Pergamon 0031-9422(94)00896-5 Phytochemistry, Vol. 39, No. 1, pp. 225-229, 1995

0031-9422/95 $9.50 + 0.00

CHEMILUMINESCENCE OF BENZOIC AND CINNAMIC ACIDS, AND FLAVONOIDS IN THE PRESENCE OF ALDEHYDE AND HYDROGEN PEROXIDE OR HYDROXYL RADICAL BY FENTON REACTION

YUMIKO YOSHIKI, KAZUYOSHI OKUBO, MASAMICHI ONUMA* and KIHARU IGARASHI*

Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1-1, Tsutsumidori, Amamiyamachi, Aoba-ku, Sendai, Miyagi 981, Japan; *Department of Bioproduction, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997, Japan

(*Received in revised form* 17 *October* 1994)

Key Word Index—Chemiluminescence; benzoic acid; cinnamic acid; flavonoid; anthocyanin; Fenton reaction; hydroxyl radical; hydrogen peroxide.

Abstract—Light emission (chemiluminescence; CL) was observed by exciting phenolic compounds with hydrogen peroxide (H_2O_2) or hydroxyl radical (HO·), formed from H_2O_2 and $FeCl_2$ by the Fenton reaction. The intensity of the CL of benzoic acid derivatives in the presence of H_2O_2 was in the order of gallic acid > caffeic acid > 3,5-dimethoxy-4-hydroxycinnamic acid > isovanillic acid = syringic acid. The CL intensity of flavonoids and related compounds, measured in the presence of HO· was in the following order; rutin > myricetin ≒ isoquercitrin > quercetin > kaempferol > isorhamnetin, for the major flavonols; nasunin > rubrobrassicin > delphinidin > cyanidin = malvin > malvidin, for anthocyanins. These results indicated that CL increased with increasing number of vicinal hydroxyl groups to that at C-4 of benzoic acid or its derivatives, and at C-4 of flavonoids. The data further indicated that glycosylation at C-3 of quercetin enhanced the CL of flavonoids, and that this was dependent on the nature of the sugar moiety, rhamnosylglucose being more effective than glucose. The order of the CL intensity of flavonoids agreed well with that of the radical scavenging activities of these compounds, as determined by ESR and by using a linoleic acid–β-carotene–lipoxygenase system.

INTRODUCTION

It has been reported that soybean seedling exhibits weak light emission (chemiluminescence, CL) characteristics and that this is significantly enhanced by the addition of various aldehydes [1, 2]. In living organisms, chlorophyll, peroxidase or α-oxidase can generate excited triplet carbonyls through the degradation of hydroxyperoxide, formed by the oxygenation of aldehydes with enzymatically-activated oxygen. Such an excited state of carbonyl is able to transfer energy to produce light from CL compounds [3]. However, CL also occurs non-enzymatically; for example in an autoclaved extract of soybean seedlings mixed with aldehyde and oxygen. H. Watanabe *et al.* [2] postulated that the CL in the soybean extracts resulted from the formation of radical derived hydroperoxide intermediates. Therefore, the measurement of CL when compounds are examined in the presence of aldehyde and hydrogen peroxide (H_2O_2) or hydroxyl radical (HO·) has been suggested to be the most effective method for detection of the scavengers. In all cases, both the occurrence and the intensity of CL were closely related to the radical reaction and the radical scavenging activity of the compounds present in the reaction mixture. In general, ESR spin-trapping and enzymatic measurements have been used for the determination of the radical scavenging activity in foodstuffs.

