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Purification and Identification of Squalene Synthase Inhibitor Isolated from Fermented Soybean Paste

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Abstract Squalene synthase (SQS) inhibitors were screened from various plants and food extracts. Effective SQS inhibitor was purified from fermented soybean paste using ethanol extraction, HP-20 column chromatography, ethyl acetate extraction, silica gel column chromatography, and crystallization. Through UV spectrometry, ^{1}H NMR, ^{13}C NMR, and mass spectrometry, SQS inhibitor was identified as daidzein with molecular mass of 254 and molecular formula of $C_{15}H_{10}O_4$. Daidzein showed IC_{50} value of 50 nmol/L against SQS, confirming its potential as therapeutic agent for hypercholesterolemia.

Key words: squalene synthase inhibitor, daidzein, fermented soybean paste, hypercholesterolemia

Introduction

The elevated plasma low density lipoprotein (LDL) cholesterol is generally accepted as a major risk factor of coronary heart disease, with a significant correlation demonstrated among the level of plasma LDL cholesterol, the incidence of coronary artery disease, and progression of atherosclerotic lesions (1). Cholesterol, a steroid composed of 27 carbons, is an essential component of animal cell membranes. It serves as a precursor of progestins, corticosteroids, and sex hormones such as androgens and estrogens. In addition, it also acts as a substrate for the biosynthesis of bile acids essential to the adsorption of fats and fat-soluble vitamins from intestines (2). Humans consume approximately one-third of the total body cholesterol (300 to 500 mg cholesterol/day) through foods, and over two-thirds (700 to 900 mg cholesterol/day) derived through the *de novo* cholesterol biosynthesis in the body.

At present the most effective therapeutic approach to reduce the level of plasma LDL cholesterol is the inhibition of cholesterol biosynthesis (3). The cholesterol biosynthetic pathway involves more than 25 enzymes. Among inhibitors of cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors, lovastatin and simvastatin, are currently the most effective cholesterol-lowering agents. The mammalian isoprenoid pathway produces sterol, in addition to generating dolicol, ubiquinone, farnesyl group of heme A, farnesyl and geranyl groups of prenylated proteins, and isopentenyl side-chain of isopentenyl adenine. Reduction in the levels of ubiquinone and possibly other noncholesterol metabolites of mevalonate has been implicated to the generation of some of the side effects of the HMG-CoA reductase inhibitors (4, 5). Squalene synthase (SQS) is the first pathway-specific enzyme and catalyzes the reductive dimerization of two molecules of farnesyl pyrophosphate to form squalene, as well as being the first committed step in sterol synthesis (6). A specific SQS inhibitor would inhibit cholesterol biosynthesis but not prevent the forma-tion of other products of the isoprenoid pathway, such as dolicol and ubiquinone. Thus, SQS offers a potential target for the safe and specific inhibition of cholesterol bio-synthesis (7), because it can reduce adverse effects of the HMG-CoA reductase inhibitors and improve efficacy for cholesterol-lowering agents.

In this study, the SQS inhibitors were screened from various plants and food extracts to discover a potential therapeutic agent for hypercholesterolemia. An effective SQS inhibitor was purified and identified from fermented soybean paste, and its molecular structure was determined.

Materials and Methods

Extraction of samples for screening of SQS inhibitor To screen the effective SQS inhibitor, 32 different kinds of plants and food materials were investigated. Samples were prepared using both solvent and hot water extractions. In solvent extraction, 100 g sample was extracted with 1 L of 80% (v/v) methanol using a waring blender at 25°C, and the mixture was centrifuged at 4,000 × *g* for 15 min. The extraction was repeated three times to increase the yield. After extraction, collected supernatant was concentrated using a vacuum evaporator (Buchi Rotavapor R-124, Buchi Labortechnik, Flawil, Swiss). The concentrates were kept in -80°C deep-freezer until use, and the samples were dissolved in a predetermined amount of methanol before the experiment. Prior to the SQS inhibition test, distilled water was added to the sample to precisely make 20% (v/v) methanol concentration, because methanol higher than 20% (v/v) may affect the enzymatic assay and at lower than 20% (v/v), the sample did not dissolve well in the solution. Hot water extraction was performed using the same procedure as described above except 80°C distilled water was used instead of 80% (v/v) methanol.

