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Chlorination of Betacyanins in Several Hypochlorous Acid Systems

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ABSTRACT: This study presents a comparative evaluation of chlorination of betanin, betanidin, and neobetanin exposed to sodium hypochlorite and myeloperoxidase (MPO)/H_2O_2/Cl^- systems. For betanin/betanidin, the chlorination takes place at the aglycone unit, but for neobetanin, no chlorinated products in the reaction mixtures can be detected. In the RP-HPLC system, monochloro-betanin/-betanidin were eluted earlier than their corresponding nonchlorinated substrates. An influence of Cl^- concentration on betanin/betanidin chlorination efficiency in sodium hypochlorite and MPO systems was investigated. At pH 3−5, the yields of formed monochloro-betanin/-betanidin decrease dramatically at higher Cl^- concentrations, indicating that generated Cl_2 is not the chlorinating agent in the presence of sodium hypochlorite. The intriguing low activity of Cl_2 in betanin/betanidin chlorination compared to HOCl and/or Cl_2O can be explained by a special position of the attack by molecules of HOCl and/or Cl_2O. In the MPO/H_2O_2/Cl^- system, the highest efficiency of monochloro-betanin/-betanidin generation is observed at pH 5.

KEYWORDS: *betanin, betanidin, neobetanin, betacyanins, betalains, myeloperoxidase, MPO, inflammation, neutrophils, chlorination, hypochlorite*

INTRODUCTION

Betalains are water-soluble plant pigments commonly used as food colorants.[1,2] A remarkable betalain subgroup includes betacyanins, which are primarily immonium conjugates of betalamic acid with glycosylated cyclo-DOPA.[1,2] Betanin (Figure 1), the principal pigment of red beet root (*Beta vulgaris* L.), is the first and most frequently studied betalain for its antioxidant activity.[3−10]

Products containing betalains have more often been used for modulation of various health conditions.[3−7] Moreover, a betalain-rich concentrate (BRC) derived from *B. vulgaris* roots standardized to a minimum content of 25% betalains and substantially free of sugars and nitrates was tested in a pilot clinical study, which showed that short-term treatment with BRC improved the function and comfort of knee joints in individuals with knee distress.[11,12]

The above-referenced studies show that hypochlorous acid may trigger several detrimental processes in the human body.

Allegra et al.[13] reported the effectiveness of the betalains, betanin and indicaxanthin, in scavenging of hypochlorous acid. In addition, both of these betalains have been reported to reduce myeloperoxidase activity and oxidation, two key components of the inflammatory process,[13−17] which also results in a reduction of hypochlorous acid generation.[14,18] In recent studies on enzymatic oxidation of betanidin with horseradish peroxidase,[19] a formation of oxidation products 2-decarboxy-2,3-dehydro-betanidin and 2,17-bidecarboxy-2,3-dehydro-betanidin indicated their generation via two possible reaction paths with two different quinonoid intermediates: dopachrome derivative and quinone methide. Both reaction pathways lead to a decarboxylative dehydrogenation of betanidin. Subsequent oxidation and rearrangement of the conjugated chromophoric system results in the formation of 14,15-dehydrogenated derivatives. Betanin (5-*O*-glucosylated betanidin) is presumably oxidized solely via generation of a quinone methide intermediate that rearranges to 2,3-dehydro- or neo-derivatives.[19] Further comprehensive nonenzymatic studies on the oxidation mechanism in neobetanin and betanin as well as its decarboxylated derivatives in the presence of ABTS cation radicals as a commonly used diagnostic oxidation agent were accomplished recently.[20]

Except for the paper of Allegra et al.[18] no studies on betalain chlorination were performed that would clarify a role of these potent antioxidants in hypochlorite scavenging. Similarly, no chlorination/oxidation products of betalains were detected or identified. Therefore, a comprehensive study of betanin, betanidin, and neobetanin (partially oxidized betanin) reacting with hypochlorous acid and a comparative evaluation of the products based on the LC-DAD-MS/MS results were performed. In addition, the chromatographic properties of the generated chloro-betalains were also studied.

MATERIALS AND METHODS

Reagents. Formic acid, LC-MS grade methanol, and water were obtained from Sigma Chemical Co. (St. Louis, MO, USA). NaOCl solution, H_2O_2, NaCl, horseradish peroxidase, and myeloperoxidase from human leukocytes (MPO) were also obtained from Sigma Chemical Co.

Preparation of Natural and Hydrolyzed Analytes. Betanin and neobetanin were isolated from a betalain-rich red beet root extract obtained from FutureCeuticals, Inc. (Momence, IL, USA).[21] The extract was dissolved in water and was separated on Sephadex DEAE A-25 gel and by solid phase extraction on C18 cartridges before HPLC preparative fractionation.[22,23] For betanidin preparation, purified

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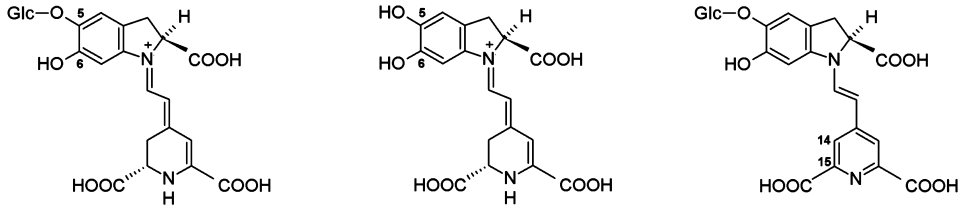

Figure 1. Chemical stuctures of the studied betalains in the hypochlorous systems.

betanin was subjected to enzymatic hydrolysis catalyzed by almond β-glycosidase[24] and the same cleanup as in the case of the natural pigments. The eluates were concentrated under reduced pressure at 25 °C.

Semipreparative HPLC. For the semipreparative isolation of betanin and neobetanin from the purified red beet root extract as well as betanidin, an HPLC system with a UVD170S detector, a series P580 pump, and a thermostat (Gynkotek Separations, H. I. Ambacht, The Netherlands) was used. The semipreparative column used was a 250 mm × 10 mm i.d., 10 μm, Luna C18(2), with a 10 mm × 10 mm i.d. guard column of the same material (Phenomenex, Torrance, CA, USA) 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 H_2O). In each case, the injection volume was 100 μL 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.

