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| 보 문 |

Effect of Interaction between Protocatechualdehyde Produced from *Streptomyces lincolnensis* M-20 and Copper Ions on Antioxidant and Pro-oxidant Activities

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Streptomyces lincolnensis M-20 균주에서 생산된 Protocatechualdehyde와 구리 이온의 상호 작용이 항 산화 및 산화 촉진 활성에 미치는 영향

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Protocatechualdehyde (PA) is phenolic compound having antioxidative and antitumor activities. PA was purified from supernatant of *Streptomyces lincolnensis* M-20. In the presence of copper ion, PA acted as pro-oxidant. The antioxidant activity was assessed with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and the pro-oxidant effect of PA on DNA damage as pBR322 plasmid DNA-cleaving agents in the presence of Cu(II) ions was investigated. The involvement of reactive oxygen species (ROS) in the DNA damage was confirmed by the inhibition of the DNA breakage by using glutathione (GSH), specific scavenger of ROS. When the increase in ROS reaches a certain level (the toxic threshold), it may trigger cell death. The formation of the PA/Cu(II) chelate complex was confirmed by reaction with ethylenediamine-tetraacetic acid (EDTA), a well-known chelating agent for metal ions, by using UV/Vis spectroscopic analysis.

***Keywords*:** *Streptomyces lincolnensis*, antioxidant, copper ions, DNA damage, pro-oxidant, protocatechualdehyde

Antioxidant activity refers to the ability of phenolic compounds to prevent damage caused by reactive oxygen species (ROS) (such as through radical scavenging) or to prevent the generation of ROS (by binding iron) (Perron and Brumaghim, 2009). Phenolic anti-oxidants, including quercetin (Yamashita *et al*., 1999), salsolinol (Jung and Surh, 2001), and resveratrol (Ahmad *et al*., 2005), were reported to induce lipid peroxidation and/or DNA damage in the presence of cupric ions. In this reaction, Cu(II) ions is reduced to Cu(I) ions by phenolic compounds and the re-oxidation of Cu(I) ions to Cu(II) ions is accompanied by the formation of ROS. Therefore, it is interesting to see how an antioxidant can switch to a pro-oxidant and its biological implications. Recent studies have demonstrated that the pro-oxidant action of various phenolic compounds is attributed to the accelerated lipid peroxidation and/or induction of DNA damage, mutagenesis, carcinogenesis, and apoptosis in cancer cells (Zheng *et al*., 2006, 2008; Wang *et al*., 2008). A previous study on the antioxidant constituents in *Streptomyces lincolnensis* M-20 reported the presence of PA (Fig. 1). PA is phenolic compound having antioxidative and antitumor activities. PA was isolated from a butanol fraction of the purple colored supernatant of *S. lincolnensis* M-20. Cu at higher concentrations causes oxidative

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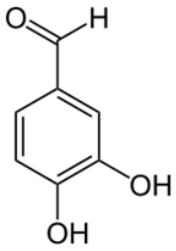

Fig. 1. Structure of protocatechualdehyde.

stress due to overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can be cytotoxic and damage important cell compounds (Maksymiec and Krupa, 2006; Kováèik and Baèkor, 2007; Posmyk *et al*., 2009). In this study, interaction of PA purified from supernatant of M-20 and Cu(II) ions was investigated. Antioxidant activity of PA was assessed with the 1,1-diphenyl- 2-picrylhydrazyl (DPPH) assay, and the pro-oxidant effect of PA on DNA damage as pBR322 plasmid DNA-cleaving agents in the presence of Cu(II) ions was investigated. The interaction between PA and Cu(II) ions and the influence of EDTA were also studied by using UV/Vis spectroscopic analysis.

Materials and Methods

Reagents and chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ethylenediamine-tetraacetic acid disodium salt (EDTA), and glutathione (GSH) were obtained from Sigma Co. (Seoul, Korea).

Strain and culture conditions

Streptomyces lincolnensis M-20 isolated from Mongolian soil was reported previously (Kim *et al*., 2003). Medium used for the production of purple pigment and antioxidant substance was following compositions (in g/L): glucose (5), K_2HPO_4 (1), Na_2HPO_4 (1.6), KCl (0.5), yeast extract (0.5), $MgSO_4 \cdot 7H_2O$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.01), pH 7.0. Cultivation was carried out at 28℃ in shaking incubator for 5–7 days.

