

Antioxidative Activity of Extracts of Aged Black Garlic on Oxidation of Human Low Density Lipoprotein

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This study was developed to assess the antioxidative activity of aged black garlic extract on lipid peroxidation and low density lipoprotein (LDL). Antioxidative activity of aged black garlic extract on human low density lipoprotein (LDL) was investigated by monitoring a barbituric acid reactive substance (TBARS). Electron donating ability (EDA) of aged black garlic, ethanol extract was higher than that alliin and water extract. Aged black garlic water and ethanol extracts inhibited the Cu^{2+} mediated oxidation of human LDL in a dose dependent manner at concentration of 10 and 20 $\mu\text{g}/\text{ml}$. Ethanol extract and water extract of aged black garlic almost completely inhibited J774 mediated LDL oxidation in electrophoretic mobility and conjugated diene. These results indicate that aged black garlic might play a protective antioxidant effects on LDL, probably affecting the structural properties for the LDL oxidation.

Key words – Aged black garlic, low density lipoprotein (LDL), antioxidant

Introduction

There are several lines of evidence for the existence of oxidatively modified low density lipoprotein (LDL) in early atherosclerotic lesions [26,27].

LDL is oxidized by free radicals generated from endothelial cells [10], monocyte-derived macrophages [16], and smooth muscle cell [19], resulting in several chemical and physical changes of LDL.

Oxidized LDL has entered the artery wall and then would affect atherosclerotic progress [20] as foam cells. Therefore, it has been hypothesized that oxidized LDL initiates and promotes atherogenesis in several ways.

It is well established that LDL is the major cholesterol carrier in the blood, and the elevation of LDL plasma level is correlated with an increased risk of atherosclerosis and cardiovascular disease [30]. LDL does not cause atherosclerotic plaques in its native form, but the oxidative modification of LDL may contribute to the pathology of atherosclerosis, leading to plaque-buildup arteries and consequently coronary heart diseases [22].

Evidence in support of the oxidized LDL hypothesis also comes from studies using antioxidants. If oxidized LDL is crucial to atherogenesis, the potential role of anti-

oxidants in the prevention of the oxidative modification of LDL assumes great importance. Therefore, inhibition of LDL oxidation has been suggested as a novel approach to impede atherogenesis. LDL carries several antioxidants, such as α -tocopherols and carotenoids, which protect them from oxidation. Dietary supplying of vitamin E inhibits LDL oxidation, and prevents oxidized LDL mediated vascular injury.

Recently, the natural antioxidants has been expected to replace in the synthetic antioxidants which are widely used at the present time [4,14]. Antioxidants from natural substances such as edible plants, spices and herbs have been widely investigated, because those natural occurring antioxidant compounds have been found to strengthen the resistance of LDL to oxidative modification *in vitro* and *in vivo* [12].

Garlics are known to have medicinal effects such as overcoming fatigue, detoxification, and anti-aging effects [3,25]. Results of the recent clinical studies reveal garlic has definite effects not only on recovery from lack of oxygen, cold, fatigue, and microwave radiation, but also on increasing the concentration and work efficiency, removing the cholesterol and preventing [15,29].

Garlic component has been paid a great attention to many researchers because of its biochemical and pharmacological importance, and now is even being added to some tonic-like beverages and health food supplements [28]. Garlic components were reported to have antibacterial

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[6] and antifungal activity [18]. Also its component was report to have antioxidative effect [14].

Following these considerations, the present study was undertaken to characterize the antioxidative activity of garlic after its incorporation *in vitro* in physiological concentrations into human LDL as estimated by measuring the formation of TBARS and electrophoretic mobility.

Materials and Methods

Preparation of aged black garlic extracts

Aged black garlic was from Food Factory of Samrangjin Nonghyup (Korea), other chemicals were purchased from Sigma Co. (St, Louis, U.S.A).

Aged black garlic were extracted by refluxing twice at 80°C with 100 ml water or 75% ethanol, freeze-dried after evaporation of ethanol and kept at -20°C until analysis.

Isolation of human low density lipoprotein (LDL)

Human LDL was isolated from blood of healthy man by ultracentrifugation and dialyzed extensively against 0.9% (w/v) NaCl, 0.004% (w/v) EDTA, pH 7.4 [9]. Prior to oxidation, LDL was dialyzed against phosphate-buffered saline, pH 7.4 to remove the EDTA.

