Original Article

DOI: 10.5582/ddt.2012.v6.1.44

A novel flow-injection analysis system for evaluation of antioxidants by using sodium dichloroisocyanurate as a source of hypochlorite anion

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ABSTRACT: A flow injection analysis (FIA) system for evaluation of the antioxidant activity of a compound capable of scavenging a hypochlorite anion (OCl^-), one of the reactive oxygen species (ROS), was developed. Aminophenyl fluorescein (APF), a fluorescence indicator of ROS, was mixed manually with the test compounds and the mixed solution was injected into the FIA system. The injected solution was reacted in-line with OCl^-, that was produced by using sodium dichloroisocyanurate in the presence of 0.1 M CH_3CO_2Na in H_2O. The fluorescence intensity of fluorescein generated from non-fluorescent APF was significantly attenuated in compounds that had a scavenging effect on OCl^-. The precision obtained by the FIA system was satisfactory (relative standard deviation < 5.0%) and a rapid assay within 0.5 min per sample was achieved. The proposed FIA system was used to demonstrate that reduced glutathione, dithiothreitol, and 3-methyl-1-phenyl-5-pyrazolone (edaravone) showed a significant scavenging effect on OCl^-. Therefore, the proposed FIA system can be used as a screening assay for OCl^--scavenging compounds.

Keywords: Screening assay, reactive oxygen species, aminophenyl fluorescein, fluorescence, reduced glutathione

1. Introduction

Reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anion, and hypochlorite anion have attracted considerable attention in the post-genome era because they are involved in posttranslational modification of proteins (*1-3*). It has been suggested that these posttranslational modifications lead to dysfunctions in biological systems, thus causing diseases such as diabetes and cancer (*4,5*). Recently, we reported that hydroxyl radicals could oxidize specifically ^{27}Met of glucagon (*6*), and furthermore that the ability of ^{27}Met sulfoxide of glucagon to produce cAMP was reduced to approximately 25% of that of native glucagon in HepG2 cells (*6*). A similar phenomenon has been observed in the case of cholecystokinin octapeptide (CCK8) (*7*), in which the oxidized CCK8 revealed *in vitro*-altered bioactivity in the LOVO cells (*8*). Similarly, oxidation of functionalized proteins or bioactive peptides by ROS could deteriorate their original and regular functions (*9,10*). Therefore, it is crucial to prevent ROS-induced oxidative stress by providing an anti-oxidant supplied from exogenous origins such as food or supplements. Research has focused on a variety of candidates for anti-oxidants capable of preventing oxidation from ROS, such as catechins, ascorbic acid, and quercetin (*11-17*).

Considering this background on the importance of anti-oxidants *in vivo*, a novel screening assay system is needed for the discovery and evaluation of compounds that can scavenge ROS. In most studies concerning ROS, Fenton's or Fenton-like reactions, which utilize Fe^{2+} or Cu^{2+} and H_2O_2 as a tool for ROS production, have been employed for *in vitro*-generation of hydroxyl radical (HO·) (*18,19*). In Fenton's reaction, however, it is difficult to regulate the concentration of hydroxyl radicals generated from Fenton's solution. Therefore, Fenton's reaction has a disadvantage in that a constant concentration of hydroxyl radical could not be provided. In order to evaluate precisely the anti-oxidant activity of a compound, it is preferable that the amount of generated ROS should remain unchanged in the reaction system. In the present study, therefore, hypochlorous acid (HOCl) was selected as the ROS. In aqueous solution, HOCl is released as hypochlorite anion (OCl^-), which is involved in the etiology of atherosclerosis (*20*). As a source of HOCl and OCl^-, sodium dichloroisocyanurate (DCCA, Figure 1)

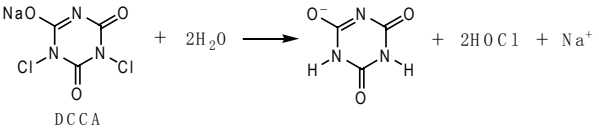

Figure 1. Reaction scheme of DCCA with H_2O.

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was used because an accurate concentration of the DCCA solution is easy to prepare due to the fact that it is a solid compound. As shown in Figure 1, DCCA can be released as OCl^- in aqueous solution (*21*). Until now, DCCA has been used for the production of OCl^-, which has in turn been utilized for chemiluminescent detection of sulfide ion in spring water (*21*). To evaluate the scavenging effect of ROS, aminophenyl fluorescein (APF) (*22*) was employed in this study. It has been reported that APF can emit fluorescence originating from fluorescein in the presence of OCl^- (*22*).

