Role of Hydrogen Peroxide in Bactericidal Action of Catechin

Hidetoshi ARAKAWA,*[,a] Masako MAEDA,[a] Sachie OKUBO,[b] and Tadakatsu SHIMAMURA[b]

[a] *School of Pharmaceutical Sciences, Department of Analytical Chemistry, Showa University; and* [b] *School of Medicine, Department of Microbiology, Showa University; Shinagawa-ku, Tokyo 142–8555, Japan.*
Received October 6, 2003; accepted December 4, 2003

Catechin (epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC) and epigallocatechin gallate (EGCg)), which occur in green tea and black tea, possess strong bactericidal action. We observed a reactive oxygen species that was generated from the catechins as the active mechanism: and this reactive oxygen was identified. EGCg reacted with the dissolved oxygen in aqueous solution, resulting in the generation of hydrogen peroxide. Hydrogen peroxide production derived from EGCg rose with increasing pH. EGCg (0.22 mmol/l) in neutral solution (0.1 mol/l phosphate buffer pH 7.0: PBS) quantitatively generated 0.2 mmol/l hydrogen peroxide after 60 min incubation. The bactericidal effect of EGCg is dependent on hydrogen peroxide levels produced by EGCg; moreover, EGCg action was inhibited by treatment with catalase. Both bactericidal effects correlated closely when the effects of EGCg and hydrogen peroxide for the bacterium (9 of 10 kinds of bacterial strains) were examined. Therefore, hydrogen peroxide, which is generated by EGCg, appears to be involved in the bactericidal action of EGCg.

Key words catechin; epigallocatechin gallate; hydrogen peroxide; reactive oxygen; bactericidal action; tea

Recently green tea, which is the traditional drink of Japan and China, has been recognized as healthful. Catechins, which are polyphenol chemical compounds found in abundance in green tea, possess physiological effects including antioxidative and bactericidal action as well as antitumor activity. Four main catechins are contained within green tea: epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC) and epigallocatechin gallate (EGCg). These catechins display strong antioxidant activity,[1] which efficiently eliminates reactive oxygen species. The antioxidant mechanism is believed to involve radical elimination by the phenolic hydroxyl group of the catechin structure.[2] EGCg and EGC in catechin have demonstrated anti-tumor growth and a strong apoptosis induction effect in the human lung tumor cells H661 and H1299 and the induction was inhibited by catalase.[3] This paper indicates that hydrogen peroxide participated in the apoptotic action of EGCg; however, the chemical mechanism has not been clarified.

On the other hand, we noted previously that catechins exhibit a wide antibacterial spectrum of gram-negative bacteria and positive fungus in terms of germicidal action.[4] The structure–activity relationship of catechin and bactericidal activity occurs in the following order: ECg>EGCg>EGC>EC.[5] Furthermore, the gallate and pyrogallol moieties are necessary with respect to this activity. Investigation of EGCg uptake of liposomal membranes[6] and ultra-structure change on sterilization of trychophyton employing electron microscopy reveals that the action is based on the membrane injury action of bacterial cells.[7] Moreover, it has been reported that the bactericidal effect of EGCg is stronger for gram-positive bacteria than for gram-negative bacteria due to the difference in the amount of EGCg absorbed by the bacterial cell. EGCg was thought to carry a net negative charge in aqueous solution (pH 6—7); furthermore, it was believed to bind to the membrane component of bacteria as a result of electrostatic interaction, which led to membrane damage.[8] However, chemical elucidation of the mechanism *via* which EGCg causes membrane injury following binding to the liposome remains unclear. We previously established a highly sensitive analytical method for catechin using peroxalate chemiluminescent reaction.[9] The principle is based on the reaction mechanism in which catechin reacts with dissolved oxygen in basic solution, resulting in the generation of active oxygen. In this study, we established that the reactive oxygen produced by catechin was hydrogen peroxide *via* chemiluminescent methodology and electron spin resonance (ESR) using a spin-trapping method. We believed that the hydrogen peroxide generation ability of EGCg was a central component with respect to bactericidal activity; consequently, examination was conducted in detail regarding the role of hydrogen peroxide-mediated bactericidal activity of EGCg and the effect of bactericidal activity of EGCg and hydrogen peroxide in terms of gram-negative (9 of 10 kinds of bacterial strains) and gram-positive bacteria (2 of 3 kinds of bacterial strains).