Spectrophotometric Monitoring of Chlorination Kinetics. The main betalain chlorination experiments in the Cl_2/HOCl/OCl^- systems were performed by their reaction with NaOCl aqueous solutions buffered with 25 mM acetate (pH 3−5) and phosphate (pH 6−8) buffers in 96-well plates of an Infinite 200 microplate reader (Tecan Austria GmbH, Grödig/Salzburg, Austria). The concentration of the NaOCl solution was determined spectrophotometrically at pH 12 (ε_{292} = 350 M/cm). Just before the measuring step, 20 μL of NaOCl solution was dispensed to each well containing 40 μM dissolved pigment, bringing the volume to 200 μL. The final concentration of the NaOCl solution in the wells was in the range of 40−120 μM for all of the tested pigments except neobetanin (10−40 μM). For the experiments with influence of NaCl on the tested reactions, the salt was added to the wells to reach the final concentration of 20−1500 mM. The mixture was then shaken for 20 s by a shaker within the reader. The spectra were collected over 30 min at a temperature of 25 °C by spectrophotometric detection at the wavelength range of 350−550 nm. For the chromatographic analyses, separate experiments were performed, and 20 μL samples after 5 min of reaction were injected directly to the LC-DAD or LC-DAD-ESI-MS/MS system without further purification. The measurements were performed in triplicate.

Spectrophotometric Monitoring of Enzymatic Chlorination Kinetics. The enzymatic chlorination experiments on 40 μM betalains in the MPO/H_2O_2/Cl^- system were performed in aqueous solutions (buffered with 25 mM acetate (pH 3−5) and phosphate (pH 6−8) buffers) during reaction catalyzed by 1 μM MPO in the presence of 50 μM H_2O_2 and 150 mM NaCl in 96-well plates of the microplate reader. Just before the measuring step, 20 μL of H_2O_2 solution was dispensed to each well containing all of the components, bringing the volume to 200 μL. For the experiments with the influence of NaCl on the tested reactions, the salt was added to the wells to reach the final concentration of 20−600 mM. The mixture was then shaken for 20 s by a shaker within the reader, and the spectra were collected over 120 min of the experiment. For the chromatographic analyses, separate experiments were performed, and each injection to the LC-DAD-ESI-MS/MS system was performed after 120 min of the reaction. The measurements were performed in triplicate.

Chromatographic Analysis. The equipment was the same as that used for the semipreparative HPLC. Data were acquired with the Chromeleon 4.32 (Gynkotek Separations) software package. Samples were eluted through a 250 mm × 3 mm i.d., 5 μm, Luna C18(2) column preceded by 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 column was thermostated at 35 °C. For the separation of the analytes, a gradient system was used (system 2): 93% A in B at 0 min; gradient to 80% A in B at 35 min (A, 2% formic acid in water; B, methanol). Online UV/vis spectra acquisition was performed using the diode array detection (DAD) mode typically at 538, 505, 480, and 440 nm. The same chromatographic conditions were applied to LC-DAD-ESI-MS/MS analyses.

LC-ESI-MS/MS Analysis. The positive ion electrospray mass spectra were recorded on an LCMS-8030 mass spectrometry system (Shimadzu, Kyoto, Japan) coupled to a LC-20ADXR pump utilizing the HPLC gradient system 2. The LC-MS system was controlled with LabSolutions software (Shimadzu), which recorded total ion chromatograms and mass spectra (electrospray voltage, 4.5 kV; capillary, 250 °C; sheath gas, N_2). Argon 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 −35 V.

RESULTS AND DISCUSSION

The possibility of a chlorination of betacyanins as well as detection of the chlorinated pigments was studied on the closely related compounds betanin, **1**; betanidin, **2**; and neobetanin, **3** (Figure 1). Betanin is a glucosylated derivative of the chromophoric structure of betanidin. The chromophoric unit of betanidin is the only one with the 5,6-dihydroxy (catechol) moiety, and consequently it possesses a high antioxidant activity.[6,9,10] Recent enzymatic oxidation experiments on betanidin at pH 3−8 confirmed the prevailing effect of the *o*-diphenol group on the oxidation pathway.[19] However, taking into account that betanin also exhibits antioxidative properties,[6,9,10] the possibility of a formation of oxidized products during betanin exposure to hypochlorous acid was investigated and verified. In addition, neobetanin, partially oxidized betanin, represents another reactive derivative, which arises during numerous oxidation processes and is a key diagnostic intermediate in the oxidation pathways.[1]

Chlorination of Betanin by Sodium Hypochlorite. The spectrophotometric monitoring of betanin, **1**, reaction induced by NaOCl at pH 3, 5, and 7.4 was performed in dependence on different NaOCl concentrations. The highest oxidation activity of the reagent is observed at pH 7.4, and it is in contrast to the results of enzymatic oxidation of betanin that indicated the highest rate of oxidation at pH 3.[19] However, analysis of the chlorination UV/vis spectra suggests completely different mechanisms of betanin reaction in acidic and close to neutral media. At pH 7.4, the main absorption band of betanin (λ_{max} 538 nm) is slightly shifted during the chemical changes (λ_{max} 534 nm), whereas at pH 3, a hypsochromic shift of λ_{max} is observed, reaching 522−524 nm at NaOCl concentration of 40 μM after 5 min of the experiment. At pH 5, a medium point of the shift is attained (λ_{max} 526 nm) at higher NaOCl

