Hypocholesterolemic Effect of Triterpene Alcohols with Soysterol on Plasma Cholesterol in Rats

Michiaki KIRIBUCHI, Kazue MIURA, Setsuko TOKUDA, and Takashi KANEDA

Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Tsutsumidori, Sendai 980, Japan
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Summary To identify the synergistic hypocholesterolemic substance found in soybean oil unsaponifiable matter, rats were fed diets containing various fractions of the unsaponifiable matter prepared by silicic acid column chromatography. The plasma cholesterol level of the group fed the alcohol fraction, which mainly consisted of triterpene alcohols, was significantly lower and the effect was synergistic with soysterol. So the effect of cycloartenol and 24-methylenecycloartanol, which are main constituents of triterpene alcohols in soybean oil unsaponifiable matter, was investigated. Both compounds were prepared from γ-oryzanol (ferulate) and were added (0.05%) respectively to the experimental diet containing 0.5% cholesterol and 1% soysterol. It was observed that both cycloartenol and 24-methylenecycloartanol in combination with soysterol greatly reduced the plasma cholesterol and enhanced cholesterol excretion. This suggests that the hypocholesterolemic activity of dietary vegetable oils may account for not only their fatty acid compositions and sterol contents but also the synergistic hypocholesterolemic effect of triterpene alcohols.

Key Words hypocholesterolemic effect, synergy, soysterol, triterpene alcohol, rat

Since the hypocholesterolemic effect of phytosterol in chickens was reported by Peterson (1), much investigation has been done using several kinds of experimental animals (2–6) and the activity of phytosterol was observed. At present, the effect of phytosterol is recognized to be caused possibly by its inhibitory effect on cholesterol absorption (7,8). In this laboratory it was suggested by Shibukawa et al. (9) that a hypocholesterolemic substance occurs in the unsaponifiable fraction of soybean oil, which acts synergistically with soysterol, but does not act without soysterol. Thus,
this study was undertaken to identify the effective substance. In expt. 1, soybean oil unsaponifiable matter was fractionated by silicic acid column chromatography and the effect of each fraction on plasma cholesterol in rats was examined. In expt. 2, whether the activity of the effective fraction was synergistic with soysterol was determined. In expt. 3, the effects of the two substances which are constituents of the effective fraction were tested. The amounts of fecal neutral and acidic steroids excretion were also determined at the same time.

**MATERIALS AND METHODS**

*Materials.* The fractions of soybean oil unsaponifiable matter (Morishita Seiyaku Co., Osaka) used in expt. 1 were prepared by silicic acid column chromatography according to the procedure of Capella et al. (10). The composition of the alcohol fraction, used in expt. 2 also was (in %): tocopherol, 6; triterpene alcohols, 61 (α-amyrin, 20; β-amyrin, 4; cycloartenol, 14; cyclolaudenol, 21) and unknowns as measured by GLC (11). Cycloartenol and 24-methylene cycloartanol used in expt. 3 were prepared from γ-oryzanol (Marusan Yuka Co., Tokyo) by the following method (12). The γ-oryzanol was extracted with hot methanol, and the insoluble fraction was crystallized repeatedly from ethylacetone: ethanol (1:1) and saponified with 2N alcoholic KOH solution. The cycloartenol was obtained by solvent extraction from the solution and its purity was 90% by GLC (13). Similarly, the 24-methylene cycloartanol was prepared first by saponification and next by repeated recrystallization from methanol. Its purity was 84%.

*Animals and diets.* The male Wistar rats (Nikon Rat Co., Saitama) weighing 145 g (expt. 1), 195 g (expt. 2) and 110 g (expt. 3) were fed ad libitum on the experimental diets for 21 days (expt. 1), 10 days (expt. 2) and 22 days (expt. 3). Each group consisted of 5–10 rats. The composition of the basal diet was (in %): casein, 22; celluflour, 3; salt mixture, 4; vitamin mixture, 0.5; choline chloride, 0.24; cotton seed oil, 5; and sucrose to 100. Cholesterol, bile salt (0.125%), soysterol and samples were added at the expense of sucrose. The composition of the soysterol (Morishita Seiyaku Co., Osaka) was (in %): sitosterol, 45.9; stigmasterol, 26.7; campesterol, 26.9; and brassicasterol, 0.5. In expt. 1, the amount of each fraction added to the diet was equivalent to the amount contained in soybean oil unsaponifiable matter as 0.375% of the diet.

