# Inhibitory Effects of S-Allylmercaptocysteine Derived from Aged Garlic on Cholesterol Biosynthesis in Hepatocytes

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**ABSTRACT** - The present study was undertaken to elucidate the mechanisms underlying the cholesterol-lowering effect of S-allylmercaptocysteine (SAMC) derived from aged garlic. Rat hepotocytes and HepG2 cells were used to determine the short-term effects of SAMC on [$^{14}$C] acetate incorporation into cholesterol, and several enzymatic steps. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and treated with 20, 40, 60 and 80 μg/ml of SAMC. At concentration of 20~40 μg/ml, no significant cells viability effect was noted during those incubation periods. However, at a concentration 60 μg/ml, cell viability decreased approximately 50% compared with the control. The treatment of cells with 5, 10, 15, and 20 μg/ml of SAMC resulted in a marked of [$^{14}$C]-acetate incorporation into cholesterol. At concentration of 15 μg/ml, the cholesterol synthesis was inhibited 79% in cells. The activities of lipogenic enzymes, fatty acid synthase (FAS), and glucose-6-phosphate dehydrogenase (G3PDH) were measured in culture hepatocytes treated with the inhibitors. The activity of FAS in cells treated with 0.95 nmol SAMC was 19% lower than that of nontreated cells, and no affected G6PDH activity, 3-hydroxy-3-methylglutaryl Co A activity was decreased at concentration dependant manner. The present study demonstrates that SAMC is effective in inhibiting cholesterol biosynthesis.

**Key words :** S-allylmercaptocysteine, hepatocytes, cholesterol biosynthesis

## Introduction

Many health-beneficial effects of garlic have been demonstrated based on its diverse biological and pharmacological effects observed experimental and clinically[1,2]. Garlic and its organosulfur compounds have been shown to reduce risk factor for cardiovascular diseases[3]. Several garlic products have been shown to decrease plasma cholesterol and triglyceride levels in human[4,5], and animal[6]. A significant reduction of cellular cholesterol synthesis also has been demonstrated after exposing primary rat hepatocytes to garlic extracts, allicin and ajoene[7]. The depression of activities of the cholesterogenic enzyme 3-hydroxy-3-methyl-glutaryl Coenzyme A (HMG-CoA) reductase as well as the lipogenic enzymes including glucose-6-phosphate dehydrogenase and malic enzyme, by garlic compounds has been reported[5,7,8]. Its therefore seems reasonable to postulate that hypolipidemic effect of garlic and its compounds results from impaired lipogenesis and cholesterogenesis.

One of the possible explanation for the discrepancy may stem from different ingredients of garlic or garlic preparations used in various studies[7]. Moreover, the active compounds of garlic responsible for the putative lipid-lowering effects are ill defined[9]. Raw garlic and garlic preparations contains lipid-soluble thiosulfinates, thiosulfinate transformation products, and sulfides. Among these compounds, allicin and vinyldithiins have been suggested as potential lipid-lowering agents[10]. In addition, garlic in general, and aged garlic extract in particular, contains a significant amount of water soluble organosulfur compounds including S-alkenyl cysteines and γ-glutamyl-S-alkenyl cysteins[11]. Some of these water soluble organosulfur compounds, such as S-methyl-cysteine sulfoxide and S-allylcysteine sulfoxide, are known to reduce cholesterol levels in the liver and plasma[12].

S-allylmercaptocysteine content in the intact garlic is small (not more than 30 μg/g-fresh weight), however, this compound is increased in a aging procedure through hydrolysis of γ-glutamyl-s-allylcysteine, which exists in raw garlic as a precursor of S-allylamino compound[13]. Thus, the physiologic role of S-allylmercaptocysteine is largely unknown, despite the fact that S-allylmercaptocysteine is the major metabolite of aged garlic compounds[11].

Recently, S-allylmercaptocysteine, water soluble a sulfur-containing amino acid derived from aged garlic, have been reported antiproliferative[14], apoptosis[15], and prostate, colon

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and breast cancers[16]

Despite extensive studies on understanding the effects of aged garlic on human lipid metabolism, little is known about the mechanisms underlying possible hypotriglyceridemic action of water soluble organosulfur compound.

