Garlic and Garlic-Derived Compounds Inhibit Human Squalene Monoxygenase

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ABSTRACT Although extracts of garlic inhibit cholesterol biosynthesis in cultured hepatocytes, the inhibitory components of garlic and the site or sites of inhibition in the cholesterol biosynthetic pathway have not been established. To elucidate potential mechanisms of inhibition, we examined the effect of fresh garlic extract and 16 water- or lipid-soluble compounds derived from garlic on purified recombinant human squalene monoxygenase. Squalene monoxygenase catalyzes the second and likely rate-limiting step in the downstream pathway for cholesterol biosynthesis. A 50% inhibitory concentration (IC50) of squalene epoxidation was achieved with 1 g/L of fresh garlic extract; of the 16 garlic compounds tested, only selenocystine (IC50 = 65 μmol/L), S-allylcysteine (IC50 = 110 μmol/L), alliin (IC50 = 120 μmol/L), diallyl trisulfide (IC50 = 195 μmol/L), and diallyl disulfide (IC50 = 400 μmol/L) substantially inhibited the enzyme. Kinetic analysis showed that the inhibition by garlic and by these compounds was slow and irreversible, suggestive of covalent binding to the enzyme; the ability of thiol-containing compounds such as glutathione and 2,3-dimercaptopropanol to prevent and reverse the inhibition indicated that the garlic compounds were reacting with sulfhydryl groups on the protein. Dithiols were better reversal agents than monothiols, further suggesting that these inhibitors bind to the proposed vicinal sulfhydryls present on this enzyme. These results indicate that squalene monoxygenase may be one of the target enzymes through which garlic inhibits cholesterol biosynthesis. J. Nutr. 131: 1662–1667, 2001.

KEY WORDS: • cholesterol biosynthesis • garlic • selenium • selenocystine • squalene epoxidase

Garlic has been reputed to possess medicinal properties since ancient times. More recently, attention has been focused on garlic's ability to decrease blood cholesterol levels, as demonstrated in clinical trials in humans (1,2), through meta-analyses of clinical studies (3–5) as well as in experimental studies with animals (6–8) and cultured hepatocytes (9–11). Efforts have been made to isolate and identify the active chemical components of garlic; S-allylcysteine (SAC),3 diallyl disulfide (DADS), and alliin and its derivatives (ajoene) all have been shown to contribute to its hypocholesterolemic actions (11–13). However, the mechanism underlying the inhibitory action of these garlic compounds has not been established. Several studies have reported that feeding garlic-supplemented diets to animals decreased the activity of several cholesterologenic enzymes, including 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (8,14–15) and acetyl-CoA synthetase (12), and it is possible that additional enzymes in the cholesterol synthesis pathway are also inhibited.

Squalene monoxygenase (EC 1.14.99.7, earlier called squalene epoxidase) is a 64-kDa FAD-containing enzyme bound to the endoplasmic reticulum of eukaryotic cells. The enzyme catalyzes the first oxidative step in cholesterol biosynthesis, the epoxidation of squalene across a C=C double bond to yield 2,3-oxidosqualene, a reaction more typical of cytochrome P450 type chemistry. Like the cytochromes P450, this flavoprotein monoxygenase also is dependent upon NADPH-cytochrome P450 reductase (CPR) for reducing equivalents. Squalene monoxygenase evidently plays an important role in the overall regulation of cholesterol biosynthesis in that the addition of cholesterol to cells in culture lowers squalene monoxygenase mRNA levels, suppresses squalene monoxygenase activity and results in the accumulation of squalene (16). Garlic can contain high levels of tellurium and selenium compounds (17–20); the possibility that these compounds contribute to the overall block in cholesterol synthesis by inhibiting squalene monoxygenase has been proposed (21) but not yet examined. Indeed, inhibition of this enzyme by tellurium was shown to block cholesterol synthesis in Schwann cells and led to a peripheral demyelinating neuropathy (22). In previous work from our laboratory, tellurium, selenium and arsenic compounds were shown to inhibit human squalene monoxygenase by binding to vicinal cysteine sulfhydryls on this enzyme (23,24). It is therefore plausible that tellurium and selenium compounds in garlic may inhibit squalene monoxygenase and thereby contribute to the hypocholesterolemic actions of this plant.

