ABSTRACT

Betacyanins are conjugates of betalamic acid and glycosylated cyclo-DOPA which bring about the creation of a specific chromophoric system, the 1,7-diazaheptamethin chain. Betanin, a betacyanin pigment from red beet (Beta vulgaris L.), as well as its decarboxylated derivatives were subjected to reduction with the use of NaBH₄. The pigment structures contain a vinyl linker which is postulated to be protonated during reaction with NaBH₄. In this study, 17-decarboxy-betanin and 2,17-bidecarboxy-betanin were obtained from betanin, which had been previously isolated from red beet root (Beta vulgaris L.). Purified pigments were tested in the presence of the reduction reagent while looking for the products of the reaction identified by LC-MS.

KEY WORDS

betanin, decarboxy-betacyanins, reduction, 1,7-diazaheptamethin, chromophore, LC-MS

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INTRODUCTION

Betanin is a natural pigment approved as a colorant for food. It is obtained from red beet root (*Beta vulgaris* L.)\(^1\), but can be found in many plant species of the **Caryophyllales** order. Natural pigments from betacyanins group have a lot of advantages in comparison with artificial food colorants. They not only are non-toxic but also have a proved antioxidant activity\(^2\). Therefore, they are prone to degradation leading to creation of decarboxy-derivatives or total decomposition with betalamic acid and cyclo-DOPA release. The decarboxy-derivatives of betanin from red beet were extensively studied. Their formation, initiated by many factors such as oxygen, high temperature and low pH, was reported by Herbach et al.\(^3\) Mono-, di- and tridecarboxybetacyanins were obtained, for instance, by heating acidified aqueous as well as ethanolic solutions at a temperature not exceeding 85°C\(^4\). One of the routes of betanin decarboxylation is presented in Figure 1.

\[ \begin{align*}
\text{betanin} & \rightarrow \text{17-decarboxy-betanin} \\
\text{17-decarboxy-betanin} & \rightarrow \text{2,17-bidecarboxy-betanin}
\end{align*} \]

![Figure 1. One of betanin decarboxylation routes](image)

A reduction reaction using NaBH\(_4\) has never been performed with betanin nor with its decarboxylated derivatives. However, a protonation of the vinyl linker was reported in previous studies as a presumable result of an inside molecular disproportionation\(^5\).

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MATERIAL AND METHODS

Isolation of betanin

Betanin was isolated from juice of red beet root (Beta vulgaris L.) by a sequence of chromatographic techniques. Firstly, preliminary purification using a C18 cartridge was carried out. The betanin-rich fractions were eluted by aqueous solution of 30% acetone, 2% formic acid (v/v). Next, a Flash chromatography was used with gradient system: 5% A in B at 0 min; gradient to 35% A in B at 30 min (A, acetone; B, 2% formic acid in water). Afterwards, a preparative HPLC system with HPLC PUMP 64 (Knauer, Germany) was applied. The semi-preparative column was Bischoff C18 (Bischoff Chromatography, Germany) 250 mm x 30 mm i.d., 10 μm, C18, with a 10 mm x 10 mm i.d. guard column with the same material. An applied gradient system was 6% A in B at 0 min; gradient to 10% A in B at 30 min (A, acetone; B, 1% formic acid in water). In each case, the injection volume was 100 mL and the flow rate was 3 mL/min. The signal detection was performed at 505 nm by a UV-Vis detector (Knauer, Germany). The temperature during chromatographic isolation was ambient.

Semi-synthesis of betanin derivatives

17-decarboxylated-betanin and 2,17-bidecarboxy-betanin were obtained by heating of betanin aqueous solution acidified with formic acid for 30 min at 85°C. The final isolation of decarboxylated pigments was carried out by the same procedure of semi-preparative HPLC as for betanin.

UV-Vis spectra measurement

The reaction was monitored by collecting UV-Vis spectra for 0.6 h using a microplate reader. For this purpose, 10 μL of each pigment was dispensed into 6 wells with buffer solutions in a pH range of 3–8. Immediately before measurement, 170 μL of NaBH₄ aqueous solution (0.85 mM) were added to each well. The spectra were recorded in the range of 350–650 nm at 25°C within a 0.6 h period. The calculated optical path-length for a volume of 200 μL in a microplate well was 0.53 cm.

LC-DAD-MS analysis

The reaction products were identified by means of positive ion electrospray mass spectra as recorded on ThermoFinnigan LCQ Advantage (electrospray voltage 4,5 kV; capillary 250°C; sheath gas: N₂) with a ThermoFinnigan LC Surveyor pump. An ONYX monolithic column 100 x 4.6 mm i.d. with a guard column (Phenomenex, Torrance, CA, USA) thermostated at 35°C was used. The
injection volume was 10 μL, the flow rate was 0.5 mL/min and the applied gradient system was: 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 controlling software, ThermoFinnigan Xcalibur (San Jose, CA, USA), was used for 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 as the collision gas for the CID experiments.