Extraction and isolation of protocatechualdehyde

Extraction and isolation of PA of *S. lincolnensis* M-20 was performed as previously described (Kim *et al*., 2008). Briefly, after filtration, the culture media of *S. lincolnensis* M-20 was freeze dried, and then partitioned between water and *n*-BuOH. The *n*-BuOH layer was subjected to Sephadex LH-20 (MeOH) and active LH-20 fractions having good antioxidant activity in the DPPH free radical scavenging assay was subjected to reversed-phase HPLC. The retention time of PA was on average 6.4 min. Concentration of PA produced from *S. lincolnensis* M-20 was determined by comparison with standard PA by HPLC.

DPPH radical-scavenging activity

The antioxidant activity was measured using a DPPH radical scavenging assay method. A 0.1 ml sample was mixed with 0.9 ml of a ethanolic 0.15 mM DPPH solution. After vortexing thoroughly and leaving to stand at room temperature for 30 min, the absorbance of the mixture was measured at 517 nm using a UV mini 1240 UV-VIS spectrophotometer. The mean of three measurements was taken. Ascorbic acid was used as the positive control. The scavenging activity was determined using the following equation:

% Scavenging Activity = 100 × (1−Abs. sample / Abs. control)

The effects of copper on the antioxidative activity of protocatechualdehyde

Antioxidative activity of PA purified from *S. lincolnensis* M-20 was measured by DPPH radical scavenging activity with or without the added copper (II) ions ($CuSO_4$ or $CuCl_2$).

UV/Vis spectral measurements

The UV/Vis spectra were measured at room temperature with UV mini 1240 UV-VIS spectrophotometer. 50 mM potassium phosphate buffer (pH 7.0) containing 100 μM of PA was kept at room temperature, and the spectral tracing was started by the addition of 100 μM $CuSO_4$. All the spectra were run against blanks containing the buffer and the metal ions. The spectra were recorded after addition of 100 μM Cu(II) ions.

Assay for oxidative DNA strand breakage

The induction of DNA strand breakage by PA was assessed by measuring the conversion of the supercoiled pBR322 plasmid DNA into linear form by gel electrophoresis. pBR322 DNA (200 ng) was incubated with PA and/or Cu(II) ions in 50 mM potassium phosphate buffer at pH 7.0 and 37℃ for 1 h in 1.5-ml microcentrifuge tubes. The total volume was 20 μl, that is, DNA (5 μl), PA (5 μl), Cu(II) (5 μl), and buffer (5 μl). In the inhibition experiments, specific scavengers of ROS (glutathione) was preincubated before addition of the Cu(II) ions. After incubation, the samples (10 μl) were mixed with gel loading buffer (2 μl 0.25% bromophenol blue and 30% (w/v) glycerol) and immediately loaded in a 1% agarose gel containing 40 mM tris(hydroxymethyl)aminomethane (Tris), 20 mM sodium acetate, and 2 mM EDTA and subjected electrophoresis in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer for 30 min The gels were stained with 0.5 μg/ml ethidium bromide for 10 min followed by destaining in water for 10 min and photographed under UV light.

Results and Discussion

The effects of copper on the antioxidative activity of protocatechualdehyde

The antioxidant activity of PA purified from supernatant of *S. lincolnensis* M-20 was measured by the DPPH method, which is one of the oldest and the most frequently used methods for evaluating antioxidant activity. In previous report (Kim *et al.*, 2008), 50 μM PA showed 55% higher DPPH radical scavenging activity than the positive control, ascorbic acid. Antioxidant activity refers to ability of phenolic compounds to prevent damage from ROS (through radical scavenging). Antioxidative activity of PA from *S. lincolnensis* M-20 was inhibited by addition of copper (II) ions. IC_{50} of Cu(II) on the DPPH radical scavenging activity of PA (50 μM) was 10 μM (Table 1). Phenolic compounds can switch from antioxidants to pro-oxidants in the presence of Cu(II) to induce ROS production and subsequently DNA damage. Cu^{2+} is reduced to Cu^{+} by phenolic compounds and the re-oxidation of Cu^{+} to Cu^{2} is accompanied by the formation of ROS. Therefore, it is interesting to see how an antioxidant can switch to a pro-oxidant and its biological implications. Growing evidence has revealed that oxidative DNA damage induced by reactive oxygen species (ROS) contributes to human tumorigenesis (Cooke *et al.*, 2003; Perwez Hussain *et al.*, 2003). There are several reports that clarify the mechanism of the pro-oxidant activity (Ahmad *et al.*, 2005; Fan *et al.*, 2009). Mechanism of prooxidant activity of PA in the presence of Cu(II) was investigated by following UA/Vis spectral change analysis.

