Rapid and Specific Detection of Hydroxyl Radical Using an Ultraweak Chemiluminescence Analyzer and a Low-Level Chemiluminescence Emitter: Application to Hydroxyl Radical-Scavenging Ability of Aqueous Extracts of Food Constituents

Chin-Hung Tsai,[†] Arnold Stern,[‡] Jeng-Fong Chiou,[§] Chi-Liang Chern,[⊥,#] and Tsan-Zon Liu*[,#,Δ]

Department of Food Nutrition, Chung-Hwa Institute of Technology, Tainan, Taiwan; Department of Pharmacology, New York University Medical Center, New York, New York; Department of Radiotherapy and Oncology, Taipei Medical College and Hospital, Taipei, Taiwan; Department of Medical Technology, Fooyin Institute of Technology, Kaohsiung Hsien 831, Taiwan; Department of Biological Science, National Sun Yat-Sen University, Kaohsiung, Taiwan; and Department of Medical Research, Yuan's General Hospital, Kaohsiung, Taiwan

With the availability of an ultraweak chemiluminescence analyzer, it is possible to monitor the production of a specific oxygen-derived reactive species, such as hydroxyl radical (•OH), whenever a suitable chemiluminescent probe is obtainable. Reported herein is the development of a rapid and specific method for detecting •OH production using a specific probe, indoxyl-*β*-glucuronide (IBG), a low-level chemiluminescence emitter. Using the Fenton reagent as a source of •OH, it was shown that IBG could elicit a very strong intensity of chemiluminescence (CL) (16200 ± 200 photon counts/s). Conversely, IBG was shown to be insensitive to either superoxide radical or hydrogen peroxide with their CL intensities nearly close to the background values (25 ± 5 and 180 ± 20 photon counts/s, respectively). Furthermore, it was also shown that this IBG-based CL production could be effectively quenched by the addition of •OH scavengers such as sodium salicylate, dimethyl sulfoxide, and penicillamine to the assay system. Taken together, these data indicate that IBG is a specific CL probe suitable for monitoring the production of •OH. This system demonstrated inhibitory activities of various aqueous extracts of food constituents on the CL of hydroxyl radicals generated by Fenton's reagents with the order of scavenging efficiencies being *Prunus mume* > *Cordyceps sinensin* > *Lilium lancifolium* > *Astragalus membranceus*.

Keywords: *Chemiluminescence emitter; indoxyl-β-glucuronide; hydroxyl radical; food constituents; scavenging effect*

INTRODUCTION

Among the oxygen-centered free radicals, the hydroxyl radical (•OH) is considered to be one of the most potent oxidizers in nature and is assumed to play a pivotal role in damaging cellular macromolecules. Numerous methods for the detection of •OH have been developed. They are spin-trapping electron paramagnetic resonance spectroscopy (*1*−*3*), gas−liquid chromatography (GLC) of ethylene produced from methional by the reaction of •OH (*4*), and high-performance liquid chromatography (HPLC) of hydroxylated substances (*5*, *6*) or methanesulfinic acid (*7*, *8*) produced by the reaction of •OH with aromatic compounds or dimethyl sulfoxide (DMSO), respectively. Some of the most sensitive methods of detection of •OH are based on measurement of the fluorescence of a chemical detector molecule produced by •OH attack on a nonfluorescent molecule (*9*). A related method that has been used for the detection of •OH employs oxidation of 2-deoxy-D-ribose by •OH with subsequent measurement of the products by their reaction with thiobarbituric acid (TBA) (*10*, *11*). Notwithstanding their merits for •OH detection, these methods are somewhat cumbersome and lengthy for use in assessing the hydroxl radical-scavenging ability of various food extracts and/or their respective ingredients.