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Preparation of pig liver microsome One hundred grams of pig liver was finely chopped and minced. Two hundred milliliters of homogenizing buffer [0.1 M potassium phosphate (pH 7.4), 0.3 M sucrose, 5 mmol/L dithiothreitol (DTT), 10 mmol/L $MgCl_2$, and 50 mmol/L KCl] was immediately added to the minced sample and homogenized. The mixture was centrifuged twice at 4,000 $\times g$ for 15 min and at 15,000 $\times$ g for 30 min. The supernatant was ultra-centrifuged at 105,000 $\times g$ for 1 hr to obtain microsome precipitates. Fifty milliliters of homogenizing buffer was added to wash and homogenize the microsome precipitates, which were then ultra-centrifuged again at 105,000 $\times g$ for 30 min. The precipitates were resuspended with the homogenizing buffer and used as an enzyme source for the measurement of SQS activity (8, 9).

Measurement of inhibitory activity to SQS Ten microliters of the inhibitor sample, 100 μL of reaction buffer [100 mmol/L potassium phosphate (pH 7.4), 5 mmol/L $MgCl_2$, 100 mmol/L KCl, 10 mmol/L DTT, and 2 mmol/L 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 mmol/L [^{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, USA) (10, 11). To estimate the inhibitory activity against SQS, differences in cpm values with and without inhibitor sample were measured.

Purification of SQS inhibitor from fermented soybean paste The purification procedure for SQS inhibitor is shown in Fig. 1. SQS inhibitor was extracted from 100 g of fermented soybean paste using 1 L of 70% ethanol. After the centrifugation, the supernatant was concentrated using a vacuum evaporator (Buchi Rotavapor R-124, Buchi Labortechnik, Flawil, Swiss). The concentrate was washed with 70% ethanol and resuspended with distilled water. The extract was adjusted to pH 4.0, adsorbed on HP-20 column, and eluted sequentially using isopropyl alcohol (IPA). The active fractions eluted with 50% IPA were concentrated under reduced pressure, adjusted to pH 3.0, and extracted three times using the same volume of ethyl acetate. The resultant was dehydrated using sodium sulfate (Na_2SO_4), filtrated through Whatman paper No. 1 (Whatman International Ltd., Maidstone, England), and concentrated. The concentrated sample was coated with silica gel and loaded into a silica gel column, which was then filled with a mixture of *n*-hexane/ethyl acetate (7/3, v/v). The elution step was performed with the stepwise gradient chromatography from 7:3 and 3:7 ratios of *n*-hexane and ethyl acetate. The active fraction was collected and concentrated under reduced pressure, and finally the active compound was purified by crystallization.

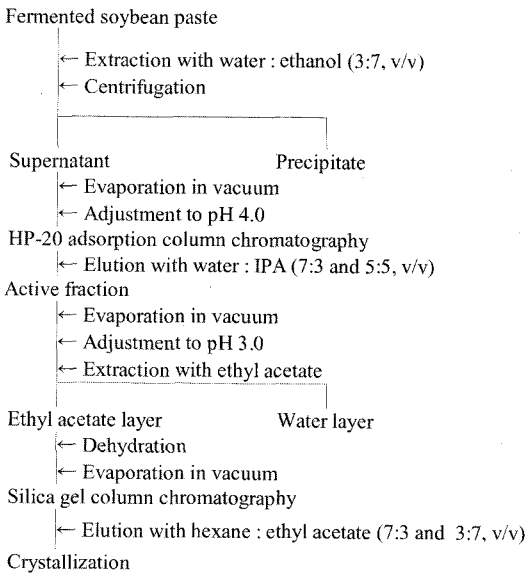

Fig. 1. Purification procedure of the squalene synthase inhibitor from fermented soybean paste.

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^{TM}486 UV detector, Waters, Milford, MA, USA). UV-visible Beckman DU series 600 spectrophotometer (Beckman Instruments, Brea, CA, USA) was used to observe the pattern of UV-VIS absorbance by scanning from 190 to 800 nm. Mass Spectrum was analyzed using EI-MS with a JMS-AX505WA mass spectrometer (JEOL Ltd. Tokyo, Japan), and NMR spectra were recorded on a Varian Unity 400 NMR (Varian Inc., Palo Alto, CA, USA) at 399.65 and 100.40 MHz for ^{1}H-NMR and ^{13}C-NMR, respectively.

