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Inhibition of Enzymatic Browning of Chlorogenic Acid by Sulfur-Containing Compounds

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Supporting Information

ABSTRACT: The antibrowning activity of sodium hydrogen sulfite ($NaHSO_3$) was compared to that of other sulfur-containing compounds. Inhibition of enzymatic browning was investigated using a model browning system consisting of mushroom tyrosinase and chlorogenic acid (5-CQA). Development of brown color (spectral analysis), oxygen consumption, and reaction product formation (RP-UHPLC−PDA−MS) were monitored in time. It was found that the compounds showing antibrowning activity either prevented browning by forming colorless addition products with *o*-quinones of 5-CQA ($NaHSO_3$, cysteine, and glutathione) or inhibiting the enzymatic activity of tyrosinase ($NaHSO_3$ and dithiothreitol). $NaHSO_3$ was different from the other sulfur-containing compounds investigated, because it showed a dual inhibitory effect on browning. Initial browning was prevented by trapping the *o*-quinones formed in colorless addition products (sulfochlorogenic acid), while at the same time, tyrosinase activity was inhibited in a time-dependent way, as shown by pre-incubation experiments of tyrosinase with $NaHSO_3$. Furthermore, it was demonstrated that sulfochlorogenic and cysteinylchlorogenic acids were not inhibitors of mushroom tyrosinase.

KEYWORDS: *Tyrosinase, polyphenol oxidase, sulfite, cysteine, glutathione, UHPLC−MS*

INTRODUCTION

Enzymatic browning is a major quality problem in the processing of fruits and vegetables. The enzymes responsible for enzymatic browning are polyphenol oxidases (PPOs), which, depending upon their source, can either catalyze the *o*-hydroxylation and subsequent oxidation of a wide range of phenolic substrates (tyrosinase, cresolase, or monophenolase activity) or only the oxidation of *o*-diphenols (catecholase or diphenolase activity).[1,2] The oxidation of *o*-diphenols results in *o*-quinones, which, in turn, react with each other and other compounds present, resulting in dark-colored pigments.[3] Additives widely used to inhibit enzymatic browning include sulfites and ascorbic acid.[2,4]

Different sulfites used as food additives are sodium sulfite (Na_2SO_3), sodium metabisulfite ($Na_2S_2O_5$), and sodium hydrogen sulfite ($NaHSO_3$, also referred to as sodium bisulfite). When dissolved, these salts yield a mixture of SO_3^{2-} and HSO_3^-.[5] Three possible mechanisms for browning inhibition by sulfite have been suggested: (i) irreversible inhibition of PPO,[6] (ii) reduction of *o*-quinones, thereby reversing the enzymatic reaction,[4] and (iii) formation of addition products between sulfite and *o*-quinones, preventing them from reacting further into brown pigments.[7]

Using sulfites in food products is controversial because of related health risks.[8] An alternative antibrowning agent is ascorbic acid. The mechanism by which ascorbic acid inhibits browning is well-known: *o*-quinones formed by PPO are reduced to their precursors, *o*-diphenols, which subsequently can be oxidized again.[9] This redox cycling continues until all ascorbic acid is consumed, after which the brown-colored pigments are still formed. Therefore, ascorbic acid can delay browning but does not inhibit enzymatic activity. Hence, much effort has been put in finding natural sulfite alternatives. Many PPO inhibitors have been identified, and often their mode of inhibition has been determined.[10] Surprisingly, the mechanism by which sulfite inhibits browning is still unclear, despite its common use for a long time.

Recently, it was found that, when extracting phenolic compounds from potato in the presence of sulfite, the expected phenolic compounds, mainly chlorogenic acid, were absent in the extracted material.[11] Sulfonated derivatives were found instead. It was proposed that PPO-catalyzed *o*-quinone formation was a prerequisite for the formation of these sulfophenolics and that formation of these compounds might be responsible for the inhibition of browning.

In the present study, the effect of sulfite on PPO-catalyzed browning was further investigated. In contrast to the previous research,[11] a model browning system was used, consisting of chlorogenic acid (Figure 1) and a commercially available mushroom PPO (tyrosinase; EC 1.14.18.1). The mechanism of action of $NaHSO_3$ is compared to that of other sulfur-containing compounds (Figure 1). Cysteine and glutathione (GSH) were selected, because these compounds are known to

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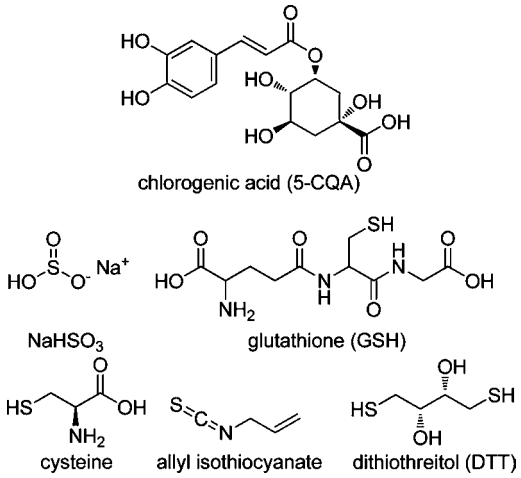

Figure 1. Structures of substrate and different sulfur-containing compounds used in this study.

form adducts with *o*-quinones.[12−15] Besides the formation of adducts with *o*-quinones, the antibrowning activity of cysteine has also been proposed to be due to irreversible inhibition of PPO.[16] Dithiothreitol (DTT) was chosen because it was reported to be an inhibitor of tyrosinase-catalyzed browning. However, different explanations have been given: DTT was found to form addition products with *o*-quinones, was found to reversibly inhibit tyrosinase, and was found to reduce the copper in the active site of tyrosinase.[17,18] It should be noted that the results for the sulfur-containing compounds mentioned above were obtained in different studies, under a variety of conditions. For example, the type of substrate, substrate concentration, inhibitor concentration, and enzyme source varied over the different studies.

