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Improved Superoxide-Generating System Suitable for the Assessment of the Superoxide-Scavenging Ability of Aqueous Extracts of Food Constituents Using Ultraweak Chemiluminescence

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In the interest of developing a simple and rapid ultraweak chemiluminescence assay for assessing the superoxide (O_2^-)-scavenging activities of various aqueous extracts of food constituents, a specific and stable O_2^--generating system was sought. Reported herein is the obtainment for the first time of a specific and stable O_2^--generating system consisting of methylglyoxal (MG), a reactive 2-oxo aldehyde and arginine, which has been shown to produce much steadier lucigenin-based chemiluminesence (LBCL) than the conventional xanthine/xanthine oxidase system running in parallel and monitoring by an ultraweak chemiluminescence analyzer. Upon mixing of MG and arginine in a phosphate-buffered saline solution, pH 7.4, steady, time-dependent increments of LBCL can be visually observed. The plateau of LBCL can be reached in approximately 10 min and retained in a steadily stable state thereafter without fluctuation for the next 15 min. The lucigenin-based LBCL generation was shown to be specific since it could be effectively inhibited by active bovine SOD, but not by heat-inactivated enzyme or catalase. Conversely, the xanthine/xanthine oxidase system can merely produce a LBCL peak rapidly but decay instantaneously. To illustrate the application of the proposed method for assessing the O_2^--scavenging ability of various food extracts, namely, *Prunus mume* (A), *Lilum lancifolium* (B), *Creataegus pinnatifida* (C), *Tremella fuciformis* (D), *Fortunella margarita* (E), and *Scutellaria baicalensis* (F), we used the following protocol: 12 min after monitoring of LBCL, 1 mg/mL of each of the test compounds was added to the assay system and various degrees of sudden drop of LBCL values were observed, indicating differences in O_2^--scavenging abilities exerted by these food extracts that can be visually compared. Consequently, the percentages of inhibition of LBCL versus the concentrations of a test compound can be constructed. It follows that the concentration needed to inhibit 50% of LBCL (IC_{50}) of a test compound can be extrapolated from the curve. Using this approach, we were able to obtain the IC_{50} values of various compounds to be tested and the order of inhibitory efficiency of the above-mentioned food extracts was ranked, being A > B > C > D > E > F, respectively.

KEYWORDS: Chemiluminescence emitter; methylglyoxal; arginine; superoxide-scavenging ability; food constituents

INTRODUCTION

Free radical scavengers and antioxidants can reduce lipid peroxidation and the generation of reactive oxygen species. The protection that fruits and vegetables provide against disease, including cancer and cardio- and cerebrovascular diseases, has been attributed to the various antioxidants contained in them (*1*−*3*). For this reason, there is considerable interest in the use of food extracts for therapeutic use, aiming to replenish the needed antioxidants to correct the deficit of prooxidant/antioxidant imbalance (*4*−*6*). A detailed assessment of a variety of foodstuffs, herbal remedies, and other agricultural products should provide insight into the antioxidative capacity of these

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substances to allow eventual identification of the most effective free radical scavengers so that a natural products antioxidant mixture can be formulated. A variety of radical-scavenging capacity assays, such as the Trolox equivalent antioxidant capacity (TEAC), total reactive antioxidant potential (TRAP), and ferric-reducing antioxidant power (FRAP), have been devised for high-throughput screening on potential antioxidant capacity of these substances. Notwithstanding their merits for measuring the total antioxidant capacity, these methodologies unfortunately lack the ability to identify what type of free radical is being specifically scavenged by the test compound.

Advances in the technology of luminometers have made the chemiluminescent detection of oxygen-derived free radicals (ODFR) a relatively simple, rapid, and inexpensive procedure. However, the light emitted by ODFR is generally insufficient to quantify without the addition of a chemilumigenic probe of high quantum efficiency, such as lucigenin (dimethyl biacridinium dinitrate) (*7*). With the availability of an ultraweak chemiluminescence analyzer which has been equipped with a high-sensitivity detector (3.3×10^{-15} W/(cm^2·count)), it is possible to monitor the production of a specific ODFR, such as superoxide anion (O_2^-), whenever a suitable chemiluminescent probe is obtainable. Lucigenin was considered to be a specific chemiluminescence probe for O_2^- (*7*), and a combined application of lucigenin along with an enhancer, *p*-iodophenol, to increase the sensitivity and stability of the emitted light for the measurement of antioxidative activity of sera from a variety of pathological conditions has been reported (*8*).

