Fluorescent Bioinspired Protein Labeling with Betalamic Acid. Derivatization and Characterization of Novel Protein-Betaxanthins.

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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.

Keywords: Betalains, Betalamic acid, Bioinspired, Fluorescence, Labeling, Pigments

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 λm = 536 nm, and yellow betaxanthins, with absorbance spectra centered at wavelengths around λm = 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 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 10K 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 5.000g. 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 5M. 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 (6,500-200,000 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, λ_m = 424 nm) to deep yellow (betaxanthins, λ_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 10K Amicon Ultra-15 (4.000g, 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, λ_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 hydrolysis, was then condensed with the protein, after reaching pH 5.0. Therefore, this process did not use any anion exchange resin to purify the betalamic acid, which was liberated after the basic hydrolysis of the betanin contained in the beet root concentrate.

2.5. C-18 Solid Phase Extraction

C-18 cartridges (35 mL, Waters, Milford, MA, USA) were conditioned with 70 mL of ethanol followed by 70 mL of purified water. Aqueous solutions of purified betalamic acid and lysine-betaxanthin were injected and bound to the column. Salts and buffers were washed off by rinsing the column with purified water. Pigments were eluted with ethanol, and the resulting fraction was evaporated to dryness under reduced pressure at room temperature. The residue was redissolved in water for further use or stored at -80 ºC.

2.6. HPLC-DAD Analysis

A Shimadzu LC-20AD apparatus (Kyoto, Japan) equipped with a SPD-M20A photodiode array detector (PDA) was used for analytical HPLC separations. Reversed phase chromatography was performed with a 250 × 4.6 mm Luna C-18(2) column packed with 5 µm particles (Phenomenex, Torrance, CA, USA). Gradients were formed with two solvents, A and B. Solvent A was H_2O with 0.05% trifluoroacetic acid (TFA); solvent B was acetonitrile with 0.05% TFA. A linear gradient was performed from 5% B to 35% B for 35 min. The flow rate was 1 mL min^{-1}, operated at 25º C. Elutions were followed at λ = 405 (betalamic acid), 480 (betaxanthins), and 536 nm (betacyanins). Injection volume was 20 µL.

2.7. Electrospray Ionization Mass Spectrometry

A VL 1100 apparatus with LC/MSD Trap (Agilent technologies, Palo Alto, CA) was used for HPLC-ESI-MS-MS analysis. Elution conditions were as described above using the same column (250 × 4.6 mm Luna C-18(2)) and decreasing the flow rate to 0.8 mL min^{-1}. Vaporizer temperature was 350 ºC, and voltage was maintained at 3.5 kV. The sheath gas was nitrogen, operated at a pressure of 45 psi. Samples were ionized in positive mode. Ion monitoring mode was full scan in the range *m/z* 60-600. The electron multiplier voltage for detection was 1350 V.

2.8. HPLC-Electrospray Ionization Time of Flight Mass Spectrometry

This technique (HPLC-ESI-TOF-MS) has been used to determine with precision the absolute molecular weight of the starting proteins and the labeled protein-betaxanthins. The experiments were carried out with an HPLC Agilent Series 1100 and an Agilent 5100 Series time of flight mass spectrophotometer equipped with an electrospray ionization source. The column used, (Zorbax 300SB-C18 Poroshell, 1x75mm, 5 µm), was appropriate for large biomolecules, such as proteins. The sample was injected into the column and eluted with a gradient of acetonitrile. Samples were ionized in positive mode. The major peak was analyzed by MS and the mass spectrum obtained was analyzed by using Agilent MassHunter Software vB.03.01. External calibration of the spectrophotometer was performed with cytochrome C as standard protein. Analyses were carried out in duplicate.

2.9. Spectroscopy

2.9.1. Absorbance spectroscopy

A Jasco V-630 spectrophotometer (Jasco Corporation, Tokyo, Japan), attached to a Tectron thermostatic bath (JP Selecta, Barcelona, Spain) was used for absorbance

spectroscopy. For the quantification of betalains, pigment concentration was evaluated taking molar extinction coefficients of ε = 65,000 $M^{-1}cm^{-1}$ at 536 nm for betanin [25]. Molar extinction coefficients of lysine-betaxanthin and BSA-betaxanthin were determined by an end point method, carrying out a set of basic hydrolysis experiments [14]. Briefly, spectra for solutions of each pigment were recorded and then submitted to hydrolysis using 1.2 M ammonia. The resulting betalamic acid solution was compared with that obtained from betanin solutions of known concentrations, and the initial pigment concentration and the molar extinction coefficient at the corresponding maximum wavelength were calculated. Measurements were made in water at 25º C.

2.9.2. Fluorescence spectroscopy

A Hitachi F-4500 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan), attached to an Ultraterm 200 thermostatic bath (JP Selecta, Barcelona, Spain) was used for fluorescence spectroscopy. Quartz cuvettes were used, and samples were diluted in water, with the final concentration of the pigment being 1 µM for BSA-betaxanthin and 3 µM for lysine-betaxanthin. Excitation and emission spectra were recorded at 25 ºC, and excitation and emission slit was set at 5 nm. Alanine derived betaxanthin at the same concentration was used as standard for the determination of relative fluorescence intensity. Excitation spectra were recorded through the emission at the maximum emission wavelength. Emission spectra were obtained by exciting at the corresponding maximum wavelength.

