldehyde such as dehydroretinal. All these require several attempts to clarify these queries.

ACKNOWLEDGEMENT

I offer my sincere thanks to the Director, Regional Forensic Science Institute, Kahilipara, Assam, for allowing me to conduct several experiments and some of the critical evaluation of certain parts of the experiments.

Thanks are also due to the Analytical Nutritional Chemistry Division of National Institute of Nutrition, (ICMR), Hyderabad and Indian Institute of Chemical Technology, CSIR, Hyderabad, for different HPLC works entitled in the present work.

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