Chemical Studies on Antioxidant Mechanism of Curcuminoid: Analysis of Radical Reaction Products from Curcumin

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In the course of studies on the antioxidant mechanism of curcumin, its radical reaction was investigated. Curcumin was reacted with radical species, which were generated from the pyrolysis of 2,2′-azobis(isobutyronitrile) under an oxygen atmosphere, and the reaction products from curcumin were followed by HPLC. The reaction at 70 °C gave several products, three of which were structurally identified to be vanillin, ferulic acid, and a dimer of curcumin after their isolation. The dimer was a newly identified compound bearing a dihydrofuran moiety, and its chemical structure was elucidated using spectroscopic analyses, especially 2D NMR techniques. A mechanism for the dimer production is proposed and its relation to curcumin's antioxidant activity discussed. The time course and gel permeation chromatography studies of the reaction were also investigated, and the results indicate that the dimer is a radical-terminated product in the initial stage.

Keywords: *Curcumin; antioxidant; radical termination; dimer; antioxidant mechanism; HPLC analysis; GPC analysis*

INTRODUCTION

Some phenolic compounds in edible plants have received much attention as powerful antioxidants not only to protect against the oxidative deterioration of food but also to reduce oxidation-induced diseases by their ingestion. A main mechanism for a phenolic antioxidant in food is the trapping and stabilizing of radical species, such as the lipid peroxyl radical, which is generated from radical chain oxidation of food components. This property is also important in biological systems for scavenging harmful oxygen radicals. The antioxidation process is thought to be divided into two stages as shown in following schemes:

(1) radical trapping stage

$$S{-}OO^{\bullet}\ (\text{or } S^{\bullet}) + AH \rightarrow S{-}OOH\ (\text{or } SH) + A^{\bullet}$$

(2) radical termination stage

$$A^{\bullet} \rightarrow \text{nonradical materials}$$

S is the substance for oxidation, AH is the antioxidant, and $A^{\bullet}$ is the antioxidant radical.

Recent mechanistic studies of a plant phenolic antioxidant have been focused on the trapping stage using a kinetic approach or a structure−activity relationship approach [for recent reviews see: Aruoma et al. (1997) and Hall and Cuppett (1997)]. The antioxidant radical, which forms in the trapping stage, should be converted to a nonradical material in the termination stage without any production of further radical species. Structural information about these nonradical products would afford important contributions to the antioxidant mechanism studies. Recently, a phenolic antioxidant bearing ortho-dihydroxyl groups was intensively investigated as an efficient antioxidant, and the produced stable *o*-quinone material plays a key role in its antioxidation (Hall and Cuppett, 1997). However, studies of the other types of phenolic antioxidants are very limited.

Curcumin (**1**) is the main yellow phenolic material of turmeric and has been widely used as a food coloring reagent (Govindarajan, 1980). Various curcumin-related phenols (curcuminoids) have also been found in edible plants, especially Zingiberaceae plants (Toda et al., 1985; Uehara et al., 1987; Masuda et al., 1992, 1993, 1994, 1995). Curcumin has a strong antioxidant activity not only in food systems but also in biological systems (Huang and Ferraro, 1992). Recently, its antioxidant activity in biological systems has received attention as it prevents some peroxidation-related diseases (Ruby et al., 1995). A study of the clinical application of curcumin as a chemopreventive anticancer drug was intensively carried out at the National Cancer Institute (Kelloff et al., 1996). Structurally, curcumin consists of two ortho-methoxylated phenols and a β-diketone moiety, and they are all conjugated. Radical trapping would first occur at a phenol position as in other phenolic antioxidants; however, the position of the radical termination has not been defined so far because of the complexity of its highly conjugated structure. The objectives of this study were to clarify the stable radical termination products from curcumin and to examine the antioxidation process of curcumin on the basis of the products (Figure 1).

MATERIALS AND METHODS

Chemicals and Instruments. Curcumin was synthesized using a previously reported method (Pabon, 1964). 2,2′-Azobis-(isobutyronitrile) (AIBN) was obtained from Tokyo Kasei Co.,

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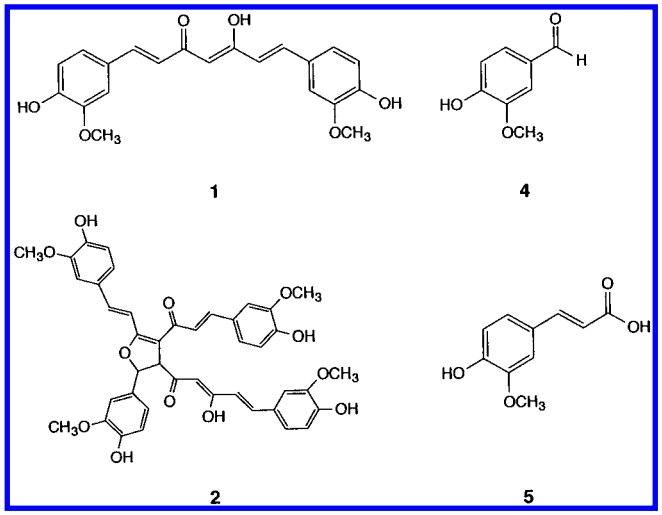

Figure 1. Chemical structures of curcumin (**1**) and the radical reaction products (**2**, **4**, and **5**).