It is well-documented that xanthine oxidase from bovine milk produces a substantial amount of O_2^- when acting aerobically on either purine or aldehyde O_2^--generating substrate, and this O_2^--generating system has widely been used in a variety of indirect methods for the measurement of SOD activity (*9*, *10*). Although it is a convenient O_2^--generating source, the xanthine/xanthine oxidase system, however, has suffered many technical drawbacks. First, at neutral pH, O_2^- production accounts for merely 15% of the total electron flow through the enzyme. Because of this shortcoming, the chemiluminescence was studied previously at high pH (>10), where the quantum efficiency was high. Second, xanthine oxidase catalyzes the oxidation of a wide range of substrates including acetaldehyde which has been shown to be a potent inhibitor for xanthine oxidase. Therefore, its existence can interfere with the enzyme activity (*11*). Third, when the concentration of the enzyme is too high, the reaction will be rapid and the chemiluminescnece peak may be passed before counting is initiated. Because of these potential troublesome problems, it is of necessity for us to search for a novel O_2^--generating system which can offset these drawbacks. Herein we report a novel O_2^--generating system consisting of methylglyoxal and arginine. Its suitability in the assessment of O_2^--scavenging ability of aqueous food extracts using an ultraweak chemiluminescence analyzer has been found to be satisfactory.

MATERIALS AND METHODS

Materials. Methylglyoxal (pyruvaldehyde; 2-oxopropanol) (6.5 mmol/L), lucigenin, arginine, xanthine, superoxide dismutase (SOD) (5100 units/mg), and xanthine oxidase 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. Six types of aqueous food extracts were prepared from raw materials of *Prunus mume*, *Lilium lancifolium*, *Crataegus pinnatifide*, *Tremella fuciformis*, *Fortunella margarite*, and *Scutellaria baicalensis*. Approximately 50 g of raw material from each of the six 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 Superoxide Radical Formation. A reaction mixture in a total volume of 2.1 mL consisted of the following components: 1.0 mL of 2.0 mM lucigenin; 1.0 mL of phosphate-buffered saline, pH 7.4; 0.05 mL of 1.0 M arginine; 0.05 mL of 1.4 μM methylglyoxal. After gently mixing the above-mentioned components, the reaction mixture was added to a quartz round-bottom cuvette in the black-box unit of the ultraweak chemiluminescence analyzer. The ultraweak photon was measured using a BJL ultraweak chemiluminescence 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 an amount of 10 000 photon counts/s at a voltage ranging between 860 and 867 V. The reproducibility of the ^{14}C-light source was <1.0%.

Superoxide Radical-Scavenging Ability of Food Extracts. At 12 min (720 s) after the initiation of the CL reaction generated by lucigenin and methylglyoxal/arginine reagents 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 six food extracts was added to the reaction mixture. As a result, varying degrees of sudden drops of CL counts could be observed. These phenomena represent different degrees of O_2^--scavenging abilities. However, if one is interested in the obtainment of IC_{50}, the concentration of a test compound needed to inhibit 50% of CL in the assay system, for a quantitative comparison, a concentration-inhibition curve can be constructed. After the percentage of inhibition of CL counts has been calculated, a comparison of IC_{50} values will permit one to determine the O_2^--scavenging efficiency of these food extracts.

