Isoflavones Found in Korean Soybean Paste as 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Inhibitors

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3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase is the rate-limiting enzyme in the biosynthesis of cholesterol in mammals. Some microbial metabolites have been found to be HMG-CoA reductase inhibitors. Korean soybean paste is a unique food fermented by many microorganisms. The catalytic domain of Syrian hamster HMG-CoA reductase was employed for the screening of HMG-CoA reductase inhibitors. Soybean paste extract was fractionated by vacuum liquid chromatography. Fractions showing relatively high HMG-CoA reductase inhibition were further purified through Sephadex LH-20 column chromatography and C18 preparative HPLC, and the inhibitory compounds were identified as genistein, daidzein, and glycitein.

Key words: Korean soybean paste; 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase; isoflavone; genistein; daidzein

Extensive epidemiological studies have shown that increased blood cholesterol level is a major cause of coronary heart disease. Each cell of the higher animals must balance external and internal sources of cholesterol so as to sustain mevalonate synthesis while avoiding sterol overaccumulation. This balance is achieved through the feedback regulation of at least two sequential enzymes in mevalonate synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase, as well as low-density lipoprotein (LDL) receptors. It is well known that HMG-CoA reductase (EC 1.1.1.34) is the rate-limiting enzyme in the biosynthesis of cholesterol in mammals. Some microbial metabolites and their analogs, which are HMG-CoA reductase inhibitors, have been synthesized, including statins, reversible competitive inhibitors of HMG-CoA reductase.

The relationship between diet and cardiovascular disease was recognized early in the 20th century. Soy food intake and its beneficial relation to blood lipid concentration and heart disease have been reported in the literature. Korean soybean paste (Doenjang) is a unique soy food, which is fermented by diverse microorganisms including fungi and bacilli during its manufacture. But not all fungi and bacilli related to the fermentation and the components of soybean paste have been identified completely.

The objective of this study was, therefore, to screen for inhibitors of HMG-CoA reductase yet unidentified in Korean soybean paste.

Materials and Methods

Extraction of soybean paste. Korean soybean paste was freeze-dried, crushed, sieved through a 35-mesh screen, and stored at −18°C until extraction. A powdered sample was extracted with a mixture of methanol and dichloroform (1:1, v/v) to achieve high extraction potential. Sample (20 g) with solvent (1:30, w/v) was homogenized at 10,000 rpm for 5 min with a disperser (ULTRA-TURRAX T25, IKA Labortechnik, Janke & Kunkel GmbH & Co. KG, Staufen, Germany), and the mixture was filtered through a filter paper (No. 1, Whatman International Ltd., Maidstone, UK). The filtrate was then concentrated using a rotary evaporator with silica gel added to the filtrate, and the temperature was kept below 40°C. The resulting powder was subjected to vacuum liquid chromatography.

Vacuum liquid chromatography. The column was prepared in a sintered glass funnel (Iwaki Glass, porosity 5–10 μm, Tokyo, Japan) using TLC-grade packing (Silica gel 60H, Merck Ltd., Darmstadt, Germany). The sample was applied uniformly at the top of the support. A stepwise gradient with organic solvents was used, and the column was allowed to run dry after the collection of each fraction. Two vacuum liquid chromatography (VLC) columns of different sizes were used for the crude purification: one of 90 mm and 50 mm and the other of 65 mm and 40 mm in diameter and

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Abbreviation: HMG-CoA, 3-Hydroxy-3-methylglutaryl coenzyme A
height respectively.

For the first chromatography with VLC, the sample (1.7 g) was fractionated using solvents in the order hexane, ethyl acetate, and methanol. The collection volume was 200 ml for each step. Each fraction was monitored on a silica gel plate (Silica gel 60F254, Merck Ltd.) developed with chloroform-methanol-acetic acid (85:10:5). For visualization of spots, two detection modes, irradiation with UV at 254 nm with a fluorescent indicator, and iodine vapor adsorption, were used. Based on the assay results and UV observation on TLC, ethyl acetate and methanol eluates with relatively high inhibitory activity were pooled and evaporated. In addition, the residues containing hydrophilic compounds were separated with water and ethyl acetate. Water-soluble materials were discarded, and the ethyl acetate layers were collected and evaporated for the next purification step.