2.10. Electrophoresis

2.10.1. Denaturing SDS-PAGE

Electrophoresis of non-derivatized proteins was carried out using the method of Laemli [26]. Samples were applied to 10% polyacrylamide gels. The samples were

prepared by 1: 4 dilution with denaturing loading buffer and heated for 5 min at 100 ° C. The slab gels of 1.5 mm thickness were run in a Miniprotean II cell (Bio Rad) at a constant current of 180 mV. Gels were stained for protein using a standard Comassie Blue method or prepared for protein-betaxanthins labeling as described below.

2.10.2. Partially Denaturing SDS-PAGE

Derivatized proteins (proteins-betaxanthins) were submitted to partially denaturing electrophoresis. The SDS-PAGE was performed as described above, but in the absence of β-mercaptoethanol and without heating, to avoid betaxanthin degradation.

After electrophoresis, gels containing protein-betaxanthins were examined with the fluorescence scanner below with no further treatment.

2.11. Fluorescent detection of proteins after electrophoresis separation

After electrophoresis under denaturing conditions, gels containing non-derivatized proteins were incubated 15 min with water at basic pH (pH=11.4). Then, gels were incubated for 15 min with purified betalamic acid (0.5 mM) at basic pH. Acetic acid was added to reach a pH value of 5.0. Gels were incubated at this acidic pH for another 15 min. Finally, gels were washed with water to remove the betalamic acid not bound to the proteins. Gels containing the protein-betaxanthins synthesized, *in situ*, were analyzed with a Typhoon 9410 fluorescence scanner (GE Healthcare, Amersham Biosciences, Germany) equipped with an analysis program ImageQuant TL. Gels were routinely scanned with 488 nm laser (excitation laser Blue 2), and emission filter of 526 nm (526SP filter). This laser and filter combination was selected to fit betaxanthins fluorescence spectra, although the emission filter of 520 nm (520BP40, ECL+) could also be used, obtaining gel images of good quality. Alternatively, gels were photographed with an ImageQuant LAS 500 system, a cooled CCD imager for

chemiluminescence, colorimetric and fluorescence image capture. To measure fluorescence, combined Blue-Epi (460 nm) and UV- Epi (365 nm) lights were used simultaneously for excitation. An orange emission filter was needed for fluorescence emissions over 560 nm. Those proteins labeled in solution and later run in electrophoresis gels were detected under the same imaging systems.

A very similar protocol was followed for fluorescent staining of the gels with raw red beet root juice. Commercial red beet root concentrate was filtered to remove proteins and pH was raised to 11.4 to release betalamic acid from the betanin present in the juice extract. Gels were incubated for 15 min into this solution and pH was then lowered to 5.0 for the condensation reaction between the released betalamic and the proteins in the gel. The rest of the steps to wash and detect protein bands were exactly the same as those described above.

2.12. Protein determination

Protein concentration was determined according to the Bradford Bio-Rad (Hercules, CA, USA) protein assay using serum albumin as standard [27].

3. Results and discussion

3.1. Protein derivatization with Betalamic Acid

Betalamic acid is the structural unit of all betalains and can be used as the starting point to obtain betalains through a Schiff condensation reaction with free amino groups (betaxanthins) or indoline-containing structures (betacyanins) (Fig.1). At the moment, more than fifty betalains have been identified in natural sources. This number is constantly increased due to a continuous search in plant extracts and to a protocol for semi-synthesis and purification of natural and previously unconsidered betaxanthins [14,28]. The application of this procedure has allowed the production of structurally related betaxanthins and the in-depth exploration of the role of different structural features in betalain properties.

Despite the increasing number of natural and synthetic betalains, as far as we know there are no references in the literature of betaxanthins obtained by condensation between the aldehyde group of betalamic acid and an amino group belonging to a protein.

3.1.1. Generation of Protein-Betaxanthins

The semi-synthesis reaction was carried out according to a modification of the protocol for semi-synthesis of betaxanthins [24]. Betanin, purified from commercial red beet juice, was used as starting material and subjected to basic hydrolysis to give betalamic acid which was further purified by anion exchange chromatography in a novel batch process described in the Experimental section. Betalamic acid was then condensed with the protein BSA (bovine serum albumin), used here as a model, after reaching pH 5.0. The generation of the derivatized protein was seen first by the change in color from

pale yellow (betalamic acid, λm = 424 nm) to deep yellow (BSA-betaxanthin, λm = 476 nm), as confirmed by the absorbance spectrum (Fig. 2). Table 1 shows the characteristics of the absorbance spectrum of BSA-betaxanthin along with the value calculated for the molar absorption coefficient of the pigment. These results are the first ones obtained for a protein derivatized with betalamic acid, and are in accordance to those described for low molecular weight betaxanthins [14,29].