MATERIALS AND METHOD

Reagents employed in the experiments included epigallocatechin-3-gallate (EGCg), epicatechin-3-gallate (ECG), (+)-catechin (CC), sodium barbital, acetate ester, dipotassium hydrogenphosphate, disodium hydrogenphosphate, dimethyl sulfoxide (DMSO), 30% hydrogen peroxide, diethylenetriamine penta acetic acid (DETAPAC) and were from Wako Pharmaceutical Industries, Inc. 8-Anilinonaphthalene sulphonate ammonium salt (ANS) was from MERCK. Bis(2,4,6-trichlorophenyl)oxalate (TCPO) was from Tokyo Chemical Industries, Inc. Bovine serum albumin (BSA), catalase and superoxide dismutase (SOD) were from Sigma Ltd.

Preparation of Standard Solutions for Catechins and Hydrogen Peroxide Standard solutions of CC, ECG and EGCg (1×10^{-3} mol/l) in H_2O were prepared and serially diluted with H_2O. A stock solution of hydrogen peroxide (0.1 mol/l) in H_2O was prepared and stored at 4 °C until use. The working solution was serially diluted with H_2O immediately prior to use.

Preparation of Sample Solution for Detection of Hy-

* To whom correspondence should be addressed. e-mail: arakawa@pharm.showa-u.ac.jp

drogen Peroxide in Tea Infusion Tea leaves (125 mg) were brewed with 25 ml distilled water (80 °C) for 5 min. Filtered DISMIC-25c extracts (10 μl) were diluted with 90 μl distilled water; subsequently, extracts were measured with the peroxalate chemiluminescence detection system. The concentration of hydrogen peroxide in the infusion was determined employing the standard curve obtained with diluted hydrogen peroxide solution.

Identification of Reactive Oxygen A sample was prepared for reactive oxygen identification. One hundred μmol/l of EGCg sample and hydrogen peroxide in 50 mmol/l phosphate buffer (PB, pH 7.8) were incubated for 30 min at 37 °C. One mmol/l CC in 0.05 mol/l NaOH was incubated for 30 min at 37 °C; subsequently, the solution (10 μl) was diluted with 90 μl of 50 mmol/l PB (pH 7.8). Scavenging enzymes of the reactive oxygen were added to EGCg, green tea infusion and hydrogen peroxide as follows: (1) O, (2) 100 U/ml, (3) 32 μg/ml SOD. Sample solutions were then incubated for 30 min at 37 °C, followed by 10-fold dilution with 50 mmol/l PB (pH 7.8). These sample solutions were measured using the peroxalate chemiluminescence method and ESR.

Peroxalate Chemiluminescence Detection Method for Hydrogen Peroxide Sample solution (100 μl) was mixed with 100 μl ANS solution (pH 9.0), which was prepared from 0.02% ANS, 0.1% BSA, 0.2 mol/l barbital and 200 μl of 5 mmol/l TCPO in ethyl acetate solution. Intensity of luminescence was measured for 15 s after a period of 6 s following mixing of the sample solution with a Luminescence Reader BLR-201 (Aloka).