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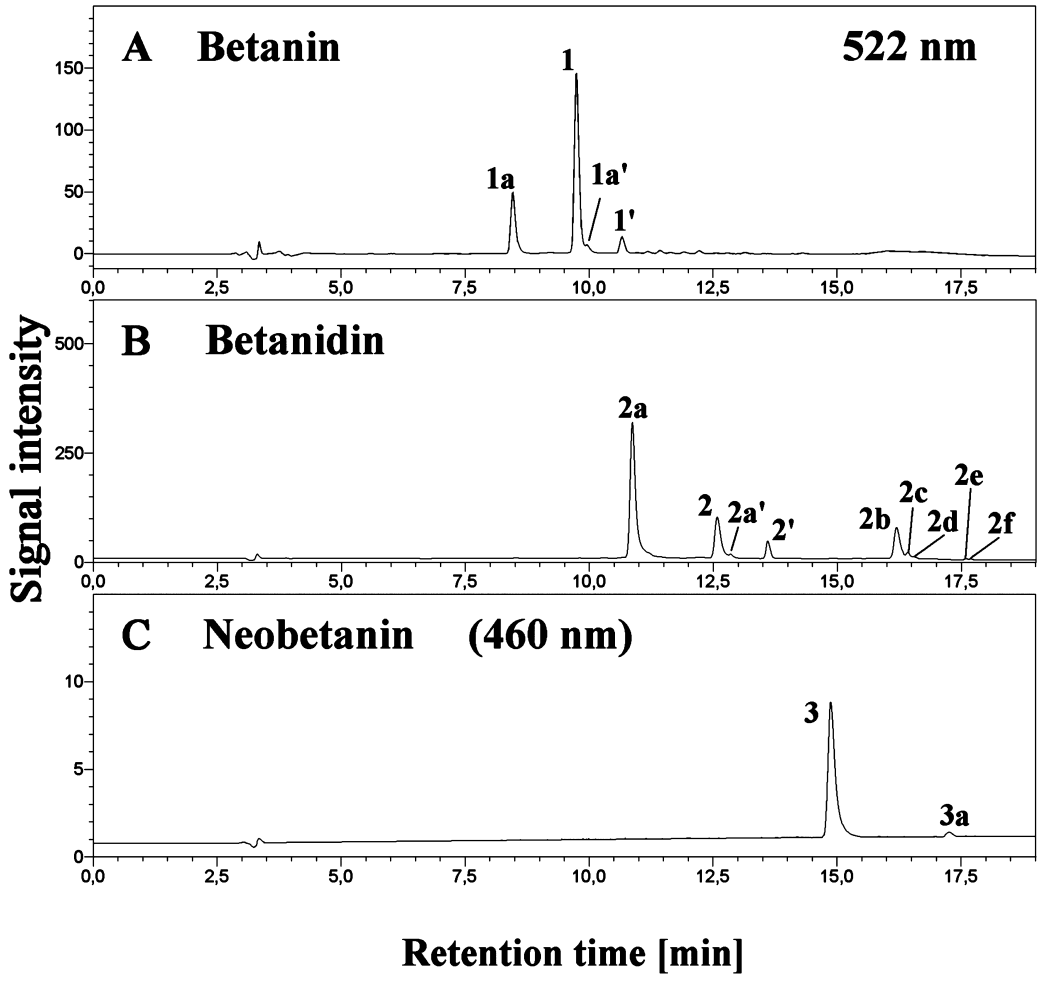

Figure 2. Chromatographic traces of the products of 40 μM betanin **1** (A), betanidin **2** (B), and neobetanin **3** (C) oxidation at pH 5 (pH 7.4 for neobetanin) by 1 μM MPO in the presence of 50 μM H_2O_2 and 150 μM NaCl and monitored at 522 nm (480 nm for neobetanin) and 25 °C. Peak numbering is presented in Table 1.

concentrations. The same results were obtained for the chlorination of isobetanin **1**′ (the diastereomeric form 15*R*) evidently with a chlorinated diastereomer **1a**′.

These data are confirmed by the HPLC-DAD analysis of the reaction mixtures revealing the formation of a main product absorbing light at λ_{max} 522 nm. In Figure 2A,B, typical chromatographic profiles of chlorinated betacyanins together with their nonchlorinated precursors obtained for enzymatic chlorination (discussed in the next section) at pH 5 are presented. The chlorinated derivatives formed were eluted from the column earlier than their precursors.

Further LC-MS/MS analytical results indicate a chlorination of betanin (characterized by λ_{max} 538 nm and m/z 551) due to the fact that the new chromatographic peak formed with λ_{max} 522 nm and m/z 585 (protonated molecular ion) suggests the substitution of a hydrogen in the molecule by one chlorine atom (m/z difference 585 − 551 = 34). The characteristic isotopic profile of the protonated molecular ions containing ^{35}Cl or ^{37}Cl isotopes confirms the presence of one chlorine atom in the molecule. A fragmentation experiment of the $[M + H]^+$ ion results in a subtraction of one glucose unit and the formation of a protonated monochloro-betanidin/isobetanidin ion (m/z difference 585 − 162 = 423). This confirms that the chlorination takes place at the aglycone unit (betanidin).

Because the chlorination of betanin is especially favored in acidic conditions, the high activity of molecular chlorine (Cl_2) generated from hypochlorous acid toward betanin at pH 3−5 was suspected to be the chlorinating factor. HOCl generated in aqueous solutions is in equilibrium with Cl_2:[25]

$$HOCl + Cl^- + H^+ \rightleftharpoons Cl_2 + 2H_2O \quad (1)$$

Therefore, the influence of Cl^- concentration on the reaction progress would result in the detection of higher concentrations of the chlorinated products, especially at higher acidity (pH 3−5). Surprisingly, this was not the case shown in the betanin chlorination experiments with increasing NaCl concentration (Figure 3A). At pH 3−5, the yield of monochlorinated betanin decreases dramatically at higher Cl^- concentrations, indicating that the generated Cl_2 is not the chlorinating agent. At pH 6−8, the yield of formed monochloro-betanin is much lower than at pH 3−5; however, it is almost independent of Cl^- concentration. At pH 3, a decreased yield of monochloro-betanin formation in comparison to pH 4−5 is also seen in the whole Cl^- concentration range.

In Figure 3B, the remaining substrate (betanin) fraction (%) analyzed by LC-MS in the reaction mixtures is presented in dependence on Cl^- concentration and pH. The highest reactivity of betanin is observed at pH 3−5 with the lowest Cl^- concentration when only <6% of unreacted betanin is