To verify the synthesis of the protein-betaxanthin, the labeled macromolecule was then analyzed by HPLC-ESI-TOF-MS, a technique that allows the accurate determination of the absolute molecular weight of a protein. The mass spectrum of BSA-betaxanthin showed two peaks with molecular masses of 66,442.08 Da and 66,652.36 Da and abundances of 50% each. The first peak corresponded to the protein bovine serum albumin and the second to the bounded protein to betalamic acid. The difference of mass between both peaks, 210.28 Da, is in agreement with the molecular mass of betalamic acid. Thus, the protein population was divided into two groups, the standard BSA and BSA linked to one molecule of betalamic acid.

In order to corroborate these results, the synthesis of betaxanthins was extended to other proteins such as ovalbumin and trypsin. These derivatized proteins were also analyzed HPLC-ESI-TOF-MS. Two peaks were also found in the mass spectrum of ovalbumin-betaxanthin with molecular masses of 44,455.96 Da and 44,665.95 Da and abundances of 50%. Analogous results were obtained for trypsin-betaxanthin; the mass spectrum showed two peaks with masses of 23,486.53 Da and 23,697.75 Da and abundances of 50%. For ovalbumin and trypsin, the difference of mass between peaks coincides with the mass of one molecule of betalamic acid. These findings confirmed the reaction of betalamic with the proteins and thus, the labeling of BSA, ovalbumin

and trypsin, generating the derived protein-betaxanthins. Moreover, the protein pigments obtained contained one molecule of betalamic acid bounded to one molecule of protein.

3.1.2. Semi-Synthesis of Lysine-Betaxanthin

In addition to the terminal amine group of the proteins, betalamic acid may react with other amine group of the side chain of the constituent amino acids of the protein. Among the amino acids with side amine groups, lysine is particularly abundant in proteins like bovine serum albumin. In fact, the content of lysine in this protein represents 10% of the total number of residues. For this reason, the semi-synthesis of lysine-betaxanthin was attempted in order to compare their properties with those of the protein betaxanthin.

Semi-synthesis of lysine-betaxanthin was started from betanin, purified from red beet juice concentrate. Basic hydrolysis of betanin released betalamic acid, whose aldehyde group was condensed with the amine group of lysine. Immediately after synthesis, lysine-betaxanthin was partially purified by solid phase extraction with a C-18 column. Next, the pigment was characterized by absorbance spectroscopy and HPLC-DAD analysis. The characteristics of the absorbance spectrum together with the value determined for the molar absorption coefficient are presented in Table 1, confirming the great similarity with the protein- betaxanthin.

The chromatogram obtained by HPLC is shown in Fig. 3. As can be seen, two major peaks appeared at t_R = 8.8 min (λ_m = 470) and 10.4 min (λ_m = 461). Electrospray ionization mass spectrometry analysis (HPLC-ESI-MS-MS) was applied to elucidate the pigment nature. The mass value determined for the parent ions of both compounds was

exactly the same (340 m/z). Since this value coincided with the corresponding protonated molecular ion ($[M + H]^+$) of lysine-betaxanthin, these two peaks have been attributed to two different forms of the betaxanthin derived from lysine. After fragmentation of the parent ions of both peaks, the most abundant daughter ions were those corresponding to mass values ($[M + H]^+$) of 296, 252 and 208 m/z, probably generated by the successive loss of the three carboxyl groups of lysine-betaxanthin. The two isomers corresponded to the two possible condensation products derived from the two amine groups available. Given that the analysis of the daughter ions obtained after fragmentation was very similar for both compounds, it was not possible to definitely assign a precise structure to either peaks. Since lysine has two amine groups, one α and one ε, the *in vitro* reaction of the amino acid with betalamic resulted in the formation of two adducts (inset in Fig. 3). A similar result was described in the analysis of pigments of plants belonging to the Amaranthaceae family, where lysine-betaxanthin was identified as one of the natural betalains present in petals of *Gomphrena globosa* L [30]. Its presence was also reported in the yellow inflorescences of the genus *Bougainvillea* (Nyctaginaceae) and in the Chenopodiaceae and Cactaceae families. The HPLC analysis of these plants extracts detected two peaks attributed to lysine-betaxanthin but their accurate structure was not elucidated [31]. Although the pigment has been identified in the natural sources mentioned, there are no previous data in the literature concerning its synthesis *in vitro* or the characterization of its most important properties.

3.2. Fluorescent Properties of Betalamic Acid Derivatives

The physicochemical properties of betalains, specially those related to their color, have been widely described in the literature. Thus, from the early work with these pigments [32,33] the absorbance spectra of different species and the limited effect of

various substituents on them have been characterized [34]. However, despite numerous studies on betalains, the existence of betaxanthins fluorescence has only recently been described and investigated [13,35,36]. Therefore, after synthesis of BSA-betaxanthin our next objective was to characterize the derivatized protein and to verify if it maintained the characteristics of betaxanthins, in particular, their fluorescent properties.

