

Note

Natural Antioxidant, Chlorogenic Acid, Protects Against DNA Breakage Caused by Monochloramine

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Chlorogenic acid prevented a stepwise conversion of plasmid pUC18 DNA, form I→form II→form III, induced by 3 mM monochloramine with a half inhibition of 67.4 μM. Chlorogenic acid reacted with monochloramine in a time-dependent manner, and the reaction rate increased with decreasing pH. These results suggest that chlorogenic acid prevents genotoxicity of monochloramine in gastric mucosa.

Key words: chlorogenic acid; DNA damage; gastric injury; monochloramine; natural antioxidant

Chlorogenic acid (3-caffeoyl-D-quinic acid; CGA) is an ester formed between caffeic acid and quinic acid, and is widely present in various agricultural products¹⁾ in substantial quantities: 3.4–14 mg/100 g fresh weight in several varieties of potatoes, 12–31 mg/100 ml of apple juice, 559–674 mg/100 g of dry tea shoots and 250 mg in a cup of coffee. CGA as an antioxidant prevents haemolysis and lipid peroxidation of erythrocytes induced by H₂O₂.¹⁾ Recently, we have reported that CGA has abilities to scavenge a nitrosating agent, nitrogen sesquioxide (N₂O₃) with a rate constant of $4.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$,²⁾ and to react with hypochlorous acid (HClO).³⁾ The second order rate constants of CGA with reactive species of oxygen and nitrogen, including O₂⁻, ·OH, ONOOH and peroxy radical, were measured.⁴⁾ These results led us to conclude that CGA is an excellent natural scavenger among many polyphenol compounds, because the one-electron oxidation product of CGA formed by the reaction with free radicals is rapidly broken down to further products that cannot generate any free radicals. This is a beneficial nature for an antioxidant, because other electron donors are not necessary for the reduction of one-electron oxidation product of CGA to end the radical reaction.

Chlorine-ammonia (*N*-chloramines) has been traditionally used for the disinfection of drinking water. *N*-chloramines are produced *in vivo* by the reaction between amines with HOCl,⁵⁾ which is the oxidation product of chloride by H₂O₂ catalyzed by myeloperoxidase in activated neutrophils.⁶⁾ Monochloramine (NH₂Cl) is specially interesting since it is lipophilic, freely penetrating the hydrophilic barrier of biological membranes,⁷⁾ and thus oxidizes membrane and intracellular components. NH₂Cl is a more stable and more potent oxidant⁸⁾ than H₂O₂ and HOCl, and may be of principal

importance as a mediator of the pathophysiology; bactericidal,⁹⁾ and mutagenic actions,^{10,11)} inhibitory effect on hexose-monophosphate pathway,¹²⁾ oxidations of hemoglobin and thiol,^{13,14)} and cytotoxicity.¹⁵⁾ NH₂Cl may be 50 times more effective than H₂O₂, HOCl and taurinemonochloramine act as oxidants for erythrocyte hemoglobin, but HOCl is 10 times more effective than NH₂Cl as a lytic agent.¹³⁾ Current interest is focused on the participation of NH₂Cl in *Helicobacter pylori*-induced gastric mucosal lesions.¹⁶⁾ *H. pylori* has a strong urease activity to produce ammonia, which can react with HOCl to form NH₂Cl.¹⁷⁾ NH₂Cl induces DNA damage in gastric cells,^{18,19)} suggesting its possible involvement in gastric carcinogenesis. Since CGA effectively scavenges many oxidants, including O₂⁻, ·OH, HOCl, ONOO⁻, peroxy radical, and N₂O₃,²⁻⁴⁾ it was of interest to examine the effects of CGA on DNA breakage induced by NH₂Cl. This study shows that NH₂Cl caused plasmid DNA breakage, and that a natural antioxidant, CGA, prevented it caused by NH₂Cl.

Figure 1A shows typical gel electrophoretic patterns indicating the double-strand plasmid DNA breakage by NH₂Cl, and the protection against the DNA breakage by CGA. Clearly NH₂Cl changed the form of plasmid DNA, causing a stepwise conversion; form I (supercoiled DNA)→form II (nicked circular DNA)→form III (linear duplex DNA). Bands of form II and form III, but not form I, disappeared with additional heat treatment (100°C, 3 min) (data not shown), indicating that the forms II and III were composed of circular and linear DNAs nicked in many points produced by NH₂Cl, respectively.