Ltd. (Tokyo, Japan). All solvents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Silica gel for column chromatography (NAM-300) was obtained from NAM Laboratory (Motosu, Japan). Silica gel TLC plates (silica gel 60 F254 PLC plates, No. 1.05744) were obtained from Merck (Darmstadt, Germany). NMR spectra were measured with a Varian Unity Plus 500 spectrometer. Mass spectra were measured with a Hitachi M-200 or a JEOL SX-102A spectrometer. A Shimadzu LC 6A system equipped with a photodiode array detector (Shimadzu, SPD-M10AP) was used for the analytical HPLC and a JASCO Gulliver Gradient LC system was used for the preparative HPLC.

Analysis of Radical Reaction Products. Curcumin (100 mg) and AIBN (100 mg) were dissolved in CH_3CN (5 mL) in a flask fitted with an oxygen balloon through a three-neck valve. After degassing and substitution with oxygen, the solution was heated at 70 °C. One hour later, 10 μL of the solution was withdrawn and diluted with 100 μL of CH_3CN. Ten microliters of the diluted solution was injected into the analytical HPLC system under the following conditions: column, Develosil CN-5 (4.6 × 250 mm, Nomura Chemical, Seto, Japan); solvent system, 2% AcOH in *n*-hexane (solvent A) and 2% AcOH in ethyl acetate (solvent B); elution, linear gradient from 14% solvent B to 71% solvent B for 40 min (after the gradient, 100% of solvent B was eluted for an additional 10 min); flow rate, 1 mL/min; detection, 420, 360, and 300 nm. The UV spectra from 280 to 480 nm for each observed peak were obtained.

For the time course studies, at 1 h intervals, 10 μL of the reaction solution was withdrawn and analyzed according to the same method. Peak area was calculated using a Shimadzu M-10A data analyzing system.

Purification Procedure for Products 2−5. A CH_3CN solution (25 mL) of curcumin (0.5 g) and AIBN (0.5 g) was degassed and heated under an oxygen atmosphere at 70 °C for 1 h. The solution was evaporated in vacuo to dryness and immediately filtered with methanol to remove most of the curcumin and AIBN. The filtrate was evaporated and the residue (~0.5 g) subjected to silica gel column chromatography eluted with 1−10% methanol in CH_2Cl_2 in the stepwise gradient mode, giving six fractions [fraction 1 (193 mg), fraction 2 (20 mg), fraction 3(175 mg), fraction 4 (44 mg), fraction 5 (27 mg), fraction 6 (25 mg)]. Fraction 2 was purified with silica gel TLC (5% methanol in CH_2Cl_2) to afford **5** (1.5 mg). From fraction 4, **2** (1.9 mg), **3** (3.2 mg), and **4** (1.3 mg) were obtained by repeated silica gel TLCs (5% methanol in CH_2Cl_2). Each isolated product was assigned to the corresponding peak in the HPLC data in Figure 2 by comparison of their retention times.

Isolation Procedure for Produt 2 Using Preparative HPLC. A CH_3CN solution (30 mL) of curcumin (0.6 g) and AIBN (0.6 g) was heated for 2 h at 70 °C in air. The solution was evaporated in vacuo to dryness and immediately filtered with methanol. After evaporation, the residue was dissolved in CH_2Cl_2 (3 mL). A 0.5 mL sample of the solution was injected into a preparative HPLC equipped with an LOP CN 24S column (Nomura Chemicals) using the following conditions: flow rate, 10 mL/min; gradient solvent system, 35% ethyl acetate in hexane containing 0.2% AcOH to 100% ethyl acetate containing 0.2% AcOH for 1 h; detection, 420 nm. A peak eluted at 26 min was collected. After the collected solution was evaporated, the residue was purified by silica gel TLC (3% methanol in CH_2Cl_2) to give **2** (11 mg) as an orange amorphous solid.