Here, we compared the antibrowning properties of the different sulfur-containing compounds under similar conditions. To investigate whether other natural sulfur-containing compounds might have the potential to function as a sulfite alternative, allyl isothiocyanate was selected. Isothiocyanates result from the hydrolysis and subsequent rearrangements of their precursors, glucosinolates, which widely occur in, for instance, the Brassicaceae plant family.[19]

MATERIALS AND METHODS

Materials. Mushroom tyrosinase, chlorogenic acid (5-*O*-caffeoylquinic acid, 5-CQA), sodium hydrogen sulfite ($NaHSO_3$), L-ascorbic acid, L-cysteine, L-glutathione (GSH), DTT, and allyl isothiocyanate were purchased from Sigma Aldrich (St. Louis, MO). Ultrahigh-performance liquid chromatography−mass spectrometry (UHPLC−MS)-grade acetonitrile (ACN) was obtained from Biosolve BV (Valkenswaard, The Netherlands). Water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA).

Purification of Mushroom Tyrosinase. Commercial mushroom tyrosinase was purified by a single gel filtration step.[20] A HiLoad 26/60 Superdex 200 column connected to an Akta Explorer system (GE Healthcare, Uppsala, Sweden) was used. A total of 50 mg of the commercial enzyme [dissolved in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer at pH 6.8] was loaded and eluted with 50 mM HEPES buffer at pH 6.8 at 4 mL/min. Fractions (5 mL) were collected, and activity was assayed by a spectrophotometric assay: 50 μL of each fraction was combined with 100 μL of 0.8 mM tyrosine in a 96-well plate, and absorbance at 520 nm was monitored in time. Active fractions were pooled and stored at −20 °C until use. Tyrosinase activity was expressed in units, according to the suppliers definition (1 unit increases the A_{280} by 0.001 min^{-1} with L-tyrosine as the substrate, at pH 6.5 and 25 °C).

Incubation of 5-CQA and Tyrosinase with Browning Inhibitors. 5-CQA (0.1 mM), $NaHSO_3$ (0.2 mM) or ascorbic acid (0.2 mM), and tyrosinase (7 units/mL) were mixed in 10 mM HEPES buffer at pH 6.5. Samples were incubated at room temperature for 1 h, centrifuged (10000*g* for 5 min at room temperature), and analyzed by reversed-phase (RP)-UHPLC. For spectrophotometric analysis, the same reaction mixtures were incubated in quartz cuvettes in a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Wavelength scans were made from 200 to 600 nm. A scan was made every 1 min, for a total of 60 min.

RP-UHPLC Analysis. Samples were analyzed on an Accela UHPLC system (Thermo Scientific, San Jose, CA) equipped with a pump, an autosampler, and a photodiode array (PDA) detector. Samples (5 μL) were injected onto a Hypersil Gold column (2.1 × 150 mm, particle size of 1.9 μm, Thermo Scientific). Water acidified with 0.1% (v/v) acetic acid (eluent A) and ACN acidified with 0.1% (v/v) acetic acid (eluent B) were used as eluents. The flow rate was 400 μL/min, and the column oven temperature was controlled at 30 °C. The PDA detector was set to measure the range of 200−600 nm. The following elution profile was used: 0−1 min, isocratic on 0% (v/v) B; 1−6 min, linear gradient from 0 to 35% (v/v) B; 6−7 min, linear gradient from 35 to 100% (v/v) B; 7−9 min, isocratic on 100% (v/v) B; 9−10 min, linear gradient from 100 to 0% (v/v) B; and 10−12 min, isocratic on 0% (v/v) B.

Electrospray Ionization−Mass Spectrometry (ESI−MS). Mass spectrometric data were obtained by analyzing samples on a LTQ-XL (Thermo Scientific) equipped with an ESI probe coupled to the RP-UHPLC system. Nitrogen was used as sheath gas and auxiliary gas. Data were collected over the m/z range of 150−1500. Data-dependent MS^n analysis was performed with a normalized collision energy of 35%. The MS^n fragmentation was performed on the most intense product ion in the MS^{n-1} spectrum. Most settings were optimized via automatic tuning using "Tune Plus" (Xcalibur 2.07, Thermo Scientific). The system was tuned with chlorogenic acid in negative ionization (NI) mode. The transfer tube temperature was 350 °C, and the source voltage was 3.5 kV. Data acquisition and reprocessing were performed with Xcalibur 2.07 (Thermo Scientific).