Alternatively, the detection of reactive oxygen species (ROS) can be amplified by measuring the chemiluminescence (CL) emitted by luminescent-generating substrates such as luminol and lucigenin. Lucigenin-derived CL has been utilized to monitor $O_2^{\bullet-}$ production, whereas H_2O_2 has been detected with luminol-derived CL (*12*−*14*). However, to the best of our knowledge, there is thus far no probe-based chemiluminescent method available for specific monitoring of the production of •OH. We report herein the development of a rapid and specific method for detecting •OH production using a probe, indoxyl-*β*-glucuronide (IBG), performed by an ultraweak CL analyzer. The proposed method offers a promising

* Address correspondence to this author at the Department of Medical Research, Yuan's General Hospital, 162 Cheng Kung First Road, Kaohsiung, Taiwan 802 (telephone 011-886-7-339-2764; fax 011-886-7-334-8380; e-mail n8898@ms21.hinet.net).

[†] Chung-Hwa Institute of Technology.

[‡] New York University Medical Center.

[§] Taipei Medical College and Hospital.

[⊥] National Sun Yat-Sen University.

[#] Yuan's General Hospital.

[Δ] Fooyin Institute of Technology.

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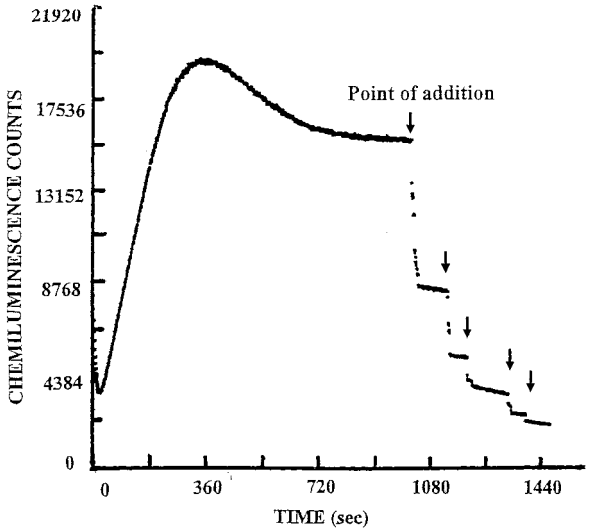

Figure 1. A representative time course of CL production via interaction of IBG and •OH generated by Fenton reagent (Fe^{2+} + H_2O_2). A sudden drop of CL counts is noted when an •OH scavenger (sodium salicylate, 1 mg) is added at a time point of 1080 s (indicated by an arrowhead). When CL counts became steady, addition of an extra amount of scavenger results in a further drop of CL counts. Inhibition (percent) versus concentration of scavenger can then be obtained. IC_{50} of a scavenger can be extrapolated from the curve.

Table 1. Specificity of IBG as the CL Emitter for Hydroxyl Radicals Generated by the Fenton Reagents

addition to or omission from the reaction mixture	CL ± SD[a] (photon count/s)
complete system[b]	16200 ± 200
− Fe^{2+}	180 ± 20
− Fe^{2+}, − H_2O_2	30 ± 5
− Fe^{2+}, − EDTA, − H_2O_2	27 ± 5
− Fe^{2+}, − EDTA, − H_2O_2, + $O_2^{\bullet-}$-generating system[c]	25 ± 5

[a] Each value represents an average of triplicate determinations. [b] The reaction mixture of the complete system is 3 μM IBG (1 mL), 1 mM Fe^{2+} (0.1 mL), 3% H_2O_2 (1.6 mL), and EDTA (50 μL) + PBS buffer, pH 7.4, to a total volume of 2.75 mL. [c] Methylglyoxal and arginine were used as the source of $O_2^{\bullet-}$ generation.

tool for rapidly assaying the hydroxyl radical-scavenging ability of various food extracts and/or their respective purified ingredients.

MATERIALS AND METHODS

Materials. IBG, ethylenediaminetetraacetic acid (EDTA), methylglyoxal, ferric chloride, arginine, H_2O_2 (30%), sodium salicylate, DMSO, and penicillamine were purchased from Sigma Chemical Co. (St. Louis, MO). Unless stated otherwise, all chemicals used were of analytical quality, and doubly distilled water was used throughout.