CGA itself did not affect the conversion of supercoiled DNA (lane 3). The form II observed in lanes 2 and 3 is present also in the purchased preparation. Even with 10 μM CGA (lane 5), form III could not be detected, and with increasing concentration of CGA, form II band decreased gradually. Maximum protection of 90% was observed with 560 μM CGA. A replot of these data (Fig. 1, B) showed that $(100 - \% \text{inhibition})^{-1}$ was proportional to the concentration of CGA ($r=0.998$), indicating that CGA and DNA compete with NH₂Cl, and the concentration of CGA required for half protection was found to be 64.7 μM.

NH₂Cl has an absorption peak at 242 nm (Fig. 2, line 1), while CGA does 324 nm (line 2). The spectrum of CGA (68 μM) was altered in a time-dependent manner

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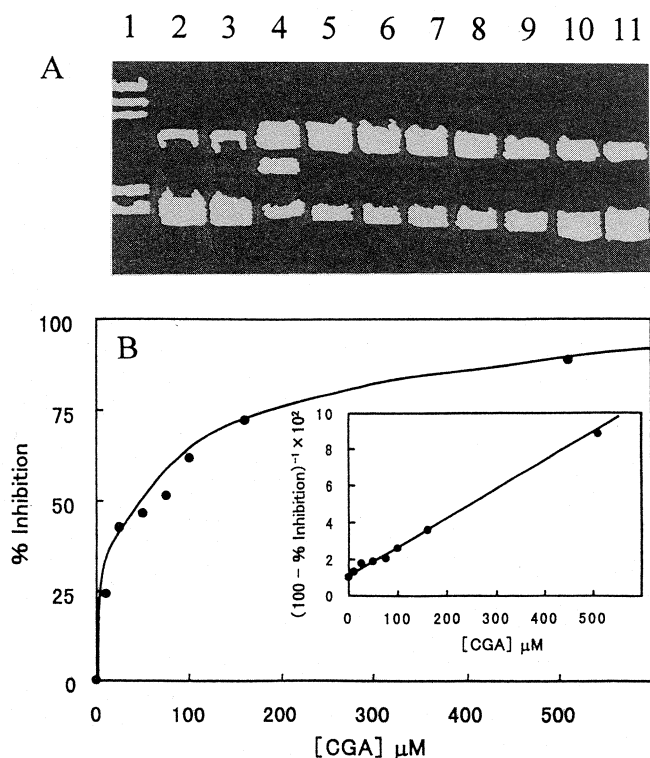


Fig. 1. Prevention by CGA of pUC18 DNA Breakage Induced by NH_2Cl .

Plasmid pUC18 DNA ($0.1 \mu\text{g}$) was treated with NH_2Cl in the presence of various concentrations of CGA for 2 h at 37°C in a total volume of $12 \mu\text{l}$ containing 167 mM K-phosphate at pH 7.0. After the incubation, $20 \mu\text{l}$ of loading buffer containing 0.05% bromophenol blue and 10% glycerol was added. The mixture was mixed for 5 s by a Vortex mixer. Electrophoresis was done by using 1% agarose gel containing $0.5 \mu\text{g/ml}$ ethidium bromide in Tris-borate buffer. A, Electrophoretic patterns of pUC18 after the treatment with CGA, or NH_2Cl in the presence of various concentrations of CGA. Lane 1, $\lambda/\text{HindIII}$ digest; lane 2, pUC18 DNA without treatment (control); lane 3, pUC18 plus 1.23 mM CGA without NH_2Cl ; lane 4, pUC18 DNA plus 3 mM NH_2Cl . CGA was added to the solution containing pUC18 and 3 mM NH_2Cl at 0.01 mM (lane 5), 0.025 mM (lane 6), 0.05 mM (lane 7), 0.075 mM (lane 8), 0.1 mM (lane 9), 0.16 mM (lane 10), and 0.56 mM (lane 11). For the preparation of NH_2Cl , a solution of 1 mM NaClO in 50 mM K-phosphate, pH 9 was added into a 100 mM $\text{NH}_3\text{-NH}_4\text{Cl}$ mixture, pH 9.1. The molar ratio of $\text{NH}_3\text{-NH}_4\text{Cl}/\text{NaClO}$ was 10, under which neither free chlorine nor dichloramine was detected, as reported²⁵ that the excess amount of ammonium and high pH suppressed the hydrolysis of NH_2Cl and formation of other chloramines. The concentration of NH_2Cl was measured spectrophotometrically²⁶ using a molar extinction coefficient; $\epsilon_{242 \text{ nm}} = 429 \text{ M}^{-1} \text{ cm}^{-1}$. B, Protection of DNA breakage as a function of CGA concentration. The change of DNA forms obtained in part A was estimated by densitometric scanning (*pd* 4200eTM scanner). The % inhibition of DNA breakage by CGA was calculated by the following equation,

