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CHARACTERIZATION OF SOME NATURAL AND SEMI-SYNTHETIC BETAXANTHINS

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Key Word Index—Betalain; betaxanthin; spectroscopic characteristics.

Abstract—Betalamic acid was conjugated with a variety of amino acids, both protein and non-protein, to yield a series of semi-synthetic betaxanthin standards. Retention characteristics for reverse phase-ion pair HPLC chromatography and optical properties are described for these standards.

INTRODUCTION

Betaxanthins are the conjugation products of betalamic acid with any amino acid (both protein or non-protein) or amine [1], although only a few have been described as occurring naturally [2-10]. Indicaxanthin, the first betaxanthin to be described [4], was extracted from the fruit of *Opuntia ficus-indica* and was characterized by a λ_{max} at 485 nm in water and a logarithmic molar extinction coefficient of 4.63 (ε_{485} = 42650). Our survey of the relevant literature indicated a lack of information concerning the chromatographic (HPLC) and spectral properties of the betaxanthins. We describe here the retention characteristics for some semi-synthetic betaxanthin standards subjected to reverse phase-ion pair HPLC and some relevant spectral properties (λ_{max} and ε values).

RESULTS AND DISCUSSION

Various methods have been proposed for the separation (purification) and identification of the betalains. The amphoteric nature of these pigments has been used to achieve separation by high tension paper electrophoresis [11], for the purpose of purification prior to structure analysis [12] and for betalain identification [13]. Preliminary separation by ion-exchange followed by purification on either polyamide [4, 13] or Sephadex [2] gels has also been proposed. HPLC as a tool for the analysis of betalain pigments was first proposed in ref. [14] and subsequently in refs [15,16]. Nevertheless, only four well documented pigments (betanin, vulgaxanthin I and II, indicaxanthin) were identified by this method.

Data for a range of natural and semi-synthetic betaxanthins are given in Table 1. According to the literature, 15 naturally occurring betaxanthins have been identified, the trivial names of which are presented in Table 1. Of the three betaxanthins present in *Amanita muscaria*, only muscaurin VII is listed. Two new pigments extracted from petals of *Portulaca grandiflora* are also listed [10]. Betalamic acid, the chromophore, and betanin, the best known member of the betacyanin family are also included in the list. In total, eight of the 15 listed pigments contain non-protein amino acids.

The molar extinction coefficient at λ= 480 nm is calculated using the following procedure. Eluting pigments (of known absorption at 480 nm) were acid hydrolysed (6 M HC1, 22 hr at 115°) and the amino acids liberated quantified according to the phenylisocyanate method [17]. The molar extinction coefficient *(ε)* was calculated according to the formula:

$$\text{molar extinction coefficient } \varepsilon_{480} = \frac{\text{betaxanthin absorbance at 480 nm}}{\text{amino acid concentration [M]}}$$

The values listed in Table 1 are the logarithm of the calculated values. Previous determinations of ε_{480} for indicaxanthin [4] differ by *ca* 17% from the values obtained using the method described here.

The R_t*s* of individual betaxanthins were only slightly altered when column packings from different batches were used, with their general order of elution being respected. Another stationary phase tested (OPTI-UP C12, Antec AG, CH-4431 Bennwil) showed similar properties regarding elution order. It was observed however, that the elution order of closely eluting peaks (i.e. those peaks eluting within the time ranges of 5.9, 8.4, 8.8-9.0, 13.7-13.9 min, Table 1) were occasionally inversed. Addition of the ion-pairing agent (triethylamine) to the mobile phase improved pigment separation, especially with early eluting signals (<7.0 min).

EXPERIMENTAL

Preparation of betaxanthin standards. Crystallized betanin and semi-synthetic or natural (gift from Dr M. Piattelli) indicaxanthin were hydrolysed in 1.5 M NH_4 . Within 10 min, the violet and deep yellow pigments, respectively, changed to a pale yellow colour (λ_{max} = 424 nm). After addition of a 10- to 25-fold molar excess of the desired amino acid, the solution was adjusted to pH 6 with HC1. The λ_{max} of this new state was around 480 nm. After lyophilization, the samples were resuspended in H_2O and analysed by reverse phase HPLC as described below. Each amino acid and amine tested gave rise to a single deep yellow

Table 1. HPLC and optical data for a range of partially synthesized betaxanthin standards, betanin and betalamic acid

