J. Microbiol. Biotechnol. (2007), **17**(12), 1970–1975

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation and Structural Determination of Squalene Synthase Inhibitor from *Prunus mume* Fruit

CHOI, SUNG-WON[1], NAM-YOON HUR[1], SOON-CHEOL AHN[2], DONG-SEOB KIM[3], JAE-KWON LEE[4], DAE-OK KIM[5], SEUNG-KOOK PARK[5], BYUNG-YONG KIM[5], AND MOO-YEOL BAIK[5]*

[1]*Department of Food and Culinary Art, Osan College, Osan 447-749, Korea*
[2]*Department of Microbiology and Immunology, and Medical Research Institute, Pusan National University College of Medicine, Pusan 602-739, Korea*
[3]*College of Natural Resources and Life Sciences, Pusan National University, Miryang 627-706, Korea*
[4]*Department of Foods and Biotechnology, Kyonggi University, Kyonggi-do 442-760, Korea*
[5]*Institute of Life Science and Resources, Department of Food Science and Biotechnology, Kyung Hee University, Yongin 446-701, Korea*

Received: April 6, 2007 Accepted: July 6, 2007

Abstract Squalene synthase plays an important role in the cholesterol biosynthetic pathway. Inhibiting this enzyme in hypercholesterolemia can lower not only plasma cholesterol but also plasma triglyceride levels. A squalene synthase inhibitor was screened from *Prunus mume* fruit, and then purified *via* sequential processes of ethanol extraction, HP-20 column chromatography, ethyl acetate extraction, silica gel column chromatography, and crystallization. The squalene synthase inhibitor was identified as chlorogenic acid with a molecular mass of 354 Da and a molecular formula of $C_{16}H_{18}O_9$ based on UV spectrophotometry, ^1H and ^{13}C NMRs, and mass spectrometry. Chlorogenic acid inhibited the squalene synthase of pig liver with an IC_{50} level of 100 nM. Since chlorogenic acid was an effective inhibitor against the squalene synthase of an animal source, it may be a potential therapeutic agent for hypercholesterolemia.

Keywords: Squalene synthase inhibitor, chlorogenic acid, *Prunus mume*, hypercholesterolemia

A number of studies have shown that the elevated serum cholesterol, especially low-density lipoprotein (LDL) cholesterol, is one of the major risk factors for coronary heart disease, and that lowering LDL cholesterol leads to a reduction in the incidence of death by coronary heart diseases [5, 11, 13, 27]. The Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT) has concluded that every 1% reduction in plasma total cholesterol leads to a 2% decrease in risk of coronary heart disease [24]. At present, the most effective therapeutic approach to reduce the level of plasma LDL cholesterol is the inhibition of cholesterol biosynthesis [16]. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.34) is a key rate-limiting enzyme involved in the cholesterol biosynthetic pathway [24]. Several inhibitors of this enzyme effectively lower plasma cholesterol levels [31, 32]. These inhibitors are statins such as lovastatin, pravastatin, simvastatin, and atorvastatin. The lipid-lowering ability of HMG-CoA reductase inhibitors has been derived from upregulation of the LDL receptor and inhibition of triglyceride secretion from the liver [12, 18, 26]. HMG-CoA reductase inhibitors occasionally induce adverse effects in liver and muscle because they suppress the production of mevalonate, which is also used for the synthesis of nonsterol products such as isopentenyl tRNA, dolichol, coenzyme Q_{10}, and isoprenylated proteins. This side effect may restrict its beneficial application in the prevention of coronary heart disease [14, 19].

Squalene synthase (SQS; E.C. 2.5.1.21) catalyzes the formation of squalene from farnesyl pyrophosphate (FPP) in the cholesterol biosynthesis [29]. This enzymatic reaction takes place after the pathway steps to produce various other isoprene derivatives such as dolichol, ubiquinones, the farnesyl group of heme A, the farnesyl and geranyl groups of prenylated proteins, and the isopentenyl side-chain of isopentenyl adenine. Since squalene synthase inhibitors might not interfere with the biosynthesis of these isoprene derivates essential for cell growth and viability, squalene synthase inhibitors might be a better arsenal for lowering cholesterol compared with the HMG-CoA reductase inhibitors currently used [4].

In this study, squalene synthase inhibitors were screened as an agent for prevention of hypercholesterolemia. A squalene synthase inhibitor was isolated and purified from *Prunus mume* fruit, and its chemical structure and biological properties were characterized.