Measurement of ESR Spectrum Fifty mmol/l PB (70 μl, pH 7.8) was mixed with 10 μl of 1 mmol/l DETAPAC solution, 10 μl of 0.5 mmol/l ferrous sulfate solution and 100 μl of sample solution. The sample solution was added strictly within 1 min following introduction of the ferrous sulfate solution. Reaction was conducted at room temperature. The reacted solution was subsequently placed within the ESR cell cavities; the spectrum was measured 40 s after the mixing step. ESR conditions were as follows on a JES-FE-2XG ESR spectrometer (Japan Electronics, Inc.): microwave power 8 mW, modulation frequency 100 Hz, modulation amplitude 0.1 mT, response time 0.03 s, gain $\times$200, sweep time 10 mT/min.

Bacterial Strains and Culture Conditions Bacterial strains used were *Escherichia* (*E.*) *coli* ATCC 25922, *Bordetella* (*B.*) *bronchiseptica* IID929, *Serratia* (*S.*) *marcescens* IID5518, *Klebsiella* (*K.*) *pneumoniae* IID5207, *Salmonella choleraesis serovar* (*S.*) *enteritidis* 87-350, *Pseudomonas aeruginosa* (*Ps. aeruginosa*) 42. *Staphylococcus* (*S.*) *aureus* ATCC25923, methicillin-resistant *Staphylococcus aureus* (MRSA) F-51, *Bacillus* (*B.*) *subtilis* ATCC 6623, and *Proteus* (*P.*) *mirabilis*.

Bacterial cells were grown overnight in 3 ml of peptone broth (pH 7.2) at 37 °C. The culture solution was diluted with 100 mmol/l phosphate buffer saline (PBS, pH 7.0) and the bacteria (1×10^6) were used in the following experiment.

Effect of Catalase on Bactericidal Activity of EGCg One hundred μl of EGCg solution (0.1 mg/ml in pH 7.0 PBS, corresponding to a final concentration of 0.22 mmol/l) was incubated for 30 min at 37 °C; subsequently, 55 μl catalase (0, 10 U, 100 U) was introduced and incubated for 30 min at 37 °C. After the incubation, reaction mixtures were boiled for 5 min to inactivate catalase, followed by cooling on ice. Preparations were then mixed with 845 μl of bacterial suspensions containing *E. coli* ATCC 25922 in PBS (approximately 10^6 CFU) and then were cultured overnight at 37 °C. A 100 μl volume of samples were spread on nutrient agar plates (Eiken Chemical Co., Tokyo, Japan). Following incubation overnight at 37 °C, the number of colonies on the plates was counted. Data are presented as CFU/ml in the form of graphs.

Effect of EGCg and H_2O_2 on Bactericidal Activity EGCg (100 μl) or H_2O_2 solution (0.22 mmol/l in pH 7.0 PBS; control is buffer) was mixed with 900 μl bacterial suspension (*E. coli* ATCC 25922, MRSA F-51, *B. bronchiceptica* IID929, *S. aureus* ATCC25923, *S. marcescens* IID5518, *K. pneumoniae* IID5207, *B. subtilis* ATCC 6623, *S. enteritidis* 87-350, *Ps. aeruginosa* 42, *P. mirabilis*) in PBS (approximately 10^6 CFU). The resulting mixtures were cultured overnight at 37 °C. A 100 μl volume of each sample was spread on nutrient agar plates; subsequently, following incubation overnight at 37 °C, the number of colonies on the plates was counted as described above.

RESULTS AND DISCUSSION

Generation of Hydrogen Peroxide by Catechin and Its Identification We established previously a highly sensitive analytical method for catechin using the peroxalate chemiluminescence method. The principle is based on the reaction in which catechin is oxidized by dissolved oxygen in basic solution, leading to the generation of reactive oxygen, which is subsequently detected by a chemiluminescent technique using TCPO. EGCg, EGC and epicatechin (EC) could be measured within a range of 10^{-7}—10^{-3} mol/l by this method. The reactive oxygen generated by EGCg was identified with ESR employing catalase and superoxide dismutase as scavenging enzymes of reactive oxygen. The results of ESR for hydrogen peroxide, EGCg and green tea infusion are shown in Fig. 1. The reactive oxygen species produced appeared to be hydrogen peroxide; consequently, spin-trapping ESR methodology (DMPO) including the Fenton reaction was applied. As a result, strong signals of OH radical were observed in the cases