3.2.1. Fluorescence of BSA-betaxanthin

Fig. 4A shows the native fluorescence of BSA-betaxanthin in water. The protein-betaxanthin exhibited a strong fluorescence in the aqueous medium. Excitation spectrum was obtained by following the emission at the maximum emission wavelength determined in a previous experiment. The emission spectrum was obtained by exciting at the corresponding maximum wavelength. As shown in this figure, excitation and emission maxima were 476 and 551 nm, respectively (Table 1). Thus, BSA-betaxanthin absorbs light in the visible area of the electromagnetic spectrum corresponding to blue light and emits green light. Table 1 also presents the rest of the data concerning the fluorescence properties of this pigment: separation between both spectra maxima (Stokes shift), width for the spectra (at half the maximum intensity), as well as the relative fluorescence intensity, determined in relation to the standard alanine-betaxanthin. There is a great similarity between these data and the results found for low molecular weight betaxanthins [14,37] indicating that the common structural motif in all betaxanthins, betalamic acid, besides being responsible for the color of the pigments, is also involved in their fluorescence.

In order to cover the entire range of possible wavelengths for both excitation and emission, three-dimensional fluorescence spectra were recorded for BSA betaxanthin. Derived excitation-emission matrix fluorescence spectroscopic data are graphically

provided in Fig. 4B. These results contribute to the full characterization of the fluorescence characteristics of BSA-betaxanthin regarding the excitation/emission maxima as well as the shape of the spectra. Therefore, these results confirm that a protein-betaxanthin has been obtained for the first time and that the characteristics of this betalamic labeled protein are similar to those presented by low molecular weight betaxanthins, including their fluorescent properties.

3.2.2. *Fluorescence of Lysine-betaxanthin*

In this work, the native fluorescence of lysine-betaxanthin has also been characterized, by using an aqueous solution of the pigment for registration of the fluorescence spectrum (Fig. 4C). As shown in this figure, the pigment showed excitation and emission maxima at 474 and 554 nm, respectively (Table 1). Table 1 also presents the rest of the values for the fluorescence properties of lysine-betaxanthin: Stokes shift, width of the spectra and relative fluorescence intensity. As expected, there is a great similarity between these data and those found in the bibliography for other betaxanthins analyzed [14,37]. To conclude the fluorescence study of lysine-betaxanthin, three-dimensional spectra were performed (Fig. 4D), thereby obtaining complete information on possible excitation and emission wavelengths and on the shape of the spectra. The matrix with the results obtained was analogous to that exhibited by BSA-betaxanthin.

3.3. Visualization of Betalamic Acid Derivatized Proteins in Electrophoresis Gels

The synthesis of BSA-betaxanthin was finally followed by partially denaturing SDS-PAGE, as shown in Fig. 5A. In order to expand the number of labelled proteins and confirm the feasibility of the method, betaxanthins derived from commercial molecular

weight markers were obtained and its electrophoretic migration is shown in Fig. 5B. Protein samples were run in polyacrylamide gels in the presence of SDS but without the addition of bromophenol blue to preserve the yellow color of the betaxanthin and in the absence of β-mercaptoethanol in order to avoid possible pigment degradation. For the same reason, samples were not heated at 100 ºC. Once the electrophoresis was finished, the gels, without any further treatment, were analyzed with a fluorescence scanner. As seen in these figures, BSA-betaxanthin and betaxanthins derived from molecular weight markers were clearly detected. These results imply once again the synthesis of protein-betaxanthins and that the fluorescent properties of the pigments remain in polyacrylamide gels; besides, fluorescence intensity was proportional to the protein amount (Fig. 5C). From this figure it can also be concluded that betaxanthin synthesis is not restricted to a limited number of proteins, the reaction is general and valid for any protein, as shown by the synthesis of fluorescent molecular weight markers (Fig.5B).

Thus far, characterization of betaxanthins fluorescence has been made in water because the pigments are highly hydrophilic and because an aqueous environment is the expected physiological one. In aqueous solutions the pigments exhibit a strong fluorescence. However, these are not the optimal conditions for the expression of fluorescence, whose intensity rises in organic solvents or in water with increasing amounts of glycerol (results not shown). In fact, the results in Fig. 5 prove that betaxanthins maintain their fluorescence in an electrophoresis gel. Taking into consideration that vibration is restrained in the gel and this may affect the fluorescence yield, a higher fluorescence intensity of the protein-betaxanthin could be expected in the gel than in water. To check this hypothesis, a polyacrylamide polymerization reaction was triggered with ammonium persulfate and TEMED in a fluorimeter cuvette

containing BSA-betaxanthin, and fluorescence intensity was measured along the time of polymerization. Unexpectedly, there was a decrease of approximately 60% in the fluorescence values rather than the expected increment (results not shown). These negative findings are probably due to the fact that the free radicals, formed during polyacrylamide polymerization, would react with the protein-betaxanthin, provoking pigment degradation. However, when agarose was used instead of polyacrylamide without the addition of any radical molecule, the fluorescence of BSA-betaxanthin rose throughout gel solidification by up to 220% of the initial fluorescence, as shown in Fig. 6. The enhancement of the fluorescence of the protein-betaxanthins in the gel implies a better visualization.