*Corresponding author
Phone: 82-31-201-2625; Fax: 82-31-204-8116;
E-mail: mooyeol@khu.ac.kr

MATERIALS AND METHODS

Preparation of Pig Squalene Synthase

One hundred g of pig liver was finely chopped and minced. Two hundred ml of homogenizing buffer [0.1 M potassium phosphate (pH 7.4), 0.3 M sucrose, 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, and 50 mM KCl] was immediately added to the minced sample and homogenized. The mixture was centrifuged at 4,000 ×*g* for 15 min and then at 15,000 ×*g* for 30 min. The supernatant was ultracentrifuged at 105,000 ×*g* for 1 h to obtain microsome precipitates. Fifty ml of fresh homogenizing buffer was added to wash the microsome precipitates, which were then homogenized and ultracentrifuged again at 105,000 ×*g* for 30 min. The precipitates were resuspended with fresh homogenizing buffer and used as an enzyme source for the measurement of SQS activity [2, 21].

Measurement of Inhibitory Activity to SQS

Ten μl of the inhibitor sample, 100 μl of reaction buffer [100 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 10 mM DTT, and 2 mM NADPH], and 50 μl of microsome suspension (1.92 mg-protein/ml) were mixed and maintained for 10 min at 37°C. Subsequently, 10 μl of 5 mM [^{3}H] farnesyl pyrophosphate was added to the mixture and reacted at 37°C for 30 min. The specific activity of [^{3}H]-farnesyl pyrophosphate was adjusted to 100 mCi/M (1 Ci=37 GBq) before use, and 200 μl of cool ethanol was added to stop the reaction. The reacted solutions were extracted three times with *n*-hexane. Finally, after the hexane layer was mixed with cocktail solutions, the enzymatic activities were measured using a scintillation counter (LS6500, Beckman Instrument Inc., Fullerton, CA, U.S.A.) [13, 20]. To estimate the inhibitory activity against SQS, differences in cpm values with and without inhibitor sample were measured [20, 22].

Purification of SQS Inhibitor from *Prunus mume* Fruit

One hundred g of *Prunus mume* fruit was extracted three times with 1 l of 70% (v/v) aqueous ethanol. After the extracts were centrifuged, the supernatants were concentrated under reduced pressure. The mixture was washed with 70% aqueous ethanol, resuspended with distilled water, and then concentrated again under reduced pressure in order to completely remove the ethanol.

The extract was adjusted to pH 4.0, adsorbed on HP-20 column chromatography, and then eluted sequentially with isopropyl alcohol (IPA). The active fractions were obtained by elution of 50% IPA, followed by concentration under reduced pressure. The pH of the solutions was adjusted to 3.0, and then the solutions were extracted three times with an equal volume of ethyl acetate. The extracts were dehydrated with sodium sulfate (Na_2SO_4), filtrated through Whatman paper (No. 1), and concentrated under reduced pressure.

The concentrated samples were coated with a silica gel, and then loaded onto a silica gel column filled with *n*-hexane. Stepwise chromatography was performed by eluting mixed solvents with 10:0 and 6:4 ratios of *n*-hexane/ethyl acetate. As a result, the active fraction was obtained by elution with a mixture of *n*-hexane/ethyl acetate (6:4). The solution was concentrated under reduced pressure, and then purified *via* crystallization (Fig. 1) [6, 7].

Identification of SQS Inhibitor

Purity of the samples was analyzed using reverse-phase high-performance liquid chromatography (Sep-Pak C18 column, Waters 510 pump system and Waters 486 UV detector; Waters, Milford, MA, U.S.A.). A UV-visible Beckman DU series 600 spectrophotometer (Beckman Instruments, Brea, CA, U.S.A.) was used to observe the pattern of UV-visible absorbance by scanning from 190 to 800 nm. Mass measurements were performed on a Micromass/ Waters LCT Premier Electrospray Time of Flight (TOF) mass spectrometer (Waters Corp., Milford, MA, U.S.A.), and NMR spectra were recorded on a Varian Unity 400 NMR (Varian Inc., Palo Alto, CA, U.S.A.) at 399.65 and 100.40 MHz for ^{1}H-NMR and ^{13}C-NMR, respectively.