*Determination of plasma and liver cholesterol.* After feeding of the experimental diets, rats were anesthetized with ether and exsanguinated by heart puncture. The liver was removed and weighed. The cholesterol in liver was extracted according to the method of Folch et al. (14). The plasma and liver cholesterol were determined by the method of Sperry and Webb (15) as modified by Abe and Kaneda (16).

*Determination of fecal neutral and acidic steroid excretion.* In expt. 3, feces were collected for the last 7 days of the feeding period. For this study, 4 rats whose plasma cholesterol levels were about the same as that of an average rat, were selected out of each group. Fecal steroids were extracted with acetone : ethanol.
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(1:1) for 24 hr in a Soxhlet's apparatus and autoclaved with 10% NaOH for 6 hr at 120°C in a teflon vial. The extracted neutral and acidic steroids were transformed into trimethylsilyl ether derivatives and methylester acetate derivatives respectively, and analyzed by GLC (17, 18). The 5-α-cholestane for the neutral steroids analysis and 5-β-cholanic acid for the acidic steroids analysis were used as internal calibration standards.

RESULTS

Growth
The food intake and body weight gain were not significantly different among all the groups in each experiment.

Plasma and liver cholesterol
Expt. 1. Plasma cholesterol levels were higher in both groups fed the hydrocarbon or the tocopherol fractions compared with the group fed 0.5% cholesterol with 1.5% soysterol (Table 1). In the group which was fed the alcohol fraction (group VI), however, it was lower in spite of the reduction in the amount of added soysterol from 1.5 to 1.125%. Therefore, the effective substance appeared to be contained in the alcohol fraction.

Expt. 2. The alcohol fraction (group V) reduced the plasma cholesterol level synergistically with soysterol, but without soysterol (group IV) it had no effect (Table 2). The liver cholesterol level of the group V which was fed the alcohol fraction with soysterol was similar to that of the group III which was fed 0.5% cholesterol with 1.125% soysterol.

Expt. 3. Cycloartenol and 24-methylene cycloartenol had greatly lowered plasma cholesterol levels in combination with soysterol, and cycloartenol was more effective (Table 3). Similar to the results in expt. 2, they did not show an additional reducing effect on liver cholesterol levels.

Fecal neutral and acidic steroids excretion
In the control group, coprostanol, cholesterol and 7-dehydrocholesterol were identified as fecal neutral steroids and the total amount was 9.2 mg/day/rat (Table 4). In the excrement, coprostanol was predominant and 7-dehydrocholesterol was the least. In the cholesterol fed group, the amount of excreted cholesterol was 42.3 mg/day/rat, while 2.6 mg/day/rat of cholesterol was excreted in the control group. This means that 41.1% of the ingested cholesterol was excreted into feces. Except for the cholesterol, an increase in the amount of excreted neutral steroids was not observed. In the case of soysterol feeding, the percentage of fecal excreted cholesterol to ingested cholesterol increased up to 62.3%. However, when cycloartenol or 24-methylene cycloartenol was fed with soysterol, it resulted in a remarkable increase in cholesterol excretion up to 73.8 and 71.0%, respectively. In the soysterol fed groups including cycloartenol and 24-methylene cycloartenol fed
| Group                                      | Plasma (mg/100 ml) | Liver (mg/g) | Liver weight (%)
|-------------------------------------------|--------------------|--------------|------------------|
|                                           | Total   | Free  | Free % | Total  | Free  | Free % | Body weight (%) |
| I. Basal + 0.5% cholesterol               | 152.0 ± 3.7    | 52.1 ± 2.1 | 34.3 | 21.16 ± 1.35 | 4.88 ± 0.25 | 23.1 | 5.07 |
| II. I + 1.5% soysterol                     | 112.9 ± 4.9    | 39.2 ± 2.3 | 34.8 | 3.59 ± 0.12  | 2.21 ± 0.04 | 61.5 | 4.91 |
| III. I + 1.125% soysterol + 0.375% unsap. matter | 113.3 ± 7.1  | 39.3 ± 1.2 | 34.5 | 4.16 ± 0.29  | 2.28 ± 0.08 | 54.8 | 5.92 |
| IV. I + 1.125% soysterol + hydrocarbon fr. | 148.5 ± 7.6    | 37.2 ± 3.3 | 25.1 | 4.61 ± 0.50  | 2.50 ± 0.11 | 54.2 | 5.24 |
| V. I + 1.125% soysterol + tocopherol fr.  | 138.8 ± 6.6    | 40.3 ± 3.8 | 29.0 | 4.57 ± 0.33  | 2.40 ± 0.12 | 52.5 | 5.85 |
| VI. I + 1.125% soysterol + alcohol fr.    | 104.3 ± 5.0    | 36.3 ± 1.5 | 34.8 | 4.20 ± 0.38  | 2.26 ± 0.08 | 53.8 | 5.49 |