3.4. Fluorescent Staining of Proteins after SDS-PAGE

The possibility of synthesizing, for the first time, protein-betaxanthins together with the results shown in Fig. 5, led us to generate a new application derived from this method of synthesis and labeling: its general use for protein detection in electrophoresis gels by fluorescent staining. Therefore, the next experiments aimed to synthesize betaxanthins from proteins previously run in electrophoresis, and later on the gel, to carry out the modification of these proteins. Non-derivatized proteins (molecular weight markers and BSA) were subjected to conventional denaturing SDS-PAGE and gels were then incubated with purified betalamic acid at basic pH. After lowering the pH value to 5.0, betalamic acid condensed with the proteins present in the gels. These were incubated at this acidic pH for 15 min and the excess of non-bounded betalamic was eliminated by successive washings with water. The resulting gels were examined with a Typhon 9410 fluorescence scanner (Fig. 7A) visualizing not only BSA but also all the proteins present in the commercial molecular weight markers. Besides, gels were

analyzed with an Image Quant LAS 500, another general system for fluorescence image capture of proteins. As shown in Fig. 7B, the protein bands were also detected, thus proving that protein-betaxanthins can be visualized using different fluorescence imager equipments.

Immediately after fluorescence scanning, gels were also stained for proteins with Coomassie blue (data not shown). Gels were then examined by image analysis software to calculate integrated optical density (IOD) and, the data obtained for each lane were plotted *versus* protein amount (Fig. 7C). Although IOD values for fluorescent staining were lower than those obtained by the standard procedure at this stage, protein amounts higher than 5 µg could be detected and the fluorescence intensity was proportional to protein content beyond the sensitivity limit.

To sum up, these results confirm that synthesis of betaxanthins is not limited to any one protein in particular, but can be extended to any protein and, last but not least, that this general synthesis procedure can be made *in situ* in a conventional electrophoresis gel. The existence of proteins bound to betalamic acid in nature under physiological conditions has never been reported nor speculated. However this possibility is now open with the demonstrated reactivity of at least one amine group of proteins towards betalamic acid and the formation of protein-betaxanthins.

3.5. Fluorescent staining of proteins with red beet root juice

In this work, protein-betaxanthins have been routinely labeled as protein derivatives starting from purified betalamic acid. To date, most of the protocols applied for purifying betalamic acid involve multiple steps and very low yields [38,39]. However, in this paper, purification of betalamic acid has been carried out by a straightforward batch method that allows pure betalamic acid to be obtained in preparative amounts.

This procedure is based on a method previously published by our group that purifies betalamic acid by column chromatography in a different anionic exchange resin [40].

Nevertheless, in order to simplify the process further, the synthesis of protein-betaxanthins was carried out starting from red beet root juice, as described in the Materials and Methods section, without any preliminary betalamic acid purification step. The synthesis of BSA-betaxanthin from red beet root juice was confirmed by partially denaturing SDS-PAGE (Fig. 8A). After fluorescence scanning, the protein band corresponding to BSA-betaxanthin was easily detected and, as can be seen, the electrophoretic pattern was similar to that shown in Fig. 5A. As expected, the background in Fig. 8A was higher, since the betaxanthins were synthesized from raw beet root extract, and not from purified betalamic acid, but this did not prevent proper band visualization.

Fluorescent detection of proteins after conventional SDS-PAGE with betalamic acid staining has been clearly demonstrated (Fig. 7). Since these experimental results were carried out by using purified betalamic acid, the next goal in this study was to simplify this detection process by directly using red beet root extracts in place of betalamic acid. As shown in Fig. 8B, fluorescent staining of proteins run in electrophoresis gels can also be achieved with raw red beet root juice. Once again, the background of the gels was higher than when staining was performed with purified betalamic acid, but all proteins were properly detected through the derived protein-betaxanthin fluorescence.

Therefore, these results show that synthesis of protein-betaxanthins can be simply performed starting from beet root juice in a general procedure and omitting any purification step. Although, the electrophoresis gels were not as clean as those obtained with purified betalamic acid, the protein bands were unquestionably detected. The

speed, simplicity and low cost of this synthesis reaction anticipate the possible use of protein-betaxanthins as fluorescent probes with multiple biological applications.

4. Conclusion

For the first time, protein-betaxanthins have been obtained. Their derivatization has been demonstrated by mass spectrometry as well as by electrophoretic techniques. The derivatized proteins exhibit the properties of betaxanthins as evidenced by absorbance and fluorescence spectroscopy. In fact, absorbance data, excitation and emission spectra together with three-dimensional fluorescence spectra confirmed the similarity of BSA-betaxanthin to low molecular weight natural and synthetic betaxanthins. Moreover, the derivatization reaction can be started from purified betalamic acid or from raw red beet root extracts.