RESULTS AND DISCUSSION

The utilization of spectrophotometric methods for the assessment of O_2^--scavenging ability of various food extracts may be simple to perform, yet the complex constituents existing in them may create an artifactual O_2^- generation. Often, O_2^- is detected by its ability to reduce cytochrome *c* or nitroblue tetrazolium (NBT). However, many other substances can reduce cytochrome *c*, such as ascorbate and thiols, and interfere with O_2^- determination by removing the indicator molecule (*12*). NBT reduction is also subjected to artifacts by the compounds that reduce it (*13*). O_2^- can also oxidize molecules, such as L-epinephrine to an adrenochrome. Again, it is difficult to relate adrenochrome formation to the actual rate of O_2^- generation by the system under testing (*13*). Along this line, one artifact that has caused some confusion is that many compounds being tested for O_2^--scavenging ability absorb strongly at 290 nm, making spectrophotometric assessment of urate production by xanthine oxidase inaccurate. On the basis of these problematic shortcomings, detection of O_2^- using spectrophotometric indicators may be potentially troublesome. Therefore, it is of necessity to recruit a more specific methodology to obviate these potential interferences.

Chemiluminescence (CL) occurs whenever a molecule emits a photon as a result of an exergonic chemical reaction that generates an intermediate or end product in an electronically excited state. The relaxation of the excited-state molecule to ground state results in the emission of a photon. Lucigenin (bis-*N*-methylacridinium nitrate) has been extensively used as a chemiluminigenic probe for assessing O_2^- (*14*−*16*). With the availability of an ultraweak chemiluminescence analyzer, a probe-based CL approach for the assessment of O_2^--scavenging

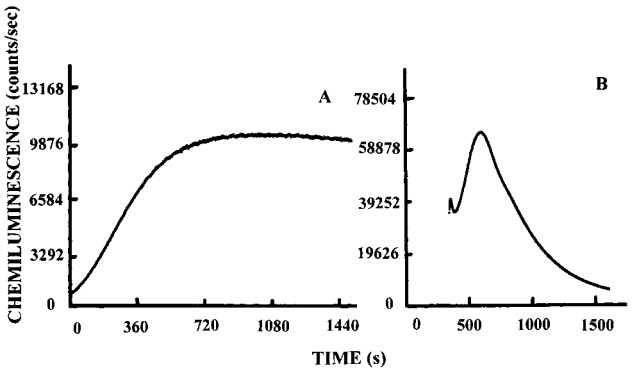


Figure 1. Representative time course of CL production via interaction of lucigenin and O_2^- generated either by methylglyoxal (MG) and arginine (A) or xanthine/xanthine oxidase (B). A reaction mixture in a total volume of 2.1 mL consisted of the following components, namely, 1.0 mL of 2.0 mM lucigenin, 1.0 mL of PBS (pH 7.4), 0.05 mL of 1.0 M arginine, and 0.05 mL of 1.4 μM MG, were used for CL monitoring by an ultraweak CL analyzer. In the xanthine/xanthine oxidase system, the components of the reaction mixture were the same as those of panel A except MG and arginine were replaced by xanthine (0.5 mL of saturated solution) and xanthine oxidase (0.087 unit/assay).

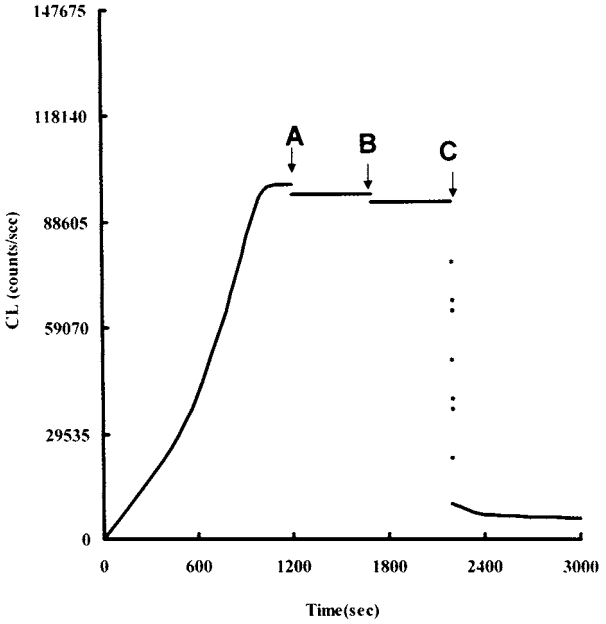
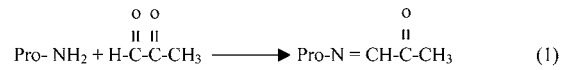