Taking these reagents together, we constructed a flow-injection analysis (FIA) system (Figure 2) in order to develop a reliable, simple, and rapid whole assay operation method. APF was mixed manually with each test compound and the mixed solution was injected into the FIA system. The injected solution was reacted in-line with OCl^- produced from DCCA. The fluorescence intensity of fluorescein generated from non-fluorescent APF would be significantly attenuated if the test compound had a scavenging effect on the OCl^-. Thus, the change of the fluorescence intensity can be used to estimate the anti-oxidant activity of the test compounds. In the present study, several compounds known as anti-oxidants were assayed by the proposed FIA system and the feasibility of the FIA system was verified.

2. Materials and Methods

2.1. *Materials*

Aminophenyl fluorescein (APF) was purchased from Sekisui Medical Co., Ltd. (Tokyo, Japan). Dichloroisocyanuric acid, sodium salt (DCCA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). *N,N*-Dimethylformamide (DMF) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Sodium hypochlorite containing 6% active chlorine was purchased from Koso Chemical (Tokyo, Japan). Water used throughout this investigation was processed by a Milli-Q Water Purification System (Millipore Corporation, Milford, MA, USA).

2.2. *Sample preparations*

A commercially available 5.0 mM APF solution (in DMF) was diluted with additional DMF to create a 10 μM APF stock solution. The APF stock solution was stable for several months in a refrigerator. Each sample solution was prepared by mixing a test compound in 500 μL of 0.1 M sodium acetate (in H_2O) with 500 μL of 10 μM APF. The blank solution was a mixture of 500 μL of 0.1 M sodium acetate and the same volume of 10 μM APF. Twenty microliters of either a sample or the blank solution were injected manually into the FIA system through a Rheodyne 7125 injector equipped with a 20 μL sample loop.

2.3. *FIA system*

A schematic diagram of the FIA system constructed in this experiment is shown in Figure 2. The two elution pumps were Shimadzu LC-10AS (Shimadzu Corp., Kyoto, Japan). All elution tubing and connectors used were of PEEK™ materials. Fluorescence intensity was monitored using a Shimadzu RF-535 fluorescence detector and the excitation and emission wavelengths were set at a constant 490 and 515 nm, respectively. The mobile phase solutions A and B were 0.1 M sodium acetate and 50 μM DCCA in H_2O, respectively. The flow rate for both solutions was 2.0 mL/min.

2.4. *Preparation of test compounds*

The test compounds dithiothreitol, reduced glutathione, and 3-methyl-1-phenyl-5-pyrazolone (MPP) were all purchased from Nacalai Tesque, Inc.

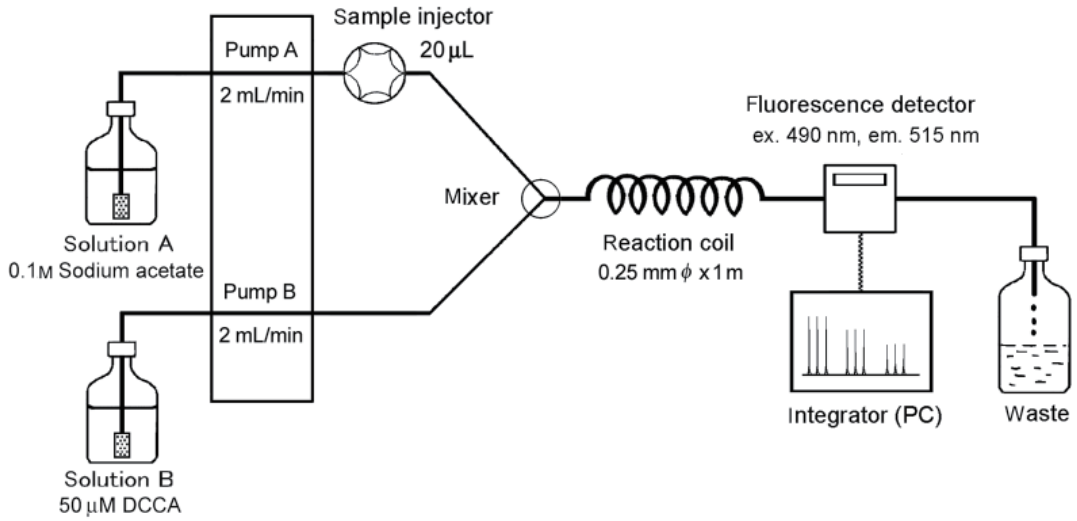

Figure 2. Block diagram of the proposed FIA system.

Quercetin dihydrate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Melatonin, quinolinic acid, and kynurenine were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds were dissolved in H_2O to prepare a 1.0 mM stock solution. In the case of the lipophilic compounds such as the pyrazolone derivatives, a water-soluble organic solvent such as DMF was used. The stock solution was diluted with 0.1 M sodium acetate in H_2O to prepare a 0.05-20 μM solution, and 500 μL of the aliquot was used for the evaluation of anti-oxidant activity by the FIA system described above.

