The Generation of Lucigenin Chemiluminescence from the Reaction of Guanidino Compounds with Phenylglyoxal under Alkaline Conditions and Its Application

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It is shown that *o*-carboxyphenylglyoxal, which is converted from ninhydrin by alkali, produces a chemiluminescent lucigenin reaction under alkaline conditions when with reacted with guanidino compounds. It is also demonstrated that phenylglyoxal, which is a model compound of *o*-carboxyphenylglyoxal, produces a strong chemiluminescent lucigenin reaction under alkaline conditions when reacted with guanidino compounds. Moreover, ESR spectra showed the presence of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)–spin adducts of superoxide anions in a mixture of phenylglyoxal and guanidino compounds under alkaline conditions. It was confirmed that the superoxide anions were generated by the reaction of phenylglyoxal with guanidino compounds under alkaline conditions, thereby causing lucigenin chemiluminescence. The chemiluminescent reaction of lucigenin in a mixture of phenylglyoxal and the guanidino compounds was applied to HPLC for guanidino compounds. The present chemiluminescence-HPLC system has a 2-fold greater sensitivity than chemiluminescence-HPLC using ninhydrin. Arginine, guanidine and methylguanidine were detected in serum from a hemodialysis patient with chronic renal failure.

Key words guanidino compound; chemiluminescence; ninhydrin; phenylglyoxal; lucigenin; HPLC

Natural guanidino compounds have been identified in animal tissues.[2—8)] Several guanidino compounds accumulate in the blood of nephritic patients, and some guanidino compounds are candidates for markers of renal dysfunction caused by uremic toxins.[7—11)] HPLC coupled with fluorometry using pre or post column derivatization with an alkaline–ninhydrin reagent has been used to determine the guanidino compounds in the blood of renal failure patients.[10—16)]

Guanidino compounds, substances that have a guanidino group, generate reactive oxygen species.[17)] However, no guanidino compounds, except for creatinine (CTN), activate the chemiluminescent reaction of lucigenin under alkaline conditions. Previously, we found that the addition of lucigenin to an alkaline solution containing ninhydrin and a mixture of guanidino compounds results in an immediate chemiluminescent reaction at room temperature.[18)] It is known that ninhydrin reacts with guanidines under alkaline conditions and results in the production of fluorescent derivatives.[19)] However, it was indicated that the lucigenin chemiluminescence is not generated by the fluorescent derivatives.[18)] Ninhydrin has two different states under alkaline conditions. The triketone ring of ninhydrin is cleaved by alkali to give *o*-carboxyphenylglyoxal, which has a dicarbonyl structure, and then the *o*-carboxyphenylglyoxal is converted to *o*-carboxymandelic acid by excess alkali (Fig. 1).[20)] In the present study, to determine the compound that participates in the generation of the chemiluminescent reaction, phenylglyoxal and mandelic acid were investigated as model compounds of the decomposition products of ninhydrin. Moreover, the elucidated substance, phenylglyoxal, was applied to HPLC with post-column chemiluminescence detection for guanidino compounds.

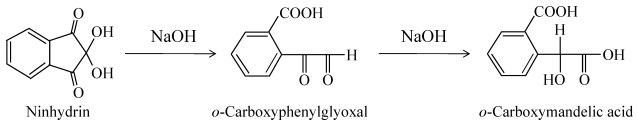

Fig. 1. Decomposition of Ninhydrin under Alkaline Conditions

The reaction of lucigenin with phenylglyoxal and guanidino compounds under alkaline conditions was presumed to produce reactive oxygen species because lucigenin is reported to generate chemiluminescence *via* reactive oxygen species.[21,22)] In the present study, it was confirmed that reactive oxygen species are generated from the reaction of phenylglyoxal and guanidino compounds under alkaline conditions by electron spin resonance (ESR) spectroscopy.