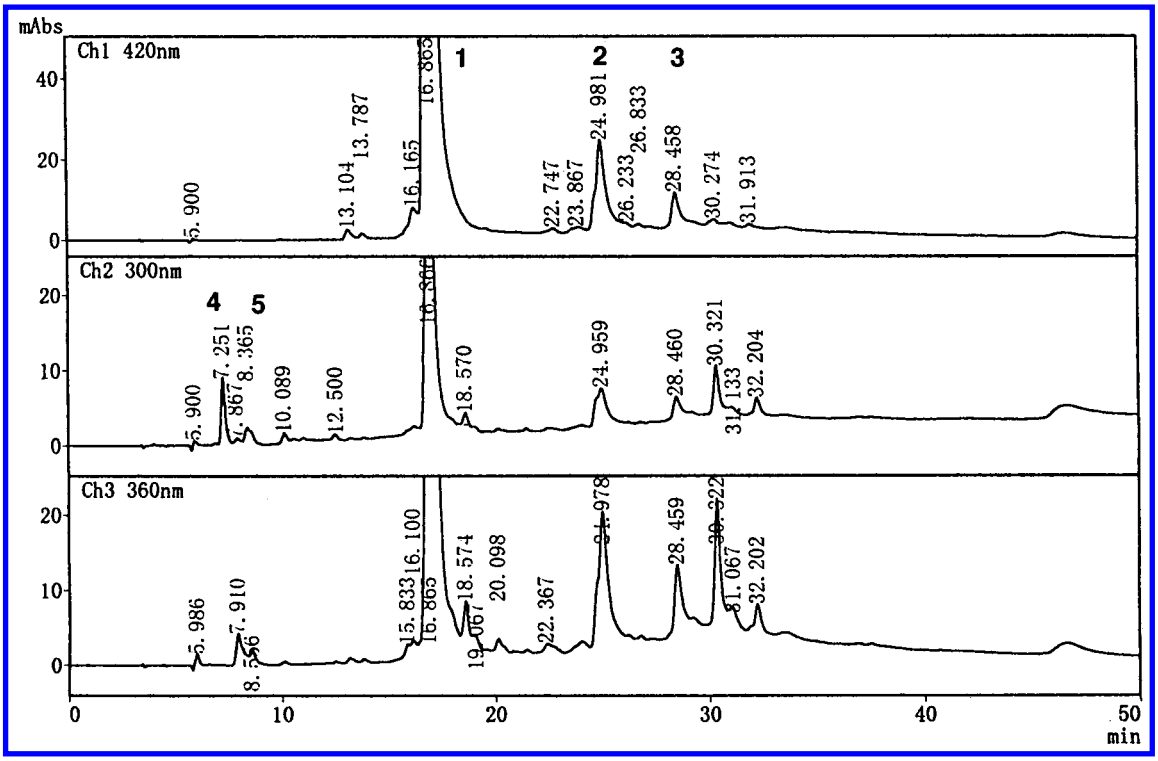

Figure 2. HPLC chromatograms of the radical reaction mixture of curcumin at 1 h.

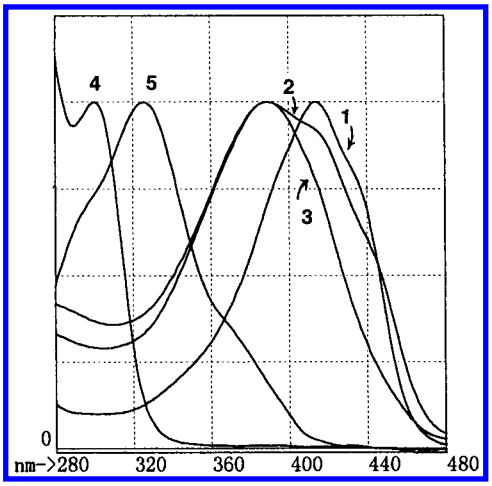

Figure 3. Comparison with each UV absorption pattern corresponding to product peaks 1−5.

GPC Analysis of Reaction Mixture. The reaction was carried out using the previously described protocol. At 1 h intervals, 100 μL of sample solution was withdrawn and concentrated to dryness in an N_2 stream. The residue was dissolved in 500 μL of THF, and 1.5 μL of the solution was then injected into an analytical HPLC equipped with the GPC column (GPC KF-803, Showa Denko, Tokyo, Japan) under the following conditions: flow rate, 1 mL/min; solvent 2% AcOH in THF; detection, 300 and 420 nm.

RESULTS

HPLC Analysis of Radical Reaction Products from Curcumin. Organic azo compounds are easily handled reagents because they are less explosive compared with organic peroxides. Azo compounds produce radicals at a constant rate by pyrolysis. Recent studies on the rate analysis of food oxidation employed them (Niki, 1987). We also employed AIBN as an azo compound to produce the curcumin radical. Pure curcumin, which was synthetically obtained, and AIBN were incubated in CH_3CN in an oxygen atmosphere, and the reaction products from curcumin were observed by the HPLC method. Although no product was observed in the reaction below 70 °C for 1 h, many products were observed in the reaction mixture at 70 °C for 1 h. The analytical data for the reaction mixture at 1 h are shown in Figure 2. With 420 nm detection, two peaks are mainly observed with retention times at 24.9 (product **2**) and 28.5 min (product **3**) along with curcumin's (**1**) peak at 16.9 min. On the other hand, with 300 nm detection, several additional peaks are observed from 6 to 13 min in addition to the 420 nm detected peaks. Curcumin has an absorption maxima at 420 nm because of its conjugated diaryl heptanoid chromophore. The two peaks at 24.9 and 28.5 min retain an absorption maxima around 400 nm as shown in Figure 3, indicating that there was no damage to the curcumin chromophore. However, the 300 nm detected peaks at 7.3 (product **4**) and 8.4 min (product **5**) do not have an absorption at 420 nm, which could be due to the damaged conjugation system of curcumin's structure.

