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Mechanism of interaction of betanin and indicaxanthin with human myeloperoxidase and hypochlorous acid

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Abstract

Hypochlorous acid (HOCl) is the most powerful oxidant produced by human neutrophils and contributes to the damage caused by these inflammatory cells. It is produced from H_2O_2 and chloride by the heme enzyme myeloperoxidase (MPO). Based on findings that betalains provide antioxidant and anti-inflammatory effects, we performed the present kinetic study on the interaction between the betalains, betanin and indicaxanthin, with the redox intermediates, compound I and compound II of MPO, and its major cytotoxic product HOCl. It is shown that both betalains are good peroxidase substrates for MPO and function as one-electron reductants of its redox intermediates, compound I and compound II. Compound I is reduced to compound II with a second-order rate constant of $(1.5 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$ (betanin) and $(1.1 \pm 0.2) \times 10^6\ M^{-1}\ s^{-1}$ (indicaxanthin), respectively, at pH 7.0 and 25 °C. Formation of ferric (native) MPO from compound II occurs with a second-order rate constant of $(1.1 \pm 0.1) \times 10^5\ M^{-1}\ s^{-1}$ (betanin) and $(2.9 \pm 0.1)\ 10^5\ M^{-1}\ s^{-1}$ (indicaxanthin), respectively. In addition, both betalains can effectively scavenge hypochlorous acid with determined rates of $(1.8 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1}$ (betanin) and $(7.7 \pm 0.1) \times 10^4\ M^{-1}\ s^{-1}$ (indicaxanthin) at pH 7.0 and 25 °C. At neutral pH and depending on their concentration, both betalains can exhibit a stimulating and inhibitory effect on the chlorination activity of MPO, whereas at pH 5.0 only inhibitory effects were observed even at micromolar concentrations. These findings are discussed with respect to our knowledge of the enzymatic mechanisms of MPO.

Keywords: Myeloperoxidase; Compound I; Compound II; Hypochlorous acid; Chlorination activity; Betalain; Indicaxanthin; Inflammation

Inflammatory diseases, including cardiovascular disease, asthma, rheumatoid arthritis, and inflammatory bowel diseases, are the major cause of morbidity and mortality in the western world, and involve inappropriate and undesirable activation of the immune system, which causes tissue damage and pathology. Especially the human heme enzyme myeloperoxidase (MPO) is known to be involved in oxygen-dependent antimicrobial action and is dominant in phagocytic neutrophils and monocytes [1]. MPO has enormous potential to inflict tissue damage through its ability to catalyse the production of a complex array of reactive oxidants including hypohalous acids (e.g., hypochlorous acid/hypochlorite), nitrogen dioxide, organic free radicals, and numerous drug metabolites [2].

In the latest years, dietary phytochemicals have attracted much attention for their benefits on human health. Flavonoids, for example, are probably the most studied and well-known natural substances present in plant foods that could provide antioxidant and anti-inflammatory effects [3]. Betalains are a class of cationized

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nitrogenous compounds, the colour of which range from the yellow betaxanthins to the violet-red betacyanins [4–6]. Betaxanthins are conjugates of betalamic acid with amino acids or the corresponding amines (including dopamine), while almost all betacyanins are derivatives of betanidin, the conjugate of betalamic acid with *cyclo*-Dopa. The hydroxyl groups at the C5 and C6 position of *cyclo*-Dopa can be either esterified or not with carbohydrate derivatives to form various betacyanins (Fig. 1A). Two betalains have been recently investigated, the betacyanin *betanin* (5-*O*-glucose betanidine) and the betaxanthin *indicaxanthin*, the adduct of betalamic acid with proline [7–12] (Fig. 1B). Due to their redox potentials [7], both molecules possess reducing properties, and are able to increase the resistance of human LDL to oxidation acting as lipoperoxyl radical scavengers [13]. Furthermore, betalains have been shown to protect vascular endothelium cells, which are a direct target of oxidative stress in inflammation, from cytokine-induced redox state alteration [14].

Based on the reports about the redox properties and antioxidative activities of these betalains as well as on the fact that class III-type plant peroxidases have been shown to be able to oxidize betanidin and betanin [15], we performed this study about the interaction of betanin and indicaxanthin with human myeloperoxidase and its major reaction product hypochlorous acid (HOCl). MPO can catalyse both two- and one-electron oxidation reactions. In the halogenation cycle, compound I, which is formed upon oxidation of ferric MPO by hydrogen peroxide (Reaction 1), is known to oxidize (pseudo-)halides (i.e., chloride, bromide, thiocyanate; X^-) via a single two-electron reaction to produce the respective cytotoxic hypohalous acids (XOH) and regenerate the native enzyme (Reaction 2).