Experimental

Materials Water was purified using a MILLI-Q Labo from Nihon Millipore Kogyo (Yonezawa, Japan). Phenylglyoxal, mandelic acid, lucigenin, sodium 1-octanesulfonate, and creatine (CT) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and guanidino acetic acid (GAA) were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium hydroxide; sodium dihydrogenphosphate dihydrate; and a guanidino compound standard mixture containing arginine (Arg), CTN, guanidine (G), GAA, guanidinobutyric acid (GBA), guanidinopropionic acid (GPA), guanidinosuccinic acid (GSA), methylguanidine (MG), and taurocyamine (TAU) were from Wako Pure Chemical Industries (Osaka, Japan). The 2-propanol of HPLC-grade was from Kanto Chemical (Tokyo, Japan).

Chemiluminescence Detection First, 0.1 ml of 0.195 mmol/l lucigenin aqueous solution, 0.1 ml of 5 mmol/l ninhydrin, phenylglyoxal, or mandelic acid in 2-propanol, 0.5 ml of 10 mmol/l GAA aqueous solution, and 1.8 ml of water were mixed in a cuvette, and then 0.5 ml of 1 mol/l sodium hydroxide aqueous solution was added to the mixture. The emission of lucigenin chemiluminescence was measured using a spectrofluorometer (Model RF-

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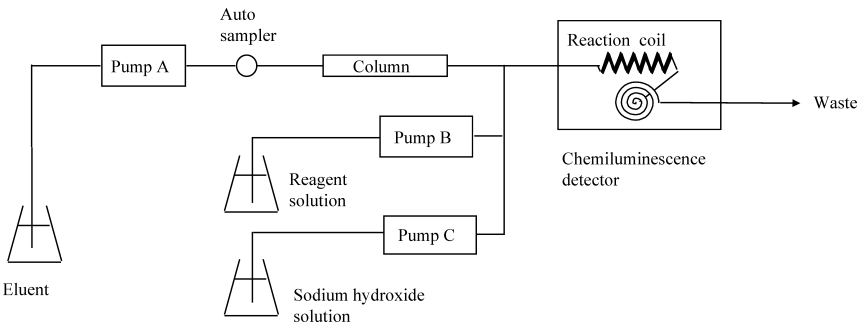

Fig. 2. A Flow Diagram of the HPLC-Chemiluminescent Detection of Guanidino Compounds

510, Shimadzu) at 465 nm, and the lamp was turned off during the measurements.

ESR Conditions Reactive oxygen species were analyzed as spin adducts of DMPO. First, 0.1 ml of 5 mmol/l ninhydrin or phenylglyoxal in 2-propanol, 0.5 ml of 10 mmol/l GAA aqueous solution, and 1.9 ml of water were mixed in a cuvette, and then 0.5 ml of 1.0 mol/l sodium hydroxide aqueous solution was added to the mixture. After being left to stand for 3 min at room temperature, 5 μl of 8.9 mol/l DMPO were added to 200 μl of the reaction mixture, dispersed with a vortex mixer, and immediately transferred to a quartz flat cell for ESR analysis. ESR spectra were measured using a spectrometer (Model JES-REIX, JEOL, Tokyo, Japan). The conditions were as follows: microwave frequency: 9.42 GHz, microwave power: 8.0 mW, magnetic field: 332.6 mT, modulation width: 0.06 mT at 300.0 kHz modulation frequency, response time: 0.3 s, and sweep time: 5.0 mT/2 min at 25 °C.

Apparatus The liquid chromatograph consisted of an HP 1100 liquid chromatograph (Yokogawa Analytical Systems Inc., Tokyo, Japan) with three isocratic pumps (Model G1310A Iso Pump, Yokogawa), an autosampler (Model G1313A ALS, Yokogawa), and an A/D converter (Model HP 35900E Dual Channel Interface, Yokogawa). Detection was performed with a chemiluminescence detector (Model CLD-10A, Shimadzu Seisakusho, Kyoto, Japan). The host computer was a Vectra XM series 3 16/90 personal computer (Yokogawa) equipped with Chem Station chromatographic software (Yokogawa).

