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Properties of Chlorogenic Acid Quinone: Relationship between Browning and the Formation of Hydrogen Peroxide from a Quinone Solution

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Chlorogenic acid is the major polyphenol in foods derived from plants and is a good substrate for polyphenol oxidase. Chlorogenic acid quinone (CQA-Q), which is an oxidative product of chlorogenic acid by polyphenol oxidase, is an important intermediate compound in enzymatic browning. CQA-Q was prepared, and its properties and the relationship with browning were examined. The quinone solution was yellow or orange, and its molecular absorption coefficient was estimated to be 1.7×10^3 for 325 nm and 9.7×10^2 for 400 nm in an acidic aqueous solution. Chlorogenic acid and H_2O_2 were spontaneously generated in the CQA-Q solution as the yellowish color of the solution gradually faded. A pale colored polymer was the major product in the reaction solution. Amino acids such as lysine and arginine added to CQA-Q solution did not repress the fading of the yellowish color of the solution. We concluded from these results that CQA-Q itself and a mixture of CQA-Q and amino acids did not form intensive brown pigments in the acidic aqueous solution. H_2O_2 spontaneously formed in the CQA-Q solution, and other polyphenols might have played an important role in the formation of the brown color by enzymatic browning.

Key words: chlorogenic acid; chlorogenic acid quinone; *o*-quinone; enzymatic browning; H_2O_2

The polyphenols in a variety of plants are oxidized to their corresponding quinones by the action of polyphenol oxidase (EC 1.10.3.1, PPO) when plant tissue is broken or crushed.[1,2] These quinones are considered to be further polymerized with quinones or amines to form a brown pigment.[3,4] This browning has been commonly observed in fruit and vegetables such as apples, bananas and lettuce, and the regulation of the browning is an important aspect for food processing and preservation.[4,5]

Molecular biological studies on PPOs have recently been making an intensive progress.[5,6] Several trials to regulate enzymatic browning by repressing the expression of PPO have been conducted.[7–10] On the other hand, clarification of the reactivity of polyphenol quinones is also important to understand the mechanism for enzymatic browning and to regulate the effect. Among the polyphenols, 5-caffeoylquinic acid (chlorogenic acid, CQA) is one of the major polyphenol in such plants as apples, coffee and lettuce. However, there is little data on the reactivity of isolated *o*-quinones or CQA quinone (CQA-Q; Fig. 1). Cilliers and Singleton have reported the products from the non-enzymatic auto-oxidation of caffeic acid as caffeicins which are dimers of caffeic

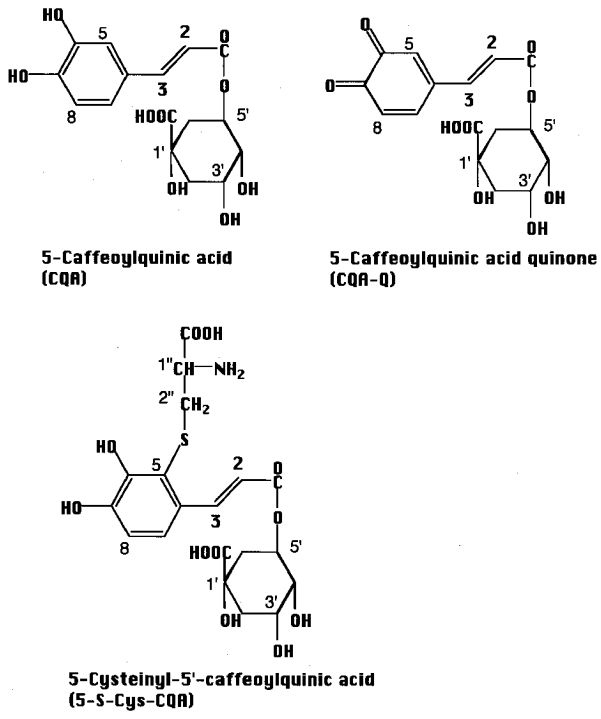

Fig. 1. Structures of Chlorogenic Acid (CQA), CQA Quinone (CQA-Q), and 5-*S*-Cysteinyl-chlorogenic Acid (5-*S*-Cys-CQA).