The goal of the present study was to determine whether S-allylmercaptocysteine suppresses cholesterol and glycerol lipid biosynthesis in hepatocytes in primary culture. To find out the nontoxic level of S-allylmercaptocysteine, the cytotoxicity was also determined at various concentration of S-allylmercaptocysteine. The human hepatoma-derived cell line HepG2 was chosen for the present study because these cells retain many normal hepatic metabolism functions including lipoprotein synthesis and cholesterol metabolism.

## Materials and Methods

### Materials

S-allylmercaptocysteine (85% S-allylmercaptocysteine, 10% methylmercaptocysteine, 5% S-allylpropenylcysteine was provided by Sejong University. HepG2 (the human hepatoma cell) was obtained from America Type cell Culture (Rockville, MD, USA). Radioactive substrates were purchased from Amersham Corp (Arlington Heights, IL, USA). Dulbecco's modified Eale's medium (DMEM), fetal bovin serum (FBS) and antibiotics were the products of GIBCO (Grand Island, NY, USA). Trypsin, ethylene diamine tetra acetic acid (EDTA), Bovine Serum Albumin (BSA), and the enzymatic cholesterol reagent kit were obtained from Sigma chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were analytical grade.

### Cell culture

The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, and antibiotics (100 units penicillin/mL and 100 μg/mL streptomycin). Stock cultures were maintained T-flask at 37°C under atmosphere of 95% air and 5% $CO_2$. The growth medium was removed and the cells were washed twice with $Ca^{2+}$, $Mg^{2+}$-free balanced Hank's set solution (BHSS). The cell were harvested by trypsinization, added immediately to DMEM containing 10% fetal bovine serum (FBS) to inhibit the tryptic activity, and centrifuged at 600xg to sediment cells. Aliquot of cell suspension in the growth medium ($2.2 \times 10^4$ cells) were seeded and grown in T-25 flask.

### Cytotoxicity of S-allylmercaptocysteine

The HepG2 cells ($1.0 \times 10^4$ mL) were seeded into each well of 24 well culture plates and grown in DMEM containing 10% FBS for 48 hr. By the time, a dense monolayer had formed. The cells were then incubated with l mL of the serum-free medium containing different concentrations of S-allylmercaptocysteine 20, 40, 60, 80 and 100 μg/mL) for 12 and 24 hr. The cells then were detached using a solution of 0.005%-trypsin-EDTA in BHSS and were immediately resuspended in DMEM supplemented with 10% FBS to inhibit the tryptic activity. The cells numbers were counted using a hemocytometer, and nonstained cells were scored a viable.

### Fatty acid/albumin mixture solution

A stock solution of a fatty acid/albumin mixture was prepared under aseptic conditions[17]. Briefly, 20 μmoles of sodium oleate, was dissolved in 1 mL of sterilized water at room temperature. Five μmoles (30 mg) of BSA was dissolved in 4 mL of DMEM. The fatty acid solution was then added to the BSA solution dropwise while stirring. The fatty acid/albumin solution prepared was optically clear. The molar ratio of fatty acid to albumin was kept at 4:1.

### Incorporation of $^{14}$C-acetate into cholesterol and fatty acid

The cells were grown in DMEM containing 10% FBS at day 6, cells were washed twice with HBSS. The cells were then incubated with 3 mL of serum free DMEM containing 3 μCi of $^{14}$C-acetate and various concentrations of S-allylmercaptocysteine.

In another study, the cells were incubated with 3 mL of the serum-free DMEM containing 1 mM of oleic acid/albumin mixture, 100 mg of S-allylmercaptocysteine, and 3 μCi of $^{14}$C-acetate. After 4 hr of incubation, the medium was collected and stored at −20°C. The cells were washed twice with BHSS, harvested by trypsinization, and centrifuge at 2,000 rpm for 5 min at 4°C and sonicated with an ultrasonic cell disrupter. The lipid from homogenate and medium were extracted by the method of Folch et al[18]. After saponification the lipids were extracted and were separated on silica gel plates as developing solvents. The cholesterol and fatty acid bands were visualized by exposure to iodine vapor. The cholesterol and fatty acid bands were then cut into vials and the radioactivity counted in a Beckman scintillation counter (Model LS 5800, U.S.A).