Chromatographic Conditions Figure 2 shows a flow diagram of the chemiluminescence-HPLC system for the analysis of the guanidino compounds. The eluent was a mixture of 5 mmol/l sodium dihydrogenphosphate solution (pH 4.6) : 2-propanol mixture (85 : 15 v/v%) and 5 mmol/l 1-octanesulfonic acid sodium salt. It was delivered from pump A at a flow rate of 1.0 ml/min. A 5 μl sample was injected *via* the autosampler. The guanidino compounds that formed ion pairs with 1-octanesulfonic acid were separated in the octadecyl silica (ODS) column (Senshu Pak PEGASIL ODS, 150×4.6 mm i.d., particle size 5 μm). The eluate from the separation column was mixed with reagent solution (5 mmol/l phenylglyoxal and 0.02 mmol/l lucigenin in a water–2-propanol mixture, 98 : 2 v/v%) and 500 mmol/l sodium hydroxide aqueous solution delivered from pumps B and C at a flow rate of 0.5 ml/min. The mixture was reacted in a coil (5 m×0.5 mm i.d.) and placed in a detector at 25 °C, and the chemiluminescence was monitored with the chemiluminescence detector. The sample solution was obtained by deproteinization of serum according to the method reported by Kobayashi *et al.*[15] A 100 μl sample of serum was vortex-mixed with 20 μl of 20% trichloroacetic acid solution for a few seconds. The mixture was centrifuged at 10000 rpm for 2 min. A 60 μl aliquot of the supernatant was mixed with 15 μl of 0.4 mol/l sodium hydroxide solution and the solution was adjusted to about pH 2.5—3.0. The serum sample of chronic renal failure patient on hemodialysis was included after informed consent had been obtained.

Results and Discussion

The Chemiluminescent Reaction of Lucigenin Induced by a Mixture of GAA and Phenylglyoxal under Alkaline Conditions The addition of lucigenin to an alkaline solution containing ninhydrin and a mixture of guanidino compounds resulted in a chemiluminescent lucigenin reaction.[18]

Table 1. The Relative Lucigenin Chemiluminescence Produced by the Reaction of Ninhydrin, Phenylglyoxal, or Mandelic Acid with GAA under Alkaline Conditions

	Relative lucigenin chemiluminescence intensity
Ninhydrin	100
Phenylglyoxal	189
Mandelic acid	N.D.

The reaction mixture contained 0.17 mmol/l ninhydrin, phenylglyoxal, or mandelic acid; 1.7 mmol/l GAA; 6.5 μmol/l lucigenin; and 170 mmol/l sodium hydroxide in 3.3% 2-propanol. The detection of lucigenin chemiluminescence was started by the addition of sodium hydroxide solution. N.D.: not detected.

During this reaction, there are two possible states of ninhydrin under alkaline conditions. Ninhydrin is converted to *o*-carboxymandelic acid *via* *o*-carboxyphenylglyoxal under alkaline conditions by excess alkali.[20]

Phenylglyoxal and mandelic acid, which are model compounds of *o*-carboxyphenylglyoxal and *o*-carboxymandelic acid, mixed with GAA were tested for theirs effects on the chemiluminescent reaction of lucigenin under alkaline conditions (Table 1). Phenylglyoxal was found to produce stronger lucigenin chemiluminescence than ninhydrin. However, mandelic acid did not produce lucigenin chemiluminescence. This finding indicates that the reaction of *o*-carboxyphenylglyoxal with guanidino compounds under alkaline conditions generates a chemiluminescent lucigenin reaction.

Production of Reactive Oxygen Species To elucidate the kind of reactive oxygen species generated by the reaction of phenylglyoxal and GAA under alkaline conditions, ESR spectra were measured. A signal was observed from the DMPO–spin adduct of superoxide anions produced by the reaction of phenylglyoxal with GAA under alkaline conditions (Fig. 3a). The difference in the signal of the DMPO–spin adduct produced by phenylglyoxal without GAA under alkaline conditions was caused by the presence of hydroxyl radicals (Fig. 3b). It was found that superoxide anions are generated by the reaction of phenylglyoxal with GAA under alkaline conditions. Therefore, these findings indicate that the superoxide anions generated by the reaction of phenylglyoxal and guanidino compounds under alkaline conditions result in lucigenin chemiluminescence (Fig. 4).