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Abbreviations: PPO, polyphenol oxidase; CQA, chlorogenic acid; CQA-Q, CQA quinone

acid.[11] Fulcrand *et al*. have prepared caffeic acid quinone and identified the products from chemical oxidation of caffeic acid under acidic conditions as two isomers of 2,5-(3′,4′-dihydroxyphenyl)tetrahydrofuran 3,4-dicarboxylic acid.[12] However, these compounds were colorless. In this present study, we prepared and isolated CQA-Q by HPLC and then examined the reactivity. We show that CQA-Q formed hardly any intense brown pigment by itself or with amino acids and that part of CQA quinone returned to CQA with the formation of H_2O_2.

Materials and Methods

Preparation of CQA-Q. One milliliter of a 100 mM CQA solution in MeOH and 1.0 ml of a 100 mM $NaIO_4$ aqueous solution were mixed and then incubated for about 3 min at room temperature. The mixture was passed through Chromatodisk (0.45 μm; Kurabo, Osaka, Japan) cartridges, before being subjected to analytical or preparative HPLC. The HPLC systems were as follow: pump, L-6320 (Hitachi, Tokyo, Japan); column, YMC pack R-ODS (Yamamura, Kyoto, Japan; 4.6 i.d. × 250 mm; for analysis) and YMC pack D-ODS (20 i.d. × 250 mm; for preparation); detector, L-4500 photodiode array (Hitachi, Tokyo; for analysis), L-4200 UV-VIS (Hitachi; for preparation); wavelength, 250–370 nm (for analysis), 325 nm (for preparation); eluent, CH_3CN and 5% aqueous acetic acid (12:88); flow rate, 1.0 ml/min for analysis and 9.99 ml/min for preparation. The eluate containing CQA-Q with a retention time of about 12 min by preparative HPLC was collected and immediately used for the subsequent experiments. CQA and CQA-Q were each determined from their peak areas at 325 nm. CQA was used as the external standard, and the CQA-Q concentration was estimated by using a conversion rate which was determined from the molecular absorption coefficients (ε) of CQA and CQA-Q at 325 nm. The ε value for CQA-Q was determined by the Cys method described later. The variation among samples was less than 10%.

Identification of CQA. The isolated CQA-Q solution was left for 3 h at room temperature and then concentrated *in vacuo*, before the concentrate was applied to preparative HPLC under the conditions just described. A peak with a retention time of about 17 min, which corresponded to a peak with a retention time of 7.2 min by analytical HPLC, was collected, concentrated *in vacuo* and dried. The residue was dissolved in CD_3OD and analyzed by NMR (Jeol JNM-GSX270). ^{1}H-NMR δ_H (ppm): 2.15 (4H, m), 3.70 (1H, dd, J=3.9, 9.2), 4.14 (1H, t, J=4.1), 5.35 (1H, t, J=8.8), 6.28 (1H, d, J=15.0), 6.75 (1H, d, J=8.0), 6.93 (1H, dd, J=1.4, 8.0), 7.03 (1H, d, J=1.4), 7.55 (1H, d, J=15.0).

Identification of 5-S-cysteinyl-CQA. About 0.8 ml of a 1 mM L-cysteine aqueous solution was added to 3 ml of the CQA-Q solution, the mixture being left for 30 min at room temperature and then concentrated *in vacuo*. The concentrate was applied to preparative HPLC, and a peak with a retention time of 12.5 min was collected, concentrated *in vacuo* and dried. This procedure was repeated several times. The residue was dissolved in D_2O and analyzed by NMR. ^{1}H-NMR δ_H (ppm): 2.15 (4H, m), 3.04 (2H, m), 3.54 (1H, dd, J=3.9, 8.5), 3.72 (1H, t, J=4.8), 4.16 (1H, t, J=3.8), 5.18 (1H, t, J=9.4), 6.25 (1H, d, J=16.0), 6.82 (1H, d, J=9.5), 7.13 (1H, d, J=9.5), 8.12 (1H, d, J=16.0).