Isolation and Structure Identification of Reaction Products. To confirm the chemical structures of the products, an isolation study was carried out. The protocol used and yield for each product are described under Materials and Methods. Structures of the isolated products were analyzed by a spectroscopic method, and the data are summarized in Tables 1−3. The MS of **4** gave a molecular ion peak at *m/z* 152. The ^{1}H NMR spectrum of **4** showed an aldehyde signal at 9.87 ppm, three substituted benzene protons at 7.26 (1H, d, $J = 8$ Hz) and 7.50 (2H, m) ppm, and one phenolic methoxyl signal at 4.00 ppm. These data indicated that **4** was vanillin. A co-injection experiment of the authentic sample confirmed the results. The MS of **5** showed a molecular ion peak at *m/z* 194, and its ^{1}H NMR spectrum revealed the signals due to three substituted benzene protons [6.92 (1H, d, $J = 8$ Hz), 7.18 (1H, dd, $J = 8$ and 2 Hz), and 7.36 (1H, d, $J = 2$ Hz) ppm], a low field shifted trans double bond [6.42 (1H, d, $J = 16$ Hz) and 7.63 (1H, d, $J = 16$ Hz) ppm], and a phenolic methoxyl group [3.98 (3H, s) ppm]. These data indicated that **5** was ferulic acid. The structure was confirmed with the authentic sample using HPLC. Product **2** was obtained as an orange amorphous solid using repeated chromatography. Although the EI and positive SI mass spectrometries gave no molecular ion peaks, the negative FAB mass spectrometry using magic bullet matrix afforded a pseudo molecular ion peak at *m/z* 733 [M − H]$^-$. The high-resolution mode of the FAB MS revealed that the molecular formula of **2** was $C_{42}H_{38}O_{12}$ (calcd for $C_{42}H_{37}O_{12}$, 733.2285; found, 733.2297), which indicated that **2** was a dimeric compound of curcumin. The fine structure of **2** was elucidated using two-dimensional

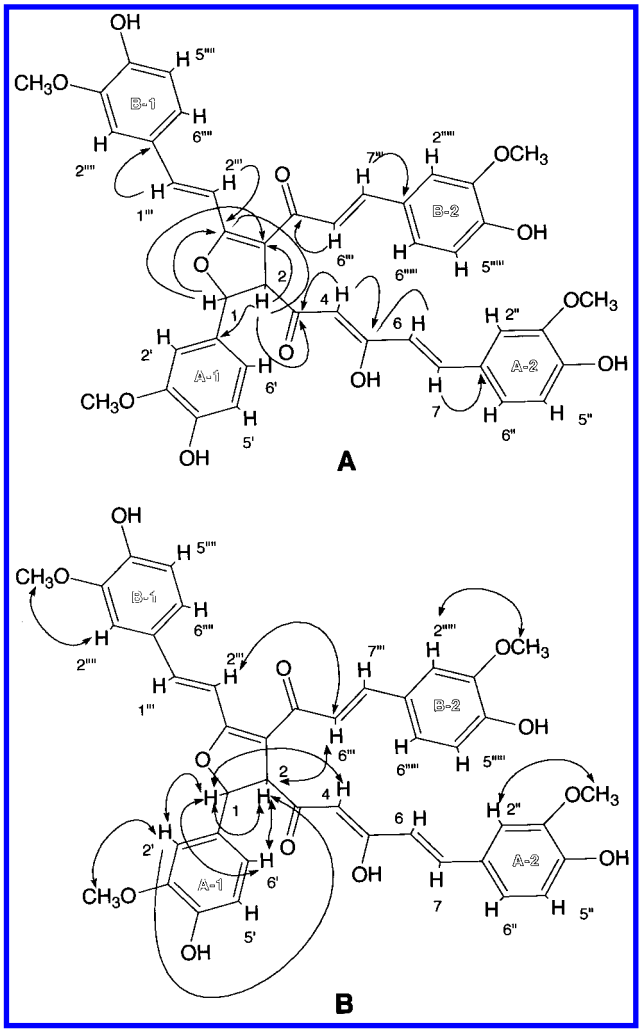

Figure 4. Selected carbon−proton long-range connectivities observed in HMBC (arrows in A) and NOE correlations observed in NOESY (arrows in B). Tentative position numberings are given on the basis of curcumin's numbering.