Figure 2. Specificity of O_2^--mediated generation of CL by MG and arginine. The components of the reaction mixture were identical to those described in Figure 1, except the concentrations of MG and argine were increased 10-fold. When heat-denatured bovine SOD (5.1 units) was added at point A, CL remained steady without fluctuation. Similarly, addition of catalase (500 units) was shown to be free of inhibition on CL (arrowhead B). However, when bovine SOD (5.1 units) was added at point C, a sudden drop of CL counts was noted, indicating that CL was actually generated from O_2^-.

ability of various food extracts is selected on the basis of the fact that lucigenin is considered to be a specific chemiluminescent probe for O_2^- and the high sensitivity of the CL analyzer.

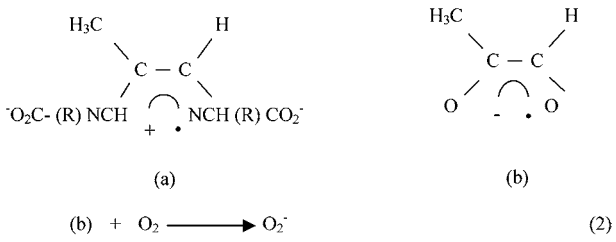
Among a variety of O_2^--generating systems documented in the literature, the xanthine/xanthine oxidase method was most commonly used. However, the xanthine/xanthine oxidase system has encountered several technical drawbacks. Besides, we also found that the lucigenin-based chemiluminescence (LBCL) provided by the xanthine/xanthine oxidase system reached a plateau rapidly and thereafter decayed very quickly (Figure 1). Thus, we reasoned that using the xanthine/xanthine oxidase system as a source of O_2^--generating system was impracticable and a specific and stable O_2^--generating system was thus necessary.

Methylglyoxal (pyruvaldehyde; 2-oxopropanol; MG), a reactive 2-oxo aldehyde, is a widely occurring compound that is produced by most glucose-metabolizing cells (*17*). MG has been shown to react with arginine, lysine, and cysteine in proteins (*18*). Recently, we showed that when serum containing MG was used as a specimen for protein electrophoresis, a concentration-dependent shift in the migration of protein bands as well as a gradual loss of Coomassie blue staining intensity was observed. These data implied that the neutralization of a positively charged amino group by the α-dicarbonyl group of MG to form a Schiff's base (reaction 1) may have occurred.



(1)

These data are in accordance with the findings of Yim et al. (*19*), who, using EPR technique, demonstrated that the interaction between a free amino acid and three-carbon α-dicarbonyl MG could generate two types of free radical species: the cross-linked dialkylimine radical cation of MG (structure **a**) and the enediol radical anion of MG (structure **b**). It followed that the interaction between **b** and molecular oxygen will generate the O_2^- radical.



(2)

Using the LBCL approach monitored by an ultraweak chemiluminescence analyzer, we were able to demonstrate that reaction 2 was evidently occurring (Figure 1). Furthermore, we found that upon mixing of MG and arginine in a PBS solution, pH 7.4, steady time-dependent increments of LBCL could be observed. The plateau of LBCL could be reached in approximately 10 min and retained steadily stable thereafter without fluctuation for the next 15 min. The specificity of the LBCL generated by MG and arginine was further confirmed by using bovine superoxide dismutase (SOD; Figure 2). Nearly all LBCL could be abolished whenever a sufficient quantity of SOD (5.1 units/assay) was added to the reaction mixture. In contrast, this phenomenon could not be demonstrated if the same amount of heat-denatured SOD or catalase (500 units/assay) was added to the system (Figure 3). These results indicated that LBCL was specifically generated from O_2^-.