Results and Discussion

Investigation of the SQS inhibitory activity A study by Biller *et al.* (1) on the SQS inhibitors based on substrate analogues described isoprenyl phosphinylformates as new inhibitors of SQS. A fungal isolate, *Phoma* sp. C2932, which produces potent inhibitors of mammalian and fungal SQS, was also discovered using a high throughput screening method based on a novel enzyme assay. These compounds were designated as squalestatins (12).

In this study, various biological resources including plants, animals, mushrooms, microorganisms, and foods were tested for the inhibitory activity against SQS. Among 32 samples, more than one third (22 samples) exhibited significant SQS inhibitory activity (Table 1). Interestingly, SQS inhibitory activity was detected in many food-related samples such as fermented soybean paste, plum juice, and paste, and various teas. Two samples, fermented soybean paste and green tea, showed the strongest pig SQS inhibitory activity at 86 and 70%, respectively. Furthermore, the SQS inhibitory activity of fermented soybean paste was not only high but also stable. Therefore, fermented soybean paste was selected for further analysis to determine the compound responsible for the SQS inhibitory activity.

Structural analysis of SQS inhibitor The active

Table 1. Squalene synthase inhibitor activities of various materials

	Samples	Inhibition*
Plant source	Kudzu vine (*Pueraria thunbergiana*)	+
	Licorice (*Glycirrhizia glabra*)	+
	Cinnamon (*Cinnamomi cortex*)	+
	Tobacco (*Nicotiana tobacum* L.)	-
	Garlic (*Allium sativum* L. var. *pekinense* MAKINO)	++
	Crataegus pinnatifida (*Crataegus pinnatifida*)	++
	Ginger (*Zinglber officinale*)	+
	Coriolus versicolor (*Coriolus versicolor*)	+
	Siberian Ginseng Extract (*Acanthopanax seticosus*)	++
	Gentiana scabra buergeri (*Gentiana scabra buergeri*)	-
	Ginkgo leaf (*Ginkgo biloba* L.)	+
	Peony (*Paeonia lactiflora* PALL)	+
	Korean Angelica (*Angelica gigas*)	-
	Balkal Skullcap (*Scutellaria baicalens*)	-
Animal source Chondroitin (from shark)	-	
	Chondroitin (from cow)	-
	Oyster extract (*Crassostrea gigas*)	-
Mushroom source	Reishi Mushroom (*Ganoderma lucidum*)	++
	Phelinus linteus	++
	Coriolus versicolor	+
	Lentinus edodes, Shiitake	+
Microbes	Brewer's yeast (*Saccharomyces cerevisae*)	-
	Glutathion yeast (*Saccharomyces cerevisae*)	-
	Spirulina (*Arthrospira platensis*)	-
Food Materials	Fermented Soybean paste	+++
	Tofu	+
	Maesil (Japanese flowering apricot) juice (*Prunus mume*)	++
	Plum juice (*Prunus salicina*)	++
	Plum paste (*Prunus salicina*)	++
	Green tea (*Camellia sinensis*)	+++
	Oolong tea (*Camellia sinensis*)	+
	Black tea (Camellia sinensis)	+

*-: Inhibition<25%, +: Inhibition 25-50%, ++: Inhibition 50-70%, +++: Inhibition>70%

compound responsible for SQS inhibition in the fermented soybean paste was purified (Fig. 1). The compound was successfully isolated from the fermented soybean paste using an HP-20 adsorption chromatography and a silica gel column chromatography, and crystallized. The molecular structure of crystallized compound was determined using UV spectrometry, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The purified SQS inhibitor showed maximum absorbances at 211 and 247 nm in UV spectrum, which indicate that it contains benzene rings in its structure. IR spectrum was examined to elucidate functional groups in the purified SQS inhibitor. Peaks presumed to correspond to the hydroxyl and carbonyl groups were observed at 3225 and 1630 cm^{-1}, respectively (Fig. 2). Based on these results, the purified inhibitor was suggested to contain hydroxyl groups, carbonyl groups, and aromatic rings. Additionally, in ^{1}H-NMR analysis, protons in the aromatic ring were observed under different conditions through a double resonance in the range of 6.8~7.4 ppm, and protons in the hydroxyl groups also were detected at 8.1 ppm (Fig. 3). From ^{13}C-NMR spectra, an aromatic ring was observed