Table 1. Spectroscopic Analysis Data for Products 2, 4, and 5

	2	4	5
MS	negative HR-FAB mode *m/z* 733.2297 $[M - H]^-$	EI mode *m/z* 152 $[M]^+$	EI mode *m/z* 194 $[M]^+$
^{1}H NMR	see Table 2	δ (acetone-d_6, 500 MHz) 4.00 (3H, s) 7.26 (1H, d, $J = 8$ Hz) 7.50 (2H, m) 9.87 (1H, s)	δ(acetone-d_6, 500 MHz) 3.98 (3H, s) 6.42 (1H, d, $J = 16$ Hz) 6.92 (1H, d, $J = 8$ Hz) 7.18 (1H, dd, $J = 8$ and 2 Hz) 7.36 (1H, d, $J = 2$ Hz) 7.63 (1H, d, $J = 16$ Hz)
^{13}C NMR	see Table 3		

Table 2. ^{1}H NMR Data and NOE Correlated Protons Observed in a Phase Sensitive NOESY of Product 2 [δ in Acetone-d_6 (500 MHz)]

H-position[a]	chemical shift[b]	correlated protons in NOESY
1	5.70, d, (5.9)	H-2, H-4,H-2′,H-6′
2	4.42, d, (5.9)	H-1, H-4, H-2′, H-6′, H-6‴
4	5.98, s	H-1, H-2, H-6
6	6.65, d, (15.6)	H-2″, H-6″, H-4
7	7.54, d, (15.6)	H-2″, H-6″
2′	7.08, d,(1.6)	H-1, H-2,3′-OCH_3
5′	6.86, d, (7.8)	H-6′
6′	6.92, dd, (7.8, 1.6)	H-5′, H-1, H-2
2″	7.30, d, (1.6)	H-6, H-7,3″-OCH_3
5″	6.80, d, (7.6)[c]	H-6″
6″	7.11, dd, (7.6, 1.6)	H-6, H-7, H-5″
1‴	7.40, d, (15.6)	H-2⁗, H-6⁗
2‴	7.78, d, (15.6)	H-6‴, H-2⁗, H-6⁗
6‴	7.00, d, (15.5)	H-2, H-2‴, H-2⁗
7‴	7.49, d, (15.5)	H-2⁗, H-6⁗
2⁗	7.26, d, (1.6)	H-1‴, H-2‴,3⁗-OCH_3
5⁗	6.87, d, (7.8)	H-6⁗
6⁗	7.15, dd, (7.8, 1.6)	H-1‴, H-2‴
2⁗	7.22, d, (1.6)	H-6‴, H-7‴,3⁗-OCH_3
5⁗	6.83, d, (7.6)[c]	H-6⁗
6⁗	7.11, dd, (7.6, 1.6)	H-6‴, H-7‴, H-5⁗
3′-OCH_3	3.82, s	H-2′
3″-OCH_3	3.85, s	H-2″
3⁗-OCH_3	3.88, s	H-2⁗
3⁗-OCH_3	3.79, s	H-2⁗
OH	7.90, 8.22, 8.22, 8.30, each br s	

[a] H-position is tentatively numbered as shown in Figure 4. [b] Coupling constants (*J* in hertz) in parentheses. [c] Assignment may be interchangeable.

Table 3. ^{13}C NMR Data and Correlated Protons in an HMBC Spectrum of Product 2 [δ in Acetone-d_6 (125 MHz)]

C-position[a]	chemical shift	correlated protons in HMBC
1	87.69	H-2′, H-6′
2	63.02	
3	200.35	H-1, H-2, H-4
4	100.17	H-2, H-6
5	179.84	H-4, H-6, H-7
6	120.28	H-4, H-7
7	142.53	H-2″, H-6″
1′	133.05	H-1, H-2, H-2′, H-6′
2′	110.30	H-1, H-6′
3′	148.57[b]	overlapped
4′	147.92	overlapped
5′	115.98[c]	overlapped
6′	119.58	H-1, H-2′
1″	127.90	H-6, H-7, H-2″, H-6″
2″	111.14	overlapped
3″	148.61[b]	overlapped
4″	150.13	overlapped
5″	116.10[c]	overlapped
6″	124.08[d]	H-7, H-2″
1‴	140.03	H-2⁗, H-6⁗
2‴	114.71	H-1‴
3‴	165.92	H-1, H-2, H-1‴, H-2‴
4‴	114.13	H-1, H-2
5‴	185.38	H-6‴, H-7‴
6‴	123.61	
7‴	141.84	H-2⁗, H-6⁗
1⁗	128.78	H-1‴, H-2‴, H-2⁗
2⁗	111.08	overlapped
3⁗	148.73[b]	overlapped
4⁗	149.61	overlapped
5⁗	116.10[c]	overlapped
6⁗	123.11	H-1‴, H-2⁗
1⁗	128.10	H-7‴, H-2⁗, H-6⁗
2⁗	111.20	overlapped
3⁗	148.74[b]	overlapped
4⁗	149.94	overlapped
5⁗	116.23[c]	overlapped
6⁗	124.16[d]	H-7‴, H-2⁗
OCH_3 (×4)	56.27, 56.70 (×2), 56.16 (×2)	overlapped

[a] C-position is tentatively numbered as shown in Figure 4. [b–d] Assignments may be interchangeable.