RESULTS AND DISCUSSION

Investigation of SQS Inhibitory Activity

The inhibitory activity of the extracts against squalene synthase was tested from thirty-two samples from plants, animals, mushrooms, microbes, and foods [8]. Fermented soybean pastes, Maesil (Japanese flowering apricot) juice, and green teas showed strong inhibitory activities against squalene synthase. *Prunus mume* fruit showing stably high inhibitory activity against pig liver squalene synthase was selected. *Prunus mume* fruit is well known to have antioxidant, antibacterial, antiallergic, antifatigue, antidiarrheal, antifever, and anticancer effects [10, 15, 17, 25, 30].

The active compound responsible for SQS inhibition in *Prunus mume* fruit was purified (Fig. 1). After *Prunus mume* fruit was extracted with ethanol, a supernatant was obtained by centrifugation of the mixture. Supernatants were concentrated under reduced pressure to remove ethanol. Crude inhibitor solutions were treated with an HP-20 absorbent resin and silica-gel column chromatography. The active fraction was further purified *via* crystallization.

Structural Analysis of SQS Inhibitor

The compound was successfully isolated from *Prunus mume* fruit. The molecular structure of crystallized compound was determined using UV, IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometries. IR absorption of the compound suggested

Prunus mume Fruit
← Extraction with water : ethanol (3:7, v/v)
← Centrifugation
Supernatant | Precipitate
← Evaporation in vacuum
← Adjustment to pH 4.0
HP-20 adsorption column chromatography
← Elution with water : IPA (7:3 and 5:5, v/v)
Active fraction
← Evaporation in vacuum
← Adjustment to pH 3.0
← Extraction with ethyl acetate
Ethyl acetate layer | Water layer
← Dehydration
← Evaporation in vacuum
Silica gel column chromatography
← Elution with *n*-hexane : ethyl acetate (10:0 and 6:4, v/v)
Crystallization

Fig. 1. Purification procedure of the squalene synthase inhibitor from *Prunus mume* fruit.

the presence of hydroxyl (3,420–3,433 cm^{-1}) and ester carbonyl (1,730–1,735 cm^{-1}) groups. In addition, the purified SQS inhibitor showed its UV absorption pattern [$UV_{\lambda max}$ MeOH (nm) 244–245, 296–300, 328–329] indicating the presence of a -CH=CH-CO_2H group linked to the phenyl ring (Table 1). Based on these results, the purified inhibitor was suggested to contain hydroxyl groups, ester carbonyl groups, carboxyl acid, and aromatic rings. Electrospray ionization mass spectrometry (ESI-MS) was also carried out to estimate the molecular weight (Fig. 2). In the ESI-MS, two peaks corresponding to [M-H]- and [M+Na]+ were observed at m/z 353 and 375, showing the molecular weight of 354 and the molecular formula of $C_{16}H_{18}O_9$. The ^1H NMR spectrum showed a spin system (6.78, 6.97, and 7.04) in the aromatic region originating from the benzene ring, one conjugated olefinic methine signal (6.18 and 7.45), and

Table 1. Physicochemical properties of the squalene synthase inhibitor isolated from *Prunus mume* fruit.

Properties	Squalene synthase inhibitor
Appearance	Yellow powder
Molecular formula	$C_{16}H_{18}O_9$
IR ν_{max} (cm^{-1})	3,420–3,433, 1,730–1,735
MS (m/z)	353
$UV_{\lambda max}$ MeOH (nm)	219, 246, 331
Solubility	Ethanol>Acetone>H_2O

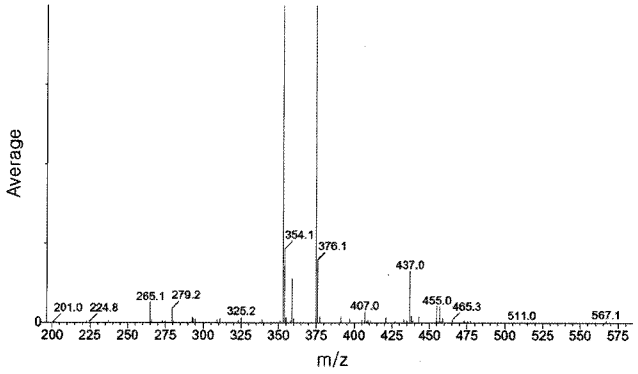

Fig. 2. ESI-Mass spectrum of the squalene synthase inhibitor isolated from *Prunus mume* fruit.

the signal of esterification on H-5 (3.92) (Fig. 3). The ^{13}C NMR spectrum showed two hydroxylated aromatic carbons (148.4 and 145.6), ester carbonyl (165.8), and carboxyl acid carbonyl (176.1) (Fig. 4). Consequently, about 16 carbons with different conditions were suggested. Based on these results, the present inhibitor was conclusively confirmed as chlorogenic acid (5-caffeoylquinic acid) with molecular weight of 354 and molecular formula of $C_{16}H_{18}O_9$ (Fig. 5, Table 1).