Preparative HPLC Purification of Sulfochlorogenic Acid. Samples of 5-CQA (1 mM) incubated with $NaHSO_3$ (2 mM) and tyrosinase (70 units/mL) were fractionated on a preparative HPLC system (Waters, Milford, MA), using a semi-preparative Hypersil Gold column (20 × 150 mm, particle size of 5 μm, Thermo Scientific) with a Hypersil guard column (20 × 20 mm, particle size of 5 μm, Thermo Scientific). Water acidified with 0.2% (v/v) acetic acid (eluent A) and ACN acidified with 0.2% (v/v) acetic acid (eluent B) were used as eluents. The flow rate was 30 mL/min. The following elution profile was used: 0−2 min, isocratic on 2% (v/v) B; 2−17 min, linear gradient from 2 to 10% (v/v) B; 17−20 min, linear gradient from 10 to 100% (v/v) B; 20−25 min, isocratic on 100% (v/v) B; 25−27 min, linear gradient from 100 to 2% (v/v) B; and 27−37 min, isocratic on 2% (v/v) B. Fractions (14 mL) were collected and pooled on the basis of ultraviolet (UV) response at 320 nm. The major reaction product was found to be 2′-SO_3H-5-CQA,[11] with a purity of 94% (on the basis of peak integration at 320 nm), which was in accordance with the MS base peak chromatogram.

Preparative Purification of Cysteinylcaffeoylquinic Acid Using Flash Chromatography. Samples of 5-CQA (1 mM) incubated with cysteine (2 mM) and tyrosinase (70 units/mL) were fractionated on a flash chromatography system (Grace, Deerfield, IL) using a Reveleris C18 flash cartridge (Grace). Water acidified with 0.1% (v/v) acetic acid (eluent A) and ACN acidified with 0.1% (v/v) acetic acid (eluent B) were used as eluents. The cartridge was equilibrated with 5 column volumes of B, followed by 5 column volumes of A. The following elution profile was used: 0−10 min, linear gradient from 0 to 40% (v/v) B; 10−14 min, linear gradient from 40 to 100% (v/v) B; and 14−16 min, isocratic on 100% (v/v) B. Fractions (25 mL) were collected and pooled on the basis of UV response at 320 nm. The major reaction product was found to be

2′-*S*-cysteinyl-5-*O*-caffeoylquinicacid,[12] with a purity of 95% (on the basis of peak integration at 320 nm), which was in accordance with the MS base peak chromatogram.

Oxygen Consumption Measurements. Oxygen consumption of tyrosinase was measured using an Oxytherm System (Hansatech, Kings Lynn, U.K.). Incubations with 5-CQA (0.5 mM) and tyrosinase (35 units/mL) with or without sulfur-containing compound (1 mM) or ascorbic acid (1 mM) were performed in a total volume of 1 mL of 10 mM HEPES buffer at pH 6.5 and 25 °C. Data acquisition and analysis were performed using Oxygraph Plus software (Hansatech).

In the case of pre-incubation experiments, tyrosinase (35 units/mL) and sulfur-containing compounds (1 mM) were incubated for different times (specified in the text) at 25 °C, after which concentrated 5-CQA (50 mM) was added to reach a final concentration of 0.5 mM.

RESULTS AND DISCUSSION

***In Vitro* Browning of 5-CQA by Tyrosinase.** To investigate the effect of different sulfur-containing compounds on 5-CQA conversion by tyrosinase, the reaction products of the *in vitro* browning of 5-CQA by tyrosinase were analyzed. The formation of reaction products was monitored by both RP-UHPLC−PDA−MS analysis and recording absorption spectra of the reaction mixture at different time intervals (Figure 2). Results were compared to those obtained in the presence of ascorbic acid, which was used as a reference antibrowning agent.

After incubation, the absorption spectra of a mixture of 5-CQA and tyrosinase (Figure 2A) showed a clear decrease in absorbance at the typical maximum for 5-CQA, 326 nm. New maxima at 250 and around 400 nm appeared, with the latter being responsible for the brown color observed after the reaction. The corresponding RP-UHPLC−PDA trace (Figure 2F) showed that different reaction products were formed. On the basis of the rise of the baseline, it seemed that a multitude of compounds eluted between 4.5 and 6.5 min, with three clear peaks standing out (peaks 1, 2, and 3). Peak 1 was identified as 5-CQA based on both MS analysis (Table 1) and comparing to RP-UHPLC analysis of the authentic standard. MS analysis of peak 2 revealed a m/z of 351, corresponding to a mass of 352, which might represent the mass of the *o*-quinone of 5-CQA, an expected reaction product of 5-CQA and tyrosinase.[21] MS^2 analysis yielded m/z 215 as the most abundant product ion, which could not be explained. Two less abundant product ions were m/z 177 and 133, which might correspond to the fragments m/z 179 and 135, respectively, found in MS^2 of 5-CQA, taking into account the loss of two protons upon oxidation of 5-CQA. Peak 3 had m/z 705, and MS^2 resulted in a predominant fragment with m/z 513, the MS^3 fragmentation of which resulted in a major product ion of m/z 339. Dimers of 5-CQA, resulting from oxidation by tyrosinase, showing the same MS fragmentation have been described before[22] and were also found in oxidized apple extracts.[23] On the basis of these data, compounds **2** and **3** (Table 1) were tentatively identified as the *o*-quinone and dimer of 5-CQA, respectively.

The absorption spectra of 5-CQA and tyrosinase incubated in the presence of ascorbic acid (Figure 2B) showed that ascorbic acid delays enzymatic browning. At time zero, besides the typical 326 nm maximum for 5-CQA, also a maximum at 265 nm was observed, caused by the ascorbic acid present. After 30 min of incubation, this maximum disappeared, whereas the 5-CQA maximum remained. After 60 min of incubation, the spectrum was similar to the spectrum of 5-CQA incubated with tyrosinase after 30 and 60 min: a decrease in absorbance at 326 nm, and new maxima formed at 250 and around 400 nm. The RP-UHPLC−PDA trace (Figure 2G) was similar to that of 5-CQA incubated with tyrosinase alone (Figure 2F). The same compounds were present, with only the ratio between them differing. These results confirmed that ascorbic acid delays browning.