NMR techniques. The H−H COSY spectrum of **2** gave the proton−proton coupling connectivities due to four sets of three substituted benzene rings (ring A-1, H2′−H5′−H6′; ring A-2, H2″-H5″-H6″; ring B-1, H2⁗-H5⁗-H6⁗; and ring B-2, H2⁗−H5⁗−H6⁗; these numbered positions and proton chemical shifts are shown in Figure 4 and Table 2, respectively), three sets of trans olefins (H6−H7, H1‴−H2‴, H6‴−H7‴), and two protons at 5.70 and 4.42 ppm, which were coupled by 5.9 Hz. In addition to these proton connections, the signals due to four phenolic methoxyl groups (3.79−3.88 ppm) and one isolated proton (H4) at 5.98 ppm were observed in the spectrum. The ^{13}C NMR spectrum of **2** gave 42 carbon signals, which consisted of 38 separated signals and two overlapped signals due to four phenolic methoxyl groups. All carbons except the quaternary carbons were assigned by the heteronuclear multiple-quantum coherence experiment (HMQC) spectrum, with the data being summarized in Table 3. The carbon−proton networks were deduced with the carbon−proton long-range couplings obtained from the heteronuclear multiple-bond coherence experiment (HMBC) spectral data. Figure 4A shows important carbon−proton correlations, which clarify all carbon−carbon linkages of structure **2** except for the C4‴−C5‴ bond. The C4‴−C5‴ linkage in **2** was determined by NOE correlations between H2−H6‴and H2‴−H6‴ in the phase sensitive nuclear Overhauser effect spectroscopy (NOESY) spectrum (Figure 4B). The substituted positions of the four methoxyl groups were determined by NOEs with ortho-protons in the benzene rings. Thus, the structure of **2** was determined as shown in Figure 1. The structure has stereocenters at the 1- and 2-positions. The relative stereochemistry of the protons on the dihydrofuran ring is difficult to determine. When protons on the dihydrofuran have appropriate NOEs resulting from the fixed conformation of the furan, the NOE data are useful for stereochemical analysis (Li et al., 1997). However, H1

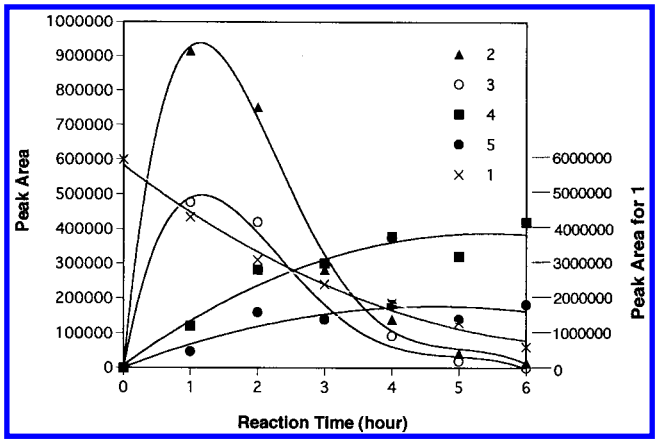

Figure 5. Time course of accumulation of the radical reaction products **2**−**5**. Each peak area was calculated from the 420 nm peak for **2** and **3**, the 300 nm peak for **4**, and the 360 nm peak for **5**.

showed NOEs with H2 and H4, and H2 also showed NOEs with H1, H2′, and H6‴ in both the NOE difference spectrum and the phase sensitive NOESY spectrum of **2**. These results revealed that **2** had some flexible conformation in its dihydrofuran ring, whereas the stereochemical relationship of H1 and H2 remained unclear. The ^{1}H NMR data and the estimated molecular size (∼750) of product **3**, which was obtained by GPC analysis, were similar to those of **2**, suggesting that **3** was also a dimer of curcumin. Unfortunately, the structure could not be obtained at this stage because of its instability and complexity.

Time Course Studies of the Product Formation. The time course of accumulation of the radical reaction products was investigated using HPLC analytical methods. The reaction was followed for 6 h, and the results are shown in Figure 5, which shows that products **2** and **3** were immediately formed after start of the reaction and that, after 1 h, the amounts of both decreased, whereas products **4** and **5** independently increased during the investigation. These results indicated that products **2** and **3** were the initial products derived from curcumin in its antioxidation process, and they were much less stable than products **4** and **5**. After the peaks of **2** and **3** disappeared, no other new peaks were observed by HPLC (data not shown).

