Note

Isoflavones Inhibit 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase *in Vitro*

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Isoflavones identified as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in soybean paste were assayed using the catalytic portion of Syrian hamster HMG-CoA reductase, and the kinetic values were measured using HMG-CoA and NADPH. The inhibition of HMG-CoA reductase by these inhibitors was competitive with HMG-CoA and noncompetitive with NADPH. K_i values for genistein, daidzein, and glycitein were 27.7, 49.5, and 94.7 μM, respectively.

Western people are exposed to very limited amounts of isoflavones, which are found almost exclusively in soybeans and other leguminous plants, in their diets, usually no more than 1–3 mg/day.[1] In contrast, East Asians (Chinese, Japanese, Koreans, *etc.*) consume an average of 25–100 mg/day isoflavones, mostly from soy food products.[2] Interest in soy ingredients has increased recently because epidemiological studies have shown that consumption of soy products may be associated with low incidence rates of hormone-dependent and -independent cancers,[1,3] and with the reduced risk of various diseases including cardiovascular problems,[4–6] osteoporosis,[7] and menopausal symptoms.[8] In these connections, soy isoflavones have been reported to have a variety of biological activities including estrogenic,[9] antioxidative,[10] anti-osteoporotic,[11] and anticarcinogenic[12] activities. Furthermore, recent studies proposed that isoflavones might be the main active ingredient responsible for the cholesterol-lowering properties of some soyfoods.[13,14]

HMG-CoA reductase, which catalyzes the reduction of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate at an early stage of cholesterol biosynthesis, represents a major target for regulation of the overall pathway. Regulation of cholesterol synthesis is exerted near the beginning of the pathway. In this study, direct inhibition effects on HMG-CoA reductase by genistein, daidzein, and glycitein (aglycone forms of isoflavones),

isolated and identified in soybean paste,[15] were investigated using HMG-CoA and NADPH.

D,L-HMG-CoA, dithiothreitol, EDTA, mevalonate, and glycitein were purchased from Sigma Chemical Co. (St. Louis, MO, USA), NADPH was from ICN Biochemicals Inc. (Cleveland, OH, USA), and genistein and daidzein from Lancaster Synthesis Ltd. (Lancashire, UK). All isoflavones were dissolved in DMSO (100 μl), to which 0.1 N NaOH (900 μl) was added. After heating at 50°C for 2 hours, each isoflavone was diluted with a mixture of 100 mM phosphate buffer (pH 6.8) and 0.1 N NaOH (1:1, v/v) for the kinetic experiments.

LC-MS analysis was done using a Hewelett Packard 1100 HPLC (Wilmington, DE, USA) coupled to a JMS-LC mate single quadrupole mass spectrometer (JEOL Ltd., Tokyo, Japan), equipped with an atmospheric pressure chemical ionization (APCI) interface that was used at an ion source temperature of 500°C. The genistein solution was injected at a flow rate of 1 ml/min with 0.1% acetic acid in acetonitrile/water (30/70, v/v) to a HPLC column of Capcell Pak UG C18 (250 mm×4.6 mm I.D., 5 μm particle size, pore size 120 Å, Shiseido Fine Chemicals, Kyoto, Japan). Molecular weight of the genistein was measured based on the positive ion mode $[M+H]^+$.

Plasmid pKFT7-21,[16] a gene encoding the catalytic domain of Syrian hamster HMG-CoA reductase, was a generous gift from Prof. V. W. Rodwell of Purdue University. HMG-CoA reductase was overexpressed in *Escherichia coli*, and the resulting enzyme was purified as described by Frimpong *et al.*[16] HMG-CoA-dependent oxidation of NADPH was monitored at 340 nm using a diode array spectrophotometer (Amersham Pharmacia Biotech, Ultrospec 4000) equipped with a cell holder maintained at 37°C. Standard assay mixtures contained 200 μM D,L-HMG-CoA, 200 μM NADPH, 100 mM NaCl, 1.0 mM EDTA, 10 mM dithiothreitol, and 100 mM Na$_x$PO$_4$ (pH 6.8) at a final volume of 150 μl.[16] Reaction mixtures containing 11.5–17.3 nmol of the purified enzyme and other components except HMG-CoA were first monitored for HMG-CoA-independent oxidation of

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Abbreviation: HMG-CoA, 3-hydroxy-3-methlyglutaryl coenzyme A

NADPH. The reaction was then initiated by adding HMG-CoA. One unit of HMG-CoA reductase was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per min.[16] The protein concentration was measured by the method of Bradford using bovine serum albumin as the standard.