It has been reported that flavonoids occurring widely in the plant kingdom exhibit antioxidant properties in lipid peroxidation systems [4, 5]. Such compounds have also been shown to inhibit the enzyme, lipoxygenase, and to act as superoxide anion (O_2^-) and hydroxyl radical (HO·) scavengers and singlet oxygen quenchers [6–10]. The HO·, easily generated by UV photolysis of H_2O_2, is highly reactive and can cause damage to biomolecules including proteins and DNA [11, 12]. It is generally thought that HO· is produced *in vivo* by Fenton-type reactions, in which ferrous ions react with H_2O_2;

$$H_2O_2 + Fe^{2+} \rightarrow HO\cdot + OH^- + Fe^{3+}.$$

Damage caused by the toxic effects of HO· can often be suppressed by the scavengers such as flavonoids. Flavonoids widely used as therapeutic agents may, therefore, be expected to act as strong HO· scavengers.

In this paper, the CL of flavonoids and related compounds were measured in the presence of aldehyde and H_2O_2 or HO·, generated by the Fenton reaction. The

relationship between the chemical structures of the flavonoids and their CL intensities and/or radical scavenging activities has also been examined.

RESULTS AND DISCUSSION

The intensity of CL of gallic acid in the presence of H_2O_2 or HO· and aldehyde system (50 mM phosphate buffer containing acetaldehyde, pH 7.0, 23°) was found to be dependent on the concentration of gallic acid; CL increased quantitatively until 25 mM (less 20 μl of injection volume) as shown in Fig. 1. The CL of gallic acid increased more than six-fold in the presence of Fenton reaction-derived HO· as compared with that measured in the presence of H_2O_2. These results indicated that measurement of CL intensity could be used for purpose of quantitative comparison. Gallic acid is similar in chemical structure to the B-ring of flavonoids or anthocyanins. Therefore, the CL intensity (50 counts of photon sec^{-1}, attn.128) of 5 mM gallic acid (10 μl) in the presence of H_2O_2 was arbitrarily set at 1000 units to compare the CL intensity of flavonoids or other related compounds.

The CL intensities of aromatic carboxylic acids which show chemical similarities to gallic acid, were measured and are presented in Table 1. When compared with gallic acid, neither isovanillic acid (the C-4 methyl ethel of gallic acid) nor syringic acid (the C-3, C-5 dimethyl ethel of gallic acid) exhibited CL in the presence of H_2O_2 or HO·. This clearly indicates that the presence of a hydroxyl group vicinal to the hydroxyl group at C-4 is required for CL.

When the CL of cinnamic acid derivatives was compared in the presence of H_2O_2, that of caffeic acid was stronger than that of 3,5-dimethoxy-4-hydroxycinnamic acid. This suggested that the hydroxyl groups at C-3 and C-4 of cinnamic acid were necessary for reaction with

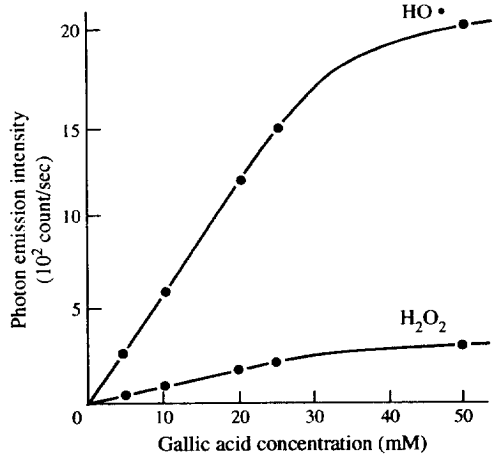


Fig. 1. Chemiluminescence intensity of gallic acid in the presence of hydrogen peroxide or hydroxyl radical and aldehyde system. Hydroxyl radicals were generated by adding 10 μl of 25 mM $FeCl_2$ (Fenton reaction).

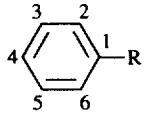
H_2O_2 or HO·. Furthermore, 3',4'-dihydroxyphenylacetic acid did not exhibit CL, suggesting that CL required the carbon of the carboxyl group to be directly or conjugationally attached to the benzene-ring (B-ring).

