Research on betanidin oxidation by ABTS radicals

Karolina Starzak, Anna Skopińska, Sławomir Wybraniec

Department of Analytical Chemistry, Institute C-1, Faculty of Chemical Engineering and Technology,
Cracow University of Technology, ul. Warszawska 24, Cracow 31-155, Poland, e-mail: kstarzak@chemia.pk.edu.pl

Betanidin is a basic betacyanin with 5,6-dihydroxyl moiety which causes its high antioxidant activity. It belongs to betalains, a group of natural, water-soluble plant pigments, which elicit a red-violet coloration of fruits and flowers. One of the most popular sources of betanidin is red beet root (*Beta vulgaris* L.). Recent studies have shown importance of betalains oxidation, because of their high natural, antiradical and antioxidant activity and potential benefits for human health [1].

An effect of oxidation of betanidin using ABTS radicals was investigated in aqueous solutions at pH 3–8 and compared to activity of horseradish peroxidase. Products of the biomimetic betanidin oxidation were monitored by high performance liquid chromatography (HPLC) coupled to optical detection and mass spectrometry (LC-DAD-ESI-MS/MS).

The presence of two main oxidation products: 2-decarboxy-2,3-dehydro-betanidin and 2,17-didecarboxy-2,3-dehydro-betanidin at pH 3 indicates their generation through two possible reaction ways with two different quinonoid intermediates: dopachrome derivative and quinone methide. Both reaction paths lead to the decarboxylative dehydrogenation of betanidin.

Keywords: betanidin, betalains, antioxidation activity, ABTS, radicals.

Introduction

Betanidin (Fig. 1) is a basic betalain with 5,6-dihydroxyl moiety which makes its high antioxidant activity. Betalains are group of natural, water soluble, plant pigments, found in the Caryophyllales order. Their main function is coloration of flowers and fruits.

Cancer diseases are one of the most common causes of death. Many different antioxidants are used in anticancer therapies recently, and scientists are still searching new compounds. Recent studies have shown some antiradical and antioxidant activity of betalains. It means, that betalains can reveal some properties with potential profits for human health. That is why studies on their oxidation mechanism and searching of new products of this reaction are of big interest.

Presence of some functional groups causes betanidin extremely high reactivity and makes it very sensitive to several factors such as oxidizing enzymes, presence of organic solvents, high and low pH or temperature. Previous studies [1–9] confirmed also its antioxidant and chemopreventive properties. Moreover, all oxidation products, formed during these reactions may also have some human health beneficial properties.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6--sulphonic) acid) is commonly used in popular technique for measurement of antioxidant activity of natural compounds. In reaction with sodium persulfate, dark green ABTS radicals are formed, which absorb light at 734 nm (Fig. 2) and later on in reaction with some antioxidants e.g. betanidin are discoloured which can be monitored by spectrophotometry (Fig. 3).

Obtained data were compared with results of oxidation catalyzed by horseradish peroxidase (HRP)

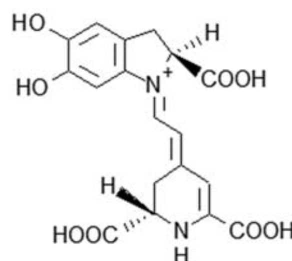

Fig. 1. Chemical structure of betanidin.