Gel Permeation Chromatography (GPC) Analysis of Reaction Mixture. GPC is one of the easiest methods to estimate the molecular size of a compound. We analyzed the reaction mixtures at 1 and 6 h (Figure 6). Figure 6A shows that curcumin's peak is observed at 10.3 min and the curcumin dimer **2** at 9.84 min. From these retention times, both molecular sizes were estimated to be ∼350 and ∼700, which were in good agreement with the MS analysis. In the 6 h data (Figure 6B), a peak observed at 420 nm showed a reading shape to the retention time of 7 min. Two peaks at 10.8 and 11.2 min were also clearly observed at the 300 nm detection (Figure 6B). For the GPC analytical conditions, a higher molecular sized material is eluted earlier. Thus, the obvious reading peak indicated that polymerization of curcumin would occur in the reaction.

DISCUSSION

The isolation work of radical reaction products from curcumin gave four compounds. Two of them were structurally fragmented compounds from curcumin. The

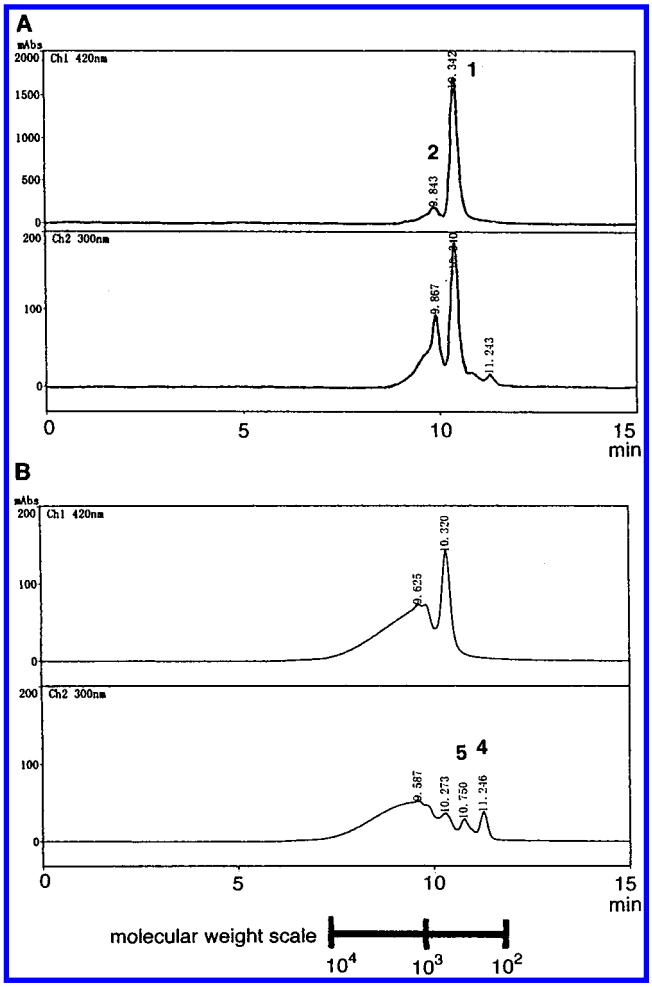

Figure 6. GPC analytical data [(A) analysis for the reaction mixture at 1 h, (B) analysis for the reaction mixture at 6 h] with an estimated molucular weight scale.

other two compounds, one of which was structurally elucidated, were complicated compounds such as a dimer. Our proposed mechanism for their production from curcumin radical is illustrated in Figure 7. In the mechanism shown, curcumin is first converted to the curcumin radical at the phenolic position (structure **I**). The radical can move to appropriate positions as represented by structures **II** and **III**. When two types of curcumin radicals (**II** and **III**) are coupled between both radical positions, the radical reaction is terminated by producing this nonradical material. The terminated product is not very stable because it has two unstable quinone methide structures. Thus, the hydroxyl group on the upside curcumin part immediately cyclizes at the benzyl position of the other curcumin part, forming a thermodynamically stable furan ring. Finally, a hydrogen on the furan ring transfers to a phenolic position to give compound **2**, which has a more stable dihydrofuran ring. The radical termination by dimerization of the antioxidant has been also observed in vitamin E (Winterle et al., 1984; Yamauchi et al., 1989) and BHT (Zhang et al., 1997). This radicalic dimerization should play an important role in curcumin's antioxidant activity. On the other hand, when curcumin radicals **II** and **III** independently react with molecular oxygen, two types of peroxyl radicals are produced. As a possible mechanism, peroxyl radicals are cyclized at adjacent benzylic or ketone positions and then decomposed to form compounds **4** and **5**, respectively. It should be noted