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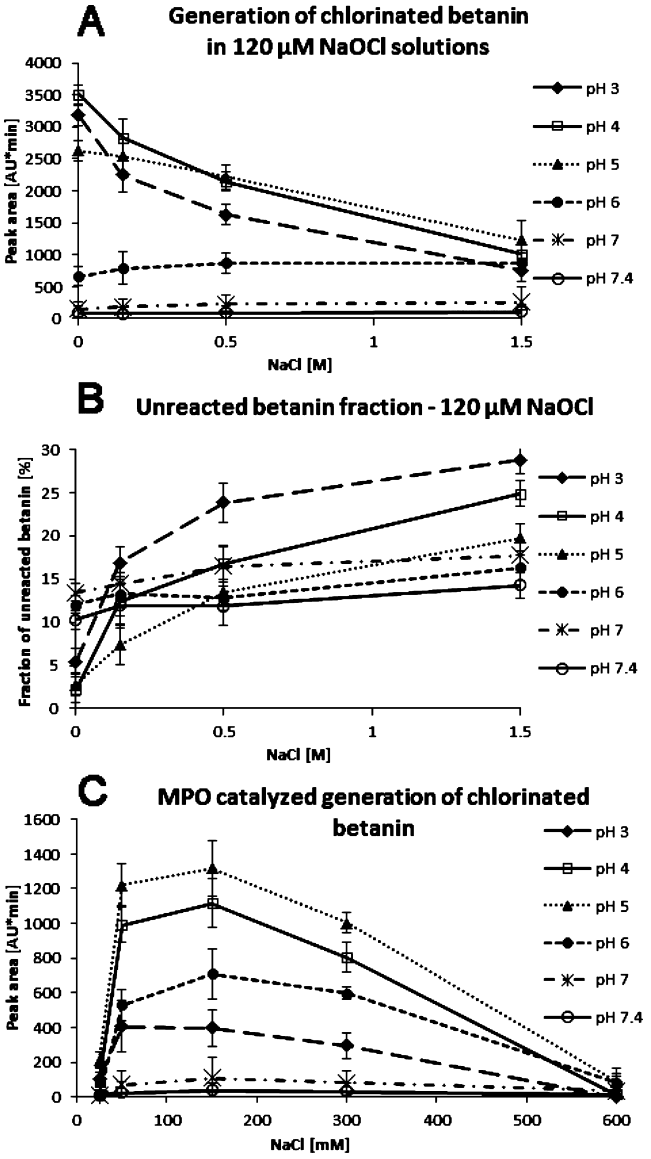

Figure 3. Influence of NaCl concentration on efficiency of betanin **1** chlorination by NaOCl (A) and stability of betanin (B) as well as myeloperoxidase-catalyzed chlorination (C) in dependence on pH. At high NaCl concentration (increased Cl_2 delivery), a diminished chlorination efficiency is noted for pH 3–5 (A, B) or in all of the MPO reaction media (C).

detected. At high Cl^- concentration (1.5 M), unreacted betanin is detected at a level of 18–28%. In contrast, the levels of remaining unreacted betanin seen at pH 6–8 do not change meaningfully with the increase of Cl^- concentration, confirming that the generation of Cl_2 is low at pH 6–8 and is not changing meaningfully. Interestingly, at pH 3, the highest concentration of betanin is detected, indicating that the reaction progress is decreased in comparison to pH 4–5.

HOCl is a weak acid (pK_a = 7.54); therefore, at physiological pH, it is present at a comparable concentration with its dissociated form OCl^-, which is presumably acting as a direct oxidant for betanin:[25]

$$HOCl \rightleftharpoons OCl^- + H^+ \quad (2)$$

In more acidic media, HOCl is partially converted to chlorine monoxide Cl_2O, which is a more potent chlorinating agent even at lower concentrations:[25,27−29]

$$2HOCl \rightleftharpoons Cl_2O + H_2O \quad (3)$$

Therefore, the influence of pH on the formation of chlorinated betanin can be explained by two factors: diminished dissociation of HOCl in acidic media and increased generation of Cl_2O from HOCl instead of chlorine in acidic media.

Both of the compounds, HOCl and Cl_2O, can have an impact on the chlorination of betanin via electrophilic substitution. As mentioned in recent literature,[25,27−29] the potency of Cl_2O in chlorination and oxidation has been recognized for over 170 years. Aqueous free chlorine equilibrium speciation for HOCl, OCl^-, Cl_2, and Cl_2O as potential chlorinating and oxidizing agents has been presented and kinetically confirmed in selected reactions.[25,27−29] The intriguing low activity of Cl_2 in betanin chlorination compared to HOCl and Cl_2O can be explained by a special position of the attack by a molecule of HOCl and Cl_2O, which is not in the aromatic ring of betanin (typical Cl_2 target) but in the carbon position C-18 with acidic hydrogen.[30] This is a carbon of enhanced negativity, frequently noted to rapidly exchange hydrogen in deuterium dioxide;[30] therefore, reactions with HOCl and Cl_2O are proposed according to a mechanism based on leaving group ability from Cl^+ in HOCl ($-OH^-$) and in Cl_2O ($-OCl^-$) (Figure 4).[25,27−29] This is also supported by the inactivity of neobetanin toward chlorination. Neobetanin is an aromatized betanin with a pyridine ring resulting from betanin oxidation. The fact that this ring cannot be chlorinated confirms that in betacyanins, only the unsaturated bond is attacked, preferably at C-18 because of its partial negative charge.

Because the leaving group ability from Cl_2O is much higher than in HOCl,[25,27−29] the chlorinating power of Cl_2O is much stronger even at lower concentration than that of HOCl. But for HOCl, an additional mechanism of chlorination based on the formation of hypochlorous acidium ion in acidic media can be proposed:[25,31]

$$HOCl + H^+ \rightleftharpoons H_2OCl^+ \quad (4)$$

This would support the chlorinating ability of HOCl toward betanin (Figure 4) and betanidin and also the oxidative properties toward betanidin.

Chlorination of Betanin by MPO System. According to the above results, hypochlorous acid generated by myeloperoxidase in the presence of chloride anions and hydrogen peroxide (reaction 5) should also chlorinate betanin throughout the entire tested pH range and with the highest efficiency at pH 3–6. However, the most optimal pH increasing the MPO activity toward HOCl formation that may occur in the phagosome is close to 5:[26]

$$Cl^- + H_2O_2 + H^+ \rightleftharpoons HOCl + H_2O \quad (5)$$

Because the activity of MPO strongly depends on the pH of the reaction medium and the enzymatic process is much slower than direct chlorination by NaOCl, a series of experiments was performed on the pH influence on the generation of chlorinated betanin and kinetic monitoring of the reaction progress. Anticipated detection of the chlorinated betanin in the MPO system at pH ≥5 was supposed to confirm the possibility of its formation during a hypochlorite scavenging process, which possibly may take place under in vivo