For the second VLC, the column was equilibrated with hexane. The ethyl acetate layer residue (0.73 g) was then dissolved in a small volume (3–5 ml) of dichloromethane and loaded on the column, and chromatographed using a stepwise gradient of n-hexane/CH2Cl2/EtOAc/MeOH. The collection volume was 100 ml for each fraction. The solvents used were hexane:CH2Cl2 (90:10), hexane:CH2Cl2 (50:50), CH2Cl2 (100), CH2Cl2:EtoAc (98:2), CH2Cl2:EtoAc (90:10), CH2Cl2:EtoAc (50:50), EtoAc (100), EtoAc:MeOH (95:5), EtoAc:MeOH (50:50), and MeOH. Among the ten fractions, based on the assay results and the similar inhibitory activities, were pooled and evaporated.

Spectrophotometric assay using Syrian hamster HMG-CoA reductase. Plasmid pKFT7-21 encoding HMG-CoA reductase was a generous gift from Professor V.W. Rodwell of Purdue University. The catalytic HMG-CoA reductase was a generous gift from Professor W.J. Sherley of the University of Rochester. Lipophilic Sephadex LH-20 chromatography. Previously prepared residue (0.12 g) dissolved in methanol was subjected to Sephadex LH-20 (20 x 400 mm, Sigma Chemical Co., St. Louis, MO, USA) column chromatography with methanol as the eluent at a flow rate of 1 ml/min. The fractions were monitored with TLC. Each fraction was divided into five subfractions. The 4th (L4 group) and 5th (L5 group) subfractions, which showed relatively high inhibition activities, were separately collected and evaporated.

HPLC analysis and purification. For HPLC analysis, HPLC-grade acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) and deionized water including 0.1% trifluoroacetic acid (TFA) (Sigma Chemical Co.) were used as the mobile phase. The mobile phase was filtered through a 0.45-μm pore-size filter (Millipore, Bedford, MA, USA). To determine the wavelength for analysis, each sample was scanned from 200 nm to 400 nm using HPLC (Agilent 1100, Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector. The sample was injected into the HPLC column (150 mm x 3.9 mm I.D. Symmetry column, thermostatted at 40 °C), packed with a 5-μm particle-size RP18 from Waters Corporation, Milford, MA, USA, injection volume 20 μl). A SymmetryPrep column (300 mm x 7.8 mm) packed with 7-μm particle-size RP18 (Waters Corporation) was used for the preparative HPLC, and the injection volume was 100 μl. The flow rates were 0.7 and 5.6 ml/min for analytical and preparative chromatographies respectively. Alternation of the eluent from 70% to 30% acetonitrile was carried out on L5 components to separate the active compound, L5-2. Additionally, L4 components were separated into two active components, L4-1 and L4-2, using 20% acetonitrile as the eluent.

Structural analysis of inhibitors. To measure the molecular weight of the inhibitors, LC/MS was performed using an Agilent 1100 HPLC system and a JMS-LCmate single quadrupole mass spectrometer (JEOL Ltd., Tokyo, Japan) equipped with an atmospheric pressure chemical ionization (APCI) interface at an ion source temperature of 500 °C. The flow rate for all analyses was 0.7 ml/min, and the entire effluent was introduced into the APCI probe.

To elucidate the structure of L5-2, NMR experiments, elemental analyses for C, H, and N (EA1110 elemental analyzer, CE Instrument, Milan, Italy), Fourier transform-infrared spectroscopy (ABB Bomem Inc., Quebec, Canada) with KBr pellet and X-ray structure determination were performed. 1H NMR (operating at 399.65 MHz), 13C NMR (operating at 100.40 MHz), 1H–1H correlation spectroscopy (COSY), 13C–1H COSY, Distortionless Enhancement by Polarization
Transfer (DEPT), Heteronuclear Multiple Quantum Correlation spectroscopy (HMOC), and Heteronuclear Multiple Bond Correlation spectroscopy (HMBC) spectra were obtained with a JEOL JNM-LA400 spectrometer (JEOL Ltd.). All NMR spectra were obtained in DMSO-d6, with chemical shifts expressed as parts per million (δ) and coupling constants (J) in Hertz. The spectra of L4-1 and L4-2 were compared with those of the authentic standards, daidzein and glycitein (Sigma Chemical Co.), respectively.