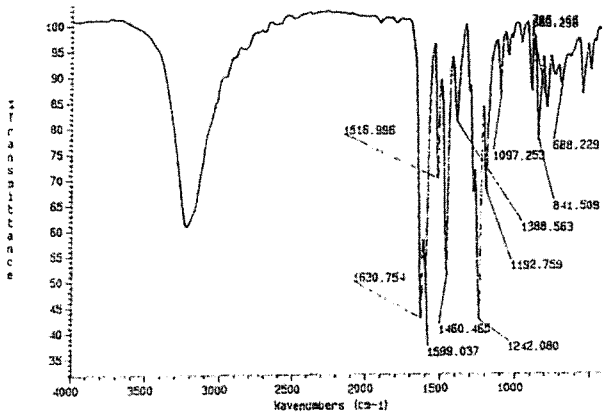

Fig. 2. IR spectrum of the squalene synthase inhibitor isolated from fermented soybean paste.

in the range of 100~160 ppm, and the peak at 180 ppm was assumed to be that of carbons in the carbonyl group. Consequently, about 15 carbons with different conditions

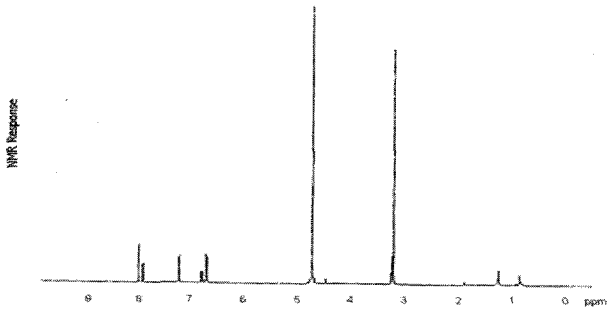

Fig. 3. ^{1}H-NMR spectrum of the squalene synthase inhibitor isolated from fermented soybean paste.

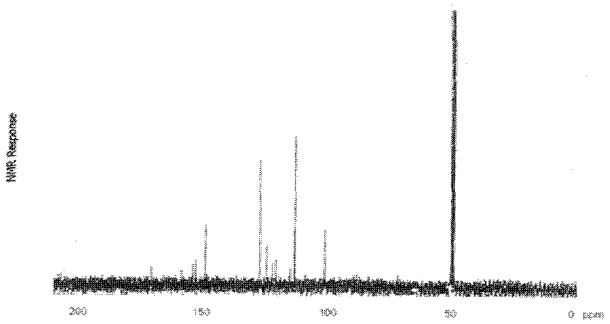

Fig. 4. ^{13}C-NMR spectrum of the squalene synthase inhibitor isolated from fermented soybean paste.

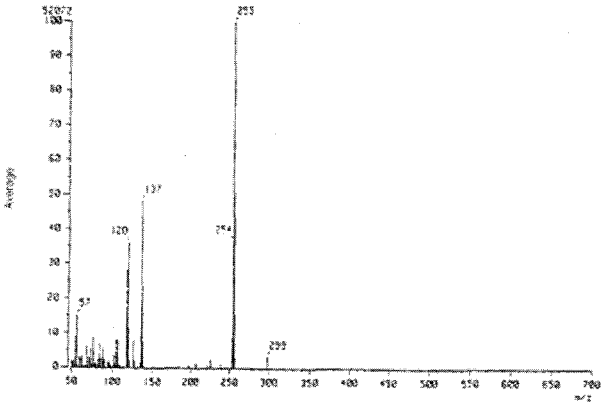

Fig. 5. EI-Mass spectrum of the squalene synthase inhibitor isolated from fermented soybean paste.

were suggested (Fig. 4). Electron-impact ionization mass spectroscopy (EI-MS) was also carried out to estimate the molecular weight. Major peaks were detected at 57, 120, and 255 m/z M, and molecular weight was estimated to be 254 (Fig. 5). Based on these results, the present inhibitor was conclusively confirmed as daidzein with molecular weight of 254 and molecular formula of $C_{15}H_{10}O_4$ (Fig. 6, Table 2).