Trivial name	Amino acid	R_f		Log molar extinction coefficient	Ref.
None*	Ser	5.3	472	4.69	
None	Asn	5.9	474	4.71	
None	Lys	5.9	472	4.63	
Muscaurin VII	His	5.95	476	4.71	[3]
Portulacaxanthin I	Hyp	6.6	484	4.70	[8]
Miraxanthin II	Asp	6.8	472	4.68	[6]
None	Cystin	8.2	476	. 4.65	
Vulgaxanthin I	Gln	8.4	474	4.67	[5]
Portulacaxanthin III	Gly	8.4	470	4.59	[10]
None	Arg	8.8	474	4.69	
None	Thr	8.9	474	4.62	
Miraxanthin I	MetSO	8.9	468	4.70	[6]
Vulgaxanthin II	Glu	9.0	474	4.68	[5]
None	Ala	9.9	470	4.70	
Betalamic acid	—	10.0	424	4.38 (ε_{424})	
None	Cys	10.2	476	4.65	
None	aAA	10.8	480	—	
Indicaxanthin	Pro	11.1	484	4.70 (4.63)	[4]
Humilixanthin	Norval	11.8	480	—	[9]
Dopaxanthin	DOPA	12.3	476	—	[7]
Betanin	CDG	12.7	536	4.75 (ε_{536})	
None	Val	13.7	472	4.70	
Portulacaxanthin II	Tyr	13.8	472	4.68	[10]
None	Met	13.9	474	4.66	
None	He	17.3	474	4.64	
None	Leu	17.6	472	4.70	
Miraxanthin V	Dopamine	18.0	472	—	[6]
None	Phe	18.9	476	—	
None	Trp	19.3	476	4.40	
Miraxanthin III	Tyramine	20.6	—	—	[6]

*Not naturally occurring; aAA = a-amino adipic acid; CDG = cyclo-DOPA-glucosyled.

pigment of characteristic R_f. All amines, including Tris buffer [tris(hydroxymethyl)-aminomethane, not listed], gave rise to compounds having the absorption spectrum of the betaxanthins. Partially synthesized betaxanthin derivatives exhibited HPLC *R,s* and absorption spectra identical to those of their natural analogues.

Preparation of betanin and indicaxanthin. Betanin, for use in the synthesis of betaxanthin standards, was prepd from the roots of red beet *(Beta vulgaris* var. Feuerball). Beet roots were homogenized in a juice mill and the resulting juice adjusted to pH 3 with glacial HOAc prior to concn (x 5) under red. pres. at 30° using a rotatory evaporator. The acidified homogenate was fractionated by reversed phase (RP) CC (6.5 x 2.5 cm, OPTI-UP C12 gel) using an aq. HOAc soln (1 mM) with a flow rate of 4.5 ml min^{-1}. Magenta coloured betanin-containing frs were collected, pooled, dried and redissolved in 0.5 ml H_2O prior to crystallization in 75% EtOH acidified with glacial HOAc (10 μl l^{-1}) at - 20°. Betanin crystals were > 97% pure as demonstrated by HPLC analysis.

Betanin crystals were hydrolysed by dissolution in aq. NH_3 soln (1.5 M) and incubated for 30 min at room temp. Pure amino acids were added in 10-25 molar excess to the hydrolysate to give the desired betaxanthin derivatives, agitated gently for 15 min, dried under red. pres. and resuspended in H_2O. Indicaxanthin prepd by this method was purified by RP CC (6.5 x 2.5 cm, OPTI-gel) using aq. HOAc (100 mM) as the eluant. Frs containing the deep yellow indicaxanthin pigment were collected, pooled and dried under red. pres. Indicaxanthin prepd by this method was determined to be >98% pure by HPLC analysis.

HPLC analysis of betalain pigments. The HPLC system (Spectra Physics) was as follows: an SP8750 organiser equipped with a ternary valve, an SP8700 XR ternary controller, a static mixer, a Rheodyne automatic injector fitted with an injection loop (20 μl), a guard column (20 x 4.6 mm i.d.) containing LiChroprep RP18 gel and a steel column (250 x 4.6 mm i.d.) (Hyperchrome, Bischoff) packed with Hypersil ODS RP18 on a 3 μm spherical support (Shandon). Eluent analysis was performed using a model HP 1040 diode array spectrophotometer (Hewlett Packard). Detection was routinely made at 280,424,480 and 536 nm. The HPLC system was used in conjunction with an HP Chemstation (Hewlett Packard) for determination of individual peak areas and data processing.

Mobile phases were prepd using HPLC grade H_2O. Separation was achieved by stepwise gradient elution. All gradient steps were evolved linearly with respect to time. Development was achieved in 30 min using the following gradient program: 100% solvent A (50 mM NaH_2PO_4,2.5 mM Et_3N, pH adjusted to 4.20

with H_3PO_4) and 0% solvent B (40% v/v MeCN) at time zero and stepped to 70% A: 30% B within 15 min; 40% A: 60% B in a further 5 min; 20% A:80% B in a further 5 min followed by isocratic elution of 20%A:80%B for a further 5 min. The system was operated at a flow rate of 1.0 ml min^{-1}.

Molar extinction coefficient (e) *determination,* e was determined indirectly by estimation of the level of free amino acids resulting from the alkaline hydrolysis [17] of betaxanthin solns (prepd using semi-synthetic, HPLC purified betaxanthin standards) of known absorbance (480 nm).

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