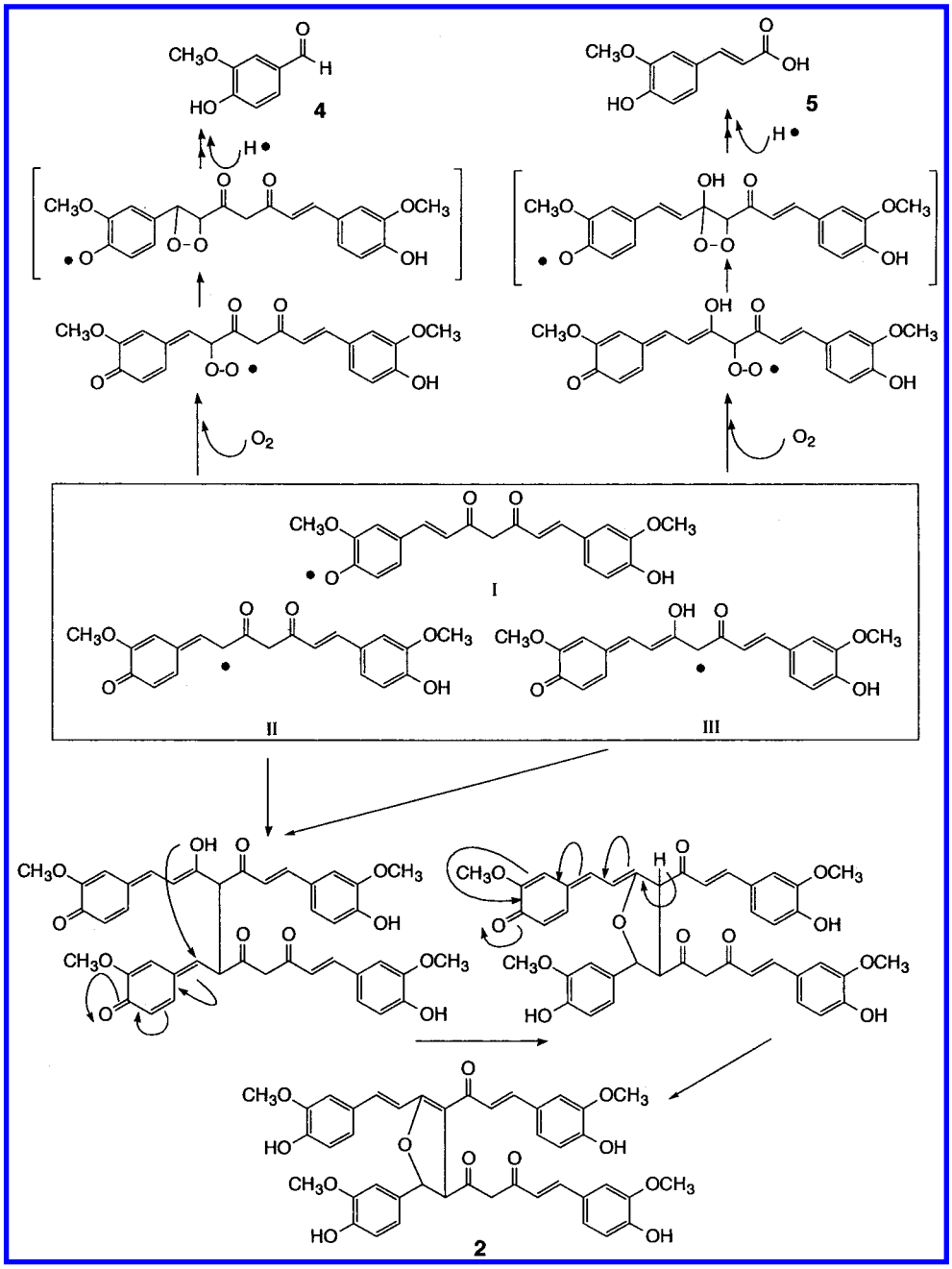

Figure 7. Proposed mechanism for formation of products **2**, **4**, and **5** in the radical reaction.

that for this pathway of **4** and **5**, the radical could not have a radical termination stage but instead required an abstraction of the hydrogen atom from the other compound. Degradation of curcumin under various conditions such as alkaline or photoirradiated conditions was examined, and compounds **4** and **5** are always observed in the degraded materials as stable compounds (Tønnesen and Karsen, 1985; Tønnesen et al., 1986; Wang et al., 1997). Antioxidant studies of the phenolic compounds also reveal that the radical trapping activity of vanillin and ferulic acid is lower than that of curcumin (Sharma, 1976; Toda et al., 1985; Brand-Williams et al., 1995), indicating that these compounds are more stable than curcumin under radical conditions and would not degrade in the presence of curcumin. Thus, **4** and **5** were probably accumulated as stable compounds under the stated conditions. The time course results indicate that the curcumin dimer is not the final terminated compound. It has an additional radical trapping ability because the reactive phenolic hydroxyl group remained. GPC analysis of the reaction mixture clearly showed the production of higher molecular weight materials than the dimer with longer reaction time. The materials would be oligomeric substances of curcumin from the analysis data and would be produced through the dimer or directly from curcumin. These results indicate that curcumin acts as a chain-breaking antioxidant due to its radical termination ability by its dimerization and following polymerization process.

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