Preparation of Aqueous Food Extract. Four types of aqueous food extracts were prepared from raw materials of *Prunus mume*, *Cordyceps sinensin*, *Lilium lancifolium*, and *Astragalus membranceus*. Approximately 50 g of raw material from each of the four foodstuffs was cut into small pieces and homogenized along with 500 mL of distilled water in a Waring blender. The whole mixture was then heated until boiling for 30 min. The heat-denatured preparation was then allowed to stand until the crude debris was precipitated. The supernatant of each food extract was then collected, and the moisture was removed by a freeze-drying technique. The resultant dried powder was sealed and stored at −20 °C until use.

CL Monitoring of Hydroxyl Radical Formation. The hydroxyl radical generating system used in this study was based on the Fenton reaction (Fe^{2+} + H_2O_2). Thus, the reaction

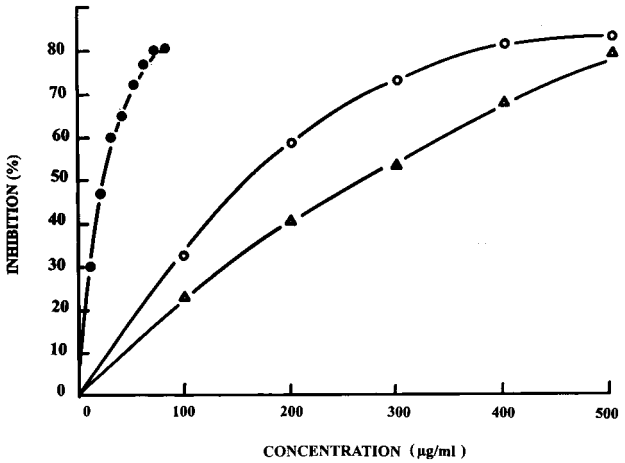

Figure 2. Parabolic relationship between the percent of inhibition of CL generation versus the concentration of a scavenger used: (●) sodium salicylate; (○) DMSO; (△) penicillamine. Each point in each set was an average value of duplicate determinations.

Table 2. Inhibitory Efficiency of Various Hydroxyl Radical Scavengers Measured by the Proposed Method

scavenger	IC_{50} (μg/mL)
sodium salicylate	14.0
DMSO	180.0
penicillamine	270.0

mixture used contains the following: 1.0 mL of 3 μM IBG [dissolved in phosphate-buffered saline (PBS), pH 7.4]; 0.1 mL of 1.0 mM $FeSO_4$; 1.6 mL of 3% H_2O_2; and 0.05 mL of 10 mM EDTA. The total volume of the reaction mixture was 2.75 mL. All of the above-mentioned reagents were added to the quartz round-bottom cuvette in the black-box unit of the CL analyzer in a sequential order of EDTA, IBG, H_2O_2, and $FeSO_4$. The ultraweak photon was measured using a BJL-ultraweak CL analyzer with a high-sensitivity detector (3.3×10^{-15} W/cm^2•count) from Jye Horn Co. (Taipei, Taiwan). This analyzer is also available from American Biologics (Chula Vista, CA). Daily calibration of the detector sensitivity was performed with a ^{14}C-light source generating 10000 photon counts/s at a voltage ranging between 860 and 867 V. The reproducibility of the ^{14}C-light source was <1.0%. Luminescence intensity was monitored in the wavelength range from 260 to 750 nm.

Hydroxyl Radical-Scavenging Ability of Food Extracts. Twelve minutes (720 s) after the initiation of the CL reaction generated by IBG and Fenton reagent (H_2O_2 + Fe^{2+}) in the CL analyzer (CL versus time relationship can be visualized on the screen of the data processing unit), 1 mg/mL of each of the four food extracts was added to the reaction mixture. As a result, varying degrees of sudden drops of CL counts could be observed. These represent the different degrees of •OH scavenging abilities. After the percentage of inhibition of CL counts had been calculated, a comparison of the •OH scavenging efficacy of each of these food extracts is possible.

