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MASS SPECTROMETRIC DETECTION OF NEW BETALAINS IN *MAMMILLARIA* FLOWERS

ABSTRACT

Betacyanins are natural, red-violet betalain pigments which can be found in plenty of plants of the *Cactaceae* family. The *Mammillaria* is the widest genus of the *Cactaceae* family of which fruit was examined for betacyanins contents. As a result of the investigation, a new pigment structure, mammillarinin, was identified. Heretofore, no betacyanins of *Mammillaria coronata* flowers has been extensively studied. In this report, the results of betacyanin analysis by LC-DAD-ESI-MS/MS in the flower extract are presented.

KEYWORDS

natural pigments, betacyanins, *Cactaceae*, *Mammillaria*, LC-MS

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INTRODUCTION

Betacyanins are a group of water-soluble, red-violet pigments occurring in plants of the *Caryophyllales* order which gain importance in regard to their pro-health, antioxidant activity¹. Many species of the *Cactaceae* family, which belong to the *Caryophyllales* order, contain betacyanins in their fruits and flowers. Within the family, *Mammillaria* genus is the largest among many others. In general, these plants have small or mid-sized globose to elongated shape, with distinctly tuberculate stem morphology. The color gradient of flowers goes from pink to white. They grow at the top of the plant creating a crown².

Hitherto, betacyanins from several species of the *Cactaceae* family like *Hylocereus* and *Opuntia* were extensively studied³. However, as regards *Mammillaria* genus, only the pigment profile of the fruits was exclusively tested⁴.

Structurally, betacyanins are immonium conjugates of betalamic acid with *cyclo*-DOPA or frequently *O*-glucosylated *cyclo*-DOPA. Glucosylation at C5 position is characteristic for betanin-type betacyanins named after betanin, the simplest betacyanin. Other types are gomphrenin-type betacyanins, which are 6-*O*-glucosylated derivatives and amaranthine-type derivatives⁵. The *O*-glycosides esterification with organic acids is very common in betacyanin structures and leads to the occurrence of many acylated derivatives, for instance: ferulic, p-coumaric, synapic and malonic acid (Figure 1).

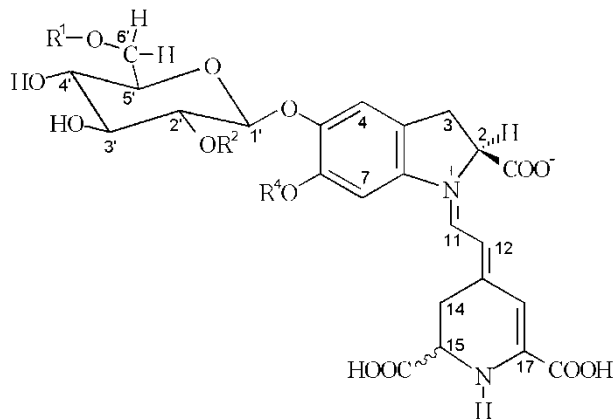
¹ D. Strack, W. Steglich, V. Wray, [in:] *Methods in Plant Biochemistry*, eds. P. M. Dey, J. B. Harborne, P. G. Waterman, "Academic Press" 1993, No. 8, p. 422; D. A. Moreno, C. Garcia-Viguera, J. I. Gil, A. Gil-Izquierdo, *Betalains in the era of global agri-food science, technology and nutritional health*, "Phytochem Rev" 2008, No. 7, pp. 261–262.

² C. A. Butterworth, R. S. Wallace, *Phylogenetic studies of Mammillaria (Cactaceae) – insights from chloroplast sequence variation and hypothesis testing using the parametric bootstrap*, "Am. J. Bot." 2004, No. 91 (7), p. 1086.

³ S. Wybraniec, I. Platzner, S. Geresh, H. E. Gottlieb, M. Haimberg, M. Mogilnitzki, Y. Mizrahi, *Betacyanins from vine cactus Hylocereus polyrhizus*, "Phytochemistry" 2001, No. 58, pp. 1209–1212; F. C. Stintzing, A. Schieber, R. Carle, *Identification of Betalains from Yellow Beet (Beta vulgaris L.) and Cactus Pear [Opuntia ficus-indica (L.) Mill.] by High-Performance Liquid Chromatography-Electrospray Ionization Mass Spectrometry*, "J. Agric. Food Chem." 2002, No. 50, p. 2302.