Crystals of L5-2 were made as follows. The sample was dissolved in the hot solvent, a mixture of methanol and ethyl acetate (1:1). A small portion of the solvent was added to the sample mixture for slow crystallization, and the mixture was then evaporated slowly at room temperature and the structure solved directly. All hydrogen atoms were located and refined isotropically.

Results and Discussion

To confirm the enzymatic method using HMG-CoA reductase of a Syrian hamster, the concentration required for 50% inhibition (IC50) was measured with lovastatin. IC50 for lovastatin was calculated to be 0.018 μM, whereas the reported value is 0.023 μM.3) Hence we concluded that the spectrometric assay method using Syrian hamster HMG-CoA reductase was proper for screening of the inhibitors.

Oily residue of the solvent extract of soybean paste was subjected to VLC to separate the active components. Spots of the active fractions observed through TLC revealed more than one active component. Furthermore, changing the solvent gradient did not result in a higher resolution than that obtained from the second VLC. Through the VLC steps, non-active or relatively inactive fractions, which have relatively high hydrophobic and hydrophilic components, were discarded. The relative inhibition activities of the fractions are shown in Table 1. 2VF and 3VF fractions that exhibited relatively high inhibition activities were pooled for a second fractionation. Among the second VLC eluates, the 6th to 8th fractions showed relatively high inhibition activities with similar Rf values detected on TLC. The resulting active fractions of the second VLC were pooled and loaded onto a Sephadex LH-20 column, and more inactive compounds were excluded. The inhibition activity levels of the L4 and L5 groups of LH-20 eluate were 63.6 ± 9.5 and 49.9 ± 12.5% at 4.5 and 3.8 μg/150 μl respectively. Thus L5 was selected for purification, and its structure was determined through LC/MS, NMR, FT-IR, elemental analysis, and X-ray diffraction. Based on the results of L5, the structures of the L4 group components were inferred from the LC/MS data and identified by comparing them with the NMR spectra of the authentic standards.

The UV absorption spectrum of L5-2 (major peak of L5) is shown in Fig. 1. The purified L5-2 had a pale yellow appearance. This UV absorption spectrum was identified as genistein.14) From the spectrum of L5-2, the analysis wavelength was determined to be 260 nm; thus, all HPLC detections were carried out at 260 nm. HPLC chromatograms of the L5 and L4 groups based on 260 nm absorption are displayed in Fig. 2. The main peak of L5-2 appeared at a retention time of 6.16 min when L5 was eluted with 30% acetonitrile with 0.1% TFA. The L4 group was found to have two main compounds. The active components purified through preparative HPLC were pale yellow, nearly white,

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Concentration (μg/150μl)</th>
<th>Relative activity of HMG-CoA reductase (%)</th>
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<tbody>
<tr>
<td>1VFb</td>
<td>30.0</td>
<td>113.4 ± 15.7</td>
</tr>
<tr>
<td>2VF</td>
<td>30.0</td>
<td>12.2 ± 0.8</td>
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<tr>
<td>3VF</td>
<td>36.0</td>
<td>6.9 ± 3.3</td>
</tr>
<tr>
<td>4VF</td>
<td>30.8</td>
<td>68.6 ± 1.1</td>
</tr>
<tr>
<td>2VFc</td>
<td>28.5</td>
<td>91.9 ± 10.0</td>
</tr>
<tr>
<td>3VF</td>
<td>24.0</td>
<td>78.2 ± 6.0</td>
</tr>
<tr>
<td>4VF</td>
<td>65.0</td>
<td>98.6 ± 10.3</td>
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<tr>
<td>5VF</td>
<td>34.5</td>
<td>105.3 ± 4.9</td>
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<tr>
<td>6VF</td>
<td>9.8</td>
<td>32.6 ± 9.1</td>
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<tr>
<td>7VF</td>
<td>11.3</td>
<td>7.4 ± 1.6</td>
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<tr>
<td>8VF</td>
<td>15.8</td>
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<tr>
<td>9VF</td>
<td>36.0</td>
<td>42.3 ± 8.7</td>
</tr>
<tr>
<td>10VF</td>
<td>36.0</td>
<td>98.9 ± 5.9</td>
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* Each value represents ± standard deviation of three replicates.
* First vacuum liquid chromatography fraction number.
* Second vacuum liquid chromatography fraction number.