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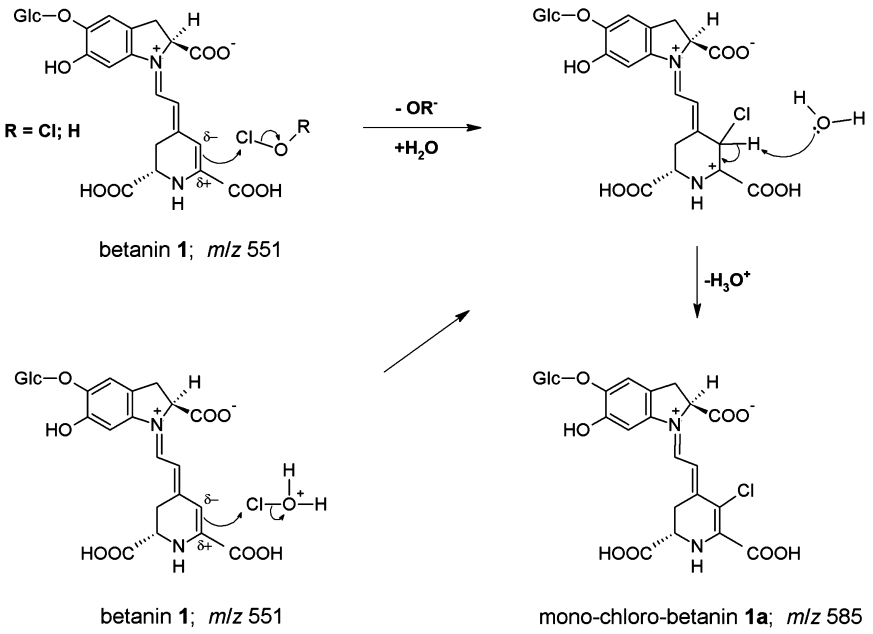

Figure 4. General reaction mechanism proposed for the chlorination of betanin **1** by hypochlorous acid (HOCl) or dichlorine oxide (Cl_2O). For HOCl, two alternative chlorination pathways are considered.[24,26−28] A similar mechanism can be assigned for betanidin **2**.

conditions. In addition, the strong oxidizing activity of HOCl could be still observable at physiological pH levels.[26]

The chromatographic traces of the reaction mixtures recorded after 2 h of incubation of MPO and H_2O_2 with the pigment at pH 5 are depicted in Figure 2. The chromatogram confirms a partial transformation of the pigment into the chlorinated derivative.

In the enzymatic system, the chlorination progress is controlled by a continuous generation of HOCl; therefore, a continuous absorption maximum shift is observed. This is in contrast to the rapid chlorination of the pigments by NaOCl at a defined reagent concentration. The observed directions of changes in the spectra are a result of a superposition of the strong betanin tendency to be chlorinated at more acidic media (pH 3−5) and the highest MPO activity at pH 5−5.5 toward the generation of HOCl. In the course of spectrophotometric changes, the main absorption maximum of betanin at pH 5 shifts toward the characteristic maximum of chlorinated betanin (522 nm) observed formerly during the direct chlorination by NaOCl.

Further experiments on MPO-mediated chlorination of betanin with increasing NaCl concentration (Figure 3C) led also to the conclusion that an overdose of NaCl (up to 600 mM) hampers the generation of monochloro-betanin as a result of Cl_2 formation (reaction 1), which is not the chlorinating agent according to the results of the above nonenzymatic chlorination experiments (Figure 3A). However, at lower NaCl concentrations, this effect is overwhelmed by a continuous production of HOCl from Cl^- catalyzed by MPO, which reacts with betanin to produce the highest quantities of chlorinated betanin within a concentration range of NaCl at 50−150 mM (Figure 3C).

In Figure 3C, as a result of the chromatographic analyses, the highest efficiency of monochloro-betanin generation is clearly indicated at pH 5. The efficiency at pH 3 is significantly diminished in contrast to the nonenzymatic chlorination. Interestingly, the MPO-mediated chlorination is still observed at pH 6−7.4 at the physiological levels of the reagents (Figure 3C), confirming that HOCl is still generated by MPO activity even at pH 7.4. In addition, the lack of the diagnostic betanin dehydrogenated and decarboxylated derivatives (possessing the characteristic chromophoric systems) in the chromatograms at any applied pH (Figure 2) suggests that no evidence of betanin oxidation can be obtained. Therefore, no peroxidase activity of MPO toward betanin is confirmed, which is in contrast to the previously observed activity of horseradish peroxidase, especially in acidic media.[19]

Chlorination of Betanidin by Sodium Hypochlorite. For betanidin, **2**, which is the only nonglucosylated betacyanin, characterized by the absorption maximum at λ_{max} 540 nm,[1,2] the analogous strong hypsochromic shifts in the spectra (down to 528 nm) are observed at pH 3. In addition, at higher NaOCl concentrations (>100 μM), the main absorption band is almost undiminished but shifted to the characteristic λ_{max} of monochloro-betanidin, whereas at pH 7.4, it gradually disappears with additional shift toward 528 nm. For pH 7.4, longer hypsochromic shifts in the spectra of the pigment are observable, starting from an NaOCl concentration of 100 μM, suggesting a substantial formation of dehydrogenated derivatives rather than chlorinated ones. This is in accordance with a high betanidin activity against various oxidation agents, especially in neutral media.[6,9,10] Therefore, except for the formation of a chlorinated derivative, the bulk of oxidized products can be expected to be generated in the reaction mixture especially in neutral media.[19]

The formation of monochloro-betanidin was confirmed by detection of a principal reaction product characterized by m/z 423 (423 − 389 = 34), suggesting the substitution of a hydrogen by chlorine in betanidin. In addition, the characteristic isotopic profile of ^{35}Cl and ^{37}Cl isotopes is observed for the protonated molecular ion, and analogous results are obtained for isobetanidin, **2′**. In addition, the generated chlorinated betanidin/isobetanidin moieties are eluted earlier than their precursors (Figure 2B). Monochloro-isobetanidin, **2a′**, exhibits a positive shift in the retention time in relation to **2a**. In addition, two chlorinated derivatives in the reaction

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pathway are detected (Table 1), monochloro-17-decarboxy-betanidin, **2d**, and monochloro-2,17-bidecarboxy-2,3-dehydro-betanidin, **2e**. The formation of **2d** in the reaction mixtures is evidently a result of a spontaneous decarboxylation of monochloro-betanidin, **2a**, in the presence of HOCl, whereas the formation of **2e** can be explained in two parallel ways: decarboxylative oxidation of monochloro-17-decarboxy-betanidin, **2d**, and/or chlorination of the already oxidized compound, 2,17-bidecarboxy-2,3-dehydro-betanidin, **2b** (Figure 5).