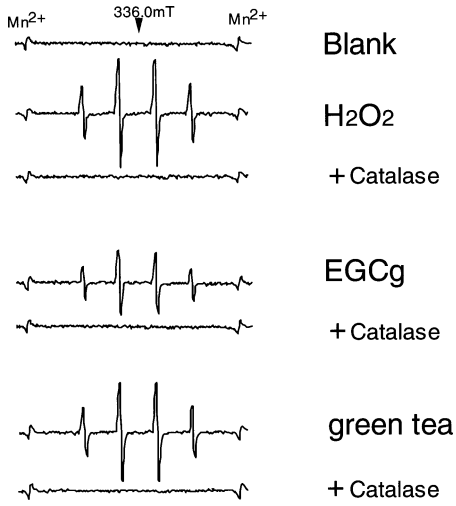

Fig. 1. ESR Spectra of H_2O_2, EGCg, and Green Tea Infusion without and with Catalase 100 U/ml

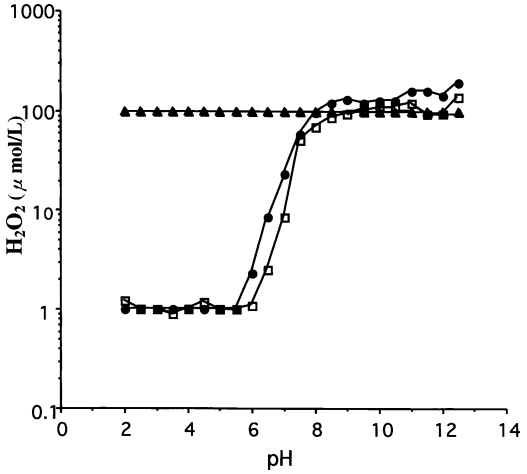

Fig. 2. Effect of pH on Hydrogen Peroxide Production in EGCg Solution and Green Tea Infusion

EGCg, green tea and H_2O_2 solution were incubated for 30 min at 37 °C under various pH conditions and measured by chemiluminescent assay. H_2O_2 solution was used as a control. ▲, 0.1 mmol/l H_2O_2 solution; ●, 0.1 mmol/l EGCg in 0.1 mol/l phosphate buffer; □, green tea infusion in 0.1 mol/l phosphate buffer.

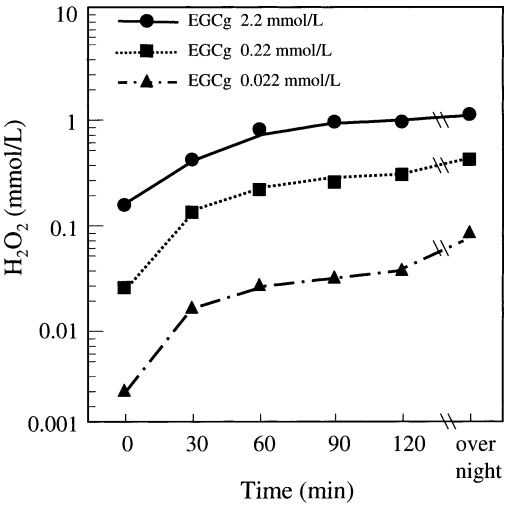

Fig. 3. Effect of Incubation Time on Hydrogen Peroxide Production in Various Concentrations of EGCg

of green tea infusion and EGCg. These ESR signals completely disappeared by addition of catalase. On the other hand, the signals were unchanged by addition of 32 μg/ml SOD (data not shown). These data demonstrated that the reactive oxygen generated from EGCg was hydrogen peroxide.