⁴ S. Wybraniec, B. Nowak-Wydra, *Mammillarinin: A New Malonylated Betacyanin from Fruits of Mammillaria*, "J. Agric. Food Chem." 2007, No. 55, pp. 8138–8143.

⁵ Y. Cai, M. Sun, H. Corke, *HPLC Characterization of Betalains from Plants in the Amaranthaceae*, "J Chromatog Sci" 2005, No. 43, pp. 455–456.



$R^1 = R^2 = R^4 = H$ Betanin (1)

$R^1 = H$ $R^2 =$ (apiosyl) $R^3 = R^4 = H$ 2'-O-Apiosylbetanin (2)

$R^1 =$ (malonyl) $R^2 = R^4 = H$ Phyllocactin (3)

$R^1 = H$ $R^2 =$ apiosyl $R^3 =$ (salicyl) $R^4 = H$ (4)

$R^1 = H$ $R^2 =$ apiosyl $R^3 =$ (feruloyl) 2'-(5''-O-E-Feruloylapiosyl)betanin (5)
 $R^4 = H$

$R^1 = H$ $R^2 =$ apiosyl $R^3 =$ (sinapoyl) 2'-(5''-O-E-Sinapoylapiosyl)betanin (6)
 $R^4 = H$

$R^1 =$ malonyl $R^2 =$ apiosyl $R^3 =$ feruloyl 2'-(5''-O-E-Feruloylapiosyl)phyllocactin (7)
 $R^4 = H$

Figure 1. Structures of several betacyanins identified in *Mammillaria coronata* flowers

The presence of acyl moiety in the betacyanin structure is related to a specific absorption maximum of hydroxycinnamoyl (ferulic, p-coumaric, synapic) moiety at ca. 330 nm⁶. Most of betacyanins have C15 diastereoisomeric isoforms⁷. This results in an immense diversity of their structures from which a large part remains unknown.

MATERIAL AND METHODS

Reference pigments for co-injection experiments

Reference samples of acylated betacyanins, used for identification of newfound betacyanins in the extract tested by co-injection experiments, were isolated from fruits of *Hylocereus polyrhizus*, *Hylocereus ocamponis*, *Hylocereus undatus* and *Phytolacca americana* berries, in accordance with the previous studies⁸.

Chromatographic analysis

For high-performance liquid chromatographic analysis, a Gynkotek HPLC system with UVD340U, Gynkotek HPLC Pump Series LPG-3400A, a thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) were used. For data collection and handling as well as chromatograph operation monitoring, the software package Chromeleon 6.0 (Gynkotek Separations) was used. An ONYX monolithic column, 100 x 4.6 mm i.d. with a guard column (Phenomenex, Torrance, CA, USA), was utilised for pigment separation. The column was thermostated at temp. 35°C. The following gradient elution system was applied: 95% A with 5% B at 0 min; gradient to 70% A with 30% B at 40 min (A, 1% (v/v) formic acid; B, acetonitrile). The injection volume was 10 µL, and the flow rate was 0.5 mL/min. The spectrometric detection was performed at 538, 505, 480, 450 and 310 nm and in the DAD (diode-array detection) mode in the case of the UV-Vis spectra acquisition. The same chromatographic conditions were applied for the LC-ESI-MS/MS analyses.

⁶ F. C. Stintzing, R. Carle, *Functional properties of anthocyanins and betacyanins in plants, food, and human nutrition*, "Trends Food Sci. Technol." 2004, No. 15, p. 23.

⁷ D. Strack, W. Steglich, V. Wray, op. cit.

⁸ S. Wybraniec, B. Nowak-Wydra, K. Mitka, P. Kowalski, Y. Mizrahi, *Minor betalains in fruits of Hylocereus species*, "Phytochemistry" 2007, No. 68, p. 251; G. Jerz, T. Skotzki, K. Fiege, P. Winterhalter, S. Wybraniec, *Separation of betalains from berries of Phytolacca Americana by ion-pair high-speed counter-current chromatography*, "J. Chromatogr. A" 2008, No. 1190, pp. 63–73.