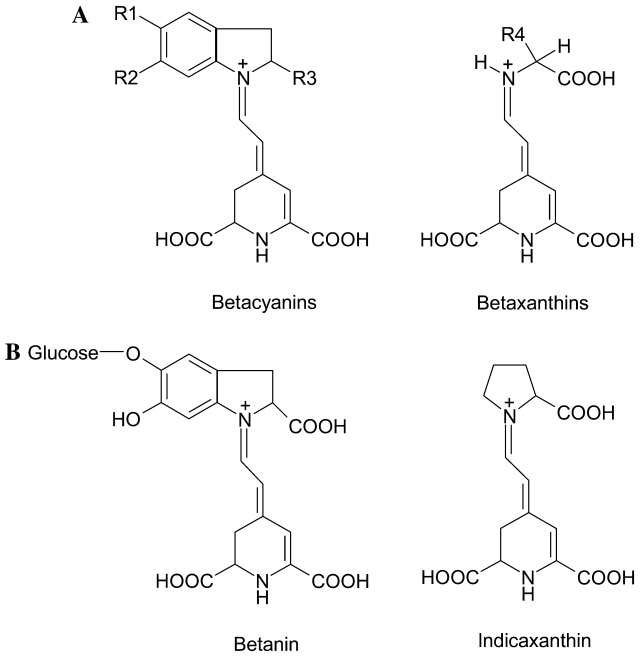

Fig. 1. Chemical structure of betalains.

$$\text{ferric MPO} + H_2O_2 \leftrightarrows \text{compound I} + H_2O \quad \text{(Reaction 1)}$$

$$\text{compound I} + X^- \leftrightarrows \text{ferric MPO} + XOH \quad \text{(Reaction 2)}$$

In the peroxidase cycle, compound I is reduced via compound II to the native enzyme (Reactions 3 and 4) thereby catalysing one-electron oxidation reactions of electron donors (AH_2) and releasing the respective radicals ($AH^{\bullet}$).

$$\text{compound I} + AH_2 \leftrightarrows \text{compound II} + AH^{\bullet} \quad \text{(Reaction 3)}$$

$$\text{compound II} + AH_2 \leftrightarrows \text{ferric MPO} + AH^{\bullet} + H_2O \quad \text{(Reaction 4)}$$

In principle, reducing compounds such as betalains could interfere with all steps in the catalytic cycle of MPO (Reactions 1–4), as well as with its major reaction product HOCl (Reaction 2). Here, by using the sequential-mixing stopped-flow technique, we investigated the electron-donating capacity of betanin and indicaxanthin with both compound I and compound II of MPO. In detail, we present actual bimolecular rate constants of Reactions 3 (k_3) and 4 (k_4) and demonstrate that low micromolar betalain concentrations enhance the chlorination activity of MPO at pH 7.0, whereas at higher concentrations they inhibit the MPO-mediated chlorination reactions by scavenging of hypochlorous acid.

Materials and methods

Highly purified myeloperoxidase of a purity index (A_{430}/A_{280}) of at least 0.85 was purchased from Planta Naturstoffe Vertriebs GmbH (http://www.myeloperoxidase.at). Its concentration was calculated using $\varepsilon 430 = 91\ \text{mM}^{-1}\ \text{cm}^{-1}$ [16]. Hydrogen peroxide, obtained from a 30% solution, was diluted shortly before use and the concentration was determined by absorbance measurement at 240 nm where the extinction coefficient is $39.4\ \text{M}^{-1}\ \text{cm}^{-1}$ [17]. All chemicals were purchased from Sigma Chemical at the highest grade available.