$$\frac{\text{area of form I (control)} - \text{area of form I (treated with } \text{NH}_2\text{Cl} + \text{CGA)}}{\text{area of form I (control)} - \text{area of form I (treated with } \text{NH}_2\text{Cl)}} \times 100$$

upon the addition of 2 mM NH_2Cl . The time-dependent decrease in absorbance at 324 nm was observed, and obeyed pseudo first-order kinetics; a straight line was obtained by plotting of $\ln A_t$ versus t , where $\ln A_t$ is the absorbance at 324 nm at time t (sec). From the slope of the

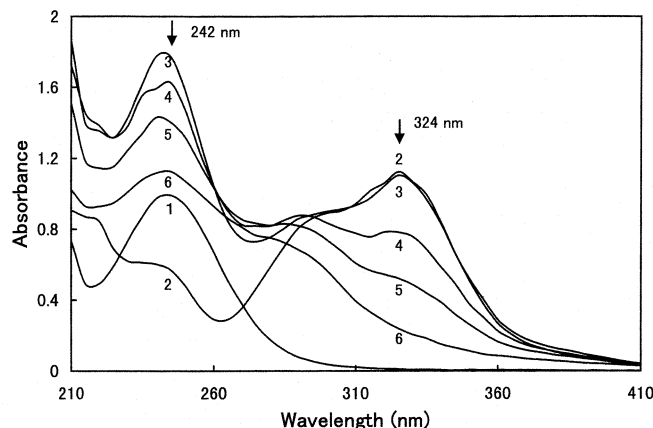


Fig. 2. Spectral Change of CGA Induced by the Addition of NH_2Cl .

The reaction mixture contained 2 mM NH_2Cl (line 1) or $68 \mu\text{M}$ CGA (line 2) in 50 mM K-phosphate, pH 7.0. The spectral changes were recorded after the addition of 2 mM NH_2Cl to $68 \mu\text{M}$ CGA at 15 sec (line 3), 30 min (line 4), 60 min (line 5), and 120 min (line 6).

line, the second-order rate constant of the reaction of CGA with NH_2Cl at pH 7.0 was estimated to be $1.88 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$. The rates increased with decreasing pH; the second-order rate constants were estimated to be 1.17×10^{-1} , 3.10×10^{-2} , and $2.16 \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$, at pH 4.0, pH 6.0, and pH 8.0, respectively.

This study showed that CGA effectively prevents the strand breakage of supercoiled DNA by NH_2Cl . Although no exact concentration of NH_2Cl or HOCl in gastric mucosa is known, in the stimulated neutrophils ($4 \times 10^6/\text{ml}$) by the incubation for 1 h at 37°C with phorbol 12-myristate 13-acetate, the concentrations of HOCl ,⁷ NH_2Cl ,¹³ and hydrophilic RNHCl derivatives⁷ are estimated to be $80 \mu\text{M}$, $20 \mu\text{M}$, and $30 \mu\text{M}$, respectively. In other reports,^{16,20} HOCl and NH_2Cl are produced by activated neutrophils in the range of $0.1\text{--}0.6 \text{ mM}$. NH_2Cl at 0.12 and 0.5 mM could convert 13% and 20% of pUC18 plasmid DNA from form I to form II, respectively (data not shown).

In addition to the NH_2Cl -inducible DNA damage in gastric cells,^{18,19} mucosal damage related to *H. pylori* may be partly caused by reactive intermediates of oxygen produced by activated neutrophils.²¹ Higher levels of oxidative DNA damage, the content of 8-hydroxydeoxy-guanaosine, in gastric mucosal DNA of *H. pylori*-positive group than that of the *H. pylori*-negative group²² indicated that reactive intermediates of oxygen were persistent in gastric mucosa due to *H. pylori* infection and could transform the chronic gastritis into gastric carcinoma. Together with a good ability of CGA to scavenge HOCl ³ as well as intermediates of oxygen,⁴ and to react with NH_2Cl more rapid at acidic pH than at neutral pH, CGA in diets may be an excellent protector for *H. pylori*-induced, and chlorinated drinking water-induced gastric mucosal lesions. However prooxidant activities of CGA and caffeic acid depending on a copper-redox cycle mechanism have been reported.^{23,24}

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