2.5. *Evaluation of anti-oxidant activity*

In the absence of a test compound, the fluorescence peak height obtained in the FIA chart was designated as the blank value (100%) (n = 5). Subsequently, a dose-response curve for each test compound was drawn by plotting the fluorescence peak height at each concentration of test compound against the concentration of the test compound (n = 5). The concentration of the test compound indicating 50% of the blank value was regarded as the EC_{50} of the compound according to previous papers (*23,24*). Thus, in the present study, the EC_{50} value (μM) was evaluated as the anti-oxidant activity of the test compound.

3. Results and Discussion

3.1. *Source of hypochlorite anion*

Among several kinds of ROS such as $HO^{\cdot}$, $O_2^{\cdot -}$, $^1O_2$, H_2O_2, $ONOO^-$, HOCl, and $ROO^{\cdot}$, in the present study, we chose HOCl as the ROS for the present FIA assay. As a source of OCl^-, we first investigated the use of sodium hypochlorite (NaOCl); however, the relative standard deviation (RSD) value of the sample was too large, and therefore stable precise data could not be obtained (data not shown). For this reason, it was speculated that the poor precision may have arisen from the physical properties of NaOCl, because NaOCl is a liquid compound. Commercially available NaOCl is a 5-7% solution, and therefore the concentration is not exact. Thus, DCCA was chosen as the source of OCl^-, because although DCCA is a solid compound, it can release OCl^- in aqueous solution (Figure 1).

3.2. *Effect of pH and kinds of base*

In the present study, APF was used for the ROS indicator. APF fluoresces, which originates from fluorescein in the presence of a hydroxyl radical or HOCl, although APF itself shows no fluorescence (*22*). The responsiveness of APF to HOCl was three times higher than it was to a hydroxyl radical, and therefore a subtle scavenging effect of OCl^- by a test compound could be detected using APF. APF can react with OCl^- to emit intense fluorescence at 515 nm in a basic solution. A pH range for the carrier solution of 8.0-11.0 was therefore recommended. For preparing the carrier solution at pH 8.0-11.0, several bases were examined as to whether sufficient data could be obtained. Table 1 lists the fluorescence peak height and the RSD of each base examined in the present study. As listed in Table 1, the use of 0.1 M sodium acetate (CH_3CO_2Na) in H_2O gave the highest peak height and the smallest RSD values. Therefore, 0.1 M CH_3CO_2Na was used as the carrier solution.

3.3. *Effect of concentrations of APF and DCCA*

Figures 3A and 3B show the change of fluorescence intensity (peak height) as a function of the concentrations of DCCA and APF, respectively. The observed fluorescence peak height increased with increasing concentration of both DCCA and APF. In the preliminary experiment, a decrease of fluorescence peak height was not dependent on the concentration of the test compound at DCCA concentrations above 50 μM in carrier solution B (Figure 2) (data not shown); therefore, the DCCA concentration was set at 50 μM. The obtained peak height was 7.0×10^5 at 5.0 μM APF; therefore, the concentration of 5.0 μM APF was sufficient for the evaluation of EC_{50} values for each test compound. Reproducibility of the FIA system was estimated using relative standard deviations of peak height for each measured point, RSD < 4.5% for DCCA (Figure 3A) and RSD < 4.4% for APF (Figure 3B).

3.4. *Effect of flow rate*

Figure 4 shows a representative FIA chart of reduced glutathione obtained at a flow rate of 2.0 mL/min. The observed peak heights apparently decreased with increasing concentration of reduced glutathione, indicating that the anti-oxidant activity of reduced glutathione could also be evaluated by the present FIA

Table 1. Effects of carrier solution on the fluorescence peak height and RSD (%)

Carrier solution	Mean value of peak height	RSD (%)
0.01 M Sodium phosphate buffer in H_2O (pH 7.4)	3,200	5.3-19.7
0.1 M Sodium acetate in H_2O (pH 8.5)	531,689	0.2-2.7
0.1 M Sodium bicarbonate in H_2O (pH 8.7)	40,700	1.5-8.7
0.1 M Sodium carbonate in H_2O (pH 10.5)	7,250	0.7-5.2

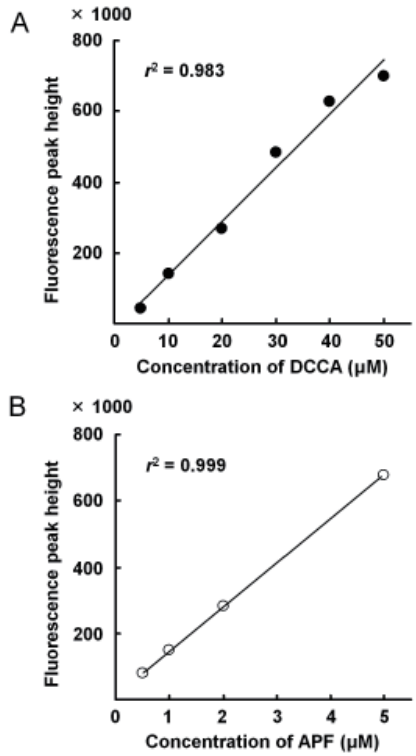

Figure 3. Fluorescence intensity of fluorescein produced from APF as a function of concentration by the present FIA system. Concentration of DCCA **(A)** and APF **(B)**.