Finally, the synthesis of protein-betaxanthins has been applied to the detection of proteins in electrophoresis gels. The fluorescence of the betaxanthins obtained *in situ* from gel proteins allowed their visualization as an alternative form to the traditional staining methods.

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LEGENDS.

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

Fig. 2. Absorbance spectrum for BSA-betaxanthin (3 µM) in water at 25 ºC.

Fig. 3. HPLC analysis profile for lysine-betaxanthin reaction media. Full scale is A=0.3 absorbance units. Elution was followed at 480 nm. 5 µL were injected with a concentration of 67 µM. Inset: Structures for the two plausible adducts of lysine-betaxanthin.

Fig. 4. (A) Excitation (solid lane) and emission (dashed line) spectra for BSA-betaxanthin. (B) Three-dimensional fluorescence spectrum for BSA-betaxanthin. (C) Excitation (solid lane) and emission (dashed line) spectra for lysine-betaxanthin. (D) Three-dimensional fluorescence spectrum for lysine-betaxanthin. Spectra were recorded in water at 25ºC, at BSA-betaxanthin concentration of 1 µM and at a lysine-betaxanthin concentration of 3 µM.

Fig. 5. Partially denaturing SDS-PAGE of protein-betaxanthins. (A) BSA-betaxanthin, at the increasing concentrations of 18, 37, 55, 74 and 110 µg. (B) Protein-betaxanthins generated from commercial molecular weight markers after betalamic acid derivatization. The specific conditions are described under Materials and Methods. (C) Integrated optical density (IOD) values corresponding to the different amounts of BSA-betaxanthin shown in A.

Fig. 6. Fluorescence increase of BSA–betaxanthin with agarose gel solidification. Excitation was carried out at λ = 476 nm and emission was recorded at λ = 552 nm. BSA-betaxanthin concentration was 1µM.

Fig. 7. Fluorescence staining of proteins in denaturing SDS-PAGE gels, after derivatization *in situ* with purified betalamic acid. (A) The gel was scanned with Typhon 9410 fluorescence scanner and (B) with an Image Quant LAS 500. The increasing concentrations of BSA in the lanes were: 2.5, 5, 10 and 15 µg. As for commercial molecular weight markers, 20 µL were applied into the gel. Specific conditions are detailed in Materials and Methods. (C) Integrated optical density (IOD) values for increasing amounts of BSA, detected by fluorescence staining (●) or by standard Coomassie blue method (○).

Fig. 8. Protein staining with raw beet root juice. (A) Partially denaturing SDS-PAGE of BSA-betaxanthin (250 µg) previously derivatized by using beet root juice, detected by fluorescence emission. (B) Fluorescent staining of BSA and molecular weight markers in denaturing SDS-PAGE after in gel derivatization with raw beet root juice. Increasing concentrations of BSA were: 2.5, 5, 10 and 15 µg. As for commercial molecular weight markers, 20 µL were applied into the gel.

Table 1.

Absorbance and fluorescence spectroscopy data obtained for BSA–betaxanthin and for lysine-betaxanthin.

Absorbance				Fluorescence					
Pigment	λ_m (nm)	ε_m (M^{-1} $\cdot cm^{-1}$)	Width (nm)[a]	$\lambda_{m\ Exc}$ (nm)	$\lambda_{m\ Ems}$ (nm)	Stokes Shift. (nm)	Relativ. Flu.Int. (%) [b]	Exc. Width (nm) [a]	Ems. Width (nm) [a]
BSA-Bx	476	46,000	62	476	551	75	663	39	27
Lysine-Bx	472	47,000	63	474	554	80	205	52	36

[a] Spectrum width was calculated at an absorbance/fluorescence intensity value half of that at the corresponding maximum.

[b] Relative fluorescence intensity values are calculated in relation to alanine-betaxanthin.