### Preparation of microsomal fraction

Microsomal fractions were prepared from liver tissue obtained from Sprague-Dawley rats (220~270 g). The tissues homogenized in the buffer containing 30 mM EDTA, 70 mM NaCl, 10 mM dithiothreitol and 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 15,000xg for 30 min. This pellet containing heavy microsomes was resuspended in the homogenizing buffer and was washed by recentrifuging at 100,000xg for 30 min. The final

pellets was used as the microsomal fraction for assay of enzymes.

### Determination of enzyme activities

The cells were cultured in 60 mm diameter culture dishes plated with $3.0 \times 10^6$ cell/dishes. The medium was removed, and the cells were washed 3 times with ice-cold phosphate buffered saline. The dishes were placed on ice and immediately the following were added: 0.5 mL of potassium phosphate buffer (PPB, 100 mmol/L, pH 7.4) containing sucrose, 250 mmol/L: EDTA, 1 mmol: and dithiothreitol (PTT), 1 mmol. The supernatant obtained was used to determine the enzyme activities and protein concentration. Fatty acid synthase activity was determined by the method of Nepokroeff et al.[19] in PPB (500 mmol/L, pH 7.0) containing malonyl CoA, 0.1 mmol/L; acetyl CoA, 0.05 mmol/L; NADPH, 0.1 mmol/L; EDTA, 1 mmol/L; and DTT 5 mmol/L. The reaction was initiated by the addition of 0.1 mL malonyl CoA to a final volume of 1 mL, and the rate of oxidation of NADPH was monitored at 340 nm by using spectrophotometer (Beckman Du-50LS 5801, Beckman Instrument).

Glucose 6-phosphate dehydrogenase (G6PDH) was measured by the methods of Deutsch[20]. The reaction mixtured contained Tris C-buffer (100 mmol/L), NADP (0.4 mmol/L), MgCl2 (5 mmol/L), and glucose 6-phosphate (G-6-p) (5 mmol/L). The reaction was initiated by the addition of 0.1 mmol/L of G-6-P to a final volume of 1 mL. The reaction of NADP was monitored at 340 nm by using spectrophotometer (Beckman Du-5025 5801, Beckman Instrument). The specific activity of enzymes was defined as nmol NADPH oxidized/mg protein/min for FAS and nmol NADP reduced/mg protein/min for G6PDH. HMG-CoA reductase was measured by the methods of Shapiro, et al.[21] Cellular protein was determined by the method of Lowry et al.[22]

### Statistics

Data are shown as the mean ± standard error of means (SEM). The data were evaluated statistically, using student's t-test or one-way analysis of variance the groups at $p$ value of less than 0.05.

## Results and Discussion

Fig. 1 is shown the effect of various concentration of S-allylmercaptocysteine on viability of HepG2 cells during incubation times at 12, and 24 hr. At concentration up to 100 µg/mL, no significant cytotoxic effect was noted during the 12 and 24 hr incubation periods. Average viable cells counts ($1.0 \times 10^3$/mL) in those concentrations in those concentrations (20~100 µg/mL) were 125 ± 5.2/control at 12 hr, and 110 ± 12.6/control at 24 hr. At concentration of 60 µg/

mL, cell viability decreased approximately 50% compared with the control ($P < 0.05$) in all two incubation times at 12, and 24 hr compared with the control. When the monolayer of cells were examined under the light microscope, the cells appeared normal at concentration up to 80 µg/mL. At 40, and 60 µg/mL, however, morphological abnormalities including cellular disintegation were very apparent. Base on cell viability and morphologic appearance, concentration up to 60 µg/mL were used as nontoxic levels of S-allylmercaptocysteine.