Estimation of ε for CQA-Q. Three methods were used for estimating the ε value of CQA-Q. The first and second methods involved leaving the CAQ-Q solution for 10–45 min, before CQA and CQA-Q were analyzed. With the first method (the absorbance method), the absorption spectrum of the quinone solution was continuously analyzed by a spectrophotometer with a photodiode array detector (MultiSpec-1500, Shimadzu, Kyoto). We assumed the following equations for this method:

$$A_{325} = \varepsilon(325, \text{CQA}) \times [\text{CQA}] + \varepsilon(325, \text{CQA-Q}) \times [\text{CQA-Q}]$$

$$A_{400} = \varepsilon(400, \text{CQA-Q}) \times [\text{CQA-Q}]$$

where [CQA] and [CQA-Q] are the concentrations of CQA and CQA-Q, respectively, and ε(325, CQA), ε(325, CQA-Q) and ε(400, CQA-Q) are the ε values for CQA at 325 nm, for CQA-Q at 325 nm, and for CQA-Q at 400 nm, respectively.

The second method (the HPLC method) involved analyzing the quinone solution by HPLC under the conditions already described. We assumed that the rate of decrease of CQA-Q was equivalent to the rate of increase of CQA. The ratio of the peak areas of CQA and CQA-Q by HPLC at 325 nm enabled the ε value of CQA-Q at 325 nm to be estimated. The ε value of CQA-Q at 400 nm was then calculated by multiplying ε of CQA-Q at 325 nm by 1.8, because the ratio of the absorbance of CQA-Q at 325 nm and that at 400 nm was 1.8.

With the third method (the Cys method), an L-Cys solution was added to the CQA-Q solution immediately after its isolation, and the absorbance at 400 nm was then measured. The equivalent mole value of CQA-Q to Cys was estimated from the Cys concentration at which the absorbance at 400 nm was zero.

Determination of H_2O_2. H_2O_2 was determined by the iodine titration and the peroxidase methods. The iodine titration method was conducted with $Na_2S_2O_3$ and KI in the usual way, while the peroxidase method was according to the method of Putter and Becker[13]

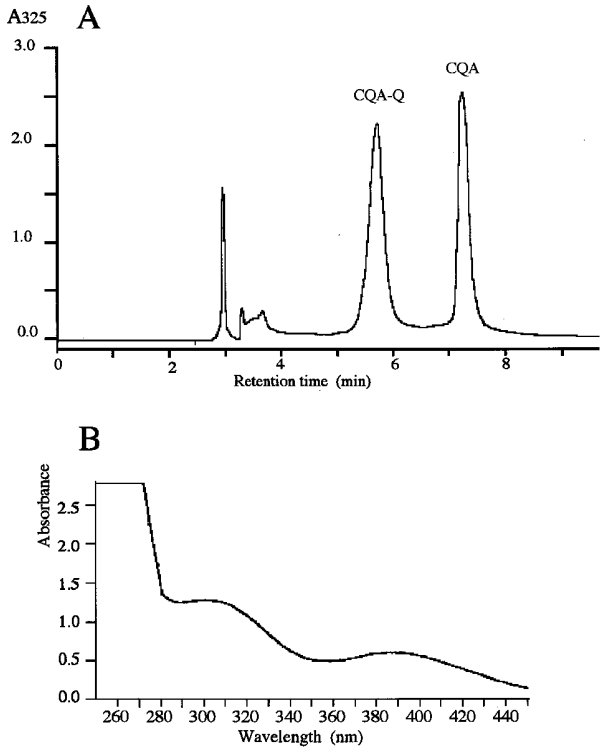

Fig. 2. Typical HPLC Trace of a Reaction Mixture of CQA and $NaIO_4$.
A, Chromatogram; B, UV Spectrum of CQA-Q.