Purification of indicaxanthin and betanin. Betanin and indicaxanthin were separated from methanolic extracts of prickly pear fruits by gel filtration on a Sephadex G-25 column (40×2.2 cm) according to Kanner et al. [18], with minor modifications. Briefly, prickly pear fruits, collected in September–November 2001 in Sicily (Italy), were obtained from a local market, and were processed within 48–72 h from collection (four different lots of fruits, at comparable ripening stages, were analysed for each cultivar). The fruits were peeled and finely chopped. The pulp was separated from the seeds, weighted, and 100 g pulp samples were homogenized with 100 mL methanol; aliquots from 2 g fresh pulp were eluted with 1% acetic acid and fractions (2.5 mL) were collected, and tested spectrophotometrically at 482 and 536 nm for the presence of indicaxanthin and betanin, respectively [9,10]. The

elution profile from the Sephadex G-25 column provided evidence that a complete separation of the two pigments was achieved. The isolated pigments were submitted to high performance liquid chromatography analysis on a Varian Microsorb C-18 column (1 × 25 cm, Varian, Palo Alto, CA, USA), eluted with a 20 min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with a flow rate of 2 mL/min [10,19]. Spectrophotometric revelation was at 536 and 482 nm for betanin (12.4 min) and indicaxanthin (13.6 min), respectively. The elution volumes relevant to indicaxanthin and betanin were collected. Samples after cryoessiccation were resuspended for measurements in phosphate buffer at suitable concentrations. Concentrations were determined by using the extinction coefficients of $\varepsilon_{536} = 65{,}000\ M^{-1}\,cm^{-1}$ (betanin) and $\varepsilon_{482} = 42{,}800\ M^{-1}\,cm^{-1}$ (indicaxanthin).

Stopped-flow spectroscopy. The sequential stopped-flow apparatus (Model SX-18MV) and the associated computer system were from Applied Photophysics (UK). For a total of 100 mL/shot into a flow cell with 1 cm light path, the fastest time for mixing two solutions and recording the first data point was ca. 1.5 ms. Because of the inherent instability of MPO compound I [16], the sequential-flow (multimixing) technique had to be used for determination of rates of the reaction of compound I with both betanin and indicaxanthin. The transition of compound I to compound II was followed at the Soret maximum of compound II (456 nm). In a typical experiment, MPO (4 μM heme) in 5 mM buffer (pH 7.0) was premixed with 40 μM H_2O_2 in distilled water. After a delay time of 20 ms, compound I was allowed to react with varying concentrations of betalains in 200 mM phosphate buffer (pH 7.0). The pH after mixing was measured at the outlet.

Reduction of compound II by betalains was performed as described recently [20]. In a typical experiment, MPO (4 μM heme) in 5 mM buffer (pH 7.0) was premixed with 40 μM H_2O_2 and 1.8 mM homovanillic acid in distilled water. After a delay time of 40 s, compound II was allowed to react with varying concentrations of betalains in 200 mM phosphate buffer (pH 7.0). The reactions were followed at 456 nm (disappearance of compound II) and 430 nm (formation of ferric MPO). Alternatively, compound II formation and reduction could be followed in one measurement. The resulting biphasic curves at 456 nm showed the initial formation of compound II and then its subsequent reaction with the betalains causing an exponential decrease in absorbance at 456 nm.

At least three determinations (2000 data points) of k_{obs} were performed for each substrate concentration and the mean value was used in a plot of k_{obs} versus substrate concentration. The final betalain concentrations were at least 10 times in excess of the enzyme to assure pseudo-first-order kinetics. All reactions were performed at 25 °C. Reduction of both compound I and compound II was also studied by using the the diode-array detector attached to the stopped-flow apparatus. Typically, the final heme concentration used in these experiments was 2 μM.

Myeloperoxidase-catalysed chlorination of monochlorodimedon. Monochlorodimedon (MCD) is a substrate often used to study the MPO-catalysed chlorination [21]. Chlorination of MCD to dichlorodimedon results in a decrease in absorbance at 290 nm. MCD (100 μM) was dissolved in 100 mM phosphate/citrate buffer (pH 5.0) or phosphate buffer (pH 7.0) containing 100 μM H_2O_2 and 100 mM chloride in the presence or absence of betalains. Reactions were started by addition of MPO (20 nM at pH 5.0 and 200 nM at pH 7.0) and performed in a Beckman DU 640 spectrophotometer equipped with a temperature controller at 25 °C.

Polarographic measurements of H_2O_2 utilization. The enzymatic activity of MPO was also determined by measuring the hydrogen peroxide consumption in the presence of betalains polarographically by using a platinum electrode covered with a hydrophilic membrane and fitted to an Amperometric Biosensor Detector 3001 (Universal Sensors, USA). At pH 7.0, the applied electrode potential was 0.65 V. The electrode filling solution was prepared freshly every day and the H_2O_2 electrode was calibrated against known concentrations of hydrogen peroxide. All reactions were performed in a thermally jacked tube at 25 °C and started by addition of 5–10 nM MPO to a 5 mL solution containing 100 μM hydrogen peroxide in 100 mM phosphate buffer, pH 7.0, and various betalain concentrations (25 °C).