The ingestion of high amounts of garlic and garlic extracts has been reported to cause toxic effects on the liver or the function of liver enzymes. Gebhardt et al[7] reported that the cytotoxity of cell death occurred at the concentrations above allicin (80 µL/mL), and ajoene (234 µL/mL).

Although the mechanism of garlic indeed cytoxity is not clearly understood, it has been suggested that ajoene could interfere with cellular sulfur hydryl (SH) hoemeostasis by et affecting the glutathione (GSH) cycle through oxidation of GSH by the sulfoxide grow[23].

Inhibition activities of S-allylmercaptocysteine on fatty acid synthase FAS and glucose-6-phosphate dehydrogenase (G6PDH) were shown as Table 1. The inhibition on fatty acid biosynthesis led to determine the effects of S-allylmercaptocysteine on the activities of lipogenic enzymes in HepG2. The cells were incubated with S-allylmercaptocysteine at the various concentrations. The activity of FAS in HepG2 cells treated with 4.0 mmol/L S-allylmercaptocysteine was 31% lower than that of control (Table 1).

On the other hand, S-allylmercaptocysteine depressed the activity of G6PDH. This data suggest that fatty and synthnase
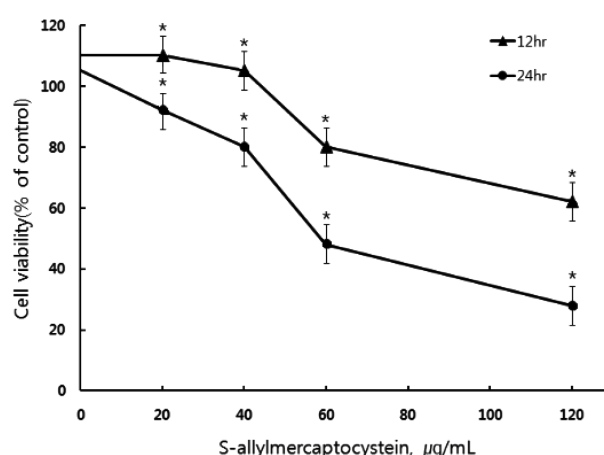


**Fig. 1.** Effects of S-allylmercaptocysteine on cell viability of HepG2 cells. The cells were incubated in a serum-free DMEM containing 20, 40, 60, 80 or 100 µg/mL of S-allylmercaptocysteine for 12 and 24 hr. Data are expressed as a percentage of the control and represent means ± SEM of six samples. *Significant difference from controls at $P < 0.05$.

**Table 1.** Inhibition activities of S-allylmercaptocysteine (SAMC) on fatty acid synthetase (FAS) and Glucose-6-phosphate dehydrogenase (G6PDH) in HepG2 cells

| Compounds | Concentration (mmol/L) | FAS[a] | G6PDH[a] |
|---|---|---|---|
| SAMC | 0 | $3.70 \pm 0.27$ | $36.63 \pm 0.72$ |
| | 0.95 | $3.00 \pm 0.20^*$ | $35.05 \pm 0.67^*$ |
| | 2.0 | $2.54 \pm 0.31^*$ | $31.54 \pm 0.76^*$ |
| | 4.0 | $2.53 \pm 0.24$ | $32.40 \pm 0.82$ |

[a]The specific activity of enzyme is defined as mmol NADPH oxidizing/mg protein/min for FAS and nmol of NADP reducing/mg protein/mg protein/min for G6PDH. Data represent means $\pm$ SEM of six sample. *Significant difference from control (nontreated) group, $P < 0.05$

depressed by the organic disulfur chain of S-allylmercaptocysteine. Earlier animal studies by other investigators showed that garlic supplemented diets decreased activities of not only lipogenic FAS but also G6PDH[8]. The reason for the discrepancy in G6PDH activity observed between the present study and that of others is not readily understood. The marked inhibition on fatty and biosynthesis led us to determine the effects of organosulfur compounds on the activities of lipogenic enzymes in cultured hepatocytes[7]. However, it is important point out that S-allylmercaptocysteine derived from aged garlic were used in the present *in vitro* experiment as compared with various garlic extracts fed to animal[9]. Garlic extracts used in the studies contained not only S-allylmercaptocysteine, but also water soluble and fat soluble sulfur containing compounds[5].