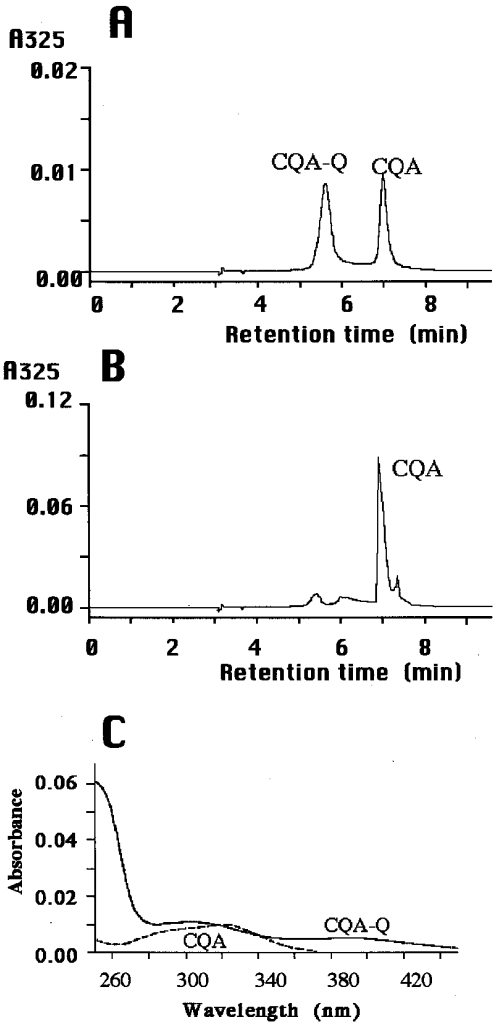

Fig. 3. Typical HPLC Traces of Isolated CQA-Q.
CQA-Q isolated by preparative HPLC was applied to analytical HPLC after 0.1 min (A) and 60 min (B) of incubation. The HPLC conditions are described in the Material and Method section. C, UV spectra of CQA and CQA-Q from A.

with some modifications. After the reaction mixture (2.2 ml) containing 1.7 mM 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid (ABTS, Sigma, St. Louis, U.S.A.) and horseradish peroxidase (20 units, Wako Chemical Co., Osaka), a 67 mM K/Na phosphate buffer (pH 6.0) and the sample solution (0.2 ml) had been incubated 30°C for 2 min, the absorbance at 405 nm was measured. Ten to 500 μM of H_2O_2 was used to produce the calibration curve.

Results and Discussion

Preparation and properties of CQA-Q

CQA-Q was prepared by the oxidation of CQA by $NaIO_4$ and isolated by HPLC. Figure 2 shows a typical chromatogram of the reaction mixture and the UV spectrum of CQA-Q. The quinone had absorption maxima at 305–310 nm and 390–400 nm. To ascertain that this quinone was CQA-Q, the fraction was collected and L-Cys was immediately added. The yellowish color of the quinone instantly disappeared. This mixture was then applied to HPLC, before the major adduct of the quinone and L-Cys was isolated. The ^{1}H-NMR spectrum showed that this compound was the adduct of CQA and L-Cys. The signals at 3.72 ppm and 5.18 ppm were derived from the Cys residue, and the other signals were from CQA moiety. The signal at position 5 of CQA had disappeared in this adduct, and coupling between the protons at positions 8 and 9 was observed. The two components were therefore connected at position 5. This adduct was thus identified as 5-*S*-cysteinyl-5′-caffeoylquinic acid (5-*S*-Cys-CQA, Fig. 1). These results enabled the quinone to be confirmed as CQA-Q. Richard *et al.* have reported 5-*S*-Cys-CQA as a single-addition compound in the reaction mixture of CQA and apple PPO.[14]