A pool of fresh plasma from normolipidemic subjects was used for isolation of LDL. EDTA (0.3 mM) was added to the plasma, the density was adjusted to 1.019 g/ml with solid NaBr, and the plasma was centrifuged at 40,000 rpm in a 50.3 Beckman rotor for 24 hr at 4°C. The top 1 ml was harvested by aspiration and discarded. The supernatant was then adjusted to 1.063 g/ml and centrifuged an additional 24 hr after which the top 1 ml representing LDL (1.019-1.063 g/ml) was collected by aspiration. The LDL were dialyzed extensively against saline, Tris (10 mM), EDTA (0.3 mM), pH 7.4 and stored, under nitrogen prior to further dialysis. LDL samples were dialyzed against phosphate buffer (50 mM, pH 7.4) prior to use.

Measurement of antioxidant activity by EDA

The electron donating ability (EDA) to DPPH (α,α -diphenyl- β -picrylhydrazyl) of aged black garlic was measured to compare the antioxidant activity by the method of Blois [2].

J774 cultivation

Transformed mouse macrophage, J774 were maintained in Ham's F-10 supplemented with 10% (v/v) foetal calf se-

rum, 0.2 g NaHCO₃ and 4 mM Hepes, pH 8.1. A series of antibiotics was included the rotation in the medium. The cells were cultured routinely in large dishes (90 mm diameter) in 10 ml of medium and plated out into smaller dishes (60 mm diameter) containing 2 ml of medium for experimentation. Cultures were maintained in a humidified incubator at 37°C and the fresh medium changed every 48 hr.

Oxidation of LDL

Oxidation was made by exposing LDL (10 µg/ml) to 10 µM Cu²⁺ in 2 mM phosphate buffer pH 7.5 containing 20 µM hydrogen peroxide or 2 mM AAPH, at 37°C. At time intervals, aliquots of the reaction mixture were taken to measure the extent of lipid peroxidation evaluating the thiobarbituric acid reactive substances (TBARS) and hydroperoxides. The entity of oxidation was expressed as malondialdehyde equivalents (MDA) using as standard MDA obtained by acid hydrolysis of tetraethoxypropane [7].

Assay of thiobarbituric acid reactive substances (TBARS)

TBARS levels were determined spectrophotometrically. To 0.1 ml aliquots of post incubation mixture and also tetramethoxypropane standards were added 1 ml of 20% trichloroacetic acid and 1 ml of 1% thiobarbituric acid containing EDTA. Tubes were placed in a boiling water bath for 30 min. After cooling, tubes were centrifuged at 1,500×g for 15 min. Absorbance of the supernatant was measured at 532 nm [31].

Detection of conjugated dienes

The formation of conjugated dienes associated with oxidized LDL was measured by monitoring the absorption at 234 nm using a UV-VIS spectrophotometer [5]. Briefly, 1 ml of LDL solution (100 µg LDL, protein/ml) in phosphate-buffered saline, pH 7.4, was incubated with 5 µM CuSO₄ at 37°C in the presence or absence of tested compound, and then the absorbance at 234 nm was measured every 30 min. The formation of conjugated dienes in control solutions containing antioxidant in the absence of LDL and 5 µM CuSO₄ was also determined.

LDL gel electrophoresis

Electrophoresis of oxidized and native LDL was carried

out on agarose gel in barbital buffer, pH 8.6. The agarose plates were then stained with Nile red [8]. Results are expressed as relative electrophoretic mobilities compared with the migration of native LDL.

Determination of protein

The protein was determined by the methods of Lowry, et al. [17].

Statistics

Data in tables and figures are mean±SE. Statistical significance was examined through oneway analysis of variance. Significance differences were accepted at $P < 0.05$.