FIGURES

Figure 1

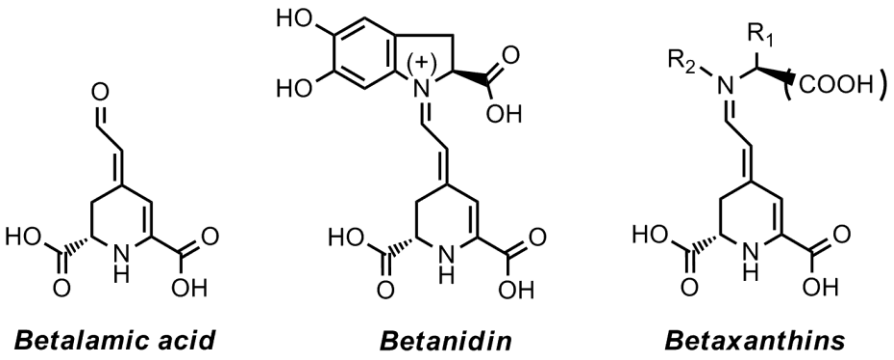


Figure2

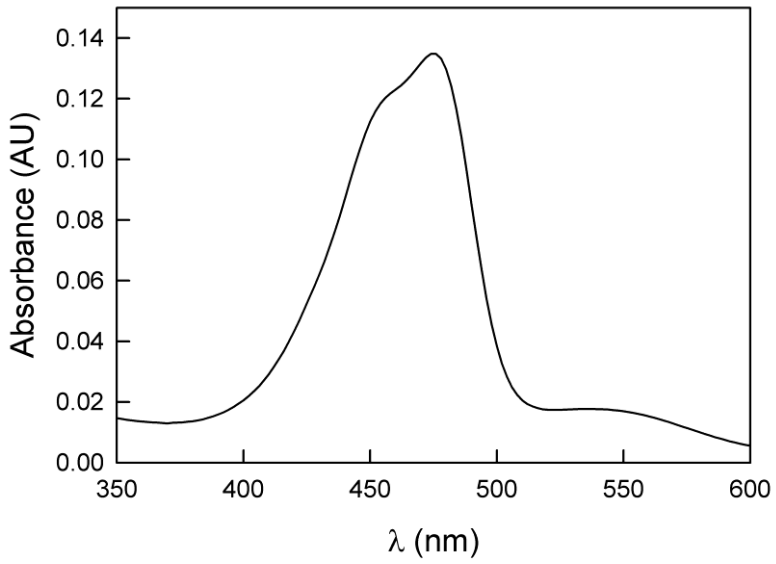


Figure 3

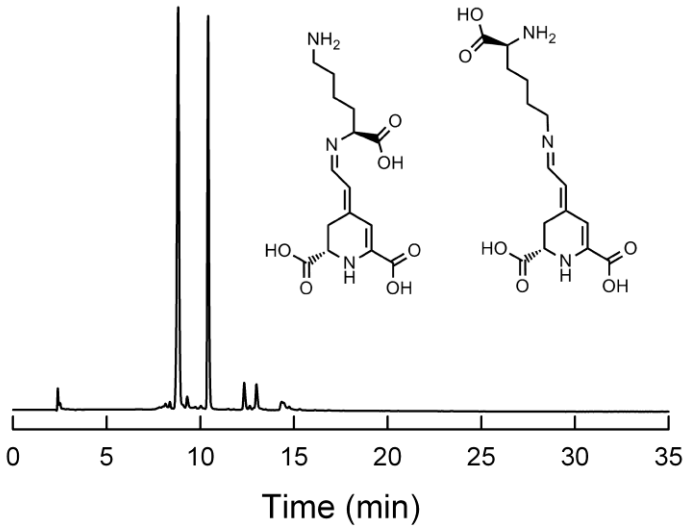
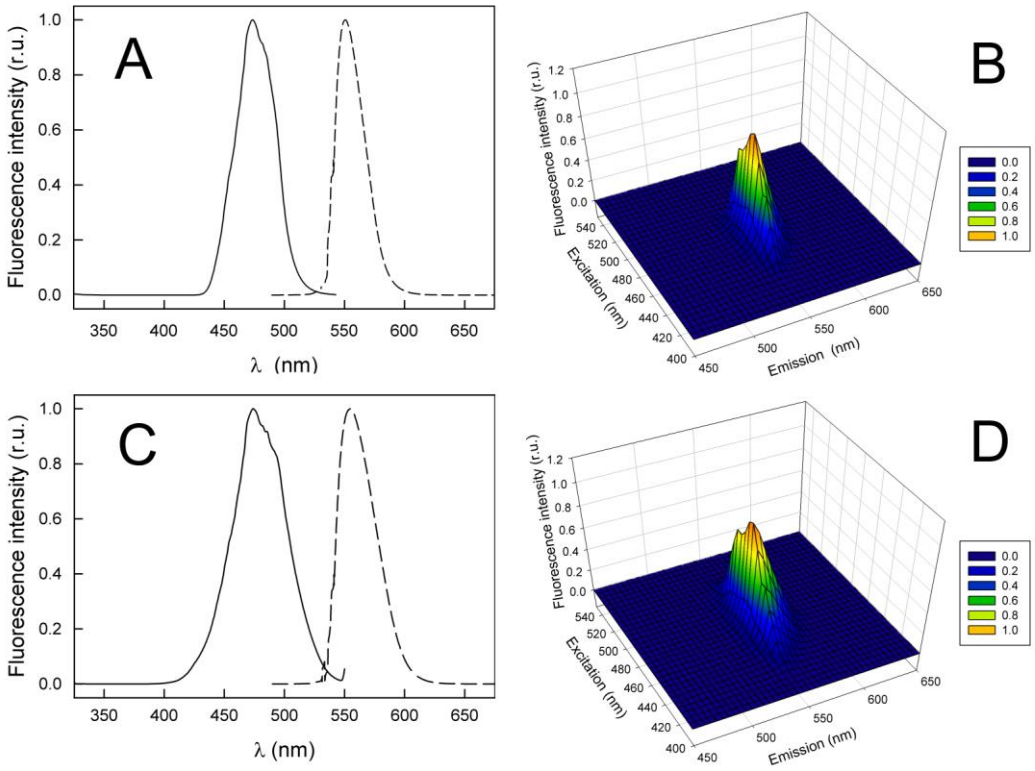