CQA-Q was very unstable, Fig. 3 showing a typical chromatogram of the isolated quinone by HPLC. In addition to the peak of quinone (a retention time of 5.7 min), a peak with a retention time of 7.2 min appeared. This new peak increased and the peak of CQA-Q decreased when the isolated quinone solution was incubated at room temperature. The retention time and the absorption spectrum of this peak corresponded with those of CQA. To ascertain that this peak was of CQA, the peak was isolated by preparative HPLC and analyzed by ^{1}H-NMR. The

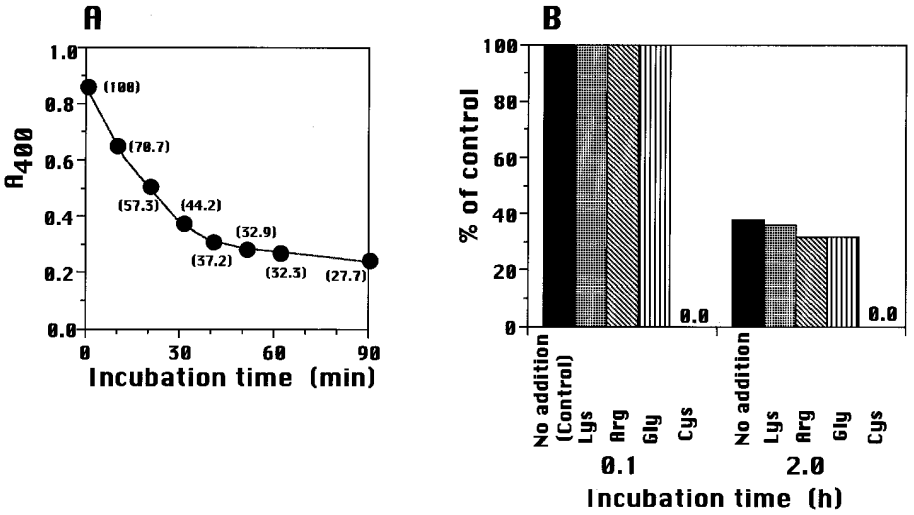

Fig. 4. Decrease in Color Intensity of the CQA-Q Solution in the Absence (A) and Presence (B) of Amino Acids. CQA-Q and the amino acids were dissolved in a mixture of MeCN and 5% AcOH (15:85).

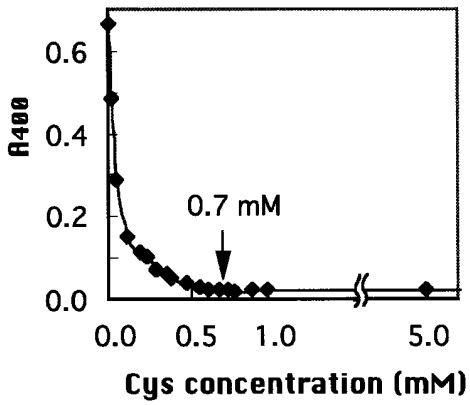

Fig. 5. Estimated ε Value of CQA-Q by the Cys Method.

NMR data were identical with those of CQA, confirming this peak to be CQA. This result shows that part of CQA-Q had been reduced to CQA in the acidic aqueous solution.

We used the isolated solution of CQA-Q without further concentration, because the quinone was very unstable. An aqueous acetic acid solution containing acetonitrile was used as the eluent. As the pH value of such fruits as apple and pear is acidic, this pH condition was suitable for identifying the change in CQA in fruit and fruit juice. The isolated solution was yellow or orange; however, the color intensity gradually reduced with the decrease in quinone and increase in CQA. Figure 4 shows the change in the absorbance at 400 nm of the CQA quinone solution. After 60 min, the absorbance had reduced by about 30% of the starting level.