Effect of Different Sulfur-Containing Compounds on 5-CQA Conversion by Tyrosinase. To investigate the influence of different sulfur-containing compounds on tyrosinase-catalyzed browning of 5-CQA, combinations of 5-CQA, tyrosinase, and $NaHSO_3$, cysteine, GSH, DTT, or allyl isothiocyanate were incubated. The formation of reaction products was monitored as described above.

The absorption spectra of 5-CQA did not change substantially upon incubation with tyrosinase and $NaHSO_3$ (Figure 2C). After 30 and 60 min, a small decrease in absorption at 326 nm was observed, with no new maxima formed. RP-UHPLC−PDA analysis resulted in five peaks (Figure 2F). Besides 5-CQA (compound **1**), four reaction products were present, which were identified by MS analysis (Table 1). Comparing the product ions found for compounds **4**−**7** with published MS fragmentation data for 5-CQA,[24−26] the fragments diagnostic for 5-CQA were observed, only with an increase of 80 atomic mass units. The product ions with m/z 259, 241, and 215 corresponded to product ions with m/z 179, 161 and 135, respectively. According to the fragmentation pattern of 5-CQA, these three product ions all contained the phenolic ring of the caffeic acid moiety of 5-CQA, indicating that the addition of HSO_3^- occurred on this phenolic ring. Compounds **4**−**7** were tentatively identified as different sulfochlorogenic acid isomers, with the main reaction product being 2′-sulfo-5-*O*-caffeoylquinic acid (2′-SO_3H-5-CQA).[11]

For the incubation of 5-CQA with tyrosinase and cysteine, the absorption spectra also did not change substantially (Figure 2D). After incubation, a small decrease in absorption at 326 nm and a small increase in absorption at 260 nm were observed. RP-UHPLC−PDA analysis revealed two major peaks (Figure 2I). Besides 5-CQA, a new peak was observed (compound **8**). MS analysis of compound **8** revealed a mass of 473, equal to the combined masses of 5-CQA (354) and cysteine (121), minus 2 for the two hydrogen atoms lost because of the formation of a covalent bond between cysteine and 5-CQA. Upon fragmentation of compound **8**, a product ion with m/z 385 was predominantly formed. The occurrence of this product can be explained by the loss of part of the cysteine moiety, with the sulfur of cysteine remaining bound to 5-CQA. MS^3 analysis of this fragment resulted in fragments with m/z 191 and 193, which could be explained as the quinic acid and dehydrated caffeoyl moiety with sulfur attached, respectively. Similar fragmentation has been observed for addition products of 5-CQA and *N*-acetyl-cysteine.[22] An addition product of cysteine and 5-CQA has been identified as 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid.[12] On the basis of these data, compound **8** was tentatively identified as 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid.

When incubated with tyrosinase and GSH, the absorption spectra of 5-CQA showed a small decrease in absorption at 326 nm and a bathochromic shift for the minimum observed at 260 nm before incubation to 275 nm after incubation (Figure 2E). RP-UHPLC−PDA analysis revealed total depletion of 5-CQA with the formation of a single reaction product, compound **9** (Figure 2J). MS analysis of compound **9** resulted in a mass of 659, corresponding to a covalent addition product of 5-CQA and GSH (354 + 307 − 2). MS^2 and MS^3 analyses resulted in the same fragments as described above for the