Natural Sciences

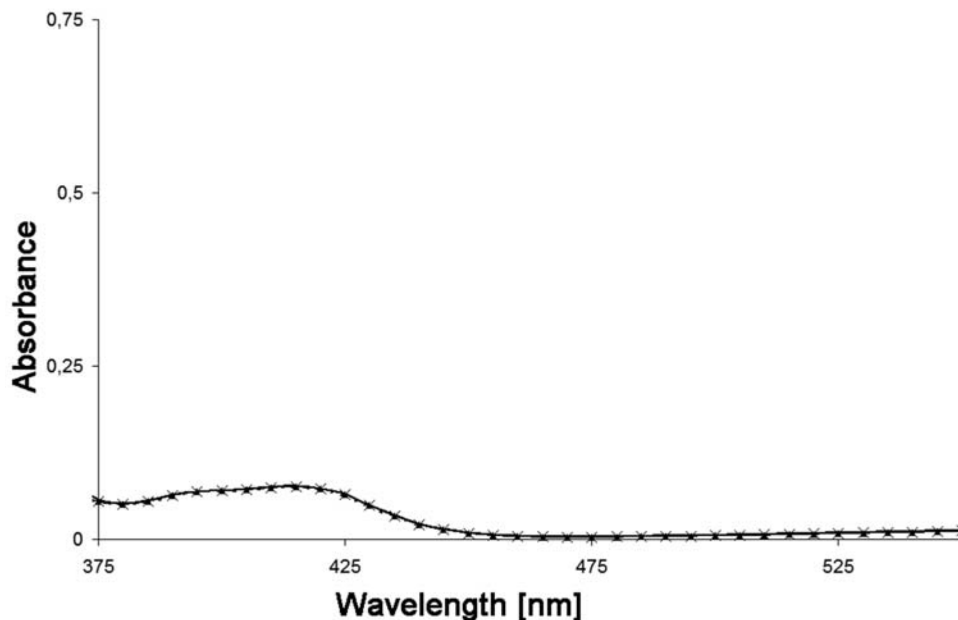

Fig. 2. Spectrum of ABTS solution.

which is a basic oxidizing enzyme [10]. All formed products were monitored by spectrophotometry and high performance liquid chromatography (HPLC) and identified by mass spectrometry MS/MS.

Material and methods

Betanidin preparation

Juice from red beetroots was obtained in a juice extractor (Zelmer, Rzeszów, Poland) and was submitted to clean--up on Sephadex DEAE A-25 gel and by solid phase extraction on C18 cartridges before HPLC preparative fractionation. Purified betanin was subjected to enzymatic hydrolysis catalysed by almond β-glycosidase and cleanup on Sephadex DEAE A-25 gel and by solid phase extraction on C18 cartridges. The elutes were concentrated under reduced pressure at 25°C and submitted to HPLC preparative fractionation.

Semipreparative HPLC

For the semipreparative isolation of betacyanins from the purified extracts a Gynkotek HPLC system with UVD170S, Gynkotek HPLC pump Series P580 and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used. The semipreparative column used was a 250 mm × 10 mm i.d., 10 mm, Luna C18(2), with a 10 mm × 10 mm i.d. guard column of the same material (Phenomenex, Torrance, CA) under the following gradient system (System 1): 6% A in B at 0 min; gradient to 10% A in B at 30 min. (A, acetonitrile; B, 4% (v/v) HCOOH in H2O). In each case, the injection volume was 100 mL and the flow rate was 3 mL/min. Detection was generally performed at 538, 505, 480 and 310 nm with a DAD UV/Vis detector. The columns were thermostated at 30°C. All fractions obtained were diluted with water and submitted to freeze-drying and analysis.

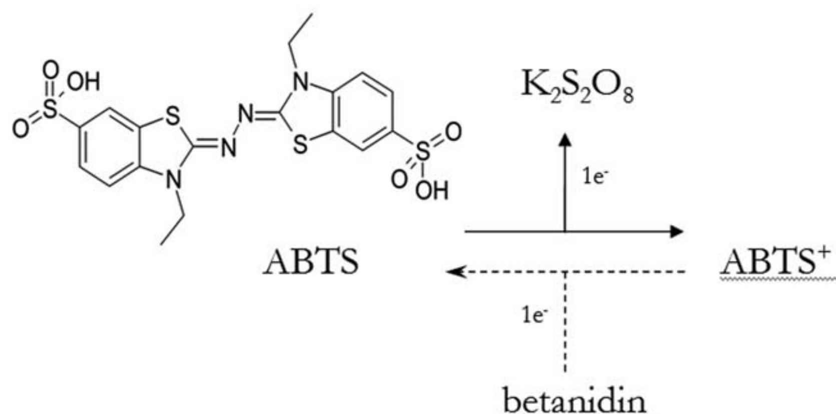

Fig. 3. Scheme of formation of ABTS⁺˙ radical, and its scavenge by betanidin.

ABTS assay

The oxidation of betanidin was performed in 25 mM acetate (pH 3–5.5) and phosphate (pH 6–8) buffers in 96-well plates of a microplate reader Tecan infinite 200 (Tecan Austria GmbH, Grödig/Salzburg, Austria). The action of 7.0 mM ABTS solutions with 2.45 mM K2S2O8, after 8 h of incubation at room temperature without light, on 25 mM pigment solutions was monitored during a period of 60 min at a temperature of 25°C by spectrophotometric detection at the wavelength range of 350–600 nm. For the chromatographic analysis, typically 70 mL reaction mixtures were sampled from the wells and injected directly to the HPLC column, without further purification.

Chromatographic system LC-DAD

A Gynkotek HPLC system with UVD170S, Gynkotek HPLC Pump Series P580 and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used for the chromatographic analysis. For the data acquisition,

Table 1. Spectrophotometric, chromatographic and mass spectrometric data of the analysed products of betanidin oxidation after 2 h of incubation with ABTS solutions.