Fig. 1. UV Absorbance Spectrum of L5-2 Recorded with a Photo-diode Array Detector.
pellet. The purified components were identified by analytical HPLC and LC/MS analysis.

Mass spectra were obtained from L5-2, L4-1, and L4-2. When acetonitrile was used as the eluent, distinct peaks in the mass spectrum of L5-2 were observed at m/z = 271 [M + H]^+, and 312 [M:CH3CN + H]^+. The exact mass spectrum of L5-2 exhibited a molecular ion by APCI-MS at 271.0563 [M + H]/C138 +, corresponding to C15H11O5 (calculated m/z = 271.0606) (Fig. 3A). The other molecular ion at 312.0876 was ambiguous when compared with the data obtained by NMR and elemental analysis. Therefore, the eluent was changed to methanol. The resulting chromatogram showed molecular ions by APCI-MS at 271 [M + H]^+ and 303 [M:CH3OH + H]^+ (Fig. 3B). An MS chromatogram showed the protonated molecular ion of L5-2, determined to be 271, and 312 m/z peak, determined to be an artefact, formed with acetonitrile. For L4-1 and L4-2 the protonated molecular weights were determined by APCI-MS with acetonitrile as eluent. From the chromatogram, the protonated molecular weights of L4-1 and L4-2 were determined to be 255 [M + H]^+ and 285 [M + H]^+, respectively.

The expected molecular formula of L5-2 from the high-resolution mass spectroscopy, C15H10O5 (MW 270), was confirmed by elemental analysis. Based on the formula weight and elemental analysis, the content of carbon and hydrogen was calculated to be 66.7 and 3.7%, and 65.2 and 3.9%, respectively.

Chemical shifts of the L5-2 protons were measured at /C14 12.95 (1H, s, HO-5), 10.86 (1H, s, HO-7), 9.58 (1H, s, HO-4), 8.29 (1H, s, H-2), 7.36 (2H, d, J = 8.27 Hz, H-20, H-60), 6.81 (2H, d, J = 8.27, Hz, H-30, H-50), 6.37 (1H, m, J = 1.96, H-8), and 6.21 (1H, m, J = 1.96, H-6). The 13C-NMR spectrum was measured by 1H broadband decoupling. Chemical shifts of carbons were measured at δ 180.21 (C-4), 164.26 (C-7), 161.99 (C-5), 157.58 (C-9), 157.42 (C-4'), 153.97 (C-2), 130.16 (C-2', C-6'), 122.27, 121.21 (C-3), 115.06 (C-3', C-5'), 104.46 (C-10), 98.96 (C-6), and 93.66 (C-8). To assign the positions of carbons and hydrogens, 1H–1H COSY, 13C–1H COSY, DEPT, HMCO, and HMBC experiments were performed. The number of hydrogen atoms bonded directly to a carbon nucleus was determined by subjecting L5-2 to a DEPT experiment. The results showed that L5-2 has chemical shifts of five CH signals and eight quaternary carbon nuclei. The C–H COSY experiment revealed five peaks belonging to the carbon nuclei that have directly bonded protons. From the H–H COSY and HMQC experiments, the resonance position of the coupled nuclei was identified. To determine the
long-range H–C connectivity, the HMBC experiment was performed. In addition to the NMR experiments and LC/MS spectra, the structure of L5-2 was determined to be genistein by X-ray diffraction analysis. After the structural determination, NMR spectra of L5-2 were compared with those of the authentic compound, genistein, in the literature. All protons and carbons of L5-2 were assigned based on the results of NMR.\textsuperscript{15,16} The molecular structure of L5-2 with the atom-numbering scheme is shown in Fig. 4. The planes defined by C1’–C6’ are almost planar, with an average atomic displacement of 0.0002 nm. The naphthalene-type ring defined by 10 atoms (C2–C10 and O1) is also nearly planar, with an average atomic displacement of 0.0019 nm. The dihedral angle between these two planes is 53.1(1)°, and the torsion angle of C8–C9–O1–C2 is very close to 180° (178.9(3)°). Furthermore, all the hydrogen atoms of the hydroxyl group are involved in the formation of hydrogen bonds of the type O–H · · · O, including one intramolecular and two intermolecular hydrogen bonds (Table 2). The data on the crystal structure are in accord with the report of Brenton et al.\textsuperscript{17}