Table 1. Effect of copper ions on the antioxidative activity of PA purified from *S. lincolnensis* M-20

Concentration of $CuSO_4$ (μM) added to the 50 μM PA[a]	DPPH radical scavenging activity (%)
0.0	100
5.0	82
10.0	50
20.0	25
30.0	10
50.0	0
60.0	0

[a] PA was purified from supernatant from *S. lincolnensis* M-20 as described in 'Materials and Methods'.

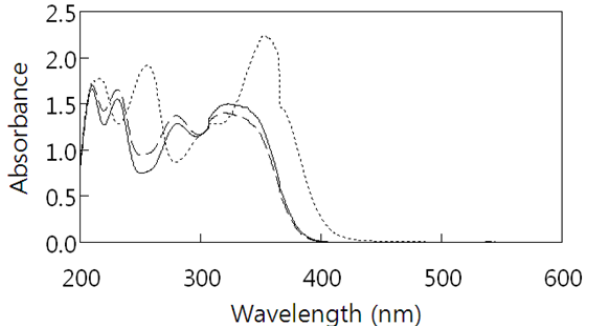

Fig. 2. Absorption spectral changes of PA (100 μM) in the absence of (——) and in the presence (· · · ·) of 100 μM Cu(II) ions in 50 mM potassium phosphate buffer (pH 7.0) for 60 min in air. The effect of EDTA (200 μM) on the absorption system of the PA/Cu(II) (– – –).

UV/Vis spectral changes of PA in the presence of Cu(II) ions and influence of EDTA on the interaction of PA and Cu(II) ions

Phenolic compounds are reducing agents and under in vitro conditions and in the presence of metal ions, such as copper or iron, they can act as pro-oxidants (Cao *et al.*, 1997; Sugihara *et al.*, 1999). PA is phenolic compound produced from *S. lincolnensis* M-20. To clarify the mechanism of pro-oxidant activity of PA, the UV/Vis absorption changes of PA upon the addition of Cu(II) ions were examined. Figure 2 was obtained when Cu(II) ions, 100 μM were added to PA, 100 μM in 50 mM phosphate buffer (pH 7.0). The rapid disappearance of the absorption bands of PA centered at 231 and 281 nm was accompanied by the appearance of bathochromic-shifted peaks at 255 and 354 nm. The formation of the PA/Cu(II) chelate complex was confirmed by reaction with ethylenediamine-

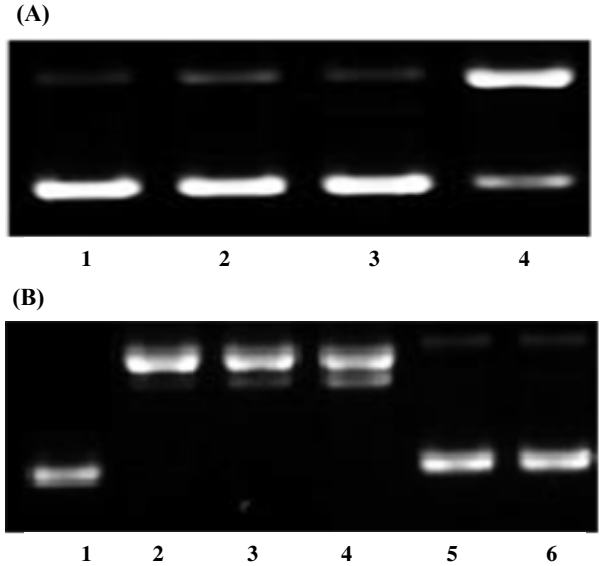

Fig. 3. (A) Effect of PA on pBR322 DNA strand breakage in the presence of Cu(II) ions. Supercoiled pBR322 DNA (150 ng) was incubated with 0.1 mM Cu(II) and 0.1 mM PA at 37℃, 1 h in 50 mM potassium phosphate buffer (pH 7.0). Lanes: 1, control; 2, Cu(II) alone; 3, PA alone; 4, PA and Cu(II). (B) Effect of glutathione (GSH) on PA and Cu(II)-mediated DNA strand breakage. The experimental conditions were the same as for (A). 1, control; 2, 0.05 mM PA plus 0.05 mM Cu(II); 3, 0.1 mM PA plus 0.1 mM Cu(II); 4–6, PA+Cu(II) together with 0.25, 0.5, and 1 mM GSH, respectively.