Results and discussion

In the first part of this study, the reactivity of betanin and indicaxanthin (Fig. 1) with the redox intermediates, compound I and compound II of human myeloperoxidase, was investigated. The sequential-mixing stopped-flow technique had to be used because of the instability of the redox intermediates of myeloperoxidase. The Soret maximum of ferric MPO is at 430 nm and upon addition of H_2O_2 (i.e., compound I formation) a hypochromicity of about 50% is observed. In the presence of one-electron donors, compound I is reduced to compound II, which has its Soret band at 456 nm and another maximum at 629 nm. A direct conversion of compound I to compound II exhibits distinct isosbestic points at 435, 489, 585, and 657 nm [20]. In our experiments, both betalains were good peroxidase substrates (see below) which allowed us to follow compound II formation and reduction in one measurement at 456 nm (absorbance maximum of compound II). The resulting biphasic curves at 456 nm showed the initial formation of compound II (Figs. 2B and 3B) and then its subsequent reduction by the betalains causing an exponential decrease in absorbance at 456 nm (Figs. 2D and 3D).

Betalain-mediated compound I and compound II reduction

Compound II formation (k_3) was followed at 456 nm (Fig. 2B). Both betanin and indicaxanthin were efficient in reduction of compound I (Figs. 2 and 3). All reactions were monophasic and the time traces could be fitted to a single-exponential function (Figs. 2B and 3B). The obtained pseudo-first-order rate constants were linearly dependent on the concentration of the electron donor and allowed calculation of the second-order rate constants (k_3) from the corresponding plots (Figs. 2A and 3A). The corresponding bimolecular rate constants for betanin and indicaxanthin were calculated to be $(1.5 \pm 0.1) \times 10^6\ M^{-1}\,s^{-1}$ (betanin) and $(1.1 \pm 0.2) \times 10^6\ M^{-1}\,s^{-1}$ (indicaxanthin) at pH 7.0 and 25 °C. Thus, betalain oxidation by compound I is about 60 times faster than chloride oxidation, which was determined to be $2.4 \times 10^4\ M^{-1}\,s^{-1}$ at pH 7.0 [16]. Thus, both betalains are excellent competitors of chloride for compound I reduction and in the presence of both betalains and chloride it depends on the actual concentrations whether MPO follows the chlorination or the peroxidase cycle.

Similarly, compound II was reduced in a one-electron process by betanin and indicaxanthin. Clear isosbestic