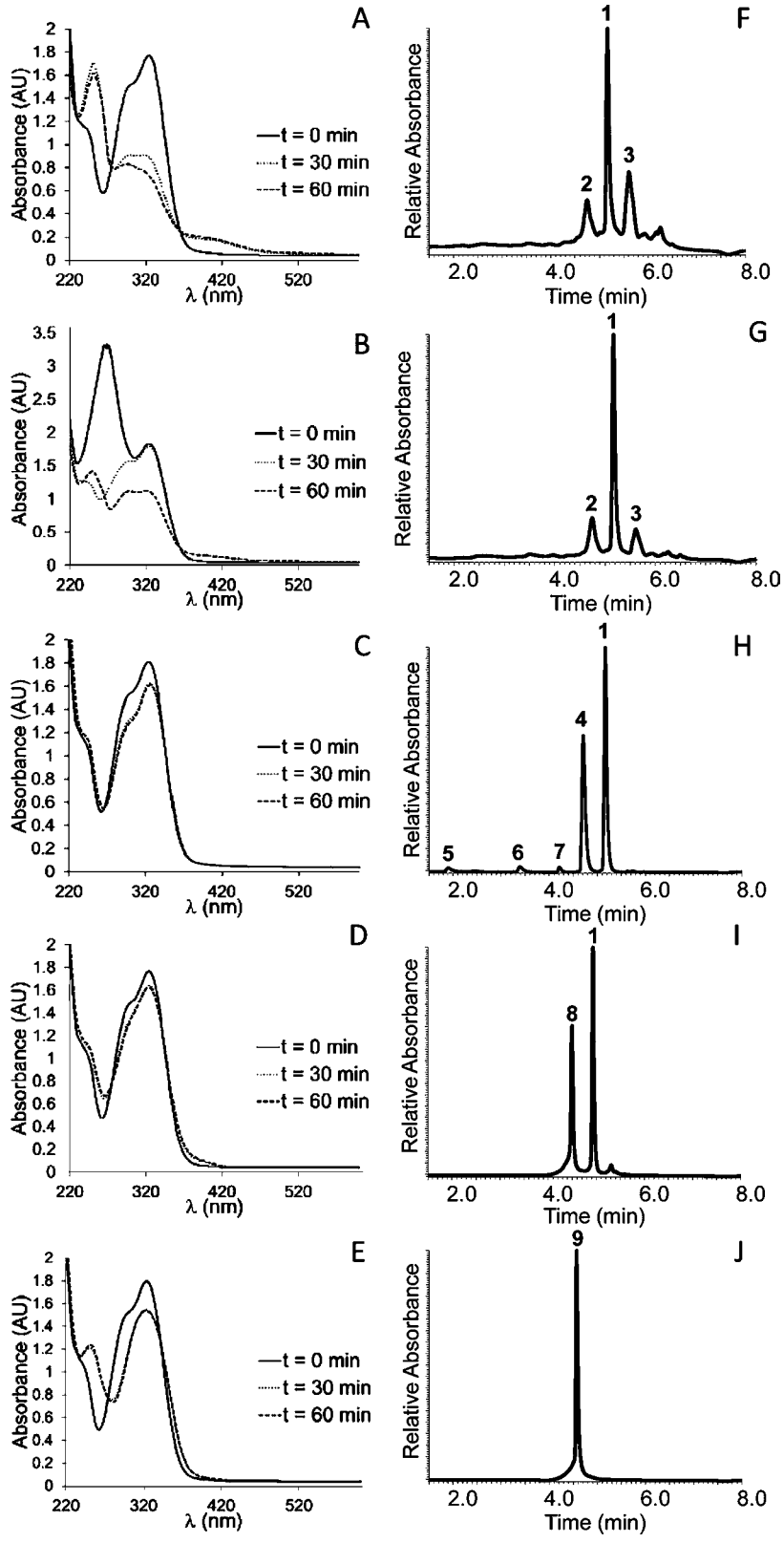

Figure 2. Absorption spectra of 5-CQA (0.1 mM) incubated with tyrosinase (7 units/mL) with and without potential browning inhibitors (A–E) at different incubation times and RP-UHPLC–PDA traces (320 nm) of the reaction mixtures after incubation (F–J): (A and F) 5-CQA with tyrosinase alone, (B and G) 5-CQA with tyrosinase and ascorbic acid (0.2 mM), (C and H) 5-CQA with tyrosinase and $NaHSO_3$ (0.2 mM), (D and I) 5-CQA with tyrosinase and cysteine (0.2 mM), and (E and J) 5-CQA with tyrosinase and GSH (0.2 mM).

fragmentation of 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid. On the basis of this and previous data,[15,27] compound **9** was tentatively identified as 2′-*S*-glutathionyl-5-*O*-caffeoylquinic acid.

Incubation of 5-CQA with tyrosinase and DTT resulted in total inhibition of 5-CQA conversion. The absorption spectra after 30 and 60 min were the same as at 0 min, and RP-UHPLC

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Table 1. Identification of Reaction Products of 5-CQA, $NaHSO_3$, Cysteine, GSH, and Tyrosinase

peak	retention time (min)	$[M - H]^-$	MS^n fragments (% relative abundance)	λ_{max} (nm)	tentative identification
1	5.11	353	MS^2 [353]: 191 (100), 179 (4), 135 (1)	326	5-*O*-caffeoylquinic acid (5-CQA)
2	4.75	351	MS^2 [351]: 215 (100), 177 (15), 195 (8), 133 (6), 307 (6), 173 (5), 123 (5), 261 (2), 155 (2), 149 (2), 191 (1)	251, 300	5-CQA quinone
3	5.61	705	MS^2 [705]: 513 (100); MS^3 [705 → 513]: 339 (100), 321 (10), 496 (7), 295 (2)	323	5-CQA dimer
4	4.45	433	MS^2 [433]: 259 (100), 415 (47), 215 (38), 241 (23), 387 (17), 161 (16) 301 (7)	240, 328	2′-sulfo-5-*O*-caffeoylquinic acid (2′-SO_3H-5-CQA)
5	1.77	433	MS^2 [433]: 353 (100), 191 (30), 241 (24), 161 (13), 415 (7), 271 (4), 179 (1); MS^3 [433 → 353]: 191 (100), 179 (6), 135 (1)	244, 302	sulfocaffeoylquinic acid isomer
6	3.16	433	MS^2 [433]: 241 (100), 415 (93), 387 (11), 259 (9), 213 (7), 416 (4), 433 (3), 242 (3), 161 (1), 133 (1); MS^3 [433 → 241]: 213 (100), 241 (56), 161 (5), 133 (3)	296, 324	sulfocaffeoylquinic acid isomer
7	4.09	433	MS^2 [433]: 259 (100), 415 (37), 215 (33), 241 (20), 387 (18), 161 (12), 301 (11)	315	sulfocaffeoylquinic acid isomer
8	4.49	472	MS^2 [472]: 385 (100), 193 (18), 191 (17), 236 (12), 280 (3), 351 (2), 298 (2), 167 (2); MS^3 [472 → 385]: 191 (100), 193 (89), 211 (1)	250, 326	2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid
9	4.57	658	MS^2 [658]: 385 (100), 466 (55), 272 (24), 193 (13), 529 (12), 191 (11), 448 (4), 379 (4), 254 (4), 337 (2), 211 (2), 340 (1), 306 (1), 293 (1), 210 (1); MS^3 [658 → 385]: 191 (100), 193 (94), 211 (1)	252, 325	2′-*S*-glutathionyl-5-*O*-caffeoylquinic acid