Daidzein was previously reported as a substance having anticancer, antioxidative, and antimutagenic activities (13-15). Recent researches showed the possibilities of soybean-derived functional materials as anticancer agents, including protease inhibitor (16), phytic acid (17), and isoflavone (15). Japanese soybean pastes, such as miso, soy sauce, and shoyu also showed the anticancer (13), antimutagenic, and antioxidant properties (14). In spite of the possibility

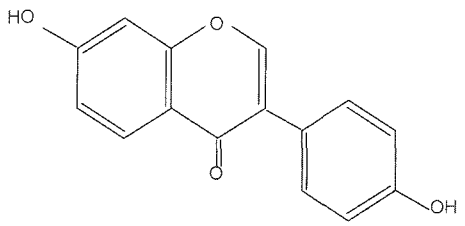

Fig. 6. Structure of daidzein isolated from fermented soybean paste.

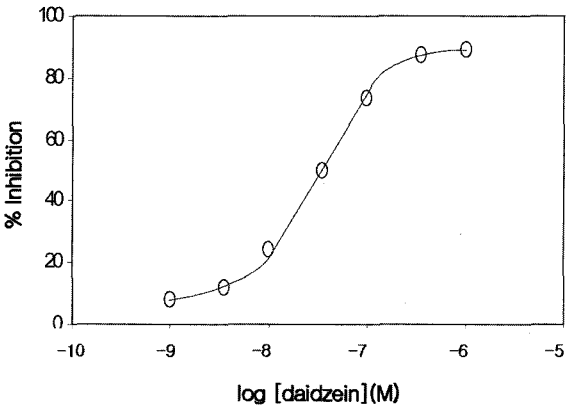

Fig. 7. Effect of daidzein concentration on squalene synthase activity.

Table 2. Physicochemical properties of the squalene synthase inhibitor isolated from fermented soybean paste

Properties	Squalene synthase inhibitor
Appearance	White powder
Molecular formula	$C_{15}H_{10}O_4$
IR ν_{max} (cm^{-1})	3225, 1630
MS (m/z)	255
UV_{max} MeOH (nm)	211, 247, 298
Solubility	
soluble	Methanol>Acetone>Ethyl acetate
insoluble	Hexane, H_2O

that Korean fermented soybean paste also have properties similar with those of Japanese soybean paste, only its taste components have been reported (18). Furthermore, although multi-functionalities of daidzein have been reported, limited information is available on the inhibitory activity of daidzein against SQS. Therefore, the inhibitory effect of daidzein against SQS was further studied.

Inhibitory effect of daidzein against SQS The IC_{50} value of daidzein from pig liver SQS was 50 nmol/L (Fig. 7). Various kinds of SQS inhibitors have been reported, such as phosphorous-containing FPP-analogues, carbocation intermediate analogues, and natural products (1, 5, 19). The compounds isolated from microbial metabolites were found to have high inhibitory activities against SQS compared to those produced by organic synthesis. Novel SQS inhibitors have been isolated from microorganisms, three squalestatins from *Phoma* sp. C2932, and three zaragozic acids from *Sporormilla intermedia* and *Leptodontium elatius* (7). The IC_{50} values of squalestatins 1, 2, and 3 against rat liver-SQS were 15.2, 15.1, and 5.9 nmol/L,

respectively (12, 19). All squalestatins have a hydrophilic core unit in common, and different alkyl side groups or fatty acyl chains. The structure and configuration of zaragozic acid A are identical with those of squalestatin 1. All members of zaragozic acid function as potent inhibitors against SQS at sub-nanomolar levels. Squalestatin 1 (zaragozic acid A) has an IC_{50} value of 39 nmol/L for cholesterol biosynthesis in freshly isolated rat hepatocytes. It also reduces serum cholesterol in marmosets, mice, and rats (7). Commercial products of zaragozic acids and squalestatins, which are produced by microorganisms, showed potent inhibitory activities against the SQS from rat livers and *Candida albicans*. The IC_{50} value of daidzein against pig SQS (50 nmol/L) was relatively low. Therefore, considering the effectiveness against pig SQS, the compound has a potential as a therapeutic agent for hypercholesterolemia, which, in turn, confirms that the Korean traditional fermented soy paste extracts suppresses the enzymatic activity of SQS.

Acknowledgments

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