RESULTS AND DISCUSSION

There is overwhelming evidence to indicate that free radicals can cause oxidative damage to lipids, proteins, and nucleic acids. Free radicals may lie at the heart of the etiology or natural history of a number of diseases including cancer and atherosclerosis. Therefore, antioxidants, which can neutralize free radicals, may be of central importance in the prevention of these disease states. A variety of food extracts contain many different antioxidants. For example, some flavonoids that are frequently components of the human diet demonstrated strong antioxidant activities (*15*−*17*). For this reason,

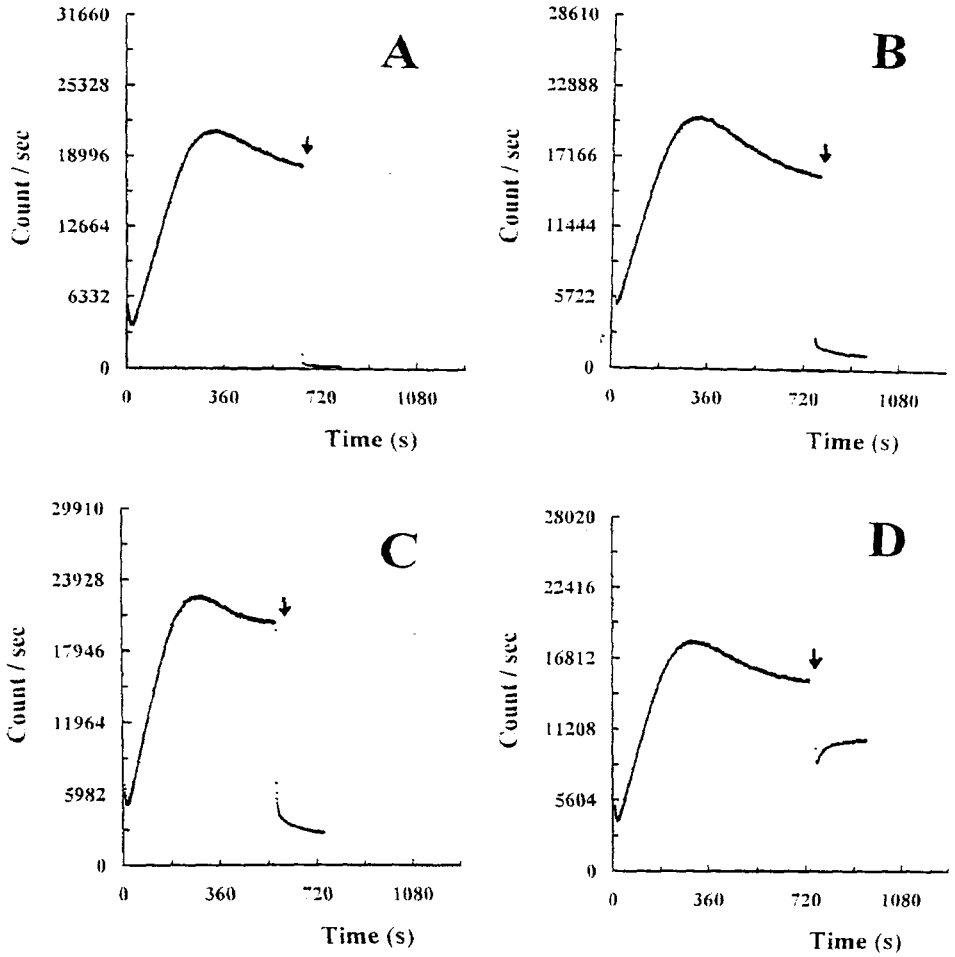

Figure 3. Hydroxyl radical scavenger abilities of four aqueous extracts of foods: (A) *P. mume*; (B) *C. sinensin*; (C) *L. lancifolium*; (D) *A. membranceus*.