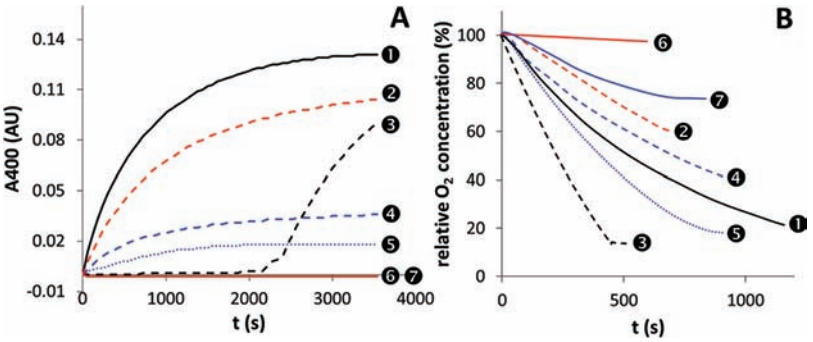

Figure 3. Development of brown color measured by (A) monitoring absorbance at 400 nm and (B) monitoring oxygen consumption in time during incubations of 5-CQA (A, 0.1 mM; B, 0.5 mM) with tyrosinase (A, 7 units/mL; B, 35 units/mL), in the presence of different sulfur-containing compounds and ascorbic acid (A, 0.2 mM; B, 1 mM): **1**, control; **2**, allyl isothiocyanate; **3**, ascorbic acid; **4**, cysteine; **5**, GSH; **6**, DTT; and **7**, $NaHSO_3$.

analysis showed that only 5-CQA was present after incubation (see panels A and C of Figure S1 of the Supporting Information). Complete inhibition of tyrosinase by DTT was found before,[18] while also adduct formation between *o*-quinones and DTT was observed.[17] Interestingly, our results did not indicate such adduct formation. Possibly, adduct formation is concentration-dependent: at relative high concentrations of DTT, tyrosinase is strongly inhibited, while at lower concentrations, tyrosinase remains (partly) active, making *o*-quinones available for the addition of DTT.

Allyl isothiocyanate seemed to have little influence on 5-CQA conversion: both absorption spectra and UHPLC–PDA trace (see panels B and D of Figure S1 of the Supporting Information) were similar to the ones obtained with the control experiment of only 5-CQA and tyrosinase (panels A and F of Figure 2).

Influence of Sulfur-Containing Compounds on Brown Color Formation and Oxygen Consumption. The rate of color formation and oxygen consumption during incubations of 5-CQA with tyrosinase and different potential antibrowning agents was determined by monitoring the absorbance at 400 nm and the oxygen consumption in time (Figure 3). It can be seen that there is not always a correlation between these parameters. While incubations with cysteine and GSH showed oxygen consumption rates comparable to the control experiment of only 5-CQA and tyrosinase, they showed much less color development. The reduced color formation corresponded to the formation of 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid and 2′-*S*-glutathionyl-5-*O*-caffeoylquinic acid, respectively (panels I and J of Figure 2). Ascorbic acid showed a higher oxygen consumption compared to the control experiment, while the onset of color formation in the presence of ascorbic acid was delayed. This confirmed the mechanism of browning inhibition by ascorbic acid, as described before. Incubation with allyl isothiocyanate resulted in both slightly reduced oxygen consumption and slightly reduced brown coloration. DTT seemed to prevent browning by inhibiting tyrosinase activity: both oxygen consumption and brown color formation were totally inhibited at the concentration used. This corresponds well with the absorption spectra and UHPLC–MS analysis of the incubation of 5-CQA with tyrosinase and DTT (see panels A and C of Figure S1 of the Supporting Information), where no conversion of 5-CQA was observed. When 5-CQA was incubated with tyrosinase and $NaHSO_3$, it was observed that

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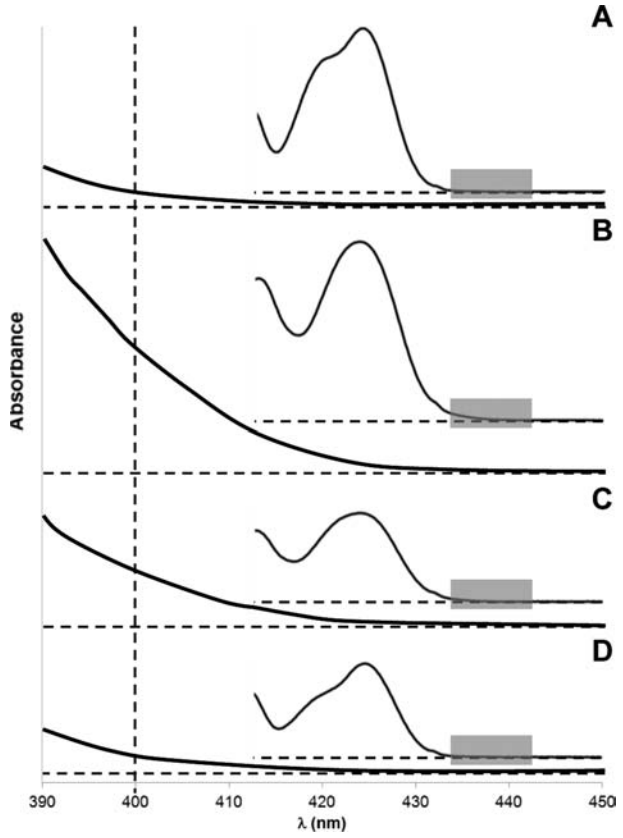

Figure 4. UHPLC–PDA absorption spectra of (A) 5-CQA, (B) 2′-*S*-glutathionyl-5-*O*-caffeoylquinic acid, (C) 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid, and (D) 2′-SO_3H-5-CQA. Absorption spectra were derived from peaks 1, 9, 8, and 4 (Figure 2), respectively. Insets show spectra of 250–500 nm, and the gray windows indicate the wavelength range (390–450 nm) of the zoom.