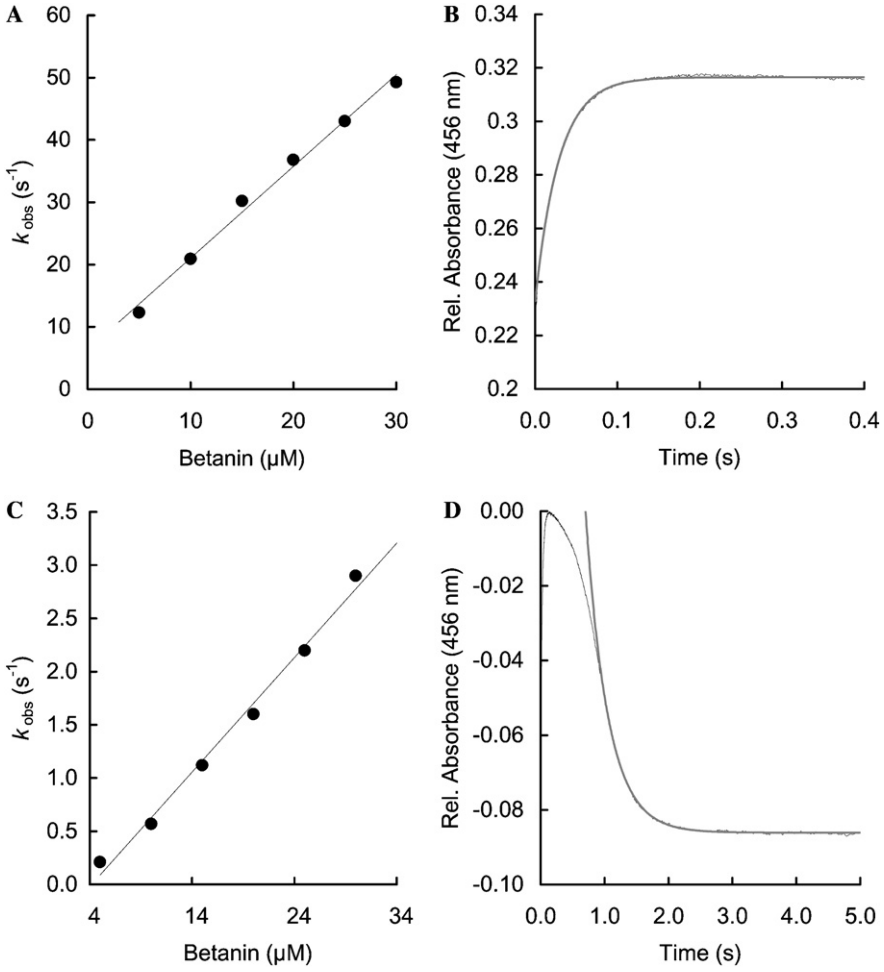

Fig. 2. Reaction of compound I and compound II of myeloperoxidase (MPO) with betanin. Experiments were carried out in the sequential-mixing stopped-flow mode. (A) Pseudo-first-order rate constants, k_{obs}, for compound I reduction plotted against betanin concentrations. (B) Time trace and single-exponential fit of the reaction between 1 μM MPO compound I and 20 μM betanin, followed at 456 nm (absorbance maximum of compound II). Conditions: 100 mM phosphate buffer pH 7.0 and 25 °C. (C) Pseudo-first-order rate constants, k_{obs}, for compound II reduction plotted against betanin concentrations. (D) Time trace and single-exponential fit of the reaction between 1 μM MPO compound II and 30 μM betanin, followed at 456 nm. Conditions as in (B).

points indicate the direct conversion of compound II back to the ferric resting enzyme. The corresponding time traces at 456 nm (disappearance of compound II) or 430 nm (formation of ferric MPO; not shown) exhibited a typical single-exponential behaviour (Figs. 2D and 3D) and yielded bimolecular rate constants (k_4) for betanin and indicaxanthin of $(1.1 \pm 0.1) \times 10^5\ M^{-1}\ s^{-1}$ and $(2.9 \pm 0.1) \times 10^5\ M^{-1}\ s^{-1}$ at pH 7.0 and 25 °C (Figs. 2C and 3C).

Both betalains are good substrates of MPO and during their steady-state oxidation by the system MPO/H_2O_2, compound II is the dominating redox intermediate (not shown), which fits well with the k_3/k_4 ratios of betanin ($k_3/k_4 = 14$) and indicaxanthin ($k_3/k_4 = 3.6$). Typically for a good peroxidase substrate, the polarographically determined hydrogen peroxide consumption was fast and depended strongly on the betalain concentration (not shown), indicating that with both betalains MPO can maintain a high H_2O_2 turnover. This clearly demonstrates that neither these betalains nor their oxidation products act as inhibitors of MPO under these conditions. By contrast, the reactivity of betalains with myeloperoxidase is comparable or even higher than that of biological substrates like tyrosine [22], ascorbate [23], indoles [20] or tryptamines [20], suggesting that there are neither thermodynamic nor kinetic restrictions. Betalamic acid derivatives can bind at the hydrophobic region at the entrance to the distal heme pocket [24] and both standard reduction potentials of the enzymatic couples, compound I/compound II (1.35 V) and compound II/ferric MPO (0.97 V) [25], have to be significantly higher than those of both betalains.

Reaction between betanin and indicaxanthin with hypochlorous acid

Both betalains are good peroxidase substrates and are oxidized by the enzyme rather than inhibiting it.