Chlorogenic acid, as a phenolic compound ubiquitously found in plants, is an *in vitro* antioxidant and metal chelator. Chlorogenic acid was previously reported as a substance having anticancer, antioxidative, and antimutagenic activities [10, 15, 17, 25, 30]. In addition, recently, chlorogenic acid has been shown to reduce the risk of cardiovascular diseases by decreasing the oxidation of low-density lipoproteins cholesterol and total cholesterol [3]. Although multiple functionalities of chlorogenic acid have been reported, limited information is available on the inhibitory activity of chlorogenic acid against SQS. Therefore, the inhibitory effect of chlorogenic acid against SQS was further studied.

Inhibitory Effect of Chlorogenic Acid Against SQS

The IC_{50} value of chlorogenic acid from pig liver SQS was 100 nM (Fig. 6). Up to now, various kinds of squalene

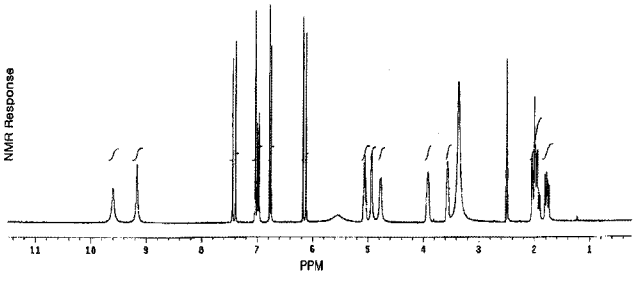

Fig. 3. ^1H-NMR spectrum of the squalene synthase inhibitor isolated from *Prunus mume* fruit.

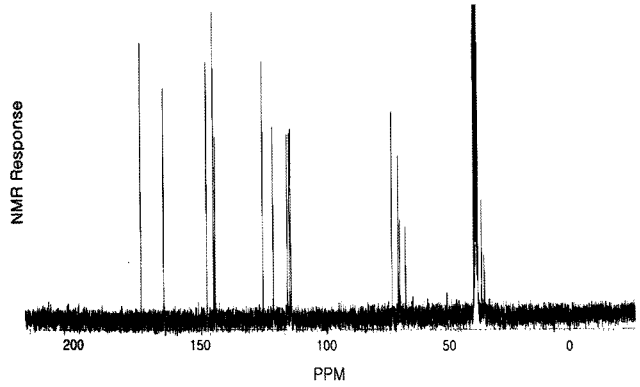

Fig. 4. ^{13}C-NMR spectrum of the squalene synthase inhibitor isolated from *Prunus mume* fruit.

synthase inhibitors have been reported and classified to analogs of farnesyl pyrophosphate (FPP), ammonium analogs of the carbocationic intermediate in the conversion of presqualene diphosphate to squalene, and natural product [3, 14]. The compounds isolated from microbial metabolites have been known to habe high inhibitory activities against squalene synthases compared with inhibitors produced by organic synthesis.

The polyanionic diphosphate group, one of FPP analog inhibitors of SQS, was crucial for substrate binding, presumably through electrostatic interactions. Phosphinylmethyphosphonates (PMP) and its derivatives, stable analogs of FPP, were shown to be effective inhibitors of rat liver squalene synthase (IC_{50}>12.2 μM), but were inactive for cholesterol biosynthesis inhibition in rat hepatocytes [5]. Other analogs, containing the less ionized phosphinylformates and phosphinylacetates, have been reported as a new class of squalene synthase inhibitors [1]. The most potent inhibitor in this class, phosphinylformate, exhibited SQS inhibition activity in the rat squalene synthase assay (IC_{50}=8.7 μM). Moreover, carbocationic intermediates are supposed to

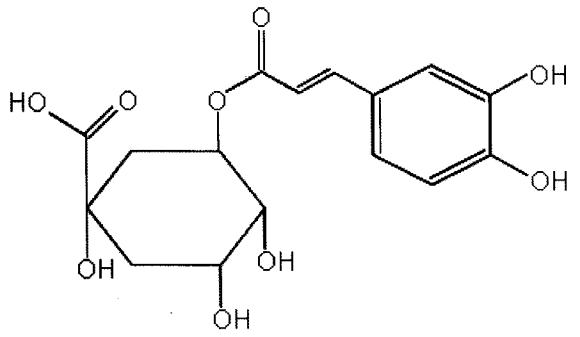

Fig. 5. Structure of chlorogenic acid isolated from *Prunus mume* fruit.