*Results are expressed as means ± SE.
Table 2. Plasma and liver cholesterol levels in expt. 2.

| Group                        | Plasma (mg/100ml) | Liver (mg/g) | Liver weight (Body weight (%) |
|-----------------------------|-------------------|--------------|-------------------------------|
|                             | Total             | Free | Free % | Total             | Free | Free % |                  |
| I. Basal                    | 96.3 ± 4.2        | 23.1 ± 1.4 | 24.0 | 3.14 ± 0.15 | 2.17 ± 0.10 | 86.3 | 4.50 |
| II. I + 0.5% cholesterol    | 154.9 ± 8.4       | 32.1 ± 2.0 | 20.7 | 16.75 ± 0.24 | 6.12 ± 0.24 | 36.5 | 5.32 |
| III. II + 1.125% soysterol  | 145.3 ± 5.7       | 38.7 ± 2.1 | 26.7 | 5.94 ± 0.36 | 3.74 ± 0.39 | 63.0 | 5.02 |
| IV. II + 0.043% alcohol fr. | 162.4 ± 12.2      | 36.0 ± 2.3 | 22.2 | 18.31 ± 1.26 | 5.45 ± 0.73 | 29.8 | 4.84 |
| V. III + 0.043% alcohol fr. | 121.7 ± 5.0b      | 38.0 ± 2.1 | 31.2 | 5.88 ± 0.45 | 2.81 ± 0.13 | 47.8 | 5.00 |

*Results are expressed as means ± SE. bSignificantly different from group III at p<0.05.*

Table 3. Plasma and liver cholesterol levels in expt. 3.

| Group                        | Plasma (mg/100ml) | Liver (mg/g) | Liver weight (Body weight (%) |
|-----------------------------|-------------------|--------------|-------------------------------|
|                             | Total             | Free | Free % | Total             | Free | Free % |                  |
| I. Basal                    | 145.6 ± 6.7       | 44.1 ± 2.9 | 30.0 | 3.55 ± 0.14 | 3.14 ± 0.19 | 88.5 | 5.45 |
| II. I + 0.5% cholesterol    | 223.2 ± 9.0       | 55.9 ± 3.0 | 25.0 | 30.43 ± 1.78 | 6.65 ± 0.60 | 21.9 | 6.57 |
| III. II + 1% soysterol      | 164.3 ± 4.9       | 42.4 ± 1.9 | 25.8 | 8.10 ± 0.85 | 5.04 ± 0.39 | 62.2 | 5.67 |
| IV. III + 0.05% cycloartenol| 118.7 ± 6.5       | 43.6 ± 3.3 | 36.7 | 8.08 ± 0.67 | 6.77 ± 0.40 | 83.8 | 5.34 |
| V. III + 0.05% 24-methylene | 134.9 ± 14.9      | 38.4 ± 5.9 | 28.5 | 10.43 ± 1.15 | 7.01 ± 0.43 | 67.2 | 5.15 |

*Results are expressed as means ± SE. bSignificantly different from group III at p<0.01.*
Table 4. Fecal neutral and acidic steroids excretion.