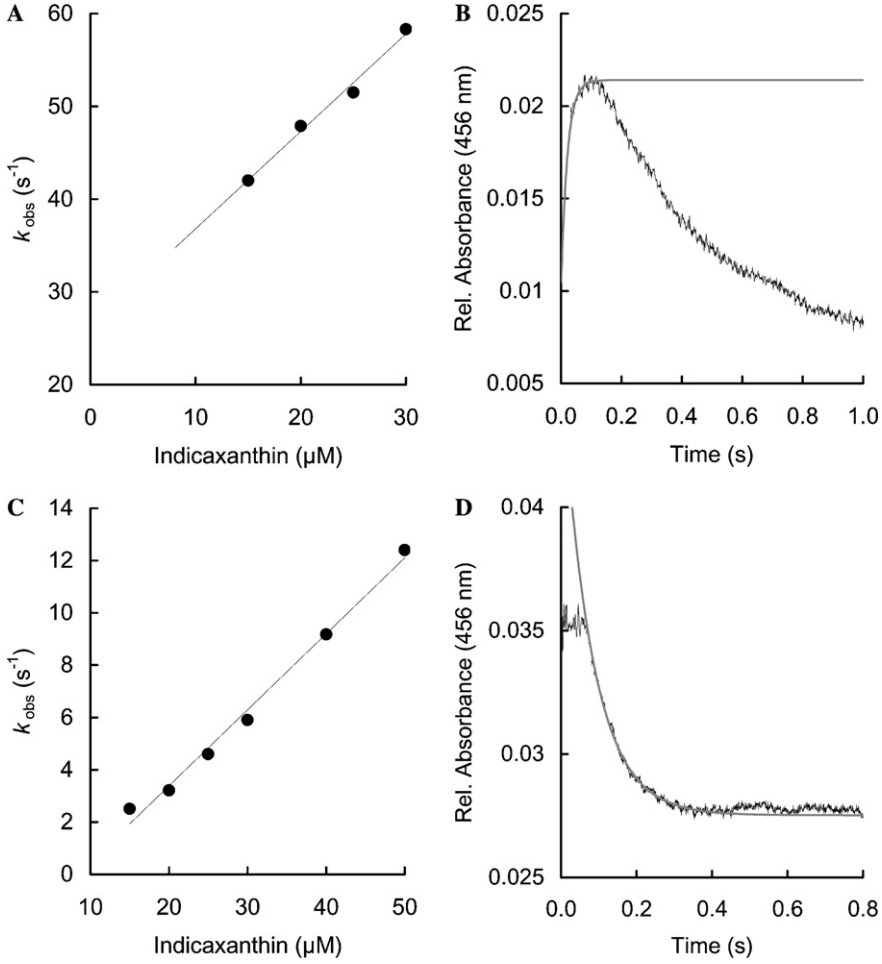

Fig. 3. Reaction of compound I and compound II of myeloperoxidase (MPO) with indicaxanthin. Experiments were carried out in the sequential-mixing stopped-flow mode. (A) Pseudo-first-order rate constants, k_{obs}, for compound I reduction plotted against indicaxanthin concentrations. (B) Time trace and single-exponential fit of the reaction between 1 μM MPO compound I and 20 μM indicaxanthin, followed at 456 nm (absorbance maximum of compound II). Conditions: 100 mM phosphate buffer, pH 7.0, and 25 °C. (C) Pseudo-first-order rate constants, k_{obs}, for compound II reduction plotted against indicaxanthin concentrations. (D) Time trace and single-exponential fit of the reaction between 1 μM MPO compound II and 50 μM betanin, followed at 456 nm. Conditions as in (B).

In order to test whether they could act as hypochlorous acid scavengers, we investigated their reactivity towards HOCl by using the conventional stopped-flow method. Figs. 4A and C show the spectral changes of betanin and indicaxanthin oxidation mediated by HOCl. In case of betanin, the absorbance at 536 nm decreased (Fig. 4A) and following this reaction in the stopped-flow apparatus at 536 nm gave monophasic time traces (Fig. 4B). From the plot of the k_{obs} values versus the hypochlorous acid concentration (Fig. 4B) the apparent bimolecular rate constant of this oxidation reaction was calculated to be $(1.8 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1}$ (pH 7.0 and 25 °C) (inset to Fig. 4B).

Upon oxidation of indicaxanthin by HOCl the maximum of absorbance at 483 nm decreased and a reaction product with an absorbance maximum at 464 nm was formed in a monophasic reaction (Figs. 4C and D). The bimolecular rate constant of this oxidation reaction was calculated to be $(7.7 \pm 0.1) \times 10^4\ M^{-1}\ s^{-1}$ at pH 7.0 and 25 °C (inset to Fig. 4D).

The obtained reaction rates of hypochlorous acid, which is the major strong oxidant generated in neutrophils, with betalains are about three orders of magnitude smaller than those with methionine and cysteine [26,27], but are comparable to those with other amino acids or biological targets [27]. Thus, in principle, by virtue of their redox properties and scavenging of hypochlorous acid produced by myeloperoxidase during phagocytosis, betalains could be effective as anti-inflammatory molecules.