Figure 4 illustrates the application of this novel O_2^--generating system for the purpose of determining the O_2^--scavenging ability of six food extracts, namely: *Prunus mume* (A), *Lilum lancifolium* (B), *Createaegus pinnatifida* (C), *Tremella fuciformis* (D), *Fortunella margarita* (E), and *Scutellaria baicalensis* (F). For a quantitative comparison, a concentration−inhibition curve can be constructed and each respective IC_{50} value converted to a SOD-equivalent unit. The order of ranking for their O_2^--scavenging ability is A (0.20 units) > B (0.14

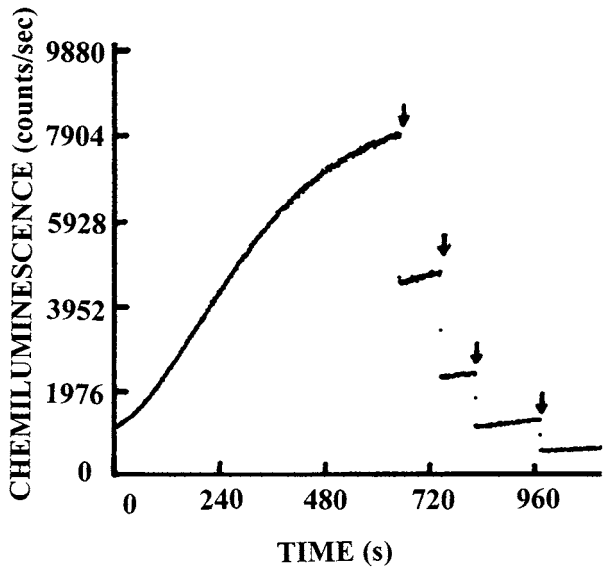


Figure 3. Inhibitory effects of superoxide dismutase (SOD) on the LBCL generated by MG and arginine. A sudden drop of CL counts is noted when SOD [10 ng (0.051 units)] is added at a time point of 600 s (indicated by an arrowhead). When CL counts became steady, addition of an extra dose of SOD resulted in a further drop of CL counts. Inhibition (percent) versus concentration of SOD can then be obtained. The IC_{50} of SOD was estimated to be 25 ng (0.13 units) (data not shown).

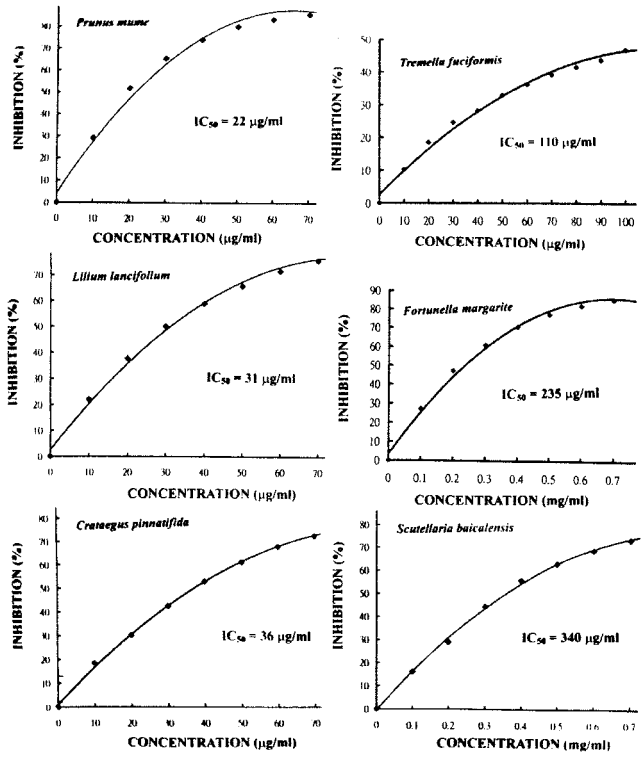


Figure 4. Concentration–inhibition curves of six aqueous extracts of foods were used to estimate the IC_{50}. The IC_{50} values obtained for all food extracts can then be compared for their scavenging efficiencies for O_2^- radicals.

units) > C (0.12 units) > D (0.04 units) > E (0.02 units) > F (0.01units), respectively.

In summary, we have developed an ultraweak, probe-based CL method for the rapid and specific detection of the O_2^- radical. The proposed method lends itself to a specific measurement of the O_2^- radical, and the source of O_2^- generated by MG and arginine produce steady and stable LBCL, which allows easy manipulation and greatly reduces the chance of committing errors. Last, this system has been found to be applicable to the determination of the relative O_2^--scavenging ability of various aqueous food extracts.

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