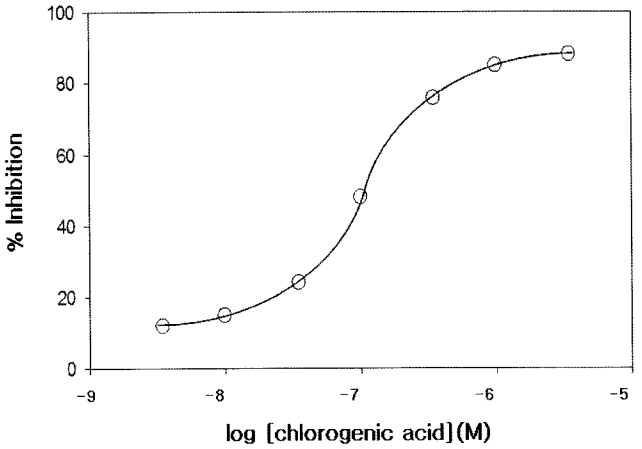

Fig. 6. Effect of chlorogenic acid concentration on squalene synthase activity.

be involved in the synthesis of presqualene diphosphate and subsequent reductive rearrangement to squalene [28]. Ammonium substituted cyclopropyl polyene compounds were reported to inhibit squalene synthase by mimicking the topological and electrostatic properties of the primary cation and tertiary cation of presqualene diphosphate. An aza analog of carbocation intermediates inhibited the squalene synthase of yeast with an IC_{50} range of 20 to 25 μM [28]. It was elucidated that the activities of inhibitors separated from microbial metabolites were higher, compared with those of inhibitors produced by organic synthesis.

Novel microbial metabolites have been discovered as potent inhibitors of squalene synthase. Squalestatins 1, 2, and 3, isolated from *Phoma* sp. C2932, inhibit rat liver microsome squalene synthase with an IC_{50} of 15.2, 15.1, and 5.9 nM, respectively [3, 9]. Common to all three compounds is the hydrophilic core unit. They differ structurally from one another in the alkyl side chain and the fatty acyl chain. Three compounds showed potent activity against both rat liver and *Candida albicans* squalene synthase. Zaragozic acids A, B, and C have been isolated from *Sporormiella intermedia* and *Leptodontium elatius* [4]. The structure of zaragozic acid A was identical to that of squalestatin 1. Each member of this family was a potent competitive inhibitor of squalene synthase at sub-nanomolar levels. Squalestatin 1 (zaragozic acid A) has an IC_{50} value of 39 nM for cholesterol biosynthesis in freshly isolated rat hepatocytes. It also reduces serum cholesterol in marmosets, mice, and rats [15]. Chlorogenic acid inhibited squalene synthase at a relatively low concentration and also had an inhibitory effect on squalene synthases derived from animal cells, which suggests a potential for a therapeutic agent for hypercholesterolemia.

Acknowledgment

This work was supported by a grant (No. 105062-3) funded from the Ministry of Agriculture, Korea.