Results and Discussion

The electron donating ability (EDA) of aged black garlic

For antioxidant activity determination of garlic by the DPPH method, the electron donating ability (EDA) of 10 µg/ml alliin and ethanol extract, water extracts of aged garlic are shown in Table 1. The percentage of EDA showed the alliin, ethanol extract and water extracts were 6.64, 11.91, and 9.53%, respectively. EDA of aged black garlic showed higher than that of alliin was known as major component of garlic, suggesting that water extract was higher than alliin in antioxidative action by the reducing power. But ethanol extract of garlic showed a similar trend to water extracts. It is believed that melanoidins [24], polyphenols [11,21] in ethanol fraction were deeply related to antioxidative activity. It suggests that prooxidant are existence in aged black garlic components for inhibition of lipid oxidation.

Table 1. Effects of alliin, ethanol extract, and water extracts of aged black garlic on the electron donating ability (EDA) *in vitro*

Components	Induction period (min)	EDA (%)
Control	0.840	-
Alliin	0.786	6.43
Aged garlic ethanol extract	0.740	11.91
Aged garlic water extract	0.734	9.53

The reaction mixtures containing 10 µg/ml extract of aged black garlic and 100 µM DPPH were kept at 37°C for 20 min to measure the absorbance at 517 min.

Alliin: main compound of garlic.

Inhibitory effects of aged black garlic extracts on human LDL oxidation

Transition metal ions including copper and iron have been shown to be strong catalysts for LDL oxidation *in vitro*. Cu^{2+} ions were found to be effective at initiating the oxidation of EDTA-free human LDL as measured by TBARS. Although the physiological significance of *in vitro* Cu^{2+} -induced LDL oxidation remains controversial, this method has been a useful model for evaluating naturally occurring antioxidant compounds [13].

To obtain the oxidized LDL, human LDL was oxidized by Cu^{2+} in a time dependent manner and the production of TBARS reached a plateau after 60 min of incubation.

As shown in Fig. 1, all data relation to Cu^{2+} presented here were obtained following 60, 120, and 180 min incubation.

80% ethanol extract of aged black garlic at 10 µg/ml was less effective in producing a prooxidant activity during the early initiation stage after 60 min incubation. Antioxidant activity showed against human LDL oxidation by reducing the formation of TBARS (Fig. 1). The prooxidant activity of 80% ethanol extract at 20 µg/ml was slightly stronger than that at 10 µg/ml. However, complete inhibition of human LDL oxidation was observed after the incubation with 20 µg/ml of 80% ethanol extracts for incubation of 120 min. Thus, these differences in the relative antioxidant activity between water and 80% ethanol extracts support that some

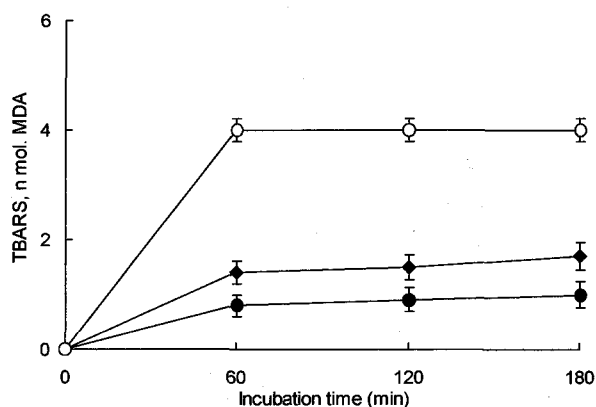


Fig. 1. Inhibitory effect of LDL oxidation by ethanol extract of aged black garlic. LDL (100 µg, protein/ml) was incubated for 60, 120 and 180 min at 37°C in phosphated buffered sharing containing 5 µM CuSO_4 in the absence (○) or presence of 10 (◆) or 20 µg/ml (●) of ethanol extract of aged black garlic. Mean±SE (n=6), Means in the same column not sharing common superscript letters are significantly different ($P < 0.05$).

lipophilic sulfur containing compounds. 80% ethanol extract could be involved in exerting the strong antioxidant activity by effectively scavenging the lipid-soluble peroxy radicals resulting from the oxidation of human LDL [31].

On the other hand, water extract of aged black garlic reduced the lag phase of human LDL oxidation at 10 $\mu\text{g}/\text{ml}$ compared to the control, thus indicating that it can contribute to a prooxidant effect in the presence of Cu^{2+} (Fig. 2). Ryu, et al. [23] demonstrated the effect of Cu^{2+} reduction in the initiation of LDL oxidation, which is consistent with our results. However, at 20 $\mu\text{g}/\text{ml}$ water extract of aged black garlic showed a weak prooxidant activity during early initiation stage, whereas a strong antioxidant activity was observed upon reducing the formation of TBARS after 120 min incubation.

