Dyes and Pigments 133 (2016) 458–466

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

Fluorescent bioinspired protein labeling with betalamic acid. Derivatization and characterization of novel protein-betaxanthins

Juana Cabanes, Fernando Gandía-Herrero, Josefa Escribano, Francisco García-Carmona, Mercedes Jiménez-Atiénzar*

Departamento de Bioquímica y Biología Molecular A, Unidad Docente de Biología, Facultad de Veterinaria, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain

ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form
6 June 2016
Accepted 22 June 2016
Available online 23 June 2016

Keywords:
Betalains
Betalamic acid
Bioinspired
Fluorescence
Labeling
Pigments

ABSTRACT

Betaxanthins are the water-soluble pigments that bestow yellow coloration to fruits, flowers and roots of plants of the Caryophyllales order and present autofluorescence after excitation with blue light. In this work, the semi-synthesis of betaxanthins derived from macromolecules is achieved for the first time by exploiting the reactivity of amine groups belonging to proteins. The synthesis of protein-betaxanthins is demonstrated by spectrophotometry and HPLC-ESI-TOF-MS mass analysis. The derivatization with betalamic acid was in a ratio 1:1 and yielded protein-betaxanthins yellow in color that exhibited fluorescent properties with a maximum excitation wavelength of 476 nm and a maximum emission wavelength of 551 nm. Moreover, staining can be started from purified betalamic acid or directly from raw red beet root extracts. The novel bioinspired labeling reaction allowed protein detection in conventional fluorescence scanning and imaging systems and opens a new perspective for betalamic acid derived molecules as fluorescent probes with multiple biological applications.

1. Introduction

Betalains are nitrogen-containing natural pigments that provide bright coloration to fruits, flowers, and roots of plants of the Caryophyllales order. They are divided into two groups: violet betacyanins, with absorbance spectra centered at wavelengths around $\lambda m = 536$ nm, and yellow betaxanthins, with absorbance spectra centered at wavelengths around $\lambda m = 480$ nm. Both groups share betalamic acid as their structural and chromophoric unit, which is condensed with *cyclo*-DOPA in the betacyanins and with amines and amino acids in the betaxanthins (Fig. 1) [1]. Betalains fulfill the role played by anthocyanins in other plants, and the two families are mutually exclusive [2].

Among the edible sources of betalains, the roots of red beet (*Beta vulgaris*) and the fruits of the cactus *Opuntia ficus-indica* are especially relevant in human diet [3–5]. The betalain-containing extracts from the roots of *B. vulgaris* are used by the food industry as a natural colorant, code 73.40 in the 21 CFR section of the Food and Drug Administration (FDA) in the USA, and code E-162 in the European Union [6–8]. Betalains are also present in nonedible parts of plants, such as bracts, stems, leaves, and flowers [9–12]. The presence of betalains in the latter is of particular importance due to the formation of colored and fluorescent patterns and their possible role in attracting animals for pollination [13]. Flowers are bright violet or yellow in coloration depending on the presence of betacyanins or betaxanthins, respectively.

Betalains are water-soluble and possess high antioxidant and free radical scavenging activities that have been described for plant extracts and purified pigments [14]. These activities support the recently discovered chemopreventive potential of betalains against different types of cancer [15–18].

Other applications of individual betalains come from their use in dye-sensitized solar cells for solar energy conversion due to their redox capacity to transfer electrons. The use of pure pigments yields energy conversion efficiencies of up to 2.7%, above that of natural photosynthesis [19,20].

Physicochemical properties of betalains have been extensively described in the literature, with special attention to their stability and color. However, fluorescent properties of betaxanthins have been recently discovered [13]. Betaxanthins exhibit spectra with excitation maxima between 463 and 475 nm and emission maxima

* Corresponding author. Dpto. Bioquímica y Biología Molecular A, Unidad Docente de Biología, Edificio Facultad de Veterinaria, Universidad de Murcia, E-30100, Espinardo, Murcia, Spain.
E-mail address: mja@um.es (M. Jiménez-Atiénzar).

http://dx.doi.org/10.1016/j.dyepig.2016.06.037
0143-7208/

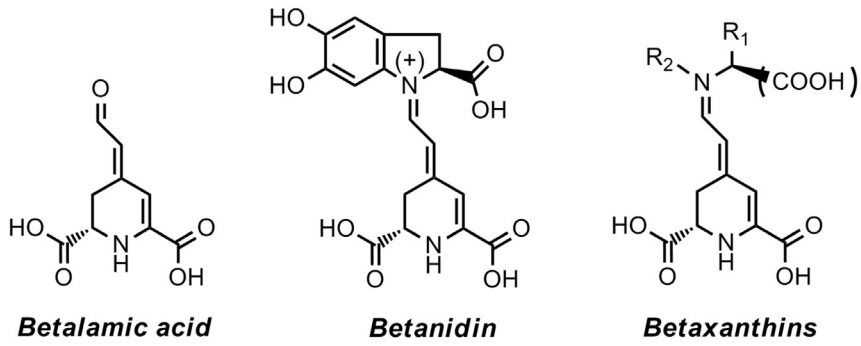

Fig. 1. The structural unit of betacyanins (betanidin) is shown together with a general structure for betaxanthins and betalamic acid.

between 548 and 554 nm. Thus, betaxanthins are able to absorb blue light and emit green light. Emission of visible light by betaxanthins is maintained when they are present in the physiological environment, inside the petal cells. This has opened up new possibilities for the study of signalling between flowers and pollinators [13,21]. Other potential applications of betalains are their use in microscopy as a new probe for live cell imaging [13,22] and as a sensor for colorimetric assays [23].