No.	Compoud	R_t [min]	λ_{max} [nm]	m/z $[M+H]^+$	m/z MS/MS of $[M+H]^+$
1	Betanidin quinonoid [a]	7.5	405; 545	387	343, 299
1’	Betanidin isoquinonoid [a]	9.5	405; 545	387	343, 299
2	Betanidin	17.9	540	389	345
2’	Isobetanidin	19.5	540	389	345
3	2,17-bidecarboxy-2,3-dehydro-betanidin [a]	23.5	475	299	255; 253
4	2-decarboxy-2,3-dehydro-betanidin [a]	24.3	500	343	299; 255; 253
5	2,17-bidecarboxy-2,3-dehydro-neobetanidin [a]	27.2	415	297	253; 251

[a] — tentatively identified.

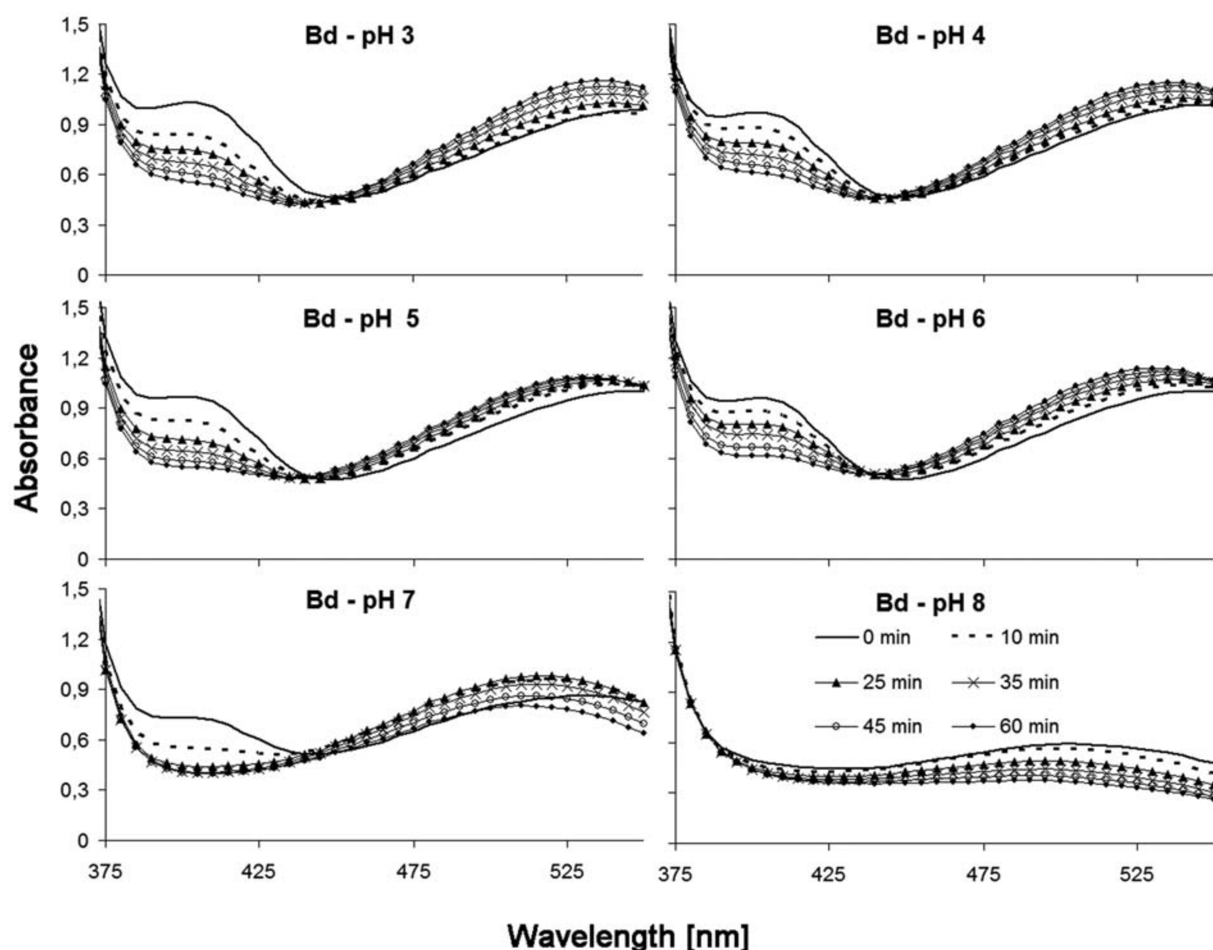

Fig. 4. Spectrophotometric results of oxidation of betanidin by ABTS radicals.