According to research papers, oxidation of LDL in the presence of copper was maximal between 2 and 3 hr of incubation; oxidation for 24 hr of incubation was almost four fold greater than at 4 hr [13]. This may reflect the level of endogenous antioxidants present in the LDL preparation, which may vary with individual donors. For example, vitamin E as dietary antioxidant protects against LDL oxidation, but LDL of donors from smokers were more susceptible to oxidation than that from non-smoker.

Effects of conjugated diene formation

In these experiments, extracts of aged black garlic were

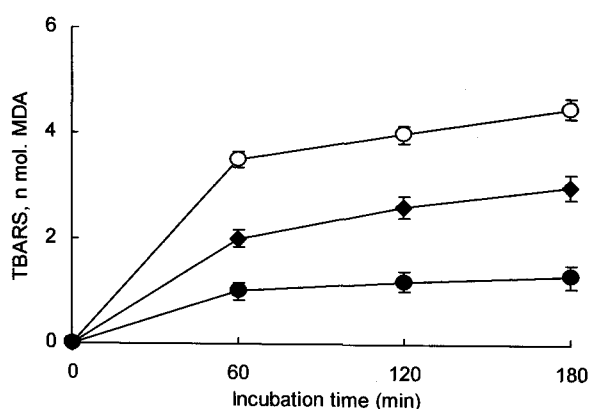


Fig. 2. Inhibitory effect of LDL oxidation water extracts of aged black garlic. LDL (100 μg , protein/ml) was incubated for 60, 120 and 180 min at 37°C in phosphated buffered sharing containing 5 μM CuSO_4 in the absence (○) or presence of 10 (◆) or 20 $\mu\text{g}/\text{ml}$ (●) of water extract of aged black garlic. Mean \pm SE (n=6), Means in the same column not sharing common superscript letters are significantly different ($P < 0.05$).

added at time Cu^{2+} at 15, 30, and 60 min after the addition of Cu^{2+} to the LDL. Using conjugated dienes, as shown in Table 2, extracts of aged black garlic inhibited the propagation of Cu^{2+} induced LDL peroxidation almost completely during the first 60 min. This data show that ethanol extracts and water extracts of aged black garlic are capable of inhibiting the initiation of LDL oxidation.

Finally, this study showed that extracts of aged black garlic inhibits not only the initiation of LDL inhibition but also the propagation. Propagation of lipid oxidation is a process less dependent on copper ions. In the presence of garlic, the increase of time in the reaction mixture coupled with a increase of the value of conjugated diene, was determined graphically by the interception of the tangents to the slow and fast increase of the diene absorption. The Cu^{2+} concentration was coupled with the decreased of conjugated diene formation, which is an index of a lipid propagation phase and dependent only on the lipid composition LDL. The presence of 10-20 $\mu\text{g}/\text{ml}$ aged black garlic in the incubation mixture delayed the reaching of high absorbance.

Effects of aged black garlic on LDL oxidation by electrophoretic mobility

Table 3 shows the effect of garlic on the electrophoretic mobility of LDL submitted to oxidative modification by J774. A increase from 5.05 \pm 0.03 to 1.16 \pm 0.02 mm in the electrophoretic mobility of LDL incubated with J774 for 24

Table 2. Antioxidative effects of ethanol extract and water extract of aged black garlic on the formation of conjugated diene observed during the oxidation of LDL

Conditions	Diene conjugates (mol/mol apoB) ^{a)}
Native LDL	0.0
LDL+Cu ²⁺	33.83 \pm 0.03 ^{b)}
LDL+Cu ²⁺ +ethanol extract at 15 min	1.45 \pm 0.03
LDL+Cu ²⁺ +ethanol extract at 30 min	1.69 \pm 0.03
LDL+Cu ²⁺ +ethanol extract at 60 min	2.43 \pm 0.02
LDL+Cu ²⁺ +water extract at 15 min	2.53 \pm 0.03
LDL+Cu ²⁺ +water extract at 30 min	3.64 \pm 0.03
LDL+Cu ²⁺ +water extract at 60 min	4.52 \pm 0.04

^{a)}LDL (100 μg , protein/ml) was incubated with 20 $\mu\text{g}/\text{ml}$ extracts of aged black garlic by the addition of 5 mM CuSO_4 and then incubation was continued at 37°C as the times indicated.