The enzyme reaction was done using inhibitors at various concentrations. The inhibition type for each inhibitor was identified by Lineweaver-Burk plots. Kinetic parameters such as V_{max}, K_m, and K_i were evaluated using the non-linear regression method based on the following inhibition equation:

$$v_i = \frac{V_{max} \times S}{K_m \left(1 + \dfrac{I}{K_i}\right) + S}$$

where v_i is the initial velocity in the absence and presence of the inhibitor; S and I, concentrations of the substrate and the inhibitor, respectively; V_{max}, the maximum velocity; K_m, the Michaelis-Menten constant; and K_i, the inhibition constant defined as [E][I]/[EI].

For the HMG-CoA inhibition study, NADPH was fixed at 200 μM, and HMG-CoA was used in the range of 9.38–50 μM. With respect to NADPH, HMG-CoA was fixed at 200 μM, and NADPH was used in the range of 28.12–150 μM. K_i values of the inhibitors were calculated based on the results.

The mass spectrum (Fig. 1) showed the peak at m/z 271.1 $[M+H]^+$, which was derived from genistein. Although isoflavones are known to be degraded by alkali,[17,18] no peaks generated by alkaline degradation were observed in this experiment.

When the enzyme reaction was done using 1.9 pmole enzyme, the V_{max} value was calculated as 17 μmol NADPH/min/mg. Under these conditions, K_m values for the two substrates, D,L-HMG-CoA and NADPH, were 8 and 55 μM, respectively. These values were similar to the values previously reported by Frimpong *et al.*[16] When the enzyme concentration was increased to 11.5–17.3 nmole, K_m values of D,L-HMG-CoA and NADPH also increased to 32±5, and 141±12 μM, respectively.

An HMG-CoA reductase inhibition study was done using genistein, daidzein, and glycitein, previously identified as inhibitors in soybean paste.[15] The Lineweaver-Burk plots (Fig. 2) were used to predict the type of inhibition for each inhibitor. All isoflavones were shown to compete with HMG-CoA, whereas they were noncompetitive towards NADPH. The inhibition constants (K_i) with regard to HMG-CoA were: 27.7±4.3 μM for genistein, 49.5±4.5 μM for daidzein, and 94.7± 11.9 μM for glycitein.

Discovery of isoflavones as HMG-CoA reductase inhibitors in soy foods is very intriguing. Although they have relatively high K_i values compared with statins, HMG-CoA reductase inhibitors with inhibition constant values in the nanomolar range, they have no HMG-like moieties and show different inhibition activities. In
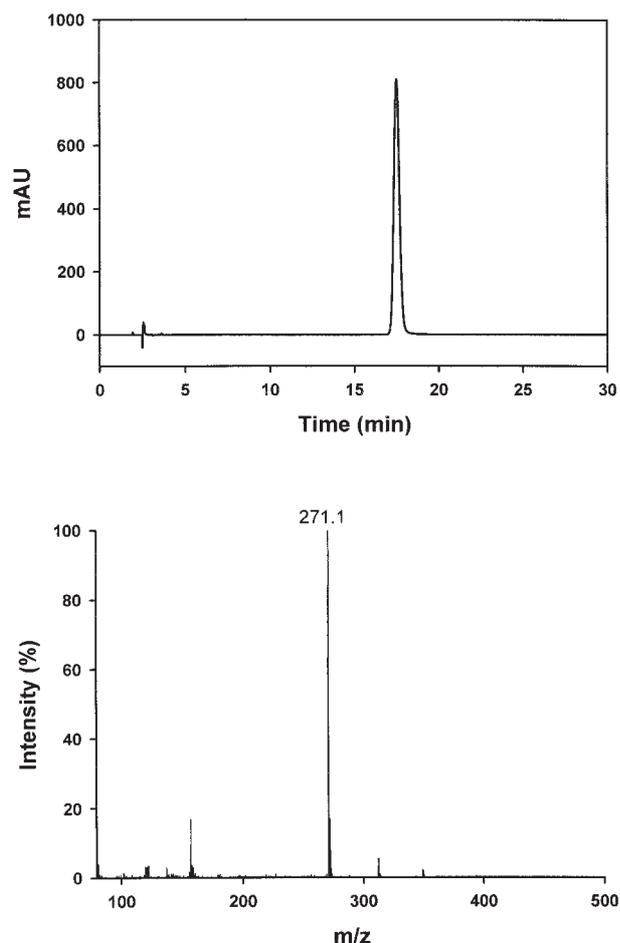