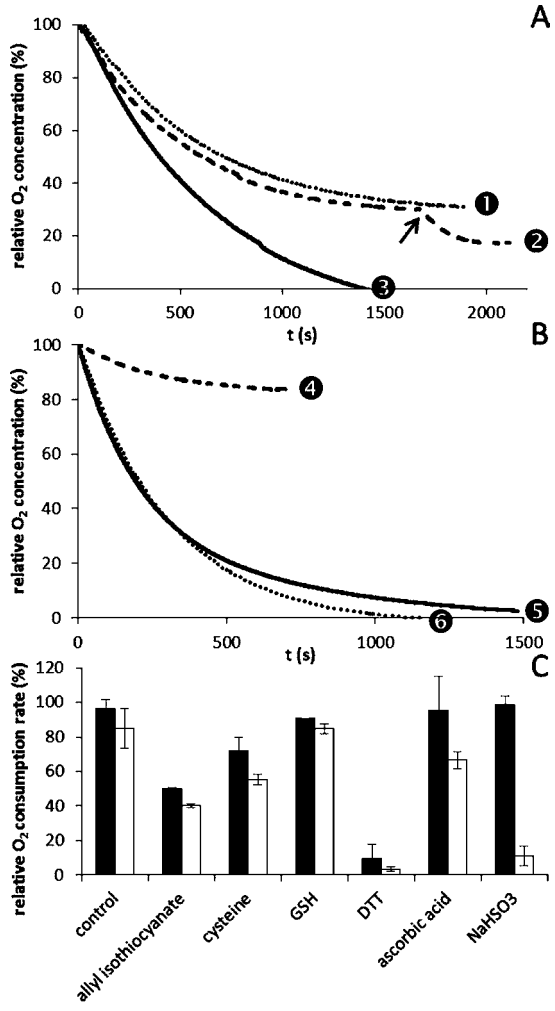

Figure 5. Oxygen consumption in time upon (A) incubation of 5-CQA (0.5 mM) and tyrosinase (35 units/mL) with and without $NaHSO_3$ (1 mM), (B) incubation of 5-CQA (0.5 mM) with 2′-SO_3H-5-CQA (0.5 mM) and 5-CQA (0.5 mM) with tyrosinase pre-incubated with $NaHSO_3$ (1 mM, 1 h, 25 °C), and (C) relative O_2 consumption rate obtained when incubating 5-CQA (0.5 mM) with different potential browning inhibitors (1 mM) and tyrosinase (35 units/mL), which was either pre-incubated with the potential browning inhibitor (15 min, 25 °C; white bars) or not pre-incubated (black bars): **1**, 5-CQA + $NaHSO_3$ + tyrosinase (single addition); **2**, 5-CQA + $NaHSO_3$ + tyrosinase (double addition); **3**, 5-CQA + tyrosinase; **4**, 5-CQA + pre-incubated tyrosinase; **5**, 5-CQA + tyrosinase; and **6**, 5-CQA + 2′-SO_3H-5-CQA + tyrosinase. The arrow indicates the second tyrosinase addition.

oxygen consumption leveled off during the experiment, to a plateau value of around 75% of the starting concentration of oxygen. This indicated that tyrosinase activity was somehow lost during the course of the incubation. In the presence of $NaHSO_3$, no color development was observed, consistent with the spectra in Figure 2C.

In contrast to incubations of 5-CQA and tyrosinase with $NaHSO_3$, those with cysteine and GSH resulted in some browning. When the UHPLC–PDA absorption spectra of the different addition products and 5-CQA were compared, it was found that 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid and 2′-*S*-glutathionyl-5-*O*-caffeoylquinic acid had a higher absorbance around 400 nm compared to 5-CQA, while that for 2′-SO_3H-5-CQA was comparable to that of 5-CQA (Figure 4). Although the absorbance at 400 nm of 2′-*S*-glutathionyl-5-*O*-caffeoylquinic acid is higher than that of 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid, for the total reaction mixtures with these compounds, one observes the opposite (Figure 3A). This suggests that the absorbance of the cysteine addition product only partially explains the observed browning. A possible explanation might be that some of the *o*-quinones formed reacted further into brown pigments prior to the reaction with cysteine.

Reduction of Tyrosinase Activity during Formation of Sulfochlorogenic Acid. $NaHSO_3$ showed a distinctly different effect compared to the other inhibitors investigated: no brown color formation was observed, and oxygen consumption leveled off during incubation with 5-CQA and tyrosinase. This observation was investigated further by monitoring the amount of 5-CQA converted by tyrosinase in time with or without $NaHSO_3$ using RP-UHPLC (see Figure S2 of the Supporting Information). It was found that, with $NaHSO_3$ present, not all 5-CQA was converted, consistent with the observation that less oxygen was consumed when 5-CQA was incubated in combination with $NaHSO_3$ and tyrosinase, as compared to an incubation of 5-CQA and tyrosinase alone. The addition of a second dose of tyrosinase restored oxygen consumption and 5-CQA conversion (Figure 5A). Markakis and Embs[28] found a similar effect of $NaHSO_3$ when following the activity of mushroom tyrosinase in a reaction mixture containing tyrosine and $NaHSO_3$.