tetraacetic acid (EDTA), a well-known chelating agent for metal ions. EDTA was added after the Cu(II) ions underwent a reaction with PA for 60 min. Upon the addition of 200 μM EDTA, the redshifted bands (255 and 354 nm) returned back to their initial position and PA recovered almost to their initial intensity at the absorption bands (231 and 281 nm). This outcome indicates unambiguously that PA chelates with Cu(II) ions.

Strand breakage of plasmid pBR322 DNA induced by PA and Cu(II) ions and the involvement of ROS

The single-strand breakage of supercoiled plasmid DNA with a relatively high electrophoretic mobility leads to the formation of an open circular conformation with a decreased electrophoretic mobility in agarose, whereas the formation of linear DNA is indicative of a double-strand breakage and has a mobility intermediate between that of the supercoiled and open circular conformation. We examined the effect of PA in combination with Cu(II) ions on the plasmid DNA breakage by using agarose gel electrophoresis (Fig. 3). Neither Cu(II) ions (0.1 mM) nor PA (0.1 mM) alone caused detectable DNA damage (lanes 2, 3 Fig. 3A). However, PA (0.1 mM) could work cooperatively with Cu(II) ions (0.1 mM) to produce a linear of fragment, which is indicative of extensive DNA damage (lane 4, Fig. 3A). PA and Cu(II) ions resulted in the conversion of the substrate DNA almost completely into linear fragmental form. It was reported that preneoplastic and neoplastic cells might be more sensitive in comparison to normal cells to copper-related redox reactions that switch an antioxidant to a prooxidant to generate ROS, thus resulting in DNA damage. DNA damage induced by antioxidants in the presence of copper might be an important pathway through which preneoplastic and neoplastic cells can be killed while normal cells survive (Antosiewicz *et al*., 2008; Hail *et al*., 2008). To analyze the role of ROS in the PA/Cu(II)-dependent DNA breakage, we used specific scavengers of activated oxygen to define the nature of the reactive species. Glutathione (GSH), a ROS scavenger provided protection against DNA strand breakage induced by PA in the presence of Cu(II) ions (Fig. 3B). GSH at concentrations of 0.5 mM completely inhibited the PA/Cu(II) mediated stand breakage. The involvement of reactive oxygen species (ROS) in the DNA damage was affirmed by the inhibition of the DNA breakage by GSH. The obtained results indicate that phenolic compounds can switch from antioxidants to pro-oxidants in the presence of Cu(II) ions to induce ROS production and subsequently DNA damage. Phenolic antioxidants, including quercetin (Yamashita *et al*., 1999), curcumin (Ahsan and Hadi, 1998), and salsolinol (Jung and Surh, 2001) were reported to act as pro-oxidants in the presence of cupric ions. If phenolic compounds such as PA and copper were absorbed by the body, they would interact with each other to generate ROS. When the increase in ROS reaches a certain level (the toxic threshold), it may trigger cell death.

적 요

Protocatechualdehyde (PA)는 항산화 활성과 항암 활성을 가진 페놀성 물질이다. *Streptomyces lincolnensis* M-20 균주에서 생산된 PA를 균주 상등액에서 분리, 정제하였다. 항산화 활성을 가진 PA가 구리 이온 존재 하에서는 산화촉진제로 작용하였다. 항산화 활성은 DPPH를 이용한 방법으로 측정하였으며, 구리 이온 존재 하에서 PA의 산화 촉진 작용은 pBR322 플라스미드의 DNA 절단 작용으로 측정하였다. DNA 손상으로 생성되는 활성 산소 종의 확인은 활성 산소종의 포집자인 글루타치온에 의해 DNA 절단이 억제되는 것으로 확인하였다. PA와 구리 이온의 복합체 형성은 금속 이온의 킬레이트인 EDTA가 존재할 경우와 존재하지 않을 경우를 자외선/가시광선 분광학적 분석법으로 비교, 확인하였다.

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