The CL of flavonoids which, on oxidative degradation produce benzoic acids corresponding to their B-rings, was measured (Table 2). In the presence of H_2O_2, CL could either not be observed or was very weak, when compared to that of gallic acid and the anthocyanins (see below); in contrast, in the presence of HO· strong CL was observed, with the intensity decreasing in the order of rutin > myricetin > isoquercitrin > quercetin > isorhamnetin 3-*O*-glucoside > dihydroquercetin > diosmetin 7-*O*-glucoside > kaempferol > rhamnetin > astilbin > isorhamnetin > luteolin 4'-*O*-glucoside. When the

Table 1. Chemiluminescence intensity of gallic acid and its related compounds in the presence of hydrogen peroxide and hydroxyl radical

Name	Substituent at C-1	C-3	C-4	C-5	CL in the presence of HOOH	HO·
Gallic acid	COOH	OH	OH	OH	1000*	6275
Isovanillic acid	COOH	OH	OMe	H	0	0
Syringic acid	COOH	OMe	OH	OMe	0	0
Caffeic acid	COOH–CH=CH	OH	OH	H	5	30
3,5-Dimethoxy-4-hydroxy-cinnamic acid	COOH–CH=CH	OMe	OH	OMe	2	0
3,4-dihydroxyphenylacetic acid	$COOH–CH_2$	OH	OH	H	0	0

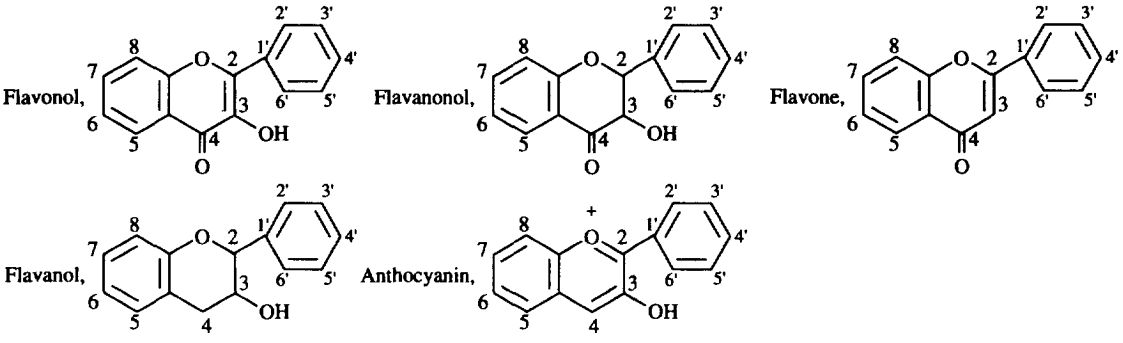
*Arbitrarily set at 1000.

In the chemical structure of 

, C-1 (R), C-3, C-4 and C-5 are substituted as shown above.

Table 2. Chemiluminescence intensity of flavonoids in the presence of hydrogen peroxide and hydroxyl radical

Name	Substituent at C-3	C-5	C-7	C-3'	C-4'	C-5'	CL in the presence of HOOH	CL in the presence of HO·
Flavonol or flavanonol:								
Kaempferol	OH	OH	OH	H	OH	H	2	243
Quercetin	OH	OH	OH	OH	OH	H	3	612
Myricetin	OH	OH	OH	OH	OH	OH	75	1197
Dihydroquercetin	OH	OH	OH	OH	OH	H	2	529
Astilbin	ORha	OH	OH	OH	OH	H	0	181
Isoquercitrin	OGlc	OH	OH	OH	OH	H	5	1155
Rutin	ORha-Glc	OH	OH	OH	OH	H	16	7101
Isorhamnetin	OH	OH	OH	OMe	OH	H	0	125
Isorhamnetin 3-*O*-glucoside	OGlc	OH	OH	OMe	OH	H	0	584
Rhamnetin	OH	OH	OMe	OH	OH	H	0	194
Flavone:								
Apigenin	H	OH	OH	H	OH	H	90	273
Flavone	H	H	H	H	H	H	23	52
Luteolin 4'-*O*-glucoside	H	OH	OH	OH	OGlc	H	0	97
Diosmetin 7-*O*-glucoside	H	OH	OGlc	OH	OMe	H	0	368
Flavanol:								
(+)-Catechin	OH	OH	OH	OH	OH	H	0	140
Anthocyanin:								
Nasunin	ORha Glc-*p*-coumaric acid	OGlc	OH	OH	OH	OH	2347	891
Delphinidin	OH	OH	OH	OH	OH	OH	794	313
Rubrobrassicin	OGlc-Glc	OGlc	OH	OH	OH	H	33	424
Cyanidin	OH	OH	OH	OH	OH	H	588	111
Malvin	OH	OGlc	OH	OMe	OH	OMe	110	111
Malvidin	OGlc	OH	OH	OMe	OH	OMe	77	83