Structurally, betaxanthins result from the condensation between the aldehyde group of betalamic acid and an amino group of an amine or amino acid. Despite the increasing interest in these molecules, there are no references in the literature of betaxanthins obtained by condensation between the aldehyde group of betalamic acid and an amine group belonging to a protein. Considering this fact, this work is aimed at exploring the generation of betaxanthins from proteins, with the amine group, therefore, being provided by the protein. Positive results have been applied to the detection of multiple proteins in electrophoresis gels by reaction of betalamic acid with the proteins present in the gel. Thus, protein-betaxanthins were synthesized *in situ* and then visualized thanks to their fluorescent properties. The results presented in this work show a new utility of betaxanthins as fluorescent probes with multiple biological applications.

2. Experimental section

2.1. Chemicals

Red beet juice concentrate (B-50-WS) was purchased from CHR Hansen (Madrid, Spain). It is a liquid formulation obtained by squeezing out, concentrating and pasteurizing the juice of beetroots, *Beta vulgaris*. The anion exchange matrix Lewatit Amberlite IRA-400 was obtained from Sigma (Madrid, Spain). Wide range molecular weight markers were also purchased from Sigma. The centrifuge filters Amicon Ultra-15 centrifugal 10 K were from Millipore (Bedford, MA, USA). Solvents were from Merck (Madrid, Spain). HPLC-grade acetonitrile was purchased from Labscan Ltd. (Dublin, Ireland). Other chemicals and reagents were obtained from Sigma. Distilled water was purified using a Milli-Q system from Millipore.

2.2. Betalamic acid purification

Red beet juice concentrate was filtered by a 10 kDa ultra filtration step (QuixStand System, General Electric Healthcare, Milwaukee, WI, USA). Betanin purified from this filtered solution was used as starting material. Basic hydrolysis (pH 11.4) of betanin (10 mL) released betalamic acid, which was then allowed to interact with the anion exchange resin Amberlite IRA-400 (8.2 g) for 15 min and then centrifuged at 5000 g. After removing the supernatant and washing the beads with water until neutral pH was reached, the betalamic acid bound to the matrix was eluted with NaCl 5 M. After elution, a C18 solid phase extraction step was performed to remove salts from the eluted acid.

2.3. Semi-synthesis of betalamic acid derivatives

2.3.1. Generation of protein-betaxanthin

Betaxanthins were obtained as immonium condensation products of betalamic acid with amines present in proteins. The labeling of proteins with betalamic acid was carried out according to a previous methodology with some modifications [24]. In short, purified betalamic acid (0.4 mM) at basic pH (approximately 11.4) was added to the protein (0.2 mM). The synthesis of betaxanthins derived from molecular weight markers (6500–200,000 Da) was carried out following the same procedure, by adding the betalamic acid directly to the commercial vial containing the markers. The corresponding betaxanthins were obtained by a condensation reaction between the betalamic acid and the proteins after reaching pH 5.0. The labeling of the protein was accompanied by a color change from pale yellow (betalamic acid, $\lambda_m = 424$ nm) to deep yellow (betaxanthins, $\lambda_m = 476$ nm). The whole process was carried out in a nitrogen atmosphere. The protein-betaxanthins synthesized were purified and concentrated by repeated washing with purified water by using centrifugal filters 10 K Amicon Ultra-15 (4.000 g, 20 min) until the not retained filtrate was colorless.

2.3.2. Semi-synthesis of lysine-betaxanthin

The pigment was obtained as the condensation product of lysine with betalamic acid obtained from *Beta vulgaris* roots. The process was carried out following a method described previously [24]. Briefly, red beet juice concentrate was filtered by a 10 kDa ultrafiltration step and the betanin from this filtered solution was used as starting material. Basic hydrolysis (pH 11.4) of betanin released betalamic acid, which was then condensed with lysine after reaching pH 5.0. The synthesis of lysine-betaxanthin occurred with the characteristic change of color (lysine-betaxanthin, $\lambda_m = 472$ nm). For pigment purification, a C-18 solid phase extraction step was performed.

2.4. Generation of BSA-betaxanthin from beet root juice

Labeling was carried out according to the method described above to synthesize protein-betaxanthins but using commercial beet root concentrate instead of purified betalamic acid. In short, red beet juice concentrate was only filtered, to remove proteins, as described, and then BSA (125 mg/mL) was added to this filtered solution prior to basic hydrolysis. Betalamic acid, released after