The fact that a second addition of tyrosinase was necessary to convert all 5-CQA in the presence of $NaHSO_3$ indicated that tyrosinase was somehow inhibited in the course of the reaction. Two explanations for the observed enzyme inhibition might be (i) $NaHSO_3$ inhibited tyrosinase directly in a rather slow, time-dependent manner or (ii) the 2′-SO_3H-5-CQA formed inhibited the enzyme. To investigate these possible scenarios of inhibition, 2′-SO_3H-5-CQA was purified from a reaction mixture to test its influence on tyrosinase activity.

The addition of 0.5 mM 2′-SO_3H-5-CQA to an incubation of 5-CQA with tyrosinase did not influence the rate of oxygen consumption (Figure 5B), showing that 2′-SO_3H-5-CQA was not a tyrosinase inhibitor. This is in contrast with addition products of cysteine and 5-CQA, which were found to be competitive inhibitors for PPO from apple.[13] On the basis of the observations with apple PPO, 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid was purified from a reaction mixture containing 5-CQA, cysteine, and mushroom tyrosinase. The addition of 0.5 mM purified 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid to an incubation of 5-CQA and tyrosinase did not result in tyrosinase inhibition (results not shown). Thus, 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid does not inhibit mushroom tyrosinase, similar to 2′-SO_3H-5-CQA. The fact that 2′-*S*-cysteinyl-5-*O*-caffeoylquinic acid was found before to inhibit apple PPO[13] might be explained by the different origins of the two enzymes.

To investigate whether $NaHSO_3$ inhibits the enzymatic activity of tyrosinase in time, tyrosinase was pre-incubated with $NaHSO_3$ (1 h). Oxygen consumption measurements with this pre-incubated tyrosinase showed a decreased activity compared to its control without prior pre-incubation with $NaHSO_3$ (Figure 5B). This experiment indicated that scenario (i) is most likely. To establish the time dependency of tyrosinase inhibition by $NaHSO_3$, tyrosinase was pre-incubated with $NaHSO_3$ for different times, after which a concentrated substrate solution was added. The initial reaction rate was determined by measuring the oxygen consumption rate (see Figure S3 of the Supporting Information). It was found that the reaction rate rapidly decreased with an increasing pre-incubation time: already after 1 min, approximately 50% of activity was lost. After 15 min of pre-incubation, activity decreased further to approximately 10% of the initial activity. Sayavedra-Soto and Montgomery[6] found that pre-incubation of pear PPO with sulfite resulted in irreversible inhibition of the enzyme: indications for modification of the protein structure were found, although the nature of the modifications was not established.

The effects of pre-incubation of tyrosinase with the other sulfur-containing compounds tested in this study were also investigated. The oxygen consumption with and without pre-incubation of tyrosinase was determined and expressed relative to a control experiment using untreated tyrosinase. Pre-incubation of tyrosinase with other sulfur-containing compounds had little effect on tyrosinase activity (Figure 5C).

$NaHSO_3$ Has a Dual Inhibitory Effect on Tyrosinase-Catalyzed Browning. In conclusion, our results show that different sulfur-containing compounds can inhibit *in vitro* browning of 5-CQA by mushroom tyrosinase in two different ways: by inhibition of enzymatic activity ($NaHSO_3$ and DTT) or formation of colorless adducts with enzymatically formed *o*-quinones ($NaHSO_3$, cysteine, and GSH). A schematic representation of enzymatic browning of 5-CQA and possible inhibitory routes is shown in Figure 6. It is evident that $NaHSO_3$ has a unique position within the group of sulfur-containing compounds investigated: it has a dual inhibitory effect on tyrosinase-catalyzed

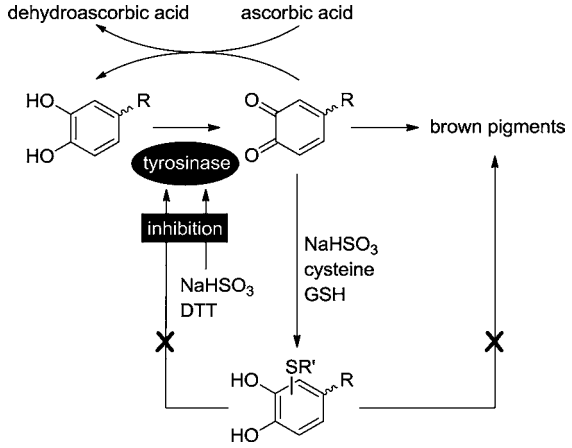

Figure 6. Schematic representation of the action of tyrosinase on 5-CQA, together with possible mechanisms of inhibition of 5-CQA browning.

browning of 5-CQA. Initially, the formation of brown pigments is inhibited by the formation of sulfochlorogenic acid, while at the same time, tyrosinase is inhibited in a time-dependent way. The exact mechanism of the time-dependent inhibition of tyrosinase by $NaHSO_3$ remains unclear. It is possibly due to covalent interactions between $NaHSO_3$ and tyrosinase. This will be the subject of further investigation.

■ ASSOCIATED CONTENT

Supporting Information

Spectral and UHPLC analysis of 5-CQA incubated with tyrosinase and DTT or allyl isothiocyanate (Figure S1), decrease of the 5-CQA concentration when incubated with tyrosinase with or without $NaHSO_3$ (Figure S2), and oxygen consumption rates of incubations of 5-CQA with tyrosinase pre-incubated with $NaHSO_3$ for different times (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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