C-3, C-5, C-7, C-3', C-4' and C-5' are substituted as above.

CL of kaempferol, quercetin and myricetin were compared in the presence of HO·, the ranking, kaempferol (243) < quercetin (612) < myricetin (1197) was observed, indicating that an increase of CL with increasing number of hydroxyl groups vicinal to that at C-4'. CL decreased following methylation of the hydroxyl groups in the B-ring (quercetin vs isorhamnetin), indicating that a vicinal hydroxyl group in the B-ring is important for CL. Such results are in good agreement with those reported by Husain *et al.* [9]. Comparison of the CL intensities of quercetin and that of dihydroquercetin showed that the presence of a double bond between C-2 and C-3 contributed only slightly to the stronger CL. When the CL intensities of quercetin, isoquercitrin and rutin, which contain the same flavonoid nucleus excepting the C-3 substituent, were compared, the observed intensity increased with increasing size of the C-3 sugar chain: hydroxyl (quercetin) < glucose (isoquercitrin) < rhamnosylglucose (rutin). On the other hand, in the presence of H_2O_2 or HO·, nasunin which contains a rhamnosylglucose moiety at C-3 exhibited more intense CL than that of rubrobrassicin which has a glucosyl glucose moiety in the same position. These results suggest that a rhamnosylglucose moiety is important for CL. The higher CL intensity of isoquercitrin and isorhamnetin 3-*O*-glucoside, compared to that of their respective

aglycones, shows that the only glucose attached to 3-hydroxyl group of the aglycone also enhances the CL. The presence of a hydroxyl group at C-3 may not be necessary for CL, since kaempferol and apigenin exhibited almost the same intensity in the presence of HO·.

In contrast to the behaviour of flavonoids, the CL of anthocyanins excepting rubrobrassicin, malvin and malvidin was greater in the presence of H_2O_2 than HO·. The reason for this phenomenon is, as yet, unknown. The difference in reactivity between flavonoids and anthocyanins in the presence of H_2O_2 or HO·, especially the higher CL of anthocyanins in the presence of H_2O_2, may suggest that anthocyanins having a pyrylium nucleus, which can be easily hydrated to produce carbinol forms under aqueous neutral condition (pH 7.0, phosphate buffer) have higher reactivity to H_2O_2, than the flavonoids having a γ-pyrone ring. It may be necessary, however, to clarify the mechanism for the reaction of anthocyanins with H_2O_2, elsewhere. In the presence of H_2O_2 or HO·, the CL intensity of delphinidin, which possesses hydroxyl groups at C'-3, C'-4 and C'-5 was greater than that of cyanidin (hydroxyl groups at C'-3 and C'-4), suggesting that an increasing number of hydroxyl groups vicinal to that at C'-4 is important for CL, as was found for the flavonoids.

It is well known that the hydroxyl radical scavenging activity of flavonoids is closely related to the number and the position of hydroxyl groups on the B-ring [9, 10]. The results obtained here clearly demonstrate that the CL intensity of the flavonoids in the presence of HO· was also influenced by the number and the position of hydroxyl groups on the B-ring, analogously to that antioxidative activity of flavonoids measured on lipid peroxidation. The CL intensity of flavonoids in the presence of HO· may also be influenced by the electro-negativity of carboxyl group moiety because the order of CL intensity of aromatic carboxylic acids was found to be $COOH- > COOH-CH=CH- > COOH-CH_2-$.