Table 1. Chromatographic, Spectrophotometric, and Mass Spectrometric Data of the Analyzed Betalains and Their Products of Chlorination by NaOCl as well as the MPO System

no.	compound	t_R (min)	λ_{max} (nm)	m/z $[M + H]^+$	m/z from MS/MS of $[M + H]^+$
1a	*monochloro-betanin*[a]	8.4	522	585	423; 379
1	betanin	9.7	538	551	389
1a′	*monochloro-isobetanin*[a]	9.9	522	585	423; 379
1′	isobetanin	10.7	538	551	389
2a	*monochloro-betanidin*[a]	10.8	528	423	379
2	betanidin	12.6	541	389	345
2a′	*monochloro-isobetanidin*[a]	12.8	528	423	379
2′	isobetanidin	13.6	541	389	345
2b	*2,17-bidecarboxy-2,3-dehydro-betanidin*[a,b]	16.2	472	299	255; 253
2c	*2-decarboxy-2,3-dehydro-betanidin*[a,b]	16.4	498	343	299; 255
2d	*monochloro-17-decarboxy-betanidin*	16.6	525	379	335
2e	*monochloro-2,17-bidecarboxy-2,3-dehydro-betanidin*	17.6	465	333	289
2f	*2,17-bidecarboxy-2,3-dehydro-neobetanidin*[a,b]	17.7	415	297	253
3	neobetanin	14.8	466	549	387
3a	*2-decarboxy-2,3-dehydro-neobetanin*[a,c]	17.3	420	503	341; 297

[a]Tentatively identified. [b]Oxidation products identified previously in ref 19. [c]Oxidation product identified previously in ref 20.

The influence of pH on the formation of the oxidized/chlorinated betanidin derivatives analyzed by LC-MS system was also studied. The contradictory effect of pH on the presence of the main chlorinated product **2a** in the reaction mixtures (prevailing at more acidic conditions) as well as the oxidized and decarboxylated compound **2b** (2,17-bidecarboxy-2,3-dehydro-betanidin) and monochloro-2,17-bidecarboxy-2,3-dehydro-betanidin, **2e** (both increasing their concentration toward more neutral media), indicates the highest chlorination efficiency of NaOCl at more acidic conditions, as in the case of betanin, as opposed to the oxidation in more neutral conditions. The direct betanidin oxidation product, 2-decarboxy-2,3-dehydro-betanidin, **2c**, is evidently less stable than **2b** because it converts spontaneously to **2b** over the whole pH range, whereas a further oxidation of **2b** is again observed at more neutral pH, resulting in the formation of 2,17-bidecarboxy-2,3-dehydro-neobetanidin, **2f**.

As in the case of betanin, the chlorination experiments on betanidin with increasing NaCl concentration confirmed that the highest efficiency of monochlorinated betanidin generation is observed for the lowest Cl^- concentration (at pH 3−5). At pH 6−7.4, the yield of formed monochloro-betanidin is almost independent of Cl^- concentration and also much lower than at pH 3−5. Similar to betanin chlorination, at pH 3, a decreased yield of monochloro-betanidin formation in comparison to that at pH 4 is visible at higher Cl^- concentration range.

Therefore, for betanidin and betanin, a similar mechanism of chlorination can be suggested, indicating that the generated Cl_2 is not the chlorinating agent but rather HOCl and/or Cl_2O.

This is partially confirmed by the reactivity test on betanidin stability, which is highest at pH 3−6 with the lowest Cl^- concentration when ca. 17−20% of unreacted betanidin is detected. However, the levels of remaining unreacted betanidin at pH 7−7.4 decrease meaningfully with the increase of Cl^- concentration to 0.5 M, which should be rather a result of increased oxidation (Figure 5) effect than of chlorination.

Chlorination of Betanidin by MPO System. In the case of betanidin myeloperoxidase-assisted chlorination, the chlorinating activity of generated hypochlorous acid by MPO in the presence of chloride anions and hydrogen peroxide (reaction 3) is strongly modulated by the oxidizing activity of HOCl (as indicated in the above NaOCl tests) as well as the peroxidase function of MPO. Both oxidizing effects were observed to prevail in neutral media.

The spectrophotometric monitoring of MPO-mediated chlorination of betanidin was also performed. As in the case of betanin, a continuous shift of the absorption maximum toward λ_{max} of the chlorinated betanidin (528 nm), as well as the highest MPO activity at pH 5, is observed. The similarity is also visible in the high reaction rate at neutral media, which should be assigned mainly to the oxidation of betanidin.

The chromatographic trace of the reaction mixture recorded after 2 h of incubation of MPO and H_2O_2 with betanidin at pH 5 is depicted in Figure 2B. The chromatogram confirms not only the partial transformation of the pigment into the chlorinated derivative but also the generation (Figure 5) of a series of the oxidized derivatives as in the case of the nonenzymatic oxidation. The presence of all of the derivatives **2a**−**2f** (Table 1) known from the previous NaOCl study was observed.

Further experiments were performed on MPO-catalyzed chlorination of betanidin with increasing NaCl concentration. As in the case of betanin, the overdose of NaCl also hampers the generation of the chlorinated pigment as a result of Cl_2 formation (reaction 1), and the highest MPO chlorinating activity is documented at pH 5. However, the chlorination of betanidin at pH 7.4 is much less efficient than that of betanin even at the physiological concentration of NaCl. This is evidently a result of the high oxidizing activity of MPO (acting at the peroxidase mode) as well as HOCl. The sole oxidizing MPO activity is also observed in the case of the lack of NaCl (no production of HOCl) in the reaction mixture.