The incorporation of [$^{14}$C] acetate into cholesterol was taken as a measure of cholesterol (Fig. 2). S-allylmercaptocysteine were tested for inhibition potency on cholesterol synthesis in cultured HepG2 cells. The treatment of cells at concentrations of 5, 10, 15, 20, 25 and 30 μg/mL of S-allylmercaptocysteine resulted in apparent inhibition of [$^{14}$C]-acetate incorporation into cholesterol in cells. S-allylmercaptocysteine exerted a concentration-dependent inhibition of cholesterol synthesis. At a concentration of 5 μg/mL, $^{14}$C-acetate incorporation into cholesterol was inhibited approximately 20% compared with then control ($P < 0.05$). The average inhibition of cholesterol synthesis was 30%, 50%, 60%, and 65% with concentrations of 15, 20, 25 and 30 μg/mL, respectively ($P < 0.05$). In present study, the treatment of cells with S-allylmercaptocysteine resulted in a significant inhibition of cholesterol synthesis. The inhibitory effect of cholesterol by S-allylmercaptocysteine was at the concentration-dependent manner and even at the concentration of 10 μg/mL, the cholesterol synthesis decreased approximately 30% compared then the control. These results suggested that organosulfur compounds exerts an inhibitory effect on cholesterol synthesis in rat hepatocytes. The inhibition of $^{14}$C-acetate incorporation into cholesterol by S-
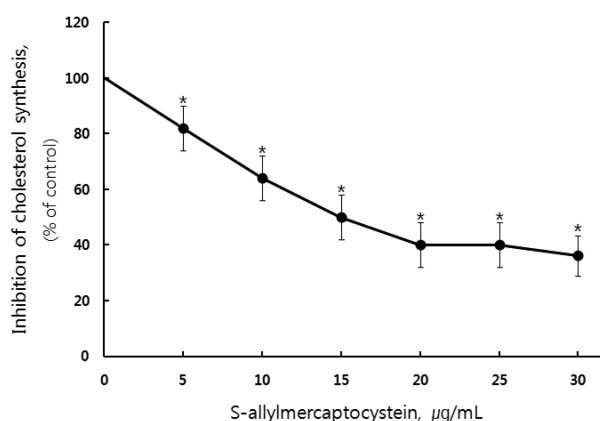


**Fig. 2.** Inhibition effects of S-allyl-mercaptocyteine on cholesterol synthesis in HepG2 cells. The cells were incubated in a DMEM medium containing various concentration of S-allylmercaptocysteine and $^{14}$C-acetate (l μCi/mL) for 4 hr. Data are expressed as a percentage of the control. Data represent mean $\pm$ SEM of six samples. *Significant difference from control at $P < 0.05$.
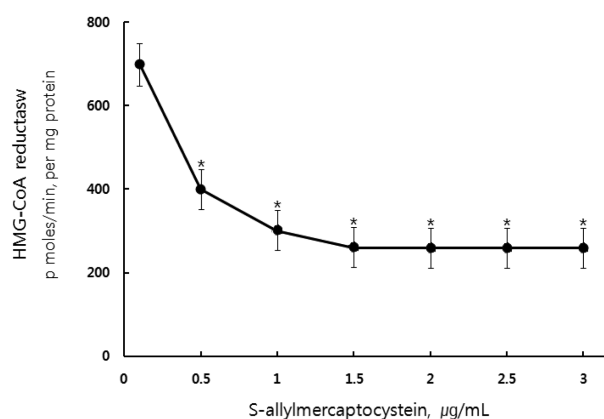


**Fig. 3.** Effects of S-allylmercaptocysteine on inhibition of HMG-CoA reductase of rat hepatocytes. Different concentrations of S-allylmercaptocysteine were preincubated with microsomal suspensions for 15 min at 37°C. The assay was carried out under standard conditions. Data represent mean $\pm$ SEM of six Samples. *Significant difference from control at $P < 0.05$.

allylmercaptocysteine could be due to the decreased activity of HMG-CoA reductase, because water insoluble garlic sulfur compounds, allicin, and ajoene have been shown to lower the activity of HMG-CoA reductase in rat hepatocytes[7,24], and the formation of protein internal disulfides, which are inaccessible for reduction by thiol agents, has been indicated to be the cause of this inactivation[25].