Mechanism of and Factors Influencing Hydrogen Peroxide Production Hydrogen peroxide generated from EGCg is strongly dependent on solution pH; in particular, generation rate increased with rising pH. Figure 2 displays the effect of pH on hydrogen peroxide production in EGCg solution and green tea infusion. Conversion from EGCg to hydrogen peroxide at pH 7—8 was 50—100%, which is a high generation rate. Hydrogen peroxide generation of EGCg is also dependent on reaction time. The production of hydrogen peroxide greatly increased by 60 min as shown in Fig. 3, then continued to increase gradually. When 0.22 and 0.022 mmol/l EGCg were dissolved in phosphate buffer (pH 7) and incubated for 60 min, 0.2 mmol/l and 0.021 mmol/l of hydrogen peroxide were generated, respectively. Furthermore, when

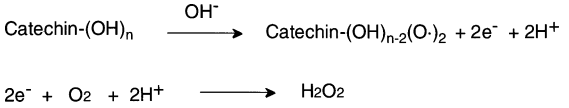

Fig. 4. A Possible Mechanism of Hydrogen Peroxide Production from EGCg

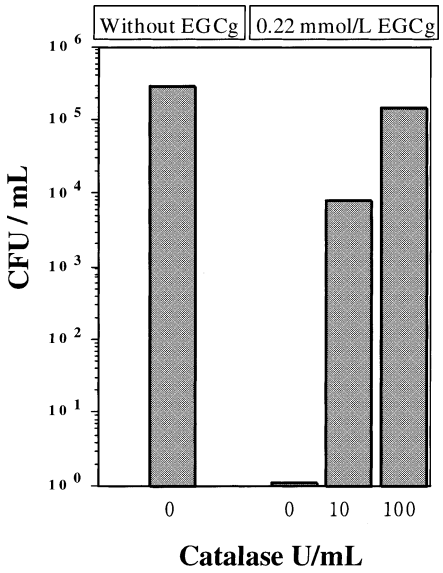

Fig. 5. Inhibitory Effect of Catalase on Bactericidal Action of EGCg

they were left overnight, 0.38 and 0.058 mmol/l of hydrogen peroxide were generated; the conversion thus corresponded to 170% and 260%. This result means that one molecule of EGCg generates hydrogen peroxide over two molecules due to the phenolic hydroxyl group of the EGCg structure.

Conversion of EGCg to hydrogen peroxide was also dependent on EGCg concentration. At higher concentrations (2.2 mmol/l), the conversion rate decreased to 50%, whereas at lower concentrations, the rate increased to over 100%. We believe that this phenomenon depends on the ratio of concentration of dissolved oxygen as well as ionization degree of the OH group of the polyphenol.

The production mechanism of hydrogen peroxide was elucidated from the aforementioned result as follows. In short, the OH moiety of EGCg dissociates H^+ in solution and an electron on the phenol reduces the oxygen; consequently, superoxide is formed. Superoxide undergoes further reduction by EGCg, which leads to formation of O_2^{2-}; additionally, the proton combines with superoxide, which generates hydrogen peroxide. EGCg produces phenoxy radical as an intermediate, which functions as an antioxidant; in turn, the quinone structure of the catechin is generated. The reaction scheme is illustrated in Fig. 4.

The concentration of hydrogen peroxide in infusions of black tea, green tea and oolong tea were measured by the chemiluminescent method. Hydrogen peroxide concentrations of 1.5×10^{-4}, 2.4×10^{-4} and 0.87×10^{-4} mol/l were detected, respectively.

Bactericidal Action of Catechin We previously clarified the bactericidal action of catechin in gram-positive and gram-negative bacteria.[5] Therefore, the inhibitory effect of catalase on the bactericidal action of EGCg was examined to assess whether this inhibition was a consequence of hydro-

gen peroxide derived from catechin. As shown in Fig. 5, it was demonstrated that the bactericidal action of EGCg decreased with increasing catalase concentration. This phenomenon was also examined to confirm the effect of catalase with respect to the level of degradation of hydrogen peroxide produced by EGCg; furthermore, bactericidal activity in the presence of catalase was compared. Results are presented in Table 1.