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E-mail: tporter@pop.uky.edu.
3 Abbreviations used: CPR, cytochrome P450 reductase; DADS, diallyl disulfide; DATS, diallyl trisulfide; DMP, 2,3-dimercaptopropanol; GSH, glutathione; HMG, 3-hydroxy-3-methylglutaryl; IC50, 50% inhibitory concentration; SAC, S-allylcysteine; SC, selenocystine.
To address this possibility, fresh garlic extract and 16 garlic-derived compounds (Fig. 1) were tested for their ability to inhibit purified human squalene monooxygenase. This work identifies five chemicals found in garlic as inhibitors of squalene monooxygenase and suggests that these compounds bind to vicinal cysteine sulfhydryls on the enzyme. Although there are several reports that selenium-containing compounds from garlic are effective in the prevention of mammary and other types of cancer (18, 19, 25), this is the first report to show that there are several reports that selenium-containing compounds inhibit purified human squalene monooxygenase. This work identifies five chemicals found in garlic as inhibitors of squalene monooxygenase. Fig. 1 activity was determined with cytochrome c spectrally using a millimolar extinction coefficient of 21.4 at 456 nm. Purification of squalene monooxygenase. Human recombinant squalene monooxygenase expressed from the pT7B4 vector was purified according to the protocol described by New England Biolabs (Beverly, MA) for expression of proteins with the IMPACT T7 system (Intein-chitin binding domain fusion proteins), as previously described (23). Protein was quantified with the Coomassie Plus Protein Assay Reagent Kit from Pierce (Rockford, IL) and was then stored at −80°C until use. Squalene monooxygenase assays. Squalene epoxidation assays were carried out as described previously (23). Standard reaction mixtures (200 µL) containing 20 mmol/L Tris-HCl (pH 7.4), 0.1% Triton X-100, 30 µmol/L FAD, 28 pmol CPR, 40 µmol/L 14C-squalene and 58 pmol squalene monooxygenase were preincubated with or without inhibitor for 30 min at 37°C. The reactions were then started by the addition of 1 mmol/L NADPH and incubated for another 30 min at 37°C. Product formation was linear for 40–60 min at 37°C, and yielded 1.1 nmol/L of 2,3-oxidosqualene/30-min reaction. Reactions were stopped by extraction into methylene chloride and then fractionated on TLC plates with 5% ethyl acetate in hexane. The developed plates were quantified by electronic autoradiography (Packard Instant Imager; Wilmington, DE). Cytochrome P450 reductase assays. Recombinant rat CPR was purified by affinity chromatography as described (26) and quantified spectrally using a millimolar extinction coefficient of 21.4 at 456 nm. Activity was determined with cytochrome c in 100 mmol/L potassium phosphate buffer (pH 7.7) with 50 µmol/L cytochrome c and 5 pmol of reductase; reaction of 50 µmol/L NADPH and followed at 550 nm for 1 min at room temperature. Preincubations were carried out with garlic extract or garlic compounds for 30 min at 37°C.