REFERENCES

1. Abe, I., J. C. Tomesch, S. Wattanasin, and G. D. Prestwich. 1993. Inhibitors of squalene biosynthesis and metabolism. *Nat. Prod. Rep.* **11:** 279–293.
2. Agnew, W. S. 1985. Squalene synthetase. *Methods Enzymol.* **110:** 359–375.
3. Baxter, A., B. J. Fitzgerald, J. L. Hutson, A. D. McCarthy, J. M. Motteram, B. C. Ross, M. Sapra, M. A. Snowden, N. S. Watson, R. J. Williams, and C. Wright. 1992. Squalestatin, a potent inhibitor of squalene synthase, which lowers serum cholesterol *in vivo*. *J. Biol. Chem.* **267:** 11705–11708.
4. Bergstrom, J. D., M. M. Krutz, D. J. Rew, A. M. Amend, J. D. Karkas, R. D. Bostedor, V. S. Bansal, C. Dufresns, F. L. V. Middleworth, O. D. Hensens, J. M. Liesch, D. L. Zinc, K. E. Wilson, J. Onishi, J. A. Milligan, G. Bills, L. Kaplan, M. O. Nallin, R. G. Jenskins, L. Huang, M. S. Meinz, L. Quinn, R. U. Burg, Y. L. Kong, S. Mochales, M. Mojena, I. Martin, F. Pelaez, M. T. Diez, and A. W. Albert. 1993. Zaragozic acids: A family of metabolites that are picomolar competitive inhibitors of squalene synthase. *Proc. Natl. Acad. Sci. USA* **90:** 80–89.
5. Biller, S. A., C. Forster, E. M. Gordon, T. Harrity, L. C. Rich, J. Marretta, and C. P. Ciosek. Jr. 1991. Isoprenyl phosphinylformates: New inhibitors of squalene synthetase. *J. Med. Chem.* **34:** 1912–1914.
6. Cho, J.-Y., G. J. Choi, S.-W. Lee, K. S. Jang, H. K. Lim, C. H. Lim, S. O. Lee, K. Y. Cho, and J.-C. Kim. 2006. Antifungal activity against *Colletotrichum* spp. of curcuminoids isolated from *Curcuma longa* L. rhizomes. *J. Microbiol. Biotechnol.* **16:** 280–285.
7. Cho, J.-Y., J.-H. Moon, H.-K. Kim, S.-J. Ma, S.-J. Kim, M.-Y. Jang, K. Kawazoe, Y. Takaishi, and K.-H. Park. 2006. Isolation and structural elucidation of antimicrobial compounds from buckwheat hull. *J. Microbiol. Biotechnol.* **16:** 538–542.
8. Choi, S. W., D. S. Kim, N. Y. Hur, C. S. Park, and M. Y. Baik. 2003. Purification and identification of squalene synthase inhibitor isolated from fermented soybean paste. *Food Sci. Biotechnol.* **14:** 89–93.
9. Dawson, M. J., J. E. Farthing, P. S. Marshall, R. F. Middleton, M. J. O'Neill, A. Shuttleworth, C. Stylli, R. M. Tait, P. M. Taylor, H. G. Wildman, A. D. Buss, D. Langley, and M. V. Hayes. 1992. The squalestatins, novel inhibitors of squalene synthase produced by a species of *Phoma*. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activity. *J. Antibiot.* **45:** 639–647.
10. Feng, R., Y. Lu, L. L. Bowman, Y. Qian, V. Castranova, and M. Ding. 2005. Inhibition of activator protein-1, NF-kappaB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. *J. Biol. Chem.* **280:** 27888–27895.
11. Ha, C. G., J. K. Cho, C. H. Lee, Y. G. Chai, Y. A. Ha, and S. H. Shin. 2006. Cholesterol lowering effect of *Lactobacillus plantarum* isolated from human feces. *J. Microbiol. Biotechnol.* **16:** 1201–1209.
12. Haria, M. and D. Mctavish. 1997. Pravastatin. *Drugs* **53:** 299–336.
13. Hiyoshi, H., M. Yanagimachi, M. Ito, I. Ohtsuka, I. Yoshida, T. Saeki, and H. Tanaka. 2000. Effect of ER-27856, a novel squalene synthase inhibitor, on plasma cholesterol in rhesus monkeys: Comparison with 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors. *J. Lipid Res.* **41:** 1136–1144.
14. Hiyoshi, H., M. Yanagimachi, M. Ito, T. Saeki, I. Yoshida, T. Okada, H. Ikuta, D. Shinmyo, K. Tanaka, N. Kurusu, and H. Tanaka. 2001. Squalene synthase inhibitors reduce plasma triglyceride through a low-density lipoprotein receptor-independent mechanism. *Eur. J. Pharmacol.* **431:** 345–352.
15. Huh, S. J., K. H. Lee, H. S. Yun, D. J. Paik, J. M. Kim, and J. H. Youn. 2007. Functions of metallothionein generating interleukin-10-producing regulatory CD4+ cells potentiate suppression of collagen induced arthritis. *J. Microbiol. Biotechnol.* **17:** 348–358.
16. Ishihara, T., H. Kakuta, H. Moritani, T. Ugawa, S. Sakamoto, S. Tsukamoto, and I. Yanagisawa. 2003. Syntheses of 3-ethylidenequinuclidine derivatives as squalene synthase inhibitors. Part 2: Enzyme inhibition and effects on plasma lipid levels. *Bioorg. Med. Chem.* **11:** 3735–3745.
17. Jee, H. J. 1999. *Health Foods from Herbs*, p. 52. Seoul National University Press, Seoul, Korea.
18. Kasim, S. E., R. C. Leboeuf, S. Khilnani, L. Tallapaka, D. Dayanada, and L. C. Jenk. 1992. Mechanisms of triglyceride-lowering effect of an HMG-CoA reductase inhibitor in a hypertriglyceridemic animal model, the Zucker obese rat. *J. Lipid Res.* **33:** 1–7.
19. Keller, R. K. and F. Vilsaint. 1993. Regulation of isoprenoid metabolism in rat liver: Near constant chain lengths of dolichy phosphate and ubiquinone are maintained during greatly altered rates of cholesterogenesis. *Biochim. Biophy. Acta* **1170:** 20–25.
20. Koo, K.-C., D.-H. Lee, J.-H. Kim, H.-E. Yu, J.-S. Park, and J.-S. Lee. 2006. Production and characterization of antihypertensive angiotensin I-converting enzyme inhibitor from *Pholiota adiposa*. *J. Microbiol. Biotechnol.* **16:** 757–763.
21. Kuswik, R. G. and H. C. Rilling. 1987. Squalene synthetase. Solubilization and partial purification of squalene synthetase, copurification of presqualene pyrophosphate and squalene synthetase activities. *J. Biol. Chem.* **262:** 1505–1510.
22. Lim, Y.-H., I.-H. Kim, J.-J. Seo, and J.-K. Kim. 2006. Tyrosinase inhibitor from the flowers of *Impatiens balsamina*. *J. Microbiol. Biotechnol.* **16:** 1977–1983.
23. Lipid Research Clinics. 1984. Lipid research clinics program. *J. Am. Med. Assoc.* **252:** 2545–2548.
24. McClellan, K. J., L. R. Wiseman, and D. McTavish. 1998. Cerivastatin. *Drugs* **55:** 415–422.