Although the mechanism and radical species involved in the formation of CL have not been elucidated, bioluminescence has already been observed in luciferase [13–15] and soybean seedling [1, 2, 16], a finding which suggests that CL may occur through the same mechanism. The CL of flavonoids may occur through the degradation of hydroperoxide, as an intermediate of the radical reaction in the presence of H_2O_2 or HO·. The direct measurement of CL in the presence of aldehyde and H_2O_2 or HO·, as reported here, with complement of existing ESR spin-trapping and enzymatic technique facilitate further investigation in this complex and challenging area.

EXPERIMENTAL

Reagents. Gallic acid was purchased from Nakarai tesque Co. Isovanillic acid, quercetin, myricetin, apigenin and flavone were purchased from Sigma. 3,5-Dimethoxy-4-hydroxy-cinnamic acid and 3,4-dihydroxyphenylacetic acid were purchased from Aldrich. Caffeic acid, rutin and catechin were supplied by Tokyo Kasei and syringic acid supplied by Kanto Chem. Isorhamnetin and rhamnetin were synthesized as described by Jurd [23]. Rhamnetin was also prepared by the methylation of the C-7 hydroxyl group of quercetin with diazomethane. Isorhamnetin 3-glucoside, isoquercitrin, and diosmetin 7-*O*-glucoside were isolated from leaves of red turnip, red clover, and carrot, respectively. Nasunin, rubrobrassicin, and malvin were isolated from eggplant, red turnip and wild grapes, respectively [24–26]. Delphinidin, cyanidin and malvidin were prepd by hydrolysing nasunin, rubrobrassicin and malvin with 18% HCl, respectively. Luteolin 4'-*O*-glucoside and astilbin were gifts from Dr M. Yasue (Yamagata Univ., Japan) and Dr K. Mizutani (Mazuzen Pharmaceuticals Co., Japan), respectively. Dihydroquercetin was synthesized according to the method of Geissman *et al.* [27]. Flavonoids were solubilized in 50% MeOH by sonification prior to use.

Chemiluminescence (CL) measurements. The CL of flavonoids and other compounds were measured by a filter-equipped photon counting-type spectrometer (CLD-110, Tohoku Electronic Ind.), connected to a Waters Model 510 pump and U6K injector. A solution of 50% MeOH diluted with 50 mM phosphate buffer containing 1.5% H_2O_2 and 5% Me CHO (pH 7.0) was used as a mobile phase; the flow rate of 1 ml min^{-1} and temp. of 23° were according to the method of Watanabe *et al.* [15]. The dispersed light at the grating was simultaneously detected on the photocathode with the image sensor set at wavelengths from 300 to 650 nm. The photons counted at the respective wavelengths were computed totally as spectral intensities (10^2 count sec^{-1}). Hydroxyl radical (HO·) was generated via the Fenton reaction by adding 25 mM $FeCl_2$ to the sample injector. Ten μl of sample was also directly injected into the sample injector with or without $FeCl_2$. The scavenging activities of benzoic and cinnamic acids, and flavonoids (5 mM, 10 μl) were measured by comparing with the CL intensity of gallic acid (5 mM, 10 μl), respectively.

Acknowledgements—This work was supported in part by a grant for Bio Renaissance Program (BRP 93-VI-B-1) from the Ministry of Agriculture, Forestry, and Fisheries (MAFF), Japan, and by a grant in aid for Scientific Research (Project No. 05660128) from the Ministry of Education, Science and Culture of Japan. We thank Dr G.R. Fenwick of the AFRC Institute of Food Research for critical reading of this manuscript.