Fig. 1. LC Chromatogram and Mass Spectrum of Genistein in the Assay Solution.

addition, it is well known that isoflavones are degraded by alkali and heat.[17] In this study, the activity of isoflavones on HMG-CoA reductase was measured by dissolving the isoflavones in 0.1 N NaOH and heating at 50°C for 2 h. Therefore, the possibility that other substances generated by degradation and cleavage of isoflavone or artifacts due to the alkaline and heat treatment existed. The assay solution of isoflavone, genistein, was analyzed by APCI$^+$ LC/MS for the confirmation of the changes in genistein. The mass spectrum only displayed the peak of genistein (Fig. 1). From this result, it was concluded that intact isoflavones inhibited HMG-CoA reductase.

Additionally, kinetic studies, which showed competitive and noncompetitive inhibitions towards HMG-CoA and NADPH, respectively, revealed that isoflavones inhibit HMG-CoA reductase by binding at the hydrophobic portion of HMG-CoA reductase where HMG-CoA binds, as statins do. Therefore, isoflavones may exert steric hindrance between HMG-CoA and HMG-CoA reductase through hydrophobic interactions. This hypothesis could explain the different inhibition constants obtained between genistein and the others as well as the difference in the inhibition mode with respect to
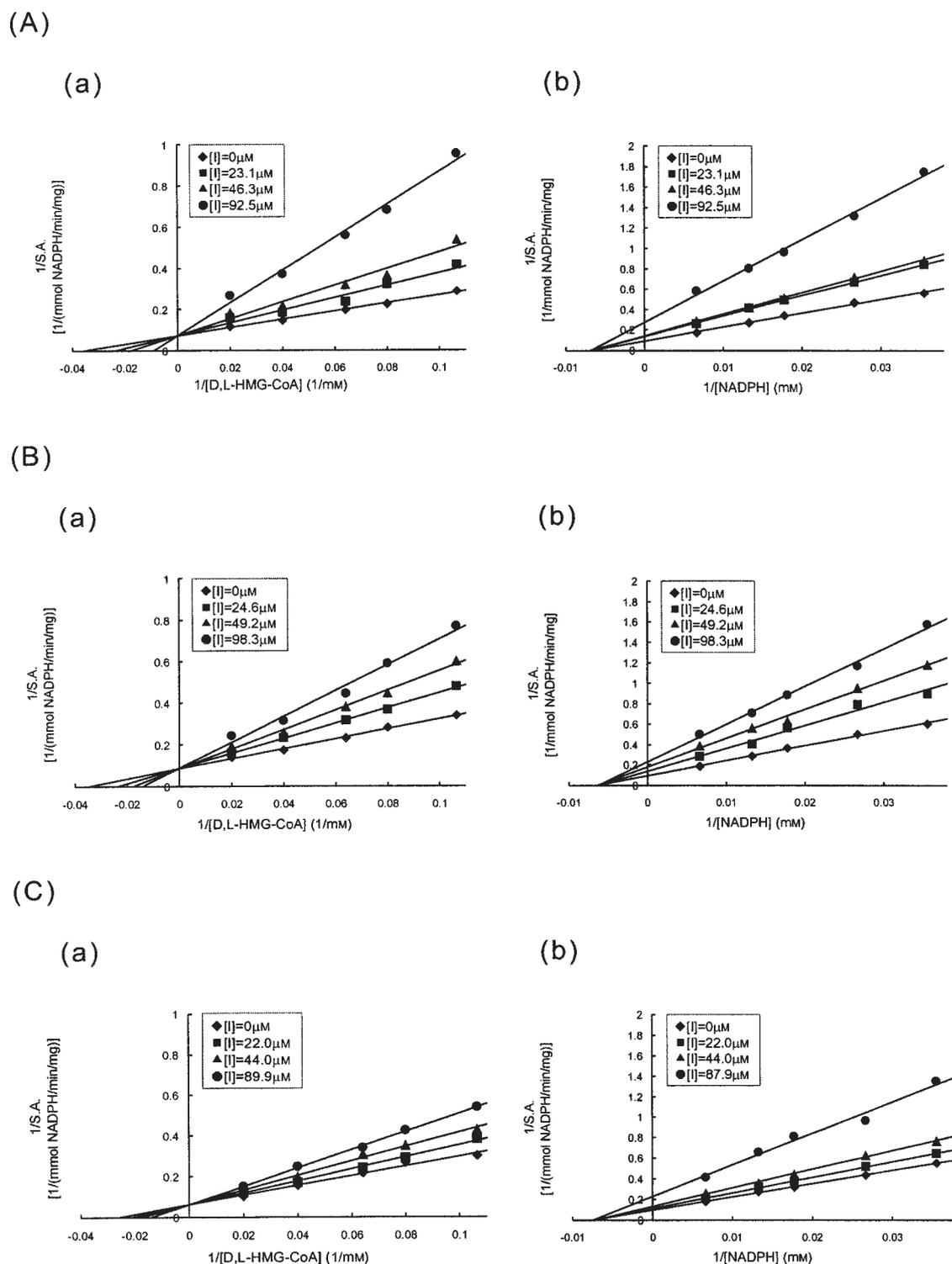
(A)

