Enzymatic oxidation of neobetanin monitored by liquid chromatography with mass spectrometric detection

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The aim of this study was monitoring of enzymatic oxidation of neobetanin, an interesting type of betalains which is a partially oxidized betacyanin. As it belongs to betalains, it is water soluble and non-toxic, but a presence of a few functional groups makes it very reactive. Oxidation reactions were performed using horseradish peroxidase followed by spectrophotometric and mass spectrometric detection (LC-DAD-ESI-MS/MS) of obtained products. Enzymatic oxidation of neobetanin leads to a formation of new decarboxy- and dehydro-derivatives. The main identified oxidation product is 2-decarboxy-2,3-dehydroneobetanin. Searching for all formed oxidation products is extremely important for elucidation of the betalains oxidation mechanism.

Keywords: neobetanin, betalains, betacyanins, horseradish peroxidase, oxidation, enzymatic, spectrophotometry, mass spectrometry, chromatography

Introduction

Betalains, next to anthocyanins, flavonoids and carotenes are one of the basic groups of plant pigments. They commonly occur in the order of Caryophyllales [1] where they are responsible for red-violet colouring of fruits, roots and petals. Taking into account their natural origin, they are widely used in food and drug industry as colorants for products which do not require thermal treatment. Neobetanin is one of their most interesting representative because it exhibits yellow colour.

High antioxidant potential of betalains was a subject of different non-enzymatic studies [2–6] and the aim of our work was enzymatic oxidation of neobetanin using horseradish peroxidase II (HRP). This enzyme, present in roots of horseradish (*Armoracia rusticana*), is very commonly used in biochemical and biotechnology applications. Depending on the tested substrate, in a presence of hydrogen peroxide, the enzyme activity results in a formation of colored products detectable by spectrophotometry. In our study we used mass spectrometry LC-DAD-ESI-MS/MS for detection and preliminary identification of the products of neobetanin enzymatic oxidation. Effects of our research let us to tentatively determine so far unknown neobetanin oxidation pathways.

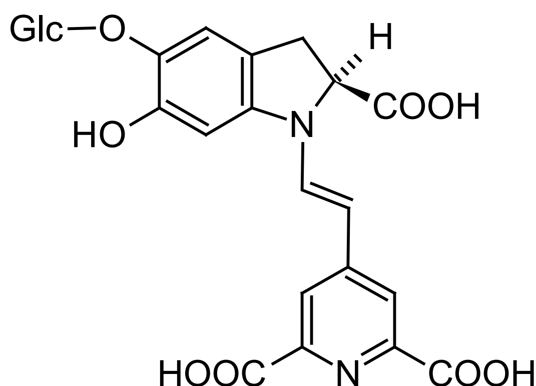

Figure 1. Neobetanin structure

Materials and methods

Reagents

Formic acid, ammonium formate, LC-MS grade methanol, acetonitrile, and water, peroxidase from horseradish type II (150250 units/mg solid (using pyrogallol), Rz = A403/A275 g 1.8, MW = 40 kDa), and almond β-glycosidase were obtained from Sigma Chemical Co. (St. Louis, MO).

Peroxidase Assay

The oxidation of neobetanin was performed in 25 mM acetate (pH 3-5.5) and phosphate (pH 6-8) buffers in 96-well plates of a Tecan infinite 200 microplate reader (Tecan Austria mbH, Gröodig/Salzburg, Austria). The action of 0.001-0.01 EU/mL peroxidase II on the 25 M pigment solutions in the presence of 1 mM H_2O_2 was monitored during a period of 120 min at a temp. of 25 °C by spec-

trophotometric detection at the wavelength range of 350-600 nm. For the chromatographic analysis, typically 50 L reaction mixtures were sampled from the wells and injected directly to an HPLC column, without further purification.

Chromatographic System LC-DAD

A Gynkotek HPLC system with UVD170S, Gynkotek HPLC pump series P580, and thermostat (Gynkotek Separations) was used for the chromatographic analysis. For the data acquisition, the software package Chromeleon 4.32 (Gynkotek Separations) was used. For the online UV-Vis spectra acquisition, the detection was performed in the diode array detection (DAD) mode. The column used was a 250 mm × 3 mm i.d., 5 µm, Luna C18(2), with a 4 mm × 2 mm i.d. guard column of the same material (Phenomenex). The injection volume was 10 µL, and the flow rate was 0.5 mL/min. The detection of analytes was performed typically at 538, 505, 480, and 440 nm. The column was thermostated at 35°C. For the separation of the analytes, two gradient systems were used: system 1, 90% (v/v) A with 10% (v/v) B at 0 min; gradient to 70% (v/v) A with 30% (v/v) B at 35 min, with (A) 20 mM ammonium formate (pH 4) and (B) methanol; system 2, 93% A with 7% B at 0 min; gradient to 80% A with 20% B at 35 min, with (A) 2% formic acid and (B) methanol. The same chromatographic conditions were applied for the HPLC-ESI-MS/MS analyses.

LC-ESI-MS/MS Analysis

The positive ion electrospray mass spectra were recorded on a ThermoFinnigan LCQ Advantage (electrospray voltage, 4.5 kV; capillary, 250°C; sheath gas, N_2) coupled to a Thermo-Finnigan LC Surveyor pump utilizing the HPLC gradient systems 1 and 2. The MS was controlled, and total ion chromatograms and mass spectra were recorded using ThermoFinnigan Xcalibur software (San Jose, CA). Heliumwas used to improve the trapping efficiency and as the collision gas for CID experiments. The relative collision energies for MS/MS analyses were set at 30% (according to relative energy scale).