After 24 h following the addition of 0.22 mmol/l EGCg in culture medium, the concentration of hydrogen peroxide was 0.25 mmol/l, and the conversion of EGCg to hydrogen peroxide was nearly 100%. The bactericidal effect with respect to *E. coli* at this concentration was 100%. Upon introduction of 10 and 100 U/ml catalase, hydrogen peroxide concentration decreased to 0.08 and 0.017 mmol/l, respectively. The rate of decrease corresponds to 68% and 93%. The bactericidal effect under this condition also decreased by 18% and 2%, respectively. This finding shows that hydrogen peroxide concentration is strongly associated with the bactericidal activity of EGCg.

Comparison of Bactericidal Activity Obtained by EGCg and Hydrogen Peroxide for Gram-Positive and Negative Bacteria Shimamura and his colleagues previously reported differences in terms of bactericidal activity of EGCg in various kinds of bacteria.[5)] Distinctions with respect to the bactericidal effect are thought to be attributable to differences in responsiveness of bacteria to EGCg. We anticipate that the bactericidal action of hydrogen peroxide also correlates with the difference relating to the effect of EGCg on bacteria if the bactericidal action of EGCg is caused by the effect of hydrogen peroxide. The bactericidal action of 0.22, 0.44 and 0.88 mmol/l hydrogen peroxide among seven types of gram-negative and two types of gram-positive bacteria (*S. aureus* 2 strains) was examined. Results are displayed in Table 2.

E. coli ATCC 25922, *S. aureus* ATCC25923, MRSA F-51, *B. bronchiceptica* IID929, *S. marcescens* IID5518, *B. subtilis* ATCC 6623, and *S. enteritidis* 87-350 demonstrated bactericidal activity in excess of 95% in 0.22 mmol/l EGCg and 95% in hydrogen peroxide of the same concentration. Bactericidal action for *K. pneumoniae* IID5207 was 94%; however, activity of 0.22 mmol/l hydrogen peroxide was not observed. In contrast, 100% bactericidal activity was evident at 0.44 mmol/l hydrogen peroxide. On the other hand, the activity of EGCg for *Ps. aeruginosa* 42 and *P. mirabilis* was weak at 57% and 17%, respectively. Bactericidal action of 0.22 mmol/l hydrogen peroxide was scarce in the case of these bacteria. However, these bacteria were sterilized when the hydrogen peroxide concentration increased to 0.44 and 0.88 mmol/l. This finding revealed that bacteria associated with EGCg-hyperresponsiveness are sterilized even in hydrogen peroxide at the same concentration, whereas bacteria associated with low EGCg-responsiveness were also weak in terms of bactericidal action of hydrogen peroxide. Furthermore, it was also shown that higher concentrations of hydrogen peroxide were required in order to obtain 100% of the bactericidal effect for low EGCg-responsiveness bacteria. Additionally, EGCg exhibited bactericidal action that was stronger than that of hydrogen peroxide of identical concentration (Table 2).

Maintenance of a constant concentration of hydrogen peroxide within the cell is thought to account for the higher bactericidal action of EGCg due to the chemical equilibrium reaction with dissolved oxygen, physical binding properties of catechin to lipid bilayers and cell membrane proteins, followed by the partial increase in intracellular hydrogen peroxide concentration. Furthermore, one molecule of EGCg generates over two molecules of hydrogen peroxide. The aforementioned finding proved that bactericidal action of the catechins and tea was attributable to hydrogen peroxide generated from catechin.