The Spectrum of Chemiluminescence Produced by the Reaction of Phenylglyoxal and GAA with Lucigenin under Alkaline Conditions The chemiluminescence spectrum produced by the reaction of phenylglyoxal and GAA in the presence of lucigenin under alkaline conditions was de-

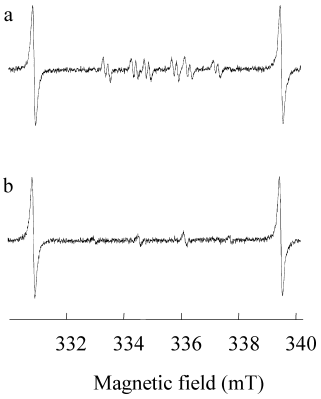

Fig. 3. The ESR Spectra of the DMPO–Spin Adducts of the Superoxide Anions and Hydroxyl Radicals from Phenylglyoxal with (a) or without (b) GAA in Alkaline Solution

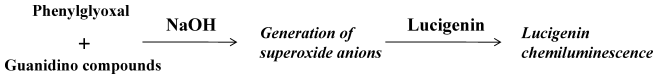

Fig. 4. The Generation of Lucigenin Chemiluminescence from the Reaction of Phenylglyoxal with Guanidino Compounds under Alkaline Conditions

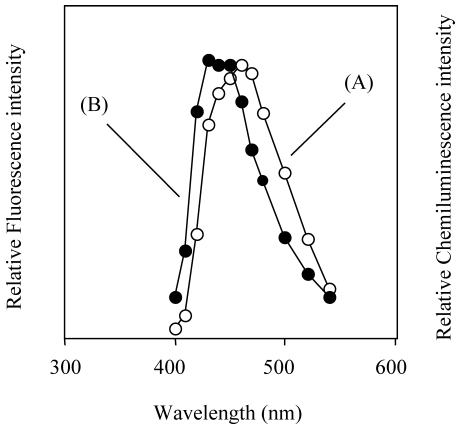

Fig. 5. The Chemiluminescent Spectrum of Lucigenin Produced by the Reaction of Phenylglyoxal and GAA under Alkaline Conditions (A) and the Fluorescent Spectrum of Lucigenin Solution under Alkaline Conditions at an Excitation Wavelength of 366 nm (B)

The reaction mixtures for the fluorescent spectrum were the same as in (A) except that they did not contain phenylglyoxal and GAA. The chemiluminescent and fluorescent intensity of the reaction mixture after 3 min was measured for each wavelength.

termined (Fig. 5A). This spectrum, which peaks at 466 nm, corresponds to the fluorescence spectrum of lucigenin solution under alkaline conditions at an excitation of 366 nm (Fig. 5B). This indicates that the chemiluminescence from the reaction of phenylglyoxal and GAA in the presence of lucigenin under alkaline conditions is due to the luminescence of lucigenin.

Chemiluminescence-HPLC for Guanidino Compounds We applied the chemiluminescent reaction of lucigenin with phenylglyoxal and guanidino compounds under alkaline conditions to HPLC for guanidino compounds. The chromatographic conditions were optimized using a standard solution of guanidino compounds. The optimal concentration of reagents for the chemiluminescent reaction was investigated over the range 0.2—10 mmol/l for phenylglyoxal, 0.004—0.027 mmol/l for lucigenin, and 200—800 mmol/l for sodium