S-allylmercptocysteine was also appeared to inhibit HMG-CoA reductase in at concentration-dependent fashion in rat hepatocytes (Fig. 3). Maximun inactivation of microsomes with the disulfide for a few minutes for HMG-CoA reductase, a concentration- dependent inhibition was found starting at the concentration as low as 0.5 μg/mL. The nature of this inhibition by garlic disulfide is presumed to be due

to rearrangement of thiol-disulfide group. Two other possibilities exist for the inactivated form; a mixed disulfide of allyl-S-S-protein or an internal protein disulfide. In either case the disulfide formed must be inaccessible for reduction[26]. Then this compound must be absorbed intact and reach the liver and the endoplasmic reticulum for inhibition of HMG-CoA reductase which then explains its capability for lowering plasma cholesterol concentration[6] and its therapeutic value in atherosclerosis. Being a natural food constituent, resistant to reduction by cellular thiol compound, S-allylmercaptocysteine will be able to regulate cholesterol biogenesis by irreversible inactivating HMG-CoA reductase, a disintinctive property of this disulfide. Its regulation represents the physiological process and S-allylmercaptocysteine is likely to be an effective dietary agent in controlling the activity of HMG-CoA reductase. The present findings concluded that S-allylmercaptocysteine inhibited the biosynthesis of cholesterol in hepatocytes thus contributing to the reduction of serum cholesterol. Although it is rather difficult to interpret the relevance of current data to dietary effect of garlic S-allylmercaptocysteine, which is a major metabolite of garlic compounds circulating in the blood, could be partly responsible for the cholesterol-lowering effect of garlic. Fresh garlic is famous for its characteristic odor, arising from allicin, therefore, aged garlic is an odorless product resulting from prolonged aging of fresh garlic oil soluble sulfur garlic compounds in fresh garlic appears to be due to toxisity as revealed by increased lactate dehydrogenase from exposed hepatocytes[12]. Water soluble sulfur compounds in aged garlic were not cytotoxic. An array of water soluble constituent, S-allylmercaptocysteine, may account for the reduced toxicity of the aged garlic compared fresh garlic.

## 요  약

마늘 숙성 중 생성되는 S-allylmercaptocysteine의 콜레스테롤 생합성 억제 효과에 대하여 Hepatocytes를 이용하여 조사하였다. HepG2 cells을 Dulbecco's modified Eagle's medium (DMEM)에 배양하여 S-allylmercaptocysteine의 농도를 20, 40, 60, 80 및 100 mL 씩 각각 첨가하여 cell viability를 살펴본 결과 20~40 μg/mL에서는 높았으며, 60 μg/mL 농도에서 약 50%가 유지되었다. S-allylmercaptocysteine을 5, 10, 15 및 20 μg/mL 농도로 [$^{14}$C]-acetate-cholesterol에서 처리하였을 경우 15 μg/mL 농도에서 cholesterol 생합성이 79%로 억제되었다. Fatty acid synthase의 활성은 0.95 nmol에서 19%의 억제효과를 나타내었으나, Glucose 6-phosphate dehydrogenase (G6PDH)의 활성에는 거의 영향을 미치지 않았다. S-allylmercaptocysteine의 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase의 활

성은 용량 의존형으로 감소하였다. 이상의 결과로 보아 마늘 숙성 과정에서 생성되는 주요 성분인 S-allylmercaptocysteine은 간 세포에서 cholesterol의 생합성을 억제하는데 기여하는 것으로 나타났다.

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