Impact of betalains on the chlorination activity of MPO

Finally, we studied the influence of betalains on the MPO-mediated chlorination reaction of monochlorodimedone (MCD). At pH 7.0, both a stimulating and inhibitory effect was observed depending on the betanin

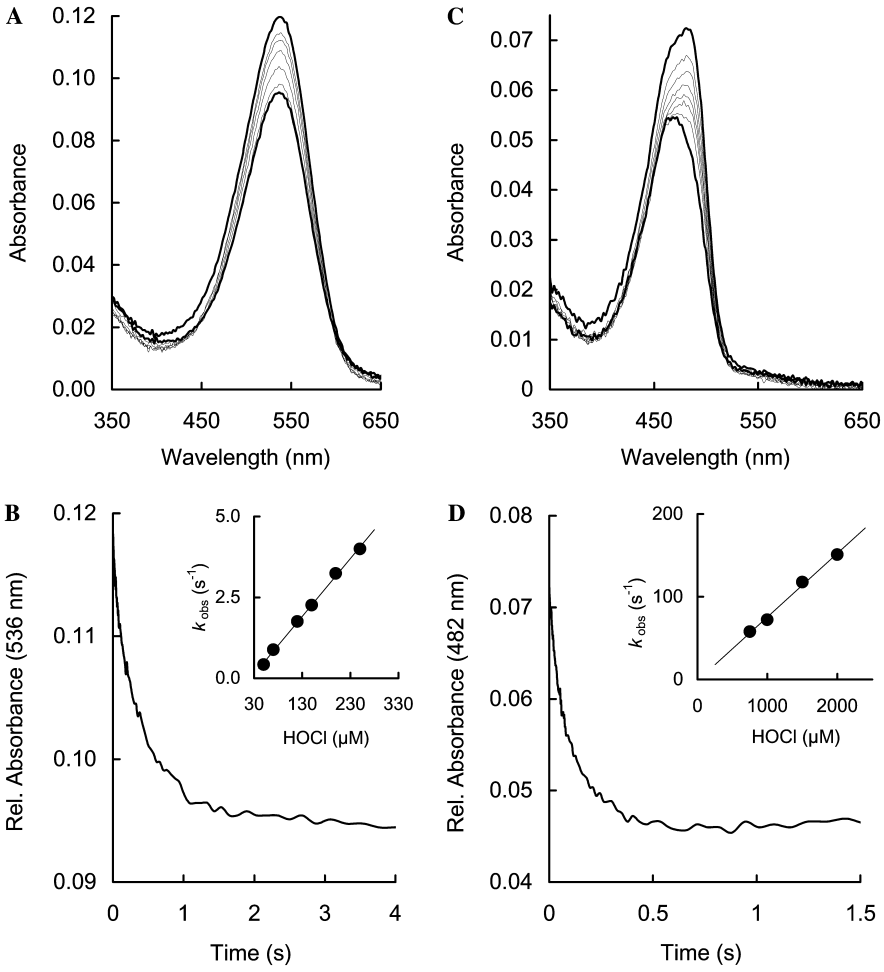

Fig. 4. Oxidation of betanin and indicaxanthin by hypochlorous acid. Spectral changes upon reaction of 2 μM betanin (A) and 2 μM indicaxanthin (B) (bold spectra) with 100 μM hypochlorous acid in 100 mM phosphate buffer, pH 7.0, and 25 °C. (A) First spectrum was recorded 1.3 ms after mixing, further spectra were selected 35, 68, 147, 378, and 943 ms, and 2.33 s (bold) after mixing. (B) First spectrum was recorded 1.3 ms after mixing, further spectra were selected 17, 32, 47, 63, 78, 117, and 283 ms (bold) after mixing. (C) Typical time trace at 536 nm selected from (A). The inset shows the pseudo-first-order rate constants, k_{obs}, plotted against the HOCl concentration. (D) Typical time trace at 482 nm selected from (B). The inset shows the pseudo-first-order rate constants, k_{obs}, plotted against the HOCl concentration.

and indicaxanthin concentration in the MCD assay (Fig. 5). At low micromolar concentrations of betalains, the chlorination activity was stimulated (Figs. 5A and B) in the beginning of the reaction, but finally MCD chlorination stopped. In case of betanin, increasing the betanin concentration up to 6 μM dramatically increased the chlorination rate of MCD (Fig. 5A). Simultaneously, the scavenging of HOCl became more evident with increasing betalain concentration thereby shortening the length of the halogenation phase and, finally, completely inhibited MCD chlorination. This effect was even more pronounced in the case of indicaxanthin (Fig. 5B), which fits well with the 4 times better HOCl scavenging capacity of indicaxanthin compared with betanin. These findings also demonstrate that both betalains have to be better scavengers of HOCl than MCD (assay concentration of MCD is 100 μM), which is commonly used to monitor the chlorination activity of MPO.