The FT-IR spectrum (KBr) displayed an absorption at 3411 (intermolecular hydroxyl), 3106 (hydroxyl), 1649 (\(\alpha, \beta\)-unsaturated carbonyl or ketone), and 1616 cm\(^{-1}\), which supports the structure of L5-2, and also is in agreement with the results of several previous reports.\textsuperscript{18,19}

L4-1 and L4-2 were deduced to be daidzein and glycitein respectively, based on the LC-MS data and those obtained for the identification of L5-2, and were directly compared with the authentic daidzein and glycitein samples. Chemical shifts of L4-1 protons were measured at \(\delta\) 10.76 (1H, br s, HO-7), 9.52 (1H, br s, HO-4’), 8.29 (1H, s, H-2), 7.97 (1H, d, \(J = 8.75\), H-5), 7.39 (2H, d, \(J = 8.55\), H-2’, H-6’), 6.94 (1H, dd, \(J = 8.89, 2.06\), H-6), 6.86 (1H, m, \(J = 1.96\), H-8), and 6.81 (2H, d, \(J = 8.55\), H-3’, H-5’), and were compared with the data in the literature.\textsuperscript{20} Chemical shifts of carbons L4-1 were measured at \(\delta\) 174.66 (C-4), 162.46 (C-7), 157.40 (C-9), 157.12 (C-4’), 152.78 (C-2), 130.04 (C-2’, C-6’), 127.26 (C-5), 123.46 (C-1’), 122.52 (C-3), 116.61 (C-10), 115.01 (C-3’, C-5’), 114.90 (C-6), and 102.06 (C-8).\textsuperscript{15} Chemical shifts of L4-2 protons were measured at \(\delta\) 10.51 (1H, br s, HO-7), 9.55 (1H, br s, HO-4’), 8.27 (1H, m, \(J = 0.48\), H-2), 7.43 (1H, s, H-5), 7.39 (2H, d, \(J = 8.31\), H-2’, H-6’), 6.93 (1H, s, H-8), 6.81 (2H, d, \(J = 8.31\), H-3’, H-5’), and 3.88 (3H, s), and were compared with data in the literature.\textsuperscript{15} Chemical shifts of L4-2 carbons were measured at \(\delta\) 174.26 (C-4), 157.05 (C-4’), 153.01 (C-7), 152.41 (C-2), 151.73 (C-9), 146.95 (C-6), 130.01 (C-2’, C-6’), 122.92 (C-3), 122.77 (C-1’), 116.09 (C-10), 114.89 (C-3’, C-5’), 104.68 (C-5), 102.75 (C-8), and 55.79 (OMe).\textsuperscript{15,21}

Soy isoflavones have been reported to show various biological activities including estrogenic,\textsuperscript{22} antioxidant,\textsuperscript{23} anti-osteoporotic,\textsuperscript{24} and anticarcinogenic\textsuperscript{25} activities. Furthermore, recent studies have proposed that isoflavones may be the main active ingredient responsible for the cholesterol-lowering properties of some soy foods.\textsuperscript{26,27} Our study of HMG-CoA reductase inhibitors in soybean paste identified the active components to be 6’-acetyldaidzin, luloactone (4’-hydroxyisoflavone, C\textsubscript{15}H\textsubscript{10}O\textsubscript{4}), daidzein (4’-dihydroxyisoflavone, C\textsubscript{15}H\textsubscript{10}O\textsubscript{4}), and glycitein (4’-dihydroxy-6-methoxyisoflavone, C\textsubscript{16}H\textsubscript{12}O\textsubscript{5}), aglycone forms of the isoflavones, which are not statins or related compounds known to be inhibitors of HMG-CoA reductase. The discovery of isoflavones as HMG-CoA reductase inhibitors, not yet reported elsewhere, in soy foods is intriguing. No structural similarity exists between statins and isoflavones. But a kinetic study of isoflavones revealed that they are competitive inhibitors of HMG-CoA\textsubscript{4},\textsuperscript{28} like statins. Additionally, a comparison of the inhibition activity of glycone, the non-fermented form, and aglycone, the fermented form, of isoflavone revealed the aglycone form to be more active.\textsuperscript{28}