LC-ESI-MS/MS analysis

The positive ion electrospray mass spectra were recorded on ThermoFinnigan LCQ Advantage (electrospray voltage 4.5 kV; capillary 250°C; sheath gas: N₂) with a ThermoFinnigan LC Surveyor pump utilizing the same HPLC gradient. The ThermoFinnigan Xcalibur software (San Jose, CA, USA) was used for MS controlling as well as total ion chromatograms and mass spectra recording. The relative collision energies for the CID experiments were set at 30% (according to a relative energy scale). Helium was used in order to improve trapping efficiency and as the collision gas for the CID experiments.

RESULTS AND DISCUSSION

The pigment identification was based on detected m/z values of protonated molecular ions $[M+H]^+$ and fragmentation ions, characteristic absorption maxima of particular pigments (λ_{\max} II) as well as retention times in comparison with co-injection data. The number of acyl moieties in the structures was deduced also from the height of the absorption band I of the hydroxycinnamoyl moieties (λ_{\max} HCA/I). All chromatographic and mass spectrometric data are presented in Table 1.

Tab. 1. Optical and mass spectrometric data of the betacyanins present in the extract

| No. | Compound | R _t [min] | λ_{\max}^a [nm] I: | λ_{\max}^b [nm] II: | Abs. Ratio II:I | m/z [M+H] ⁺ | m/z from MS/MS of [M+H] ⁺ |
|-----|--|-------------------------|----------------------------------|-----------------------------------|-----------------------|-----------------------------|--|
| 1 | Betanidin 5- <i>O</i> - β -glucoside (betanin) | 7.9 | - | 535 | - | 551 | 389 |
| 2 | 2'- <i>O</i> -Apiosyl-betanin | 8.6 | - | 536 | - | 683 | 551; 389 |
| 1' | Isobetanidin 5- <i>O</i> - β -glucoside (isobetanin) | 9.1 | - | 535 | - | 551 | 389 |
| 2' | 2'- <i>O</i> -Apiosyl-isobetanin | 10.2 | - | 536 | - | 683 | 551; 389 |
| 3 | 6'- <i>O</i> -Malonyl-betanin (phylloactin) | 9.9 | - | 536 | - | 637 | 619; 593; 551; 389 |
| 3a | 4'- <i>O</i> -Malonyl-betanin | 10.4 | - | 536 | - | 637 | 619; 593; 551; 389 |

| | | | | | | | |
|------------|--|------|-----|-----|--------|-----|--------------------|
| 3' | 6'- <i>O</i> -Malonyl-isobetanim (isophyllocactin) | 10.6 | - | 536 | - | 637 | 619; 593; 551; 389 |
| 3a' | 4'- <i>O</i> -Malonyl-isobetanim | 11.1 | - | 536 | - | 637 | 619; 593; 551; 389 |
| 4 | 5''- <i>O</i> -Salicyl-2'- <i>O</i> -apiosyl-betanim ^c | 11.5 | - | 542 | - | 803 | 683; 551; 389 |
| 4' | 5''- <i>O</i> -Salicyl-2'- <i>O</i> -apiosyl-isobetanim ^c | 12.4 | - | 542 | - | 803 | 683; 551; 389 |
| 5 | 5''- <i>O</i> - <i>E</i> -feruloyl-2'- <i>O</i> -apiosyl-betanim | 16.1 | 326 | 547 | 1:0.46 | 859 | 683; 551; 389 |
| 6 | 5''- <i>O</i> - <i>E</i> -sinapoyl-2'- <i>O</i> -apiosyl-betanim | 16.3 | 327 | 548 | 1:0.53 | 889 | 683; 551; 389 |
| 5' | 5''- <i>O</i> - <i>E</i> -feruloyl-2'- <i>O</i> -apiosyl-isobetanim | 16.4 | 327 | 547 | 1:0.49 | 859 | 683; 551; 389 |
| 6' | 5''- <i>O</i> - <i>E</i> -sinapoyl-2'- <i>O</i> -apiosyl-isobetanim | 16.6 | 328 | 548 | 1:0.48 | 889 | 683; 551; 389 |
| 7 | 5''- <i>O</i> - <i>E</i> -Feruloyl-2'- <i>O</i> -apiosyl-phyllocactin | 17.7 | 328 | 551 | 1:0.49 | 945 | 769; 683; 551; 389 |
| 7' | 5''- <i>O</i> - <i>E</i> -Feruloyl-2'- <i>O</i> -apiosyl-isophyllocactin | 18.2 | 331 | 550 | 1:0.53 | 945 | 769; 683; 551; 389 |