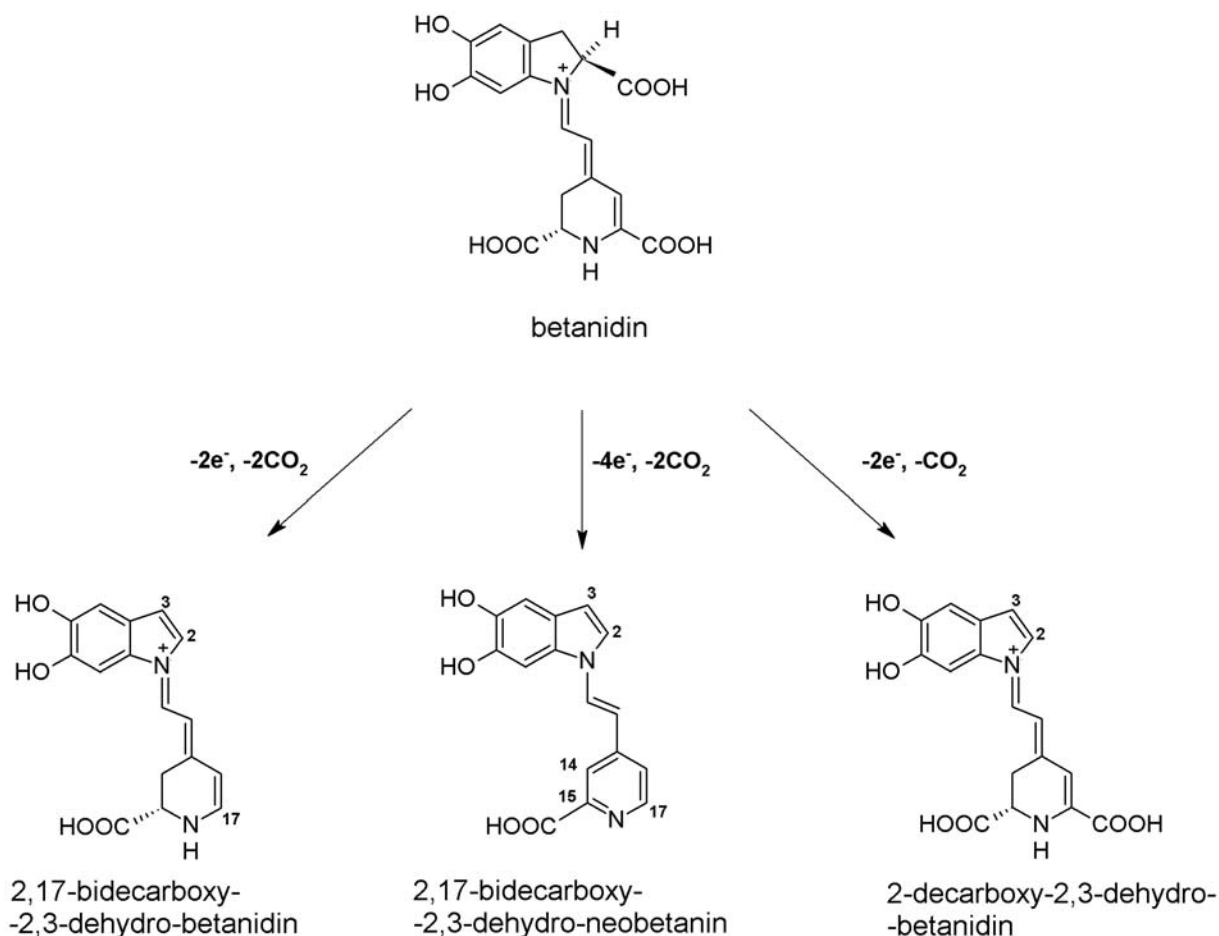

Fig. 5. Products of the reaction.

the software package Chromeleon 4.32 (Gynkotek Separations) was used. For the on-line UV/Vis spectra acquisition, the detection was performed in the DAD (diode-array detection) mode. The column used was a 250 mm × 3 mm i.d., 5 mm, Luna C18(2), with a 4 mm × 2 mm i.d. guard column of the same material (Phenomenex, Torrance, CA). The injection volume was 10 mL, 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), 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, B — methanol. The same chromatographic conditions were applied for the HPLC-ESI-MS/MS analyses.