In soy foods, each of the three isoflavones, daidzein, glycitein, and genistein, exists in four forms, and in nature 6’-O-malonyl-\(\beta\)-glucoside is the predominant form.\textsuperscript{29} 6’-O-Malonylgenistin and 6’-O-malonyldaidzin are the most abundant isoflavones of soybean hypocotyl and cotyledon, whereas 6’-O-acetyldaidzin and 6’-O-acetyldaidzin are the major isoflavones in texturized vegetable proteins.\textsuperscript{29,30} The 6’-O-acetyl-\(\beta\)-glucoside-, \(\beta\)-glucoside-, and aglycone forms are derived from 6’-
glycosid when heated to over 60°C in aqueous solvents. Less than 5% of the isoflavones in natural soybeans are aglycones, but they are the main forms present after fermentation.

Genistin and daidzin constitute 99% of all the isoflavones in dry soybeans. Isolated soy protein and textured vegetable protein consist of a mixture of all three types of isoflavone conjugates. In unfermented soy foods such as soybean milk powder, aglycones are present mainly in the glucoside forms and make up only 4–5% of the isoflavones. However, among fermented commercial soybean products, higher aglycone contents are observed. The liberation of aglycones from glucosides during fermentation occurs through the hydrolytic action of β-glucosidase from fungi and bacilli. These compositional differences may be important with regard to metabolism and bioavailability due to marked differences in the polarity of conjugated and unconjugated isoflavones.

Most daidzins and genistins were hydrolyzed after 4 weeks of miso (Japanese soybean paste) fermentation. When the soybeans were soaked in water, the daidzein and genistin contents increased, whereas those of daidzin and genistin decreased. Matsuura and Obata partially purified three isoflavones of β-glucosidase from soybean cotyledons. But because these enzymes from soybeans were destroyed during steam-cooking, microorganisms including A. oryzae probably produced new β-glucosidase in the soybean paste fermentation. Riou et al. purified a glucose-tolerant β-glucosidase from A. oryzae found in wine and juice processings.

The chemical forms of isoflavones in food should be taken into consideration when evaluating their availability for absorption through the diet. Recently, Piskula et al. demonstrated that daidzein and genistein, but not their glucosides, are readily absorbed from the stomach of rats. Similar results were observed in humans. Isoflavone aglycone-rich products may be more effective than glucoside-rich products in preventing chronic diseases, such as coronary heart disease. Not only do fermented soybean foods at the least increase the bioavailability of soy isoflavones, because glycosidic forms are degraded into unconjugated forms before absorption in the intestine, but they can also be consumed in large quantities. Therefore, fermented soy foods such as soybean paste are good sources of aglycone isoflavones for the prevention of cardiovascular disease through inhibition of HMG-CoA reductase.

Conclusions

In this study, aglycone isoflavones in Korean soybean paste have been identified as HMG-CoA reductase inhibitors, even though there is no similarity in structure to statins. Isoflavones mainly exist in glycone forms in non-fermented soy foods, whereas, in fermented soy foods such as soybean paste, they exist in aglycone forms. Aglycone forms of isoflavones have higher bioavailability and are more active in many cases than the conjugated forms. Our study suggests that the hypocholesterolemic effects of soy foods previously reported in several epidemiological studies are due in part to the inhibition of HMG-CoA reductase by isoflavones. Therefore, soybean paste is a good source of aglycone isoflavones which can prevent cardiovascular diseases.

Acknowledgments

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