It is generally believed that CQA-Q is very reactive and automatically polymerizes by itself or with CQA and amino acids to form a brown pigment, because CQA turns brown when it is incubated with PPO in the presence of oxygen. However, our result clearly shows that CQA-Q was partly reduced to CQA and the yellowish color faded during incubation. Fulcrand *et al.* have also reported that an aqueous solution of caffeic acid quinone was unstable and that caffeic acid was the major product in the aqueous solution of the quinone.[12]

We next tried to determine the molecular absorption coefficient (ε) of CQA-Q for a qualitative determination of the quinone by HPLC. We could not isolate the quinone as a solid; therefore, the coefficient was estimated by three methods. The absorbance method and the HPLC method enabled the ε value to be estimated from the proportional decrease in the quinone and increase in CQA. We assumed for these two methods that the proportional decrease in CQA-Q and increase in CQA were the same, because these two compounds were the major ones by HPLC. With the absorbance method, ε was estimated from the decrease in the absorbance at 400 nm, which was the absorption maximum for CQA-Q, and the increase in the absorbance 325 nm, which was the absorbance maximum for CQA. With the HPLC method, ε was estimated from the peak areas of CQA and CQA-Q by HPLC, this being monitored by the absorbance at 325 nm. The ε value of CQA could be measured, enabling the ε value of CQA-Q to be calculated from these data. The difference in ε value estimated by these two methods was less than 15%, the mean value of ε being 1.5×10^4 for 325 nm and 8.5×10^3 for 400 nm in this acidic aqueous solution.

The Cys method involved L-Cys being added to the CQA-Q solution immediately after its isolation. Figure 5 shows the relationship between added Cys and the absorbance of CQA-Q at 400 nm. We assumed that the Cys concentration at the point where the absorbance of CQA-Q was nearly zero was equivalent to the original CQA-Q concentration. The ε value of CQA-Q was estimated to be 1.7×10^3 for

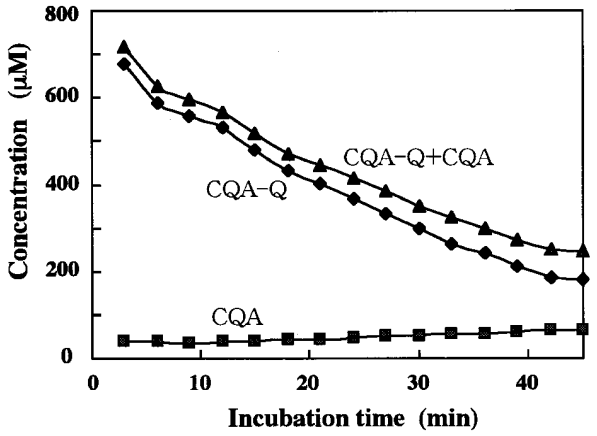

Fig. 6. Decrease in CQA-Q and Formation of CQA in the Solution of CQA-Q.

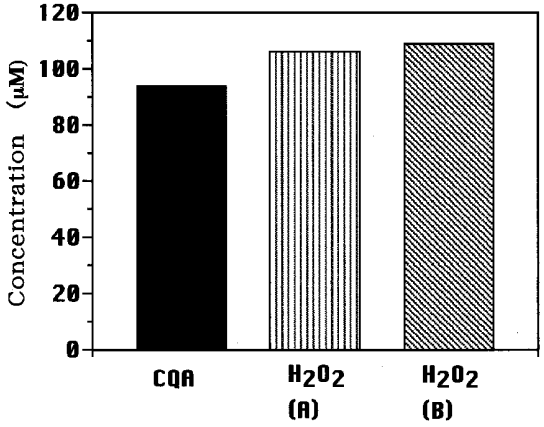

Fig. 7. Formation of CQA and H_2O_2 in the CQA-Q Solution. The CQA-Q solution was left for 30 min, before the concentrations of CQA (HPLC method) and H_2O_2 (A, iodine titration method; B, peroxidase method) were determined.