there is considerable interest in the use of food extracts for therapeutic use aiming to replenish the needed antioxidants to correct the deficit of pro-oxidant−antioxidant imbalance (*18*−*20*). Radical scavenging capacity assays, such as the Trolox equivalent antioxidant capacity (TEAC), total reactive antioxidant potential or total radical-trapping antioxidant parameter (TRAP), and ferric reducing antioxidant power (FRAP), have been devised for high-throughput screening on potential antioxidant capacity of biological matrices, such as plasma, single compounds, food components, or food extracts (*21*, *22*). However, these methods possess certain drawbacks that may be difficult to resolve. First, potential synergisms between the various antioxidants cannot be clearly ruled out. Second, FRAP reaction conditions may be far from physiological, and it must be noted that in vitro testing of plasma may not reflect in vivo hierarchies or activity (*23*). Third, methodology suited for the measurement of total antioxidative capacity cannot be used to distinguish what type of free radical is being specifically inhibited by the test compound. To circumvent these problems, we developed a CL method for the specific detection of •OH measured by an ultraweak CL analyzer. During an extensive literature search, we found only one published paper dealing with the detection of •OH by using luminol as a CL probe (*24*). As indicated previously, luminol has been used extensively as a CL probe for H_2O_2; thus, its utilization in the detection of •OH should be considered nonspecific (*12*−*14*). Furthermore, the paucity of papers dealing with the detection of •OH by the CL approach can understandably be due to the lack of a suitable probe.

Various fluorescent compounds accumulate in the plasma and hemodialysate of patients with chronic renal failure (*25*, *26*). Agatsuma et al. (*27*) reported that plasma from hemodialysis patients evoked weak photon emission (chemiluminescence) in a characteristic emission spectrum with a peak at 430 nm, attributed to attack by •OH generated from the iron-catalyzed breakdown of H_2O_2 (Fenton reaction). The compound responsible for this unique phenomenon has subsequently been purified and identified to be IBG (*28*). With the availability of this potential CL probe for •OH radical and an ultraweak CL analyzer in our laboratory, we developed a rapid and specific probe-based CL method for detecting •OH radical. Using Fenton reagent as the source of •OH, we demonstrated that indeed IBG could elicit very strong intensity of CL (16200 ± 200 photon counts/s) (Figure 1). Conversely, IBG was shown to be insensitive to either superoxide anion or H_2O_2 with their CL intensities close to background values (25 ± 5 and 180 ± 20 photon counts/s, respectively) (Table 1). Furthermore, we also showed that this IBG-elicited CL production could be effectively quenched by the addition to the assay system of •OH scavengers such as sodium salicylate, DMSO, and penicillamine (Figure 2; Table 2). These data clearly indicate that IBG is a specific CL probe suitable for monitoring the production of •OH.