MATERIALS AND METHODS

Materials. Garlic bulbs were purchased from a local grocery. Alliin, S-methylcysteine, S-ethylcysteine, S-propylcysteine and SAC were graciously provided by Wakunaga of America (Mission Viejo, CA). DADS and diallyl sulfide were purchased from Fluka Chemika (St. Louis, MO); dipropyl disulfide, dipropyl sulfide, allyl mercaptan, allyl methyl sulfide and selenomethionine were purchased from Acros Organics (Geel, Belgium); methallyl sulfide was purchased from Aldrich (Milwaukee, WI); diallyl trisulfide (DATS) was purchased from ICN Biomedicals; glutathione (GSH) was purchased from Boehringer Mannheim (Indianapolis, IN), GmbH; Se-(methyl)selenocysteine, L-selenocystine (SC), nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), FAD, 2,3-dimercaptopropanol (DMP) and cytochrome c were purchased from Sigma Chemical, St. Louis, MO. Radiolabeled squalene (2.6 X 10 Bq) was synthesized by the Chemical Synthesis Facility, Department of Medicinal Chemistry, University of Utah. Precast silica TLC plates were obtained from Whatman (Clifton, NJ). All garlic derivatives were stored at 4°C and stocks of the water-soluble compounds were made fresh every time before use. Lipid-soluble garlic derivatives were dissolved in dimethyl sulfoxide to a final concentration of 200 µmol/L.

Preparation of garlic extracts. Garlic cloves were peeled and homogenized with a small amount of quartz sand in 20 mmol/L Tris-HCl buffer (pH 7.4), and the debris was removed by centrifugation at 21,000 × g for 20 min at 25°C. The clear supernatant was divided into aliquots and stored at −80°C.

RESULTS

Fresh garlic extract and 16 different water- or lipid-soluble compounds present in garlic (Fig. 1) were tested for their ability to inhibit human squalene monooxygenase. A 50% inhibitory concentration (IC50) was achieved using 1 g/L garlic extract and activity was completely inhibited at 150 g/L (Fig. 2A). Of the eight water-soluble compounds, SC, SAC and alliin were the most potent inhibitors, with IC50 values of 65, 110 and 120 µmol/L, respectively (Fig. 2B). Inhibition by SC was nearly complete at 500 µmol/L, whereas inhibition by SAC and alliin was still incomplete. S-methylcysteine, S-
that electron transfer from the reductase is not rate limiting in reduced squalene monooxygenation by only 10–15%, indicating determined. A 70% decrease in reductase concentration re-
inhibition of cytochrome squalene monooxygenase reactions, the effect of a 70% decrease in reductase concentration had no effect on inhibition by these agents (data not shown). The protection afforded by squalene suggests that these compounds bind in the substrate-binding pocket of the enzyme and supports the conclusion that inhibition of CPR does not contribute significantly to the inhibition of squalene monooxygenation.

Garlic extract, SC, SAC, allin and DADS were slow, time-dependent inhibitors with $t_{1/2}$ values (half-life for inactivation) of 13, 22, 36, 41 and 91 min, respectively (Fig. 4). These results suggest that these inhibitors bind irreversibly to squalene monooxygenase. To address this possibility, enzyme-inhibitor dilution experiments were carried out as described previously (24). In these experiments, enzyme was preincubated with either garlic extract or one of its inhibitory components for 30 min and then diluted 12-fold before the addition of 1 mmol/L NADPH to start the reaction. No recovery of activity was seen after dilution of garlic or the four garlic derivative-containing incubations, indicating that dilution did not release these compounds from the enzyme and that these inhibitors bind irreversibly to squalene monooxygenase (Fig. 5).

Thiol-containing compounds were tested for their ability to protect against and reverse the inhibition of squalene monooxygenase by garlic and by four of the inhibitory compounds GSH, a monothiol, or DMP, a dithiol, was added before or after the 30-min preincubation. Addition of either thiol reduced the effect of the garlic components. The protection afforded by squalene suggests that these compounds bind in the substrate-binding pocket of the enzyme and supports the conclusion that inhibition of CPR does not contribute significantly to the inhibition of squalene monooxygenation.

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the garlic compounds could also be partially reversed by the thiol reagents (Fig. 6B). DMP was a significantly more effective reversal agent than GSH, an effect most noticeable with DADS and SC. This difference between the di-thiol and the monothiol was significant for three of the five substances tested, i.e., alliin, DADS and SC. Similar results were obtained with dithiothreitol and \( \beta \)-mercaptoethanol, confirming that di-thiols were more effective reversal agents (data not shown). These results are similar to those obtained previously in studies on the inhibition of squalene monooxygenase by tellurite (24) and are consistent with the garlic compounds reacting with vicinal cysteine sulfhydryls on squalene monooxygenase.