Figure 4



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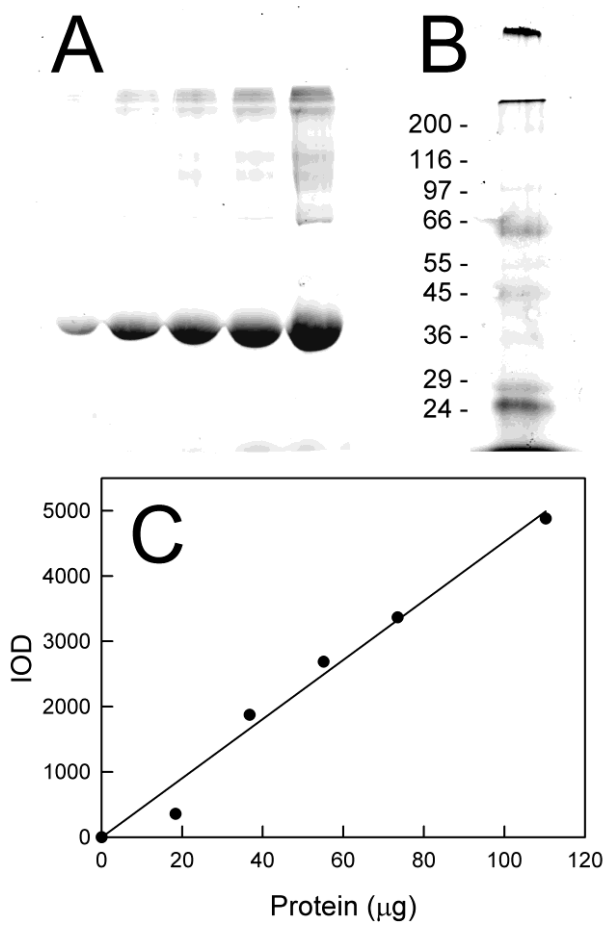


Figure 6

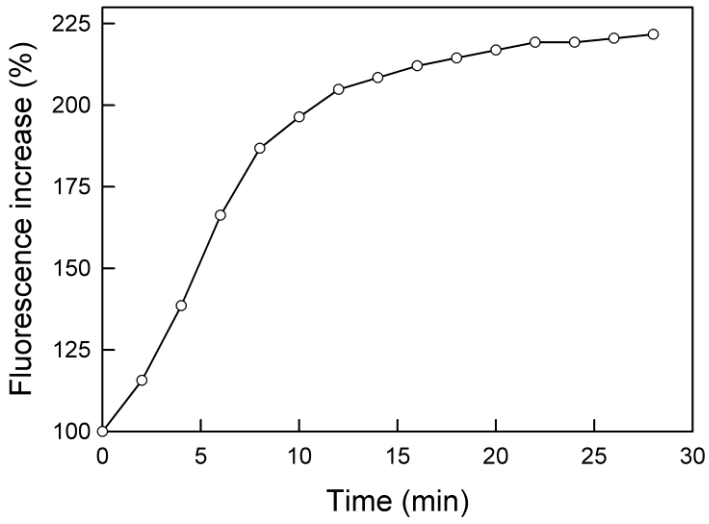
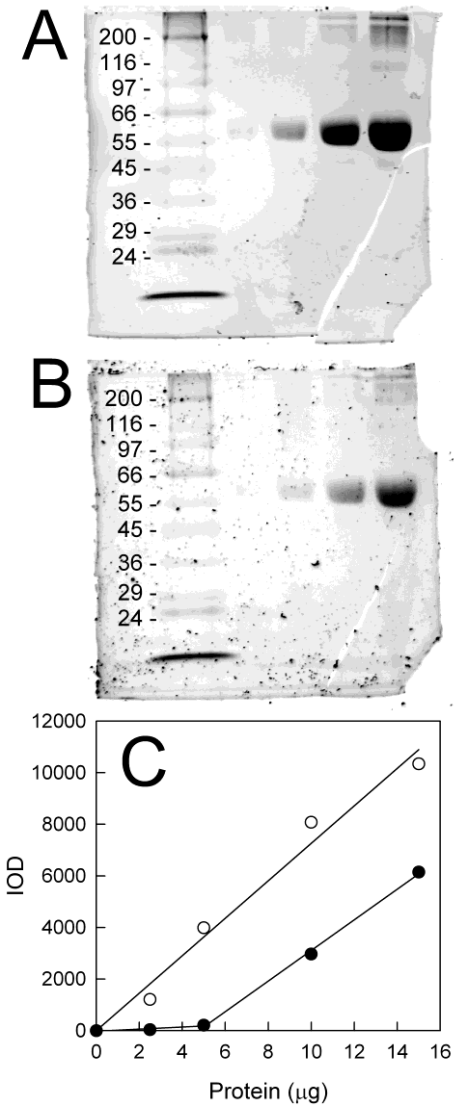


Figure 7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0
2
4
6
8
10
12
14
16
Protein (μg)

Figure 8