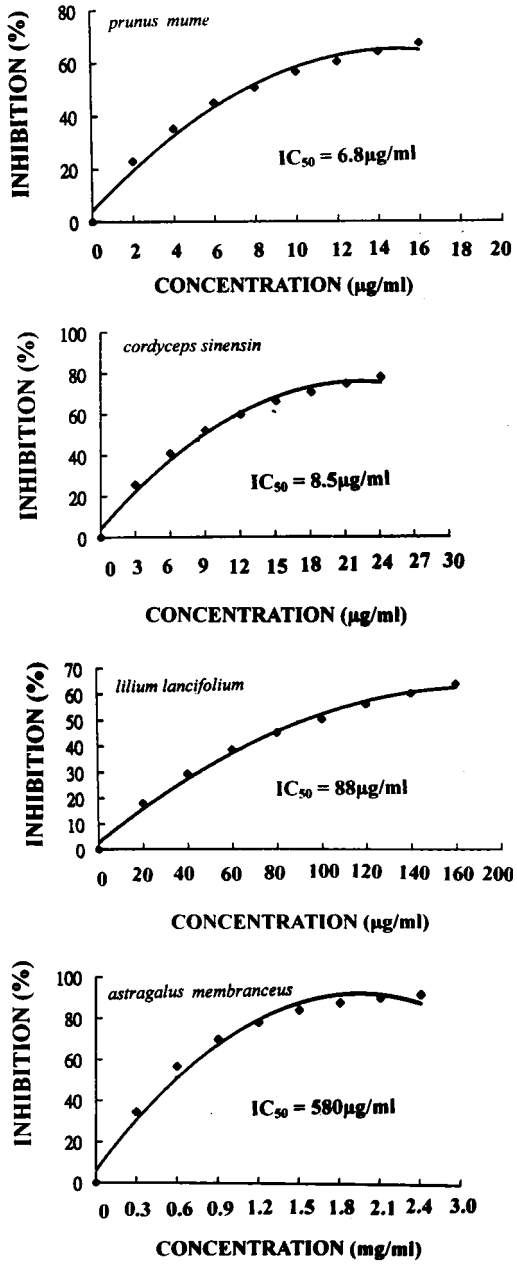

Figure 4. Concentration−inhibition curves of four aqueous extracts of foods used to estimate the IC_{50}. The IC_{50} values obtained for all food extracts can then be compared for their scavenging efficiencies for hydroxyl radicals.

One of the features of this proposed method for •OH detection is its capability of obtaining a series of inhibitory percentages of CL exerted by various concentrations of a test compound. Consequently, the percentages of inhibition of CL versus the concentrations of a test compound can be constructed. It follows that the concentration needed to inhibit 50% of CL in the assay system (IC_{50}) of a test compound can be extrapolated from the curve (Figure 2). As an example, using this approach, we were able to obtain the IC_{50} values of various •OH scavengers, the order of inhibitory efficiency being sodium salicylate > DMSO > penicillamine (Table 2).

To illustrate the application of the proposed method for the hydroxyl radical-scavenging ability of various food extracts, namely, *Prunus mume* (A), *Cordyceps sinensin* (B), *Lilium lancifolium* (C), and *Astragalus membranceus* (D), we carried out the following experiment. Twelve minutes after the monitoring of CL produced due to •OH generated by Fenton reagent and IBG, 1 mg/mL of each of the test compounds was added to the assay system. Various degrees of a sudden drop of CL values were observed, indicating differences in the •OH scavenging abilities exerted by these food extracts can be visually compared with the order being A > B > C > D (Figure 3). However, if one is interested in obtaining the IC_{50} of each food extract for a quantitative comparison, a concentration−inhibition curve can be constructed as exemplified by the information provided in Figure 4. A detailed assessment of a variety of foodstuffs, herbal remedies, and other agricultural products by CL using IBG as a probe should provide insight into the antioxidative capacity regarding hydroxyl radical scavenging of these substances. This might be useful for the eventual identification of the most effective hydroxyl radical scavengers in foodstuffs and perhaps the eventual formulation of a natural products antioxidant mixture.

In summary, we have developed an ultraweak, probe-based CL method for the rapid and specific detection of •OH radical. The proposed method lends itself to a specific measurement of •OH radical because the probe selected is insensitive toward other oxygen reactive species, such as superoxide and hydrogen peroxide. Most importantly, we find that the proposed method can now be used with relative ease of assessing the hydroxyl radical-scavenging ability of a pure ingredient or a food extract.

ABBREVIATIONS USED

IBG, indoxyl-β-glucuronide; TBA, thiobarbituric acid; CL, chemiluminescence; TEAC, Trolox equivalent antioxidant capacity; TRAP, total reactive antioxidant potential; FRAP, ferric reducing antioxidant power; DMSO, dimethyl sulfoxide.

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