The generation of betanidin oxidation and chlorination products at 150 mM NaCl in dependence on pH was also studied. The highest efficiency of MPO-mediated monochloro-betanidin generation at pH 5 is indicated; however, it is overwhelmed by the presence of the most indicative oxidation (2,17-bidecarboxy-2,3-dehydro-betanidin, **2b**, and 2-decarboxy-2,3-dehydro-betanidin, **2c**) and double-oxidation (2,17-bidecarboxy-2,3-dehydro-neobetanidin, **2f**) products. Furthermore, these compounds are generated over the whole tested pH range of 3−7.4 in substantial quantities, which contrasts with the nonenzymatic activity of NaOCl but is similar to the overall

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

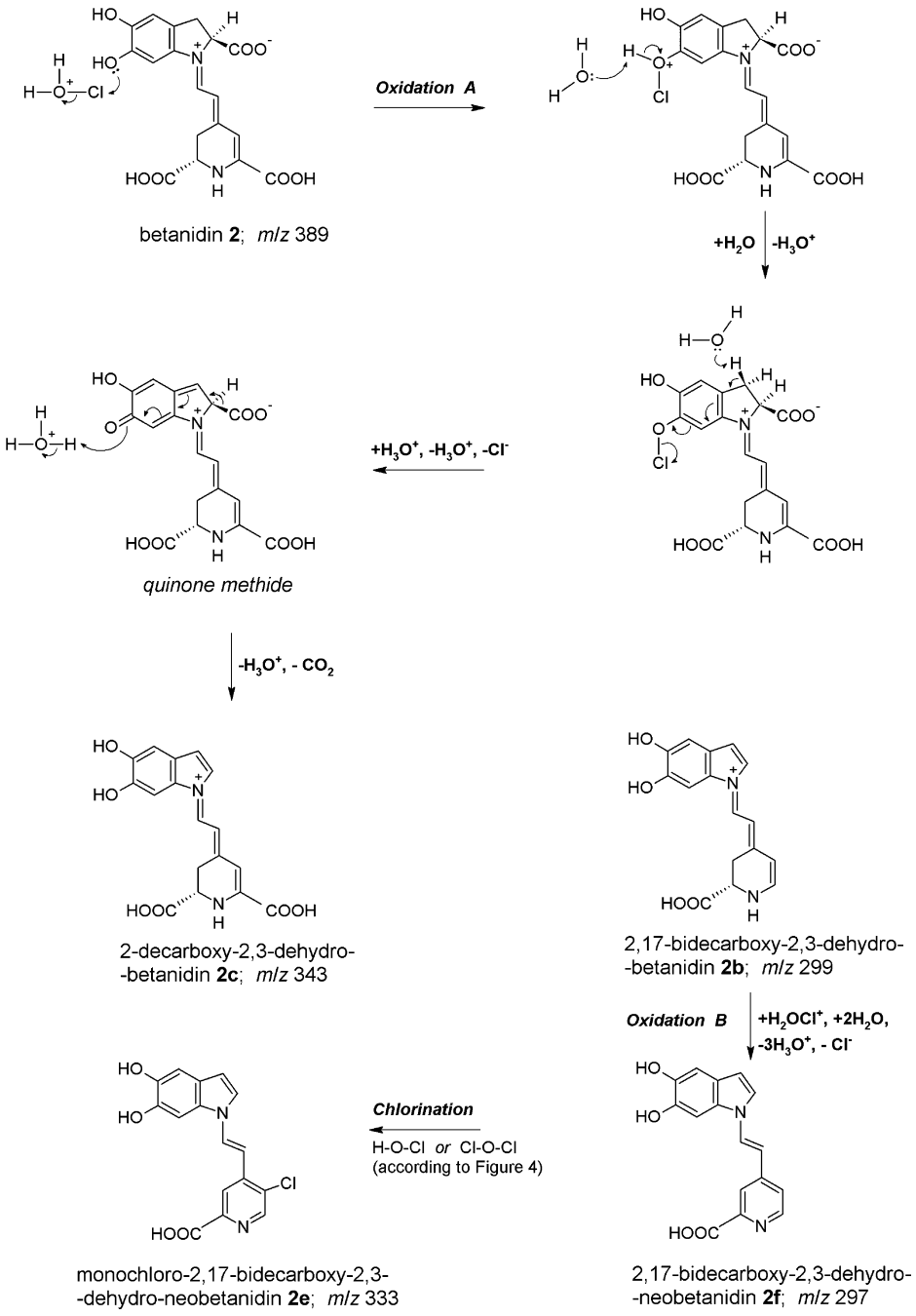

Figure 5. Mechanism of betanidin **2** oxidation by hypochlorous acid (HOCl) generated in the MPO or NaOCl system. Oxidation proceeds via a quinone methide intermediate[19] and is accompanied by a chlorination of the resulting product **2f**.

activity of horseradish peroxidase over the whole pH range.[19] In addition, the oxidation efficiency of betanidin at pH 7.4 is so high that even the oxidation products are converted to other nondetectable compounds not possessing the characteristic chromophoric systems.

Oxidation of Neobetanin by Sodium Hypochlorite. Further chlorination experiments were performed on neobetanin, **3**, (Figure 1), which is the only partially oxidized betanin derivative known in the plant kingdom.[1,2] Because of the dehydrogenation of the dihydropyridine ring in betanin, **1**, the chiral center at carbon C-15 disappears in the resulting neobetanin. Its chemical structure is different from that of betanin by the presence of a pyridine ring that completely changes the chemical properties of the compound. This is also observed in the obtained results within which no chlorinated products in the reaction mixtures can be detected by mass spectrometry for any pH values. In addition, the applied NaOCl at concentrations of 80−120 μM completely bleaches the pigment in the whole pH range tested. Therefore, we reduced the experimental concentration range of NaOCl a few times (to 5−40 μM) to enable the detection of any intermediate of the reaction. Under these conditions, a gradual decrease of the main absorption bands can be observed in all of the tested samples. The highest reaction rate is noted at pH 3−4 as well as pH 7−7.4 and the lowest rate at pH 5−5.5. The LC-MS analyses resulted in the detection of minute amounts of 2-decarboxy-2,3-dehydro-neobetanin, **3a**, at pH 7−7.4 (a chromatogram after enzymatic oxidation is presented in Figure