RESULTS AND DISCUSSION

UV-VIS spectra of betanin, 17-decarboxy-betanin and 2,17-bidecarboxy-betanin during reaction with NaBH₄, presented in Figure 2, were measured for 0.6 h at different pH. In acidic solutions (pH 3–5), no significant changes were observed. Meanwhile, at pH 6, a second minor band in the range of 350–370 nm appears for each studied pigment which is more evident at higher pH. An even stronger effect for 17-decarboxy-betanin (17-dBt) is observed at pH 8 (Figure 2H). The new band may suggest the formation of a reduction product.

The LC-MS data are presented in Figures 3–6 as well as in Table 1 including all measured details. The spectra recorded over LC-DAD-MS analysis prove arising of new absorption maxima in the range of 290–340 nm for each of the pigments (Figure 3, for 17-dBt). After experiment (Figure 3b) a distinct diminishing of absorption maxima at 505 nm of 17-decarboxy-betanin in agreement with an observation of a fading sample colour is shown. In the case of betanin and 2,17-bidecarboxy-betanin, similar results were noticed.
Reduction of Selected Betacyanins

Figure 3. LC-DAD-MS spectra of 17-decarboxy-betanin (a) and 17-decarboxy-11,12,17,18-tetrahydro-betanin, the product of reduction (b)

Table 1. Chromatographic and mass-spectrometric data of analysed pigments subjected to reduction

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>R_t</th>
<th>m/z</th>
<th>m/z from MS/MS of [M+H]^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>betanin</td>
<td>19.9</td>
<td>551</td>
<td>389</td>
</tr>
<tr>
<td>1b</td>
<td>11,12,17,18-tetrahydro-betanin^a</td>
<td>19.1</td>
<td>555</td>
<td>393</td>
</tr>
<tr>
<td>2a</td>
<td>17-decarboxy-betanin</td>
<td>20.3</td>
<td>507</td>
<td>345</td>
</tr>
<tr>
<td>2b</td>
<td>17-decarboxy-11,12,17,18-tetrahydro-betanin^a</td>
<td>19.1</td>
<td>511</td>
<td>349</td>
</tr>
<tr>
<td>3a</td>
<td>2,17-bidecarboxy-betanin</td>
<td>22.2</td>
<td>463</td>
<td>301</td>
</tr>
<tr>
<td>3b</td>
<td>2,17-bidecarboxy-11,12,17,18-tetrahydro-betanin^a</td>
<td>10.7</td>
<td>467</td>
<td>305</td>
</tr>
</tbody>
</table>

^a Tentatively identified
In LC-MS data for betanin after reduction by NaBH₄ in comparison to reference betanin (retention time R₁ 19.9 min, m/z of protonated ion 551, Figure 4a), there are traces of two coeluting compounds at R₁ 19.1 min, m/z of [M+H]+ 555 (Figure 4b). This indicates a product of 4 hydrogen atoms addition to betanin. The fragmentation ion at m/z 393 confirms deglycosylation of the compound (Δm/z: 555 – 393 = 162), which proves a 4 H addition into aglycone of betanin.

In the case of the product of a 17-decarboxy-betanin reduction, a single peak at R₁ 19.1 is present (Figure 5b). Its protonated molecular ion is also increased by about a 4 hydrogen atoms mass. The fragmentation ion at 349.1 in comparison with corresponding ion m/z = 345.1 of reference ion is increased as well (Figure 5c). This suggests a similar position of a reduction in the structure of betanin and 17-decarboxy-betanin.
Reduction of Selected Betacyanins

Figure 5. Chromatographic traces of 17-decarboxy-betanin (a), 17-decarboxy-11,12,17,18-tetrahydro-betanin (b) and MS spectrum of 17-decarboxy-11,12,17,18-tetrahydro-betanin (c).

For 2,17-bidecarboxy-betanin, a similar addition of 4 hydrogens is noted (Table 1). Interestingly, the product of 2,17-bidecarboxy-betanin reduction differs from that of betanin and 17-decarboxy-betanin, because of the shorter retention time, $R_t$ 10.7 min (Figure 6), which may suggest a higher polarity. Further investigation is necessary to explain the fact.

Figure 6. Chromatographic traces of 2,17-bidecarboxy-betanin (a) and 2,17-bidecarboxy-11,12,17,18-tetrahydro-betanin (b).
CONCLUSIONS

Presumably, the positions of the addition of hydrogen atoms over the reduction by NaBH₄ are C₁₁ and C₁₂ in the vinyl linker as well as C₁₆ and C₁₇. This suggestion, based on the previous study of Mabry et al., is shown in Figure 7⁶. Thus reduction of betacyanin pigments resulted in the destruction of 1,7-diazaheta-methin chromophoric system in their structures, which is proved by decreasing of the main absorption maxima. The proposed products of betanin, 17-decarboxy-betanin and 2,17-bidecarboxy-betanin are: 11,12,17,18-tetrahydro-betanin, 17-decarboxy-11,12,17,18-tetrahydro-betanin and 2,17-bidecarboxy-11,12,17,18-tetrahydro-betanin (Figure 7), respectively.

Figure 7. The presumable products of tested pigments reduction by NaBH₄

⁶ Ibidem.
REFERENCES