It is well known that in the MPO/H_2O_2/chloride system compound II, which is outside the halogenation cycle, accumulates with time due to compound II formation from compound I mediated by H_2O_2 [28]. In addition, as it has been demonstrated above, compound I is efficiently reduced by both betalains. Since chloride, H_2O_2, and both betalains are competitors for compound I, differences in their concentrations can lead to different pseudo-first-order rate constants and as a consequence a stimulatory or an inhibitory effect on the HOCl formation can be observed. The chloride concentration in the MCD assay as well as in human blood is very high (100 mM), and in the presence of betalains compound II is efficiently reduced. As a consequence, in the presence of betalains compound II does not accumulate and at low betalain concentrations the chlorination activity of MPO is even enhanced by regaining MPO from its compound II form for the chlorination cycle. The bimolecular rate constants

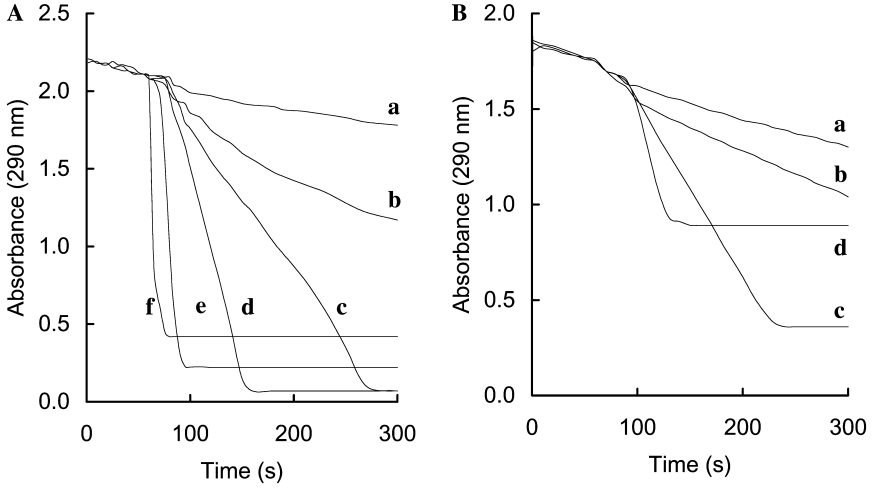

Fig. 5. Effect of betanin and indicaxanthin on the monochlorodimedone chlorination by the MPO/H_2O_2/chloride system at pH 7.0. Kinetics traces in the absence (a) or in the presence (b–g) of betanin (A) or indicaxanthin (B). Indicaxanthin and betanin in the assay were 0.5 μM (b), 1.0 μM (c), 1.5 μM (d), 3.0 μM (e), and 6.0 μM (f).

obtained in this work strongly suggest this view. At increasing concentrations of betalains, the HOCl scavenging effect of both substances becomes evident and the chlorination of MCD becomes inhibited.

At pH 5.0, no stimulating effect of the MPO-mediated MCD chlorination could be observed and the chlorination of MCD decreased upon increasing the betalain concentration from 1.5 to 8 μM and, finally, was completely inhibited (not shown). This fits well with the known fact that at decreasing pH compound II formation becomes less important since the rate of the two-electron reduction of compound I exceeds that of the H_2O_2-mediated compound II formation [16]. Therefore, at pH 5.0 low betalain concentrations may not be able to compete with chloride for compound I reduction, but can still scavenge HOCl and thus inhibit MCD chlorination.

Summing up, we have shown that betanin and indicaxanthin are good electron donors for MPO compound I and II. They interfere with the catalytic cycle of the enzyme and can enhance its chlorination activity at very low concentrations and pH 7.0. This activity is, however, modulated by the ability of both betalains to scavenge HOCl. At pH 5.0, where there is maximum release of HOCl by MPO [2] both betalains act as scavengers already at low micromolar concentrations. Betalains, therefore, may deserve a better investigation as modulators in all the inflammatory conditions in which HOCl plays a key role. It may be interesting to mention that recent studies in humans showed that these phytochemicals are bioavailable [29].

Acknowledgment

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