^a λ_{\max} of hydroxycinnamoyl moiety (HCA/I), -, no absorbance band

^b λ_{\max} of betacyanins in the visible range (II)

^c Tentatively identified

Identification of betacyanins in the flowers of *Mammillaria coronata* reveals an occurrence of betanim, the simplest polar betacyanin, which was previously detected in *Hylocereus* species [3]. However, the dominant pigment in the extract is 2'-*O*- β -apiosyl-betanim/isobetanim **2/2'**. Its presence is suggested by the protonated molecular ion ($[M+H]^+$), which is found at m/z 683. The fragmentation ion indicates a deficit of apiosyl moiety ($\Delta m/z$: 683 – 551 = 132). Another abundant pigment is 6'-*O*-malonyl-betanim/isobetanim **3/3'** (m/z 637). As a result of fragmentation, an ion without malonyl moiety ($\Delta m/z$: 637 – 551 = 86) is detected. The trivial name of **3/3'** is phyllocactin/isophyllocactin; it was previously detected in *Mammillaria* fruit⁹. The MS detector registered two small additional peaks at m/z 637, after the corresponding phyllocactin/isophyllocactin peaks, respectively. These isomeric pigments were identified as phyllocactin positional

⁹ S. Wybraniec, B. Nowak-Wydra, op. cit.

isomers (**3a/3a'**) which are acyl migration products of phyllocactin/isophyllocactin. The malonyl migration in phyllocactin presumably proceeds from the C'-6 to the C'-4 carbon. The phenomenon of acyl migration in betacyanins has been recently noticed, and further studies supported this finding. In each case, the resulting equilibrated pigment composition favours the 6'-*O*-malonylated forms. The formation of an intermediate strainless cyclic ortho ester structure between the glucosidic *O*-4' and *O*-6' hydroxyls, which was reported frequently in many cases of acyl migration in acylated β -D-glucosides, can also be responsible for the interconversion between these isomers¹⁰.

The results suggest also the occurrence of 5''-*O*-salicyl-2'-*O*-apiosyl-betanin/isobetanin **4/4'** ($[M+H]^+$ m/z 803) based on the fragmentation ion without salicyl moiety ($\Delta m/z$: 803 – 683 = 120), 5''-*O*-*E*-feruloyl-2'-*O*- β -apiosyl-betanin/isobetanin **5/5'** (m/z 859; $\Delta m/z$: 859 – 683 = 176) and 5''-*O*-*E*-sinapoyl-2'-*O*- β -apiosyl-betanin/isobetanin **6/6'** (m/z 889; $\Delta m/z$: 889 – 683 = 206) as well as 5''-*O*-*E*-feruloyl-6'-*O*-malonyl-2'-*O*- β -apiosyl-betanin/isobetanin **7/7'** (m/z 945). The second absorption maximum, at 330 nm, confirms the presence of feruloyl and sinapoyl derivatives of betacyanins. Additionally, the absorption ratio II:I of all hydroxycinnamoylderivatives confirms an occurrence of a single moiety in each structure. These acylated pigments were not detected in *Mammillaria* fruits but their presence was previously revealed in *Hylocereus* species or *Phytolacca americana*¹¹.

CONCLUSIONS

Eight pairs of betacyanin diastereoisomers were identified in the extract of *Mammillaria coronata* flowers. The profile of the flower extract was compared with the fruit extracts studied previously. Four of the identified acylated betacyanins were not identified before in *Mammillaria* fruits. Their acyl moieties were tentatively defined by means of MS/MS analysis. Interestingly, phyllocactin positional isomers, 4'-*O*-malonyl-betanin/isobetanin were detected indicating malonyl moiety migration.

¹⁰ S. Wybraniec, B. Nowak-Wydra, K. Mitka, P. Kowalski, Y. Mizrahi, op. cit.

¹¹ Ibidem; G. Jerz, T. Skotzki, K. Fiege, P. Winterhalter, S. Wybraniec, op. cit.

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