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

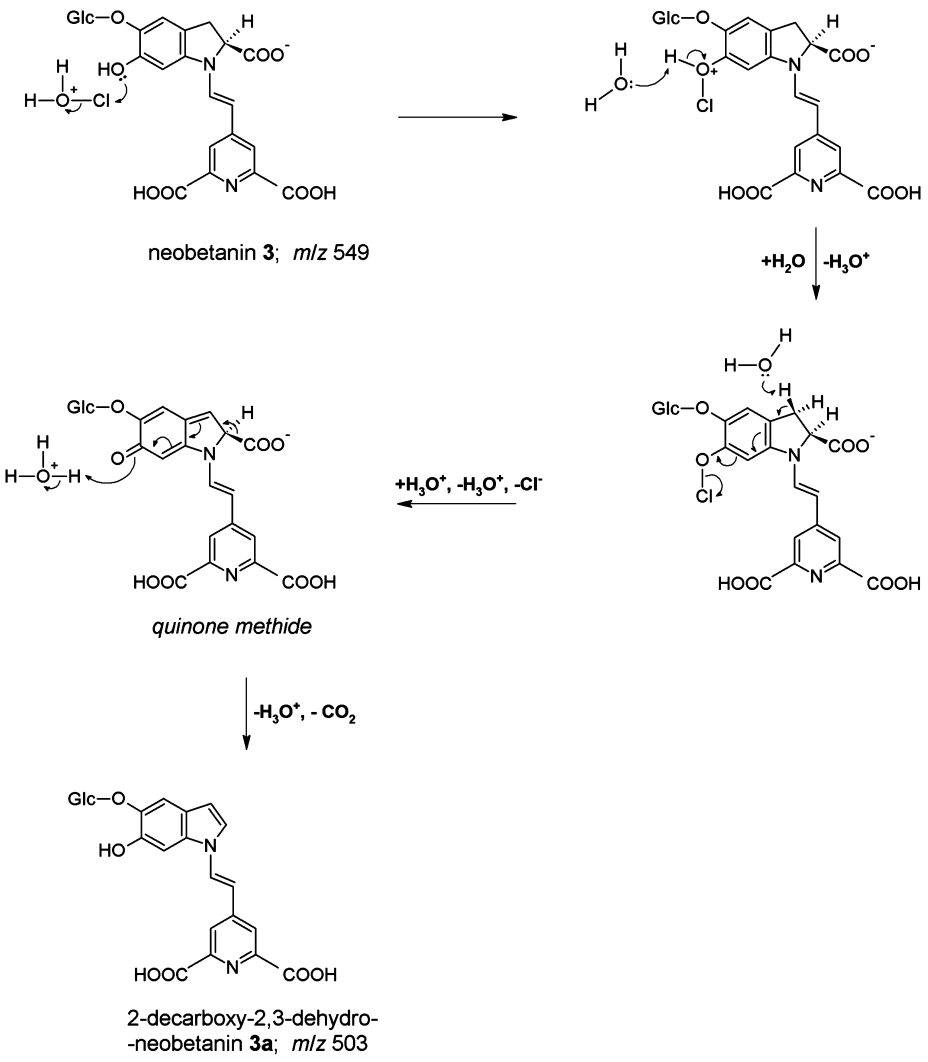

Figure 6. Mechanism of neobetanin, **3**, oxidation by hypochlorous acid (HOCl) generated in the MPO or NaOCl systems. The oxidation proceeds via a quinone methide intermediate.[19,20]

2C), a reaction product detected in previous studies on enzymatic and chemical oxidation of betalains.[20] Presumably, this compound is directly generated from neobetanin, **3**, through a quinone methide intermediate and its subsequent rearrangement with decarboxylation at carbon C-2 (Figure 6).[20] However, the high bleaching rate of neobetanin indicates that other undetected compounds are being generated that do not possess the characteristic chromophoric system. In addition, the only detectable product, **3a**, presumably reacts further with the hypochlorite reagent; therefore, it is present at low concentration levels in the reaction mixtures.

The bleaching experiments on neobetanin with increasing NaCl concentration revealed an interesting pH influence on the reactivity of the pigment. In the absence of NaCl, the highest rate of reaction is observed at pH 3, whereas the addition of NaCl to the reaction mixture results in a meaningful decrease of the reaction rate at pH 3 (the highest level of unreacted neobetanin). Instead, at pH 7−7.4, increasing NaCl concentration results in an acceleration of the bleaching rate. This suggests that in more acidic media the main reacting form is HOCl, as well as Cl_2O, because at higher NaCl concentrations generated Cl_2 is not reactive toward neobetanin. At neutral pH, generation of Cl_2 is diminished anyway; therefore, increasing the bleaching rate is not an effect of the generation of Cl_2.

Chlorination of Neobetanin by the MPO System. As in the case of NaOCl, no chlorinated products were detected in any media during MPO-catalyzed bleaching of neobetanin. Even if at pH 3 a fast decay of the substrate is recorded, no shift of the main absorption maximum occurs, which would indicate a new chlorinated compound. The results are confirmed by chromatography of the reaction products. A chromatogram obtained at pH 7.4 (Figure 2C) indicates the formation of a minute amount of oxidation product 2-decarboxy-2,3-dehydro-neobetanin,[20] as in the case of NaOCl chlorination, but no other bulk products of the bleaching reaction can be detected.

Increasing NaCl concentration significantly enhances the reactivity of neobetanin at pH 7−7.4 over almost the whole NaCl concentration range, whereas at pH 3−5, where a higher bleaching rate is observed in the absence of NaCl, the influence is manifested only at lower concentrations of NaCl (25−50 mM). At a higher dose of NaCl, an increase of neobetanin stability is noted (higher concentration of less reactive Cl_2, similar to betanin and betanidin). In any case, the increase of the pigment reactivity at low NaCl concentration can be explained by the action of MPO.

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

This is the first report on chlorinated betalains that may be generated by in vivo chlorination of these ingested pigments as a result of inflammatory processes. In particular, it explains for the first time a possible function of betalains in inhibiting the effects of chlorination in the blood and increasing the comfort of joints in individuals, as had been observed recently.[11,12]

The mechanism of betacyanin chlorination and oxidation is of significant interest because of recent appreciation of these pigments as highly active natural compounds with antioxidative properties and their potential benefits to human health as well as a lack of toxicity, which suggests that they may function well as candidates for use in numerous health applications.

In addition, betalains can presumably serve as indicators of hypochlorite in inflamed tissues because their chlorination can be confirmed by the applied analytical methods. From the first chlorination experiments, it can be deduced that monochloro-betanin is more stable than its precursor and should be easily monitored in blood because it retains its chromophoric structure after chlorination and does not degrade in the reaction media. Further bioavalaibility studies should confirm the formation of chlorinated betalains in humans after the consumption of betalains, especially under pro-inflammatory conditions.

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