REFERENCES

1. Watanabe, H., Kobayashi, M., Suzuki, S., Usa, M., Agatsuma, S., Saeki, R. and Inaba, H. (1991) *Biochem. Biophys. Acta* **1058**, 209.
2. Watanabe, H., Nagoshi, T., Suzuki, S., Kobayashi, M., Usa, M. and Inaba, H. (1992) *Biochem. Biophys. Acta* **1117**, 107.
3. De Mello, M. P., Nascimento, A. T. O., Bohne, C. and Cilento, G. (1988) *Photochem. Photobiol.* **47**, 457.
4. Aruoma, O. I. and Halliwell, B. (1993) *Food and*

Cancer Prevention: Chemical and Biological Aspects (Waldron, K. W., Johnson, I. T. and Fenwick, G. R., eds), pp. 119–124. Royal Society of Chemistry, Cambridge.
5. Sudina, G. F., Mirzoeva, O. K., Pushkareva, M. A., Korshunova, G. A., Sumbatyan, N. V. and Varfolorneev, S. D. (1993) *FEBS Letters* **329**, 21.
6. Baumann, J., Wurm, G. and Bruchhausen, F. V. (1980) *Arch. Pharm.* **313**, 330.
7. Sorata, Y., Takahama, U. and Kimura, M. (1984) *Biochem. Biophys. Acta* **799**, 313.
8. Puppo, A. (1992) *Phytochemistry* **31**, 85.
9. Husain, S. R., Cillard, J. and Cillard, P. (1987) *Phytochemistry* **26**, 2489.
10. Bors, B., Heller, W., Michel, C. and Saran, M. (1990) *Methods in Enzymology*, Vol. 186, pp. 343–355. Academic Press, New York.
11. Aruoma, I. O., Halliwell, B., Gajewski, E. and Dizdaroglu, M. (1989) *J. Biol. Chem.* **264**, 20 509.
12. Nakayama, T., Kuno, T., Hiramitsu, M., Osawa, T. and Kawakishi, S. (1993) *Biosci. Biotech. Biochem.* **57**, 174.
13. Watanabe, T. and Nakamura, T. (1976) *J. Biochem.* **79**, 489.
14. Kurfurst, M., Ghisla, S. and Hastings, J. W. (1983) *Biochemistry* **22**, 1521.
15. Branchaud, P. B. and Walsh, T. C. (1985) *J. Amer. Chem. Soc.* **107**, 2153.
16. Saeki, R., Kobayashi, M., Usa, M., Yoda, B., Miyazawa, T. and Inaba, H. (1989) *Agric. Biol. Chem.* **53**, 3311.
17. Kemal, C. and Bruice, T. C. (1976) *Proc. Natl. Acad. Sci.* **73**, 995.
18. Alspach, J. and Rasmussen, R. (1976) *Biochem. Biophys. Res. Commun.* **73**, 465.
19. Baumstark, A. L., Cline, T. W. and Hastings, J. W. (1979) *Arch. Biochem. Biophys.* **193**, 449.
20. Dumm, D. K., Michaliszyn, G. A., Bogacki, I. G. and Meighen, E. A. (1973) *Biochemistry* **12**, 24.
21. Kondou, Y., Kawai, Y., Miyazawa, T. and Mizutani, J. (1993) *Biosci. Biotech. Biochem.* **57**, 1575.
22. Zhuang, H., Hamilton, R. T., Andersen, A. R. and Hildebrand, F. D. (1992) *Plant Physiol* **100**, 80.
23. Jurd, L. (1962) *J. Org. Chem.* **27**, 1294.
24. Igarashi, K., Yoshida, T. and Suzuki, E. (1993) *Nippon Shokuhinkogyo Gakkaishi* **40**, 138.
25. Igarashi, K., Abe, S. and Satou, J. (1990) *Agric. Biol. Chem.* **54**, 171.
26. Igarashi, K., Takanashi, K., Makino, M. and Yasui, T. (1989) *Nippon Shokuhinkogyo Gakkaishi* **36**, 852.
27. Geissman, T. A. and Lischner, H. (1952) *J. Amer. Chem. Soc.* **74**, 3001.