**DISCUSSION**

Experimental data from animal studies (7,14), clinical trials (1,2), meta-analyses (3–5) and cell culture studies (9–11) have supported the postulate that garlic extracts and garlic-derived compounds can lower cholesterol levels. Although several studies have indicated that one site of inhibition in the cholesterol biosynthetic pathway is HMG-CoA-reductase (14,15), additional downstream enzymes have also been suggested as targets for garlic and its components (12). In this work, we report the inhibition of purified recombinant human squalene monooxygenase by garlic and its derivatives. Assay mixtures were preincubated for 30 min at 37°C in the presence of 5 g/L of garlic extract or 100 \( \mu \text{mol/L} \) of S-allylcysteine (SAC), alliin, selenocystine (SC) or diallyl disulfide (DADS). The reactions were started by the addition of squalene monooxygenase and incubated for the indicated times at 37°C. Data are expressed as means \( \pm \text{SEM} \), \( n = 3 \) independent experiments. Half-life \( (t_{1/2}) \) values of inactivation for garlic, SC, SAC, alliin and DADS were 13, 22, 36, 41 and 91 min, respectively.

**FIGURE 5** Irreversible inhibition of human squalene monooxygenase by garlic and its derivatives, S-allylcysteine (SAC), alliin, selenocystine (SC) and diallyl disulfide (DADS). Assay mixtures were preincubated for 30 min at 37°C in a volume of 17 \( \mu \text{L} \) containing either fresh garlic extract or garlic derivative. After preincubination, the volume was increased to 200 \( \mu \text{L} \) and inhibitor concentration was held constant at 5 g/L of garlic extract or 0.1 \( \text{mmol/L} \) garlic derivative (constant concentration), or diluted from 60 to 5 g/L of garlic extract, or from 1.2 to 0.1 \( \text{mmol/L} \) garlic derivative (diluted concentration). The concentration of all other reaction components was held constant and NADPH was added to start the reaction. Reactions were carried out for 30 min at 37°C. Data are expressed as means \( \pm \text{SEM} \), \( n = 3 \) independent experiments.

**FIGURE 6** Effect of sulfhydryls on inhibition of squalene monooxygenase by garlic and its derivatives. (A) Protection experiments: assay mixtures were preincubated at 37°C with 5 g/L of garlic extract, 65 \( \mu \text{mol/L} \) selenocystine (SC), 110 \( \mu \text{mol/L} \) S-allylcysteine (SAC), 1.2 \( \mu \text{mol/L} \) alliin or 400 \( \mu \text{mol/L} \) diallyl disulfide (DADS) in the absence or presence of 1 \( \text{mmol/L} \) of either 2,3-dimercaptopropanol (DMP) or glutathione (GSH). After 30 min, the reactions were started by the addition of NADPH and incubated for an additional 30 min. Both thiol treatments afforded significant protection from the garlic compounds \( (P < 0.05, \text{one-way ANOVA with Dunnett’s multiple comparisons test}) \). (B) Reversal experiments: after a 30-min preincubation with garlic extract or derivative, 1 \( \text{mmol/L} \) DMP or GSH was added along with NADPH to start the reactions. Asterisks indicate the points at which reversal by DMP was significantly greater than that produced by GSH \( (P < 0.05, \text{one-way ANOVA with Bonferroni’s multiple comparison test}) \). No significant reversal by either thiol compound was obtained with the garlic extract, and GSH did not reverse the inhibition by DADS or SC. The formation of 2,3-oxididosqualene in the presence of DMP but no inhibitor was 1.76 \( \text{nmol/L} \) over 30 min; in the presence of GSH and no inhibitor, it was 1.38 \( \text{nmol/L} \). Data are expressed as means \( \pm \text{SEM} \), \( n = 3 \) independent experiments.
concentrations of 100–200 μmol/L DADS were effective inhibitors of cholesterol synthesis at squalene monooxygenase in the present study. Similarly, DATS and a concentration that yielded 50% inhibition of squalene monooxygenase with an IC50 value of 65 μmol/L. Se-(methyl)selenocysteine, selenomethionine or cystine were not inhibitory, indicating that the diseleno-bond was necessary for inhibition.