Mass spectrometric 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 ThermoFinnigan 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). Helium was used to improve 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

Betanidin has only one absorption maximum in 540 nm (Table 1) which means that formation of new maxima is caused by a presence of new compounds; products of pigment oxidation. The sets of experiments with ABTS radicals at different pH's were performed to investigate changes in betanidin UV-Vis spectra. Obtained spectra (Fig. 4) were compared with results of previous studies with oxidizing enzymes [10].

Spectrophotometric analyses revealed presence of new absorption maxima. In first few minutes of reaction,

decrease of the main absorbance maximum with a shift of λ_{max} from 540 nm to 545 nm was observed as well as an increase of absorbance was noticed at λ 405 nm. These data confirmed results of previous studies and suggested a formation of *o*-quinone as a one of the products.

Further course of the reaction revealed subsequent decrease of second absorption maximum at λ_{max} 405 nm, and hypsochromic shift of λ_{max} of the main absorbance band. Moreover, almost the same trend of reaction was observed for whole range of tested pH's.

LC-DAD-MS/MS analysis enabled identification of more compounds (Fig. 5). Two main additional products **3** and **4** were identified as 2,17-bidecarboxy-2,3--dehydro-betanidin and 2-decarboxy-2,3-dehydro--betanidin, respectively. Formations of these two compounds are result of rearrangements of betanidin *o*-quinone. The last product **5**, identified as 2,17-bidecarboxy-2,3-dehydro-neobetanidin was noticed after long time of reaction.

Based on obtained results, a main oxidation mechanism through *o*-quinone derivative can be postulated. As a second step of the reactions, are internal rearrangements leading to a formation of new decarboxylated and dehydrogenated finale products. Comparison of obtained results with effects of betanidin oxidation by horseradish peroxidase confirmed our suggestion [10].

Conclusions

The experiments resulted in oxidation of betanidin by ABTS radicals. Obtained oxidation products were detected and later on identified as decarboxylated and dehydrogenated betanin derivatives: 2,17-bidecarboxy--2,3-dehydro-betanidin and 2-decarboxy-2,3-dehydro--betanidin. Comparison of results with effects of previous research with horseradish peroxidase leads us to a conclusion, that betanidin is oxidized in a similar same way by both oxidants.

Acknowledgement

This research was financed by Polish Ministry of Science and Higher Education for years 2007–2010 (Project No. N312 3268 33).

References

[1] Tesoriere, L., et al. "Distribution of betalain pigments in red blood cells after consumption of cactus pear fruits and increased resistance of the cells to ex vivo induced oxidative hemolysis in humans". *J. Agric. Food Chem.* 53 (2005): 1266–1270.

[2] Martinez-Parra, J. and R. Munoz. "Characterization of beta-cyanin oxidation catalyzed by a peroxidase from *Beta vulgaris* L. roots". *J. Agric. Food Chem.* 49 (2001): 4064–4068.

[3] Kapadia, G. J., et al. „Chemoprevention of DMBA--induced UV-B promoted, NOR-1-induced TPA promoted skin carcinogenesis, and DEN-induced phenobarbital promoted liver tumors in mice by extract of beetroot". *Pharmacol. Res.* 47 (2003): 141–148.

[4] Kanner, J., S. Harel, and R. Granit. "Betalains — A new class of dietary cationized antioxidants". *J. Agric. Food Chem.* 49 (2001): 5178–5185.

[5] Escribano, J., et al. "Characterization of the antiradical activity of betalains from *Beta vulgaris* L. roots". *Phytochem. Anal.* 9 (1998): 124–127.

[6] Butera, D., et al. "Antioxidant activities of Sicilian prickly pear (*Opuntia ficus — indica*) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin". *J. Agric. Food Chem.* 50 (2002): 6895–6901.

[7] Pavlov, A., et al. "Biosynthesis and radical scavenging activity of betalains during the cultivation of red beet (*Beta vulgaris*) hairy root cultures". *Z. Naturforsch C.* 57 (2002): 640–644.

[8] Cai, Y., M. Sun, and H. Corke. "Antioxidant activity of betalains from plants of the Amaranthaceae". *J. Agric. Food Chem.* 51 (2003): 2288–2294.

[9] Gandía-Herrero, F., J. Escribano, and F. García-Carmona. "Structural implications on color, fluorescence, and anti-radical activity in betalains". *Planta* 232 (2010): 449–460.

[10] Wybraniec, S. and T. Michałowski. "New pathways of betanidin and betanin enzymatic oxidation". *J. Agric. Food Chem.* 59 (2011): 9612–9622.