25. Nardini, M., F. Natella, V. Gentili, M. Di-Felice, and C. Scaccini. 1997. Effect of caffeic acid dietary supplementation on the antioxidant defense system in rat: An *in vivo* study. *Arch. Biochem. Biophys.* **342:** 157–160.
26. Nishimoto, T., Y. Amano, R. Tozawa, E. Ishikawa, Y. Imura, H. Yukimasa, and Y. Sugiyama. 2003. Lipid-lowering properties of TAK-475, a squalene synthase inhibitor, *in vivo* and *in vitro*. *Br. J. Pharmacol.* **139:** 911–918.
27. Park, J. K., H. J. Cho, Y. H. Lim, Y. H. Cho, and C. H. Lee. 2002. Hypocholesterolemic effect of CJ90002 in hamsters: A potent inhibitor for squalene synthase from *Paeonia moutan*. *J. Microbiol. Biotechnol.* **12:** 222–227.
28. Poulter, C. D. 1991. Biosynthesis of non-head-to-tail terpenes. Formation of 1'-1 and 1'-3 linkages. *Acc. Chem. Res.* **23:** 70–77.
29. Poulter, C. D. and H. C. Rilling. 1981. *Biosynthesis of Isoprenoid Compounds*, pp. 413–441. Wiley Publisher, New York, U.S.A.
30. Rodriguez, de Sotillo D. V. and M. Hadley. 2002. Chlorogenic acid modifies plasma and liver concentrations of cholesterol, triacylglycerol, and minerals in (fa/fa) Zucker rats. *J. Nutr. Biochem.* **13:** 717–726.
31. Shepherd, J., S. M. Cobbe, I. Ford, C. G. Isles, A. R. Lorimer, P. W. MacFarlane, J. H. McKillop, and C. J. Packard. 1995. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. *N. Engl. J. Med.* **333:** 1301–1307.
32. Scandinavian Simvastatin Survival Study Group. 1994. Randomized trial of cholesterol-lowering in 4444 patients with coronary heart disease: The Scandinavian Simvastatin Survival Study (4S). *Lancet* **344:** 1383–1389.