The present work implicates the involvement of cysteine sulphydryls in the inhibition of squalene monooxygenase by garlic and garlic-derived compounds. In previous work from this laboratory, evidence was presented that the inhibition of squalene monooxygenase by tellurium compounds results from binding to critical sulphydryl groups on the enzyme (24). Similarly, N-ethylmaleimide, a sulphydryl-specific reagent, was shown to inhibit squalene monooxygenase in rat liver microsomes (22). Thus, squalene monooxygenase is highly sensitive to inhibition by chemicals that react with sulphydryls. The inhibition of squalene monooxygenase by garlic and garlic-derived compounds could be prevented by preincubation with either GSH or DMP, indicating that these inhibitors react with enzyme thiol groups. Addition of GSH or DMP after the preincubation also partially reversed the inhibition, further supporting the thesis that cysteine sulphydryls on squalene monooxygenase are the target of binding by these compounds.

The present work further suggests that there are differences between the ability of monothiols and dithiols to reverse the inhibition of squalene monooxygenase by garlic compounds. DMP and dithiothreitol (dithiols) were more effective than GSH or β-mercaptoethanol (monothiols) in reversing the inhibition, suggesting that the garlic compounds were reacting with vicinal cysteines on the enzyme. The presence of vicinal cysteines was proposed to explain the unusual sensitivity of squalene monooxygenase to tellurium compounds (24). It should be noted that heating the garlic extract or the four garlic derivatives in a boiling water bath for 2 min completely inactivated these compounds and prevented the inhibition, indicating that they are relatively labile. Several other studies have noted that raw garlic was significantly more effective than cooked garlic in blocking cyclooxygenase activity in the prevention of thrombosis (30,31).

Although we have identified five garlic-derived chemicals that can inhibit squalene monooxygenase, we do not know whether these are the only inhibitory chemicals in garlic. Whether these are the only inhibitory chemicals in garlic extract, or whether they mediate the inhibition of squalene monooxygenase by garlic extract. Garlic contains a variety of organosulfur and organoselenium compounds, with SAC and allin as the most prominent. SAC appears to be a precursor to allin; when garlic is crushed, allinase is released to cleave allin to yield sulfenic acid (CH3=CHCH2SOH), which converts to form allicin. Allicin is also highly unstable, and rapidly degrades to a variety of organosulfur compounds, including DADS, DATS and ajoene. These compounds give rise to allyl mercaptan and, secondarily, allyl methyl sulfide; allyl mercaptan has been suggested to be the ultimate active chemical species derived from garlic consumption (10). Although allyl mercaptan is not inhibitory to squalene monooxygenase, several of the precursors and intermediates leading to this compound are inhibitory; most notably, SAC is one of the more effective inhibitors found in this study. SAC is considered to be a principal active ingredient in commercial garlic preparations; it is stable and readily absorbed from the gastrointestinal tract (32).

The present work demonstrates that the inhibition of squalene monooxygenase by garlic is slow, irreversible and likely to involve binding to sulphydryls in the squalene binding site of the enzyme. Garlic extract, as well as four of the garlic-derived components, inhibited squalene epoxidation in a time- and concentration-dependent manner, on the basis of the protection and reversal by monothiols and dithiols, it is likely that these inhibitors react with the proposed vicinal cysteine sulphydryls on squalene monooxygenase. The inhibition by the organoselenium compound, SC, is of particular interest because overconsumption of selenium by pigs and...
cattle is associated with a variety of pathologies suggestive of disruption of lipid metabolism in neural tissue (33). The effect of garlic and its components on the intracellular flux of cholesterol intermediates in vivo is the subject of current investigations.

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LITERATURE CITED