Table 1. Comparison of Bactericidal Activity and Hydrogen Peroxide Level Generated from EGCg in the Presence of Various Catalase Concentrations

	H_2O_2 concentration generated by 0.22 mmol/l in medium		Bactericidal action
	After incubation for 30 min	After incubation overnight	
Control (no catalase)	1.6×10^{-4} mol/l	2.5×10^{-4} mol/l	100% (Viable cells 0%)
Catalase 10 U/ml	5.4×10^{-5} mol/l	8×10^{-5} mol/l (68% reduction)	18% (Viable cells 82%)
Catalase 100 U/ml	5.4×10^{-5} mol/l	1.7×10^{-5} mol/l (93% reduction)	2% (Viable cells 98%)

Table 2. Comparison of Bactericidal Activities Obtained by EGCg and Hydrogen Peroxide for Gram-Positive and Negative Bacteria

Organism (1×10^6/ml)	Bactericidal action			
	EGCg 0.22 mmol/l	H_2O_2 (mmol/l)		
		0.22	0.44	0.88
E. coli (ATCC 25922)	100%	95%		
MRSA (F-51)	100%	100%		
B. bronchiceptica (IID929)	100%	99%		
S. aureus (ATCC25923)	100%	98%		
S. marcescens (IID5518)	99%	98%		
K. pneumoniae (IID5207)	94%	0%	100%	
B. subtilis (ATCC 6623)	100%	99%		
S. enteritidis (87-350)	99%	99%		
Ps. aeruginosa (42)	57%	0%	70%	100%
P. mirabilis	17%	0%	0%	100%

CONCLUSION

The chemiluminescent method and ESR measurement confirmed that EGCg efficiently generated hydrogen peroxide. The hydrogen peroxide derived from EGCg is strongly dependent on solution pH; the generation rate increased with rising pH. These results indicate that hydrogen peroxide is generated from catechin by one electron reduction to dissolved oxygen.

The present investigation also confirmed the strong dependence of bactericidal action on the generation of hydrogen peroxide upon examination of the bactericidal effect of *E. coli* employing EGCg, hydrogen peroxide and scavenging enzymes such as catalase. Both bactericidal effects correlated to 10 kinds of bacterium. Subsequently, hydrogen peroxide concentrations in infusions of black tea, green tea and oolong tea were measured, and hydrogen peroxide levels of 0.150,

0.24 and 0.087 mmol/l were detected, respectively. The sterilizing effect of drinks such as black tea and green tea was shown to be effective; the concentrations in the tea are equal to hydrogen peroxide levels that display bactericidal action. Based on the aforementioned findings, bactericidal action of catechin is due to hydrogen peroxide generated from the catechin; additionally, the intensity of action appears to be dependent on the sensitivity of bacterium for reactive oxygen and adsorption ability with respect to catechin of the bacterium.

REFERENCES

1) Stoner G. D., Mukhtar H., *J. Cell Biochem.*, **22**, 169—180 (1995).
2) Yoshioka H., Sugiura K., Kawahara R., Fujita T., Makino M., Kamiya M., Tsuyumu S., *Agric. Biol. Chem.*, **55**, 2717—2723 (1991).
3) Yang G., Liao J., Kim K., Yurkow E. J., Yang C. S., *Carcinogenesis*, **19**, 611—616 (1998).
4) Toda M., Okubo S., Hiyoshi R., Shimamura T., *Lett. Appl. Microbiol.*, **8**, 123—125 (1989).
5) Toda M., Okubo S., Ikigai H., Shimamura T., *Jpn. J. Bacteriol.*, **45**, 561—566 (1990).
6) Ikigai H., Nakae T., Hara Y., Shimamura T., *Biochim. Biophys. Acta*, **1147**, 132—136 (1993).
7) Toyoshima Y., Okubo S., Toda M., Hara Y., Shimamura T., *J.J.A. Inf. D.,* **68**, 295—303 (1994).
8) Ikigai H., Hara Y., Otsuru H., Shimamura T., *Jpn. J. Chemother.*, **46**, 179—183 (1998).
9) Arakawa H., Kanemitsu M., Tajima N., Maeda M., *Anal. Chim. Acta*, **472**, 75—82 (2002).