^{b)}Mean \pm SE, Means in same column not sharing common superscript letters are significantly different ($P < 0.05$).

Table 3. Inhibition effects of aged black garlic on LDL oxidation by J774 as assessed by electrophoretic mobility

Incubation conditions	Relative electrophoretic mobility ^{a)}	P
Native LDL	1.0±0.02	
LDL+cell+vehicle (control)	4.95±0.03 ^{b)}	
LDL+cell+ethanol extracts 5 µg/ml	2.50±0.03	
LDL+cell+ethanol extracts 10 µg/ml	1.14±0.02	<0.05
LDL+cell+water extracts 5 µg/ml	2.30±0.02	<0.05
LDL+cell+water extracts 10 µg/ml	1.26±0.02	<0.01

^{a)}LDL (100 µg/ml) was incubated for 24 hr in 35 mm dishes containing J774 in the presence or absence of aged black garlic extract. The electrophoretic mobility of LDL was determined in agarose gel as described in the text.

^{b)}Mean±SE, Means in same column not sharing common superscript letters are significantly different (P <0.05).

hr implies lipid peroxidation of LDL and an increase in negative charges on the LDL molecule. Aged black garlic reduced the relative electrophoretic mobility of LDL dose dependently. LDL oxidized by CuSO₄ displayed a greater electrophoretic mobility in agarose gels compared to native LDL. When LDL was incubated with 5~10 µg/ml extracts aged black garlic the electrophoretic mobility of oxidized LDL was only slightly greater than native LDL. Aged black garlic inhibited the cell-induced oxidation of LDL as measured by lipoperoxide content of the electrophoretic mobility of LDL in agarose gels. Steinbrecher et al. [27] demonstrated that LDL can be modified by the addition of fatty acid peroxidation in the absence of cells. This modified LDL possesses an enhanced electrophoretic mobility without the lipid constituents of LDL being oxidized. It is possible that oxidation of LDL mediated by J774 can also contribute to the modification of the LDL protein as determined by the enhanced electrophoretic mobility.

This study was conceivable that an effective level of aged black garlic extract for inhibition of LDL oxidation may be attainable *in vivo*, sulfur containing components of aged black garlic may have antiatherogenic effects. This novel action of aged black garlic would increase its therapeutic value as an LDL oxidation, since lipid peroxidation.

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초록 : 숙성 흑마늘 추출물의 Low Density Lipoprotein (LDL)의 항산화 효과

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마늘을 발효시켜 만든 흑마늘의 지질 산화 억제 및 사람 Low Density Lipoprotein (LDL)에 대하여 항산화 활성을 실험하였다. 표준품으로서 마늘의 주성분인 alliin과 흑마늘의 에탄올과 물 추출물의 전자공여능을 각각 비교하여 측정된 결과 흑마늘 에탄올 추출물이 효능이 가장 높았다. 사람 LDL을 Cu^{2+} 유도 LDL로 산화시킬 때 그 항산화능은 각 시료를 10 $\mu\text{g/ml}$ 및 20 $\mu\text{g/ml}$ 씩 첨가하여 TBARS를 측정된 결과 에탄올 추출물이 항산화 활성이 가장 높았으며 용량 의존형으로 나타났다. 흑마늘의 에탄올과 물추출물을 10 $\mu\text{g/ml}$ 및 20 $\mu\text{g/ml}$ 첨가한 후 J774 유도 산화에 대한 항산화 효과를 측정된 결과 각 추출물은 항산화 효과가 있었고 용량 의존형의 항산화 활성을 나타내었다. 흑마늘의 각 추출물을 이용한 실험에서 전기영동 이동상은 대조구에 비하여 10 $\mu\text{g/ml}$ 및 20 $\mu\text{g/ml}$ 에서 산화를 억제하였고 공액 2중 결합에 의한 실험에서도 항산화 효과가 있었으며 20 $\mu\text{g/ml}$ 의 농도에서 거의 억제되었다.