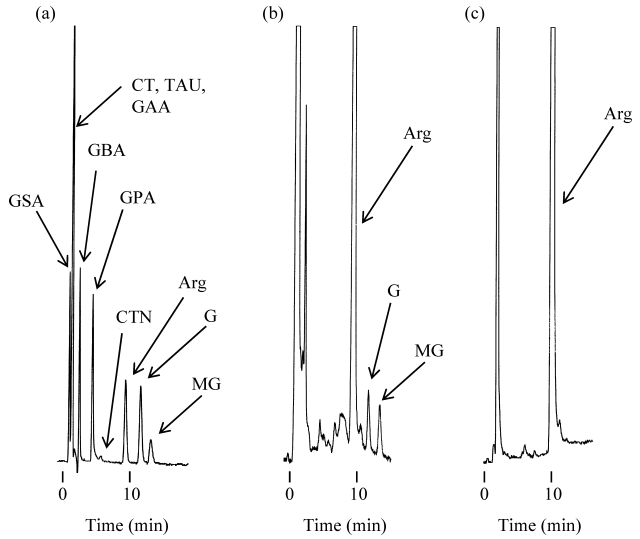

Fig. 6. A Chromatogram of a Standard Mixture of Guanidino Compounds (GAA, GPA, and MG: 50 pmol/Injection; CT, GBA, GSA, and TAU: 100 pmol/Injection; Arg and G: 200 pmol/Injection; CTN: 500 pmol/Injection) (a), a Chromatogram of the Serum of a Chronic Renal Failure Patient Receiving Hemodialysis (b), and a Chromatogram of a Normal Healthy Volunteer Serum (c)

hydroxide. The maximum peak areas for the chemiluminescence of all guanidino compounds were obtained at concentrations of 5 mmol/l phenylglyoxal, 0.02 mmol/l lucigenin, and 500 mmol/l sodium hydroxide. The effect of a 2-propanol dissolving reagent was examined over the range 0—8% (v/v). The addition of 2-propanol was effective at inducing the chemiluminescent reaction, and the maximum peak area was observed at a concentration of 2% (v/v). The reaction coil length and temperature were investigated over the range 4—8 m and 20—35 °C. The maximum peak area was observed at a coil length of 5 m and a temperature of 25 °C. The eluent composition and the concentration (5 mmol/l sodium dihydrogenphosphate solution and 5 mmol/l 1-octanesulfonic acid sodium salt) were selected because they did not affect the chemiluminescent reaction.

Figure 6a shows a chromatogram of the 10 guanidino compounds obtained using chemiluminescent detection, and GSA, GBA, GPA, CTN, Arg, G, and MG were clearly separated and detected. The detection limits (S/N=3) were as follows: GPA and MG: 3.8 pmol; Arg, G, GSA, and GBA: 7.5 pmol; CTN: 37.5 pmol. The present chemiluminescence-HPLC system has a 2-fold greater sensitivity than chemiluminescence-HPLC using ninhydrin.[18] Each guanidino compound showed a linear response in the range of 10—250 pmol (creatinine: 50—500 pmol). The coefficients of variation from 5 analyses of the guanidino compound standard mixture were as follows: GPA and MG (25 pmol): 8.6% and 7.4%, GSA and GBA (50 pmol): 2.2% and 2.9%, Arg and G (100 pmol): 1.7% and 2.3%, and CTN (250 pmol): 1.9%.

Figure 6b shows a chromatogram of serum from a hemodialysis patient with chronic renal failure. Arg, G, and MG were detected clearly, but G and MG were not found in the serum of a normal healthy volunteer (Fig. 6c). The concentrations of G and MG in the patient (Fig. 6b) were 2.9 μmol/l and 2.4 μmol/l, respectively. They were in accor-

dance with the values reported previously.[3] The present method is easier and simpler than fluorophotometric detection using post column fluorescent derivatization as it does not require the derivatization of the guanidino compounds.

Conclusion

It was found that the reaction of phenylglyoxal with guanidino compounds under alkaline conditions generates strong chemiluminescent lucigenin reactions; however, mandelic acid is not involved in the generation of lucigenin chemiluminescence. The lucigenin chemiluminescence intensity produced by phenylglyoxal was higher than that produced by ninhydrin. Moreover the chemiluminescent reaction of lucigenin is caused by the generation of superoxide anions *via* the interaction of phenylglyoxal with guanidino compounds. These findings are useful for the development of more sensitive chemiluminescence-HPLC methods for guanidines.

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