Results and discussion

Recent studies show that the oxidation of betanin proceeds through a quinone methide intermediate [5]. The spectrum of neobetanin has one main absorption maximum at λ_{max} 470 nm. Spectrophotometric monitoring of the enzymatic oxidation reaction let us observe some chemical changes during the process, suggesting a formation of new compounds or decomposition of the substrate. Previous studies [5] indicated the highest enzymatic oxidation activity of neobetanin at pH 3. Figure 2 shows spectrophotometric analysis results of enzymatic oxidation of pigment by horseradish peroxidase II at pH 3.

LC-DAD-ESI-MS/MS data collected after 2 h of reaction are presented in Table 1. All obtained products are decarboxy- and dehydro-derivatives of neobetanin. Chromatographic traces of products (Fig. 3) indicate that **4**, tentatively identified as 2-decarboxy-2,3-dehydroneobetanin, because of protonated molecular ion $[M + H]^+$ at *m/z* 503, is the main formed product which means that the first step during the oxidation is a decarboxylation at C-2 and a double dehydrogenation at C-2 and C-3.

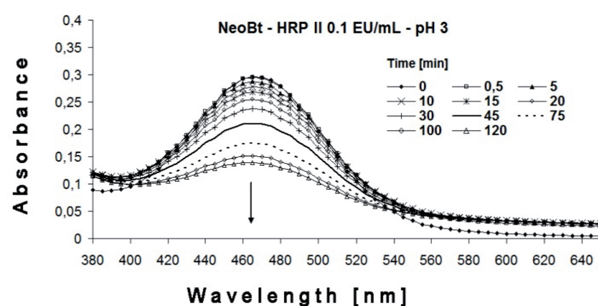

Figure 2. Visible spectra recorded at 25°C during oxidation of 20 µM neobetanin by 0.01 EU/mL horseradish peroxidase II in the presence of 1 mM H2O2 at pH 3

Table 1. Chromatographic, spectrophotometric, and mass spectrometric data of the analyzed products of neobetanin oxidation

No.	Compound	r_t [min]	λ_{max} [nm]	m/z $[M+H]^+$
1	2,15,17-tridecarboxy-2,3-dehydroneobetanin	21.2	395	415
2	neobetanin	21.4	470	549
3	2,17-bidecarboxy-2,3-dehydroneobetanin	24.1	405	459
4	2-decarboxy-2,3-dehydroneobetanin	27.4	420	503

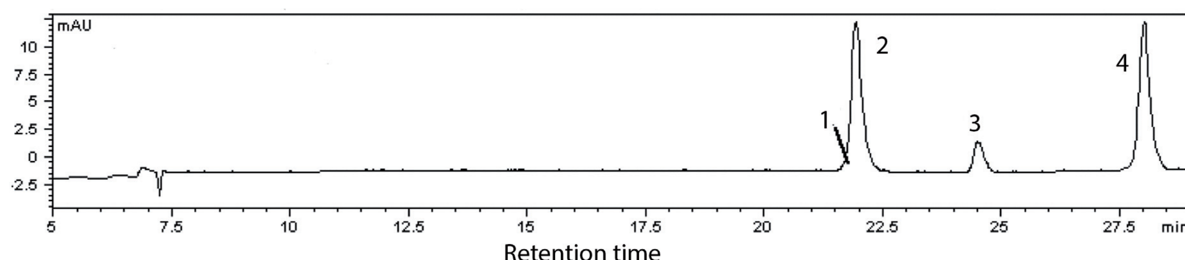

Figure 3. Chromatographic traces of the products of enzymatic neobetanin oxidation at pH 3

Another principal formed product with the highest chromatographic signal is 2,17-bidecarboxy-2,3-dehydroneobetanin (**3**), and another 2,15,17-tridecarboxy-2,3-dehydroneobetanin (**1**). A high signal detected for unreacted neobetanin (**2**) suggests that in a further course of reaction, a formation of more products may be expected, either as a products of oxidation, or degradation of the substrate. However, obtained results will be useful for our future studies on betalains and oxidation process of natural compounds in general.

Conclusions

A series of enzymatic oxidation reactions of neobetanin was performed. The progress of reactions was monitored spectrophotometrically and all the formed products were tentatively identified by mass spectrometry as decarboxy- and dehydro-neobetanin derivatives. However, we cannot exclude a formation of other products in a further course of reaction. Performed experiments will be useful for our future studies on betalains and oxidation process of natural compounds in general. Neobetanin and its partly oxidized derivatives are medically active compounds which can be potentially used in pharmacy because of their antioxidant properites.

Bibliography

[1] Strack D., Steglich W., Wray V.: Betalains, Methods in Plant Biochemistry, 8, pp. 421-451, 1993

[2] Cai Y., Sun M., Corke H.: Antioxidant activity of betalains from plants of the Amaranthaceae, Journal of Agricultural and Food Chemistry, 51, pp. 2288-2294, 2003

[3] Gandía-Herrero F., Escribano J., García-Carmona, F.: Structural implications on color, fluorescence, and antiradical activity in betalains, Planta, 232, pp. 449-460, 2010

[4] Gandía-Herrero F., Escribano J., García-Carmona, F.: Purification and antiradical properties of the structural unit of betalains, Journal of Natural Products 75, pp. 1030-1036, 2012

[5] Wybraniec S., Michałowski, T.: New pathways of betanidin and betanin enzymatic oxidation, Journal of Agricultural and Food Chemistry, 59, pp. 9612-6922, 2011

[6] Wybraniec S., Starzak K., Skopińska A., Nemzer B., Pietrzkowski Z., Michałowski T.: Studies on Nonenzymatic Oxidation Mechanisms in Neobetanin, Betanin, and Decarboxylated Betanins, Journal of Agricultural and Food Chemistry, 61, pp. 6465-6476, 2013

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