(a)

(b)

(B)

(a)

(b)

(C)

(a)

(b)

Fig. 2. Lineweaver-burk Plots for the Inhibition of HMG-CoA Reductase by Genistein (A), Daidzein (B), and Glycitein (C).

Shown are double-reciprocal plots of the dependence of initial velocity on the concentration of D,L-HMG-CoA (a) or NADPH (b). All analyses were conducted in triplicate at pH 6.8. Throughout the analyses, competitive and noncompetitive inhibitions with respect to HMG-CoA and NADPH, respectively, were observed.

HMG-CoA and NADPH. Genistein has more hydrophobic characteristics than other isoflavones, which was confirmed by the resonance structure shown in X-ray diffraction study.[19] This structural feature may allow genistein to bind more strongly to HMG-CoA reductase than other isoflavones such as daidzein and glycitein. However, to prove the structural correlation between isoflavone and HMG-CoA reductase, this hypothesis needs to be tested through crystallographic studies on the binding of isoflavone to the catalytic portion of

HMG-CoA reductase.

Numerous studies have reported inverse associations between soy protein intake and plasma cholesterol concentrations. Currently, the active component(s) and the mechanisms of action remain unclear, although the role of isoflavones has recently gained much attention.[20–22]

In the kinetic study of isoflavones regarding HMG-CoA, addition of genistein resulted in similar K_m ($32\pm5\,\mu$M) and K_i ($27.7\pm4.3\,\mu$M) values. In comparison with other literature reporting the characteristics of HMG-CoA reductase and its inhibitors, the higher value of K_m than other literature values was consistent with the result for rat liver HMG-CoA reductase, in which higher concentrations resulted in higher K_m values.[23] It is also in accord with the K_m values of HMG-CoA reductase in the micromole range ($1–10\,\mu$M) previously reported.[16] Therefore, the results of this experiment suggest isoflavones could function as HMG-CoA reductase inhibitors under physiological conditions ($0.1–6\,\mu$M) of the Asian people.[24–26]

In summary, we showed that isoflavones inhibit HMG-CoA reductase *in vitro* and exert competitive inhibition with HMG-CoA. Our study suggests that the hypocholesterolemic effects of soy foods previously reported in several epidemiological studies are in part due to the inhibition of HMG-CoA reductase by isoflavones.

Acknowledgments

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