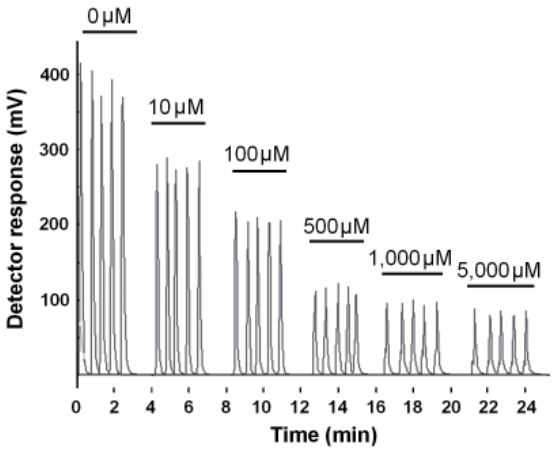

Figure 4. Representative FIA chart of 0, 10, 100, 500, 1,000, and 5,000 μM reduced glutathione, obtained at a flow rate of 2.0 mL/min. Numerals in this figure represent the concentrations of reduced glutathione.

system. When the flow rate was slow (0.5 mL/min), the peak top was divided. Although the reason why the peak shape deteriorated is not yet understood, a clear peak shape would be required for an accurate evaluation. In addition, a higher flow rate can contribute to a reduced analysis time. Thus, considering the peak shape and total analysis time, a flow rate of 2.0 mL/min was chosen in this study.

3.5. *Anti-oxidant activity of test compounds*

Figure 5 shows a representative dose-response curve

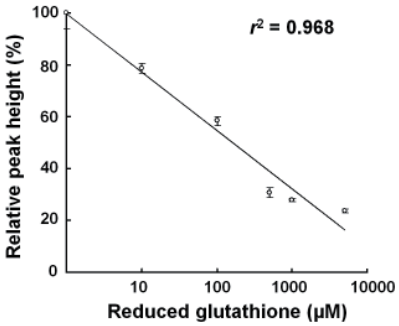

Figure 5. Dose-response curve of reduced glutathione evaluated by the present FIA system.

Table 2. EC_{50} values of test compounds, obtained by the proposed method

Test compounds	EC_{50} (μM)
Ascorbic acid	307.2
Reduced glutathione	162.6
Quercetin dihydrate	1,024
Dithiothreitol	211.1
Edaravone (3-methyl-1-phenyl-5-pyrazolone)	203.1
Quinolinic acid	1,723
Kynurenine	934.2
Melatonin	1,517

of reduced glutathione evaluated by the present FIA system. At concentrations of reduced glutathione above 1,000 μM, the plotted point was excluded from the linear regression line; therefore, only the points within the linear regression line were adopted to determine the EC_{50} value. Similarly, several compounds were tested for anti-oxidant activity using the present FIA system.

In the present study, the test compounds other than reduced glutathione were ascorbic acid, quercetin dihydrate, dithiothreitol, edaravone, quinolinic acid, kynurenine, and melatonin. The EC_{50} values for each compound were calculated and are listed in Table 2. Among them, reduced glutathione exhibited the smallest EC_{50} value. To our knowledge, thus far there have been no reports revealing that the anti-oxidant activity against OCl^- of reduced glutathione was more effective than that of ascorbic acid, which is a well-known antioxidant. In the case of scavenging OCl^- in an *in vitro* experiment, our studies indicated that reduced glutathione might be more effective than ascorbic acid. Based on these studies, it is speculated that reduced glutathione might also be efficacious for scavenging OCl^- *in vivo*.

4. Conclusion

By using APF as an ROS indicator, an FIA system for the evaluation of antioxidants using DCCA as a source of OCl^- was developed. The anti-oxidant activity of several compounds was satisfactorily evaluated by the present FIA system, demonstrating that it can be useful as a screening assay for anti-oxidant activity against OCl^-.

Acknowledgements

The authors gratefully thank Dr. Ishigami, Toho University, for his valuable comments in this study, and Miss Rie Ikeda, Miss Ayako Tsutiya, Miss Kahori Kobayashi, and Mr. Takahisa Kanayama for their technical assistance.

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(Received January 6, 2012; Revised January 27, 2012; Accepted January 28, 2012)