325 nm and 9.7×10^2 for 400 nm. These values seem to be more reliable than the values estimated by other two methods, because they are both lower. With the absorbance and HPLC methods, it was assumed that the amount of CQA formed was equal to the amount of CQA-Q lost, any polymerized or decomposed products of CQA-Q being neglected, because no clearly defined peaks, apart from CQA appeared by HPLC. Therefore, the ε values estimated by these two methods seem to be much larger than the real values.

These results suggest that most of CQA-Q was converted to pale colored polymers with the generation of H_2O_2 and CQA. In fact, polymers with a pale yellowish color were obtained from the faded solution of CQA-Q in a yield of about 90% (data not shown). Figure 6 shows the changes in the concentrations of CQA and CQA-Q estimated by the Cys method. The CQA-Q concentration decreased and was partly converted to CQA, the yield of CQA from CQA-Q being about 10%. These results show that CQA-Q did not form intense brown pigments, but pale colored polymers, and partly generated CQA.

Reactivity of CQA-Q with amino acids

The reactivity of CQA-Q with amino acids was examined. After the quinone solution had been incubated with lysine, glycine, and arginine, the absorbance at 400 nm was monitored. The absorbance decreased with incubation time, and this profile was almost identical with that of the CQA-Q solution (Fig. 4(B)). This characteristic shows that CQA-Q formed hardly any intense brown pigment with such amino acids such lysine and arginine. Yabuta *et al.* have reported the formation of a green pigment from a mixture of methyl caffeate and a primary amino compound under alkaline conditions.[15,16] There seems to have been a definite difference between the reactivity of CQA-Q in the acidic condition and that of CQA in the alkaline condition. On the other hand, the color of the quinone solution instantly disappeared when L-Cys was added. As already mentioned, 5-*S*-Cys-CQA was identified in the solution.

Sanoner *et al.* have reported that CQA-Q reacted with CQA to produce 5-CQA dimers.[17] The compound in this solution might have been formed, but the amount would have been very low. They used polymer-supported periodate[18] for the oxidation of CQA, and the CQA solution was used without further purification. The difference in methods might have led to different results.

Generation of H_2O_2 in the CQA-Q solution

CQA-Q spontaneously generated CQA in this acidic solution, so we examined what kind of oxidized product was formed. We could not detect any clearly defined peak of an oxidized product by HPLC, but did detect H_2O_2. The amount of H_2O_2 was measured by both the iodine titration method and peroxidase method. The amount of H_2O_2 formed was almost equivalent to that of CQA formed (Fig. 7). We don't know the generation mechanism for H_2O_2, but the small amount of metal ions contaminating this solution might have been linked with the formation of H_2O_2. The generation of H_2O_2 has been reported during enzymatic browning,[19,20] and our observations might explain this phenomenon. PPO generally shows some peroxidase activity, and this peroxidase activity and H_2O_2 might play an important role in enzymatic browning. For example, CQA is the major phenolic in apple[21] and is the best substrate for apple PPO.[22] During the incubation of apple juice, CQA gradually decreases and completely disappears within 1 h, and its brown color deepens. This suggests that regenerated CQA is successively used by the browning reaction. The second major polyphenol in apple is catechins. Catechins form an intense brown color by the action of apple PPO.[21] The properties of catechin quinone and the interac-

tion between CQA-Q and catechins will be further examined. Richard-Forget *et al*. have pointed out the involvement of the peroxidase in the enzymatic browning of pear.[23] Pear peroxidase did not react with phenolics, but in the presence of polyphenol oxidase, peroxidase enhanced the phenol degradation. Considering our results, H_2O_2, the substrate of peroxidase, seems to have been generated through CQA-Q formed in the reaction mixture of pear PPO and phenolics.

We conclude from these results that CQA-Q itself and the mixture of CQA-Q and amino acids did not form the intense brown pigment in the acidic aqueous solution. H_2O_2 spontaneously generated from CQA-Q and polyphenols other than CQA might have played an important role in forming the intense brown pigments by enzymatic browning.

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