| Group          | Ingested cholesterol (mg/day/rat) | Excreted cholesterol [other sterols]* (mg/day/rat) | Excreted Ingested (%) | Total excreted bile acids (mg/day/rat) |
|----------------|-----------------------------------|---------------------------------------------------|----------------------|---------------------------------------|
| I. Basal diet  | —                                 | 2.6                                               | —                    | 1.35 ± 0.4b                           |
| II. I+0.5% cholesterol | 96.5                              | 42.3                                              | 41.1 ± 4.7           | 11.7 ± 0.6                            |
| III. II+1% soysterol | 88.4                              | 57.7                                              | 62.3 ± 4.1           | 10.2 ± 1.0                            |
| IV. III+0.05% cycloartenol | 93.6                              | 71.7                                              | 73.8 ± 1.2d          | 12.0 ± 1.0                            |
| V. III+0.05% 24-methylenecycloartenol | 92.3                              | 68.1                                              | 71.0 ± 3.3           | 10.1 ± 1.4                            |

*a Coprostanol and 7-dehydrocholesterol are noticed. b Results are expressed as means ± SE. c Not detectable. d Significantly different from group III at p<0.05.

groups, about 85% of the ingested soysterol was excreted in feces. As for acidic steroids, 1.35 mg/day/rat of acidic steroids was excreted in the feces of the control group and the identified constituents were hyodeoxycholic acid (30.4%), deoxycholic acid (24.9%), Ï±-muricholic acid (17.6%), lithocholic acid (16.4%) and cholic acid (7.2%). In the cholesterol fed groups including the soysterol, cycloartenol and 24-methylenecycloartenol groups, the total amounts of excreted acidic steroids were increased up to about 11 mg/day/rat mainly caused by the increased cholic acid excretion (about 50% of the total acidic steroids).

DISCUSSION

When the presence of a synergistic hypocholesterolemic agent was demonstrated in soybean oil unsaponifiable matter, tocopherol was thought to be the effective substance because much tocopherol was contained in it with the sterols. But an experiment using a preparation of tocopherol (Tama Seikagaku Co., Tokyo) showed that the tocopherol had no synergistic effect, and this agreed with the results of expt. 1. Cho et al. (19) reported that the plasma cholesterol was not necessarily reduced by increasing tocopherol levels in the diets. In expt. 1, fractions of the unsaponifiable matter separated by differences of polarity were examined, and it was observed that the effective substances were contained in the alcohol fraction. This fraction consisted of a large amount of triterpene alcohols with a small amount of tocopherol. Since it was shown previously that tocopherol has no synergistic effect, one or more of the triterpene alcohols were thought to be the effective substance.

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Figure 1 shows the principal triterpene alcohols in soybean oil (11): cycloartenol and cyclolaudenol, which are tetracyclic, and \( \alpha \)-amyrin and \( \beta \)-amyrin, which are pentacyclic.

It is generally accepted that cycloartenol is a precursor of sterol in plants as is lanosterol in animals (20). Thus, the chemical structure of cycloartenol resembles that of sterol.

In expt. 2, the plasma cholesterol level of the group fed a soysterol supplemented diet was compared with that of the group fed a diet free of soysterol to determine if the effect of the alcohol fraction was synergistic with soysterol. The synergy of the fraction was confirmed; that is, the plasma cholesterol level of the group fed the soysterol supplemented diet with the alcohol fraction (group V) was significantly lower compared with that of group III fed the diet supplemented with soysterol alone, but that of the group IV fed the diet supplemented with alcohol fraction alone was rather higher than that of group II fed cholesterol. \( \alpha \)-Amyrin, a major component of the alcohol fraction, exhibited no effect in an experiment using a preparation of \( \alpha \)-amyrin (K & K Co., U.S.A.), so cycloartenol and 24-methylene cycloartanol were tested in expt. 3 and identified to be the effective substances. Thus the similarity of the chemical structure to that of sterol appeared to be responsible for this activity of these compounds. In the present study, cycloartenol and 24-methylene cycloartanol were tested because they are widespread in plants and easily prepared from \( \gamma \)-oryzanol, but other triterpene alcohols may have the same effect. At present, the mechanism of the synergistic effect of these compounds is not clear. However, at least the enhanced fecal cholesterol excretion seems to participate in the effect.

It is known that phytosterol feeding leads to an increase in fecal cholesterol.
excretion, but cycloartenol and 24-methylene cycloartanol feedings caused a further increase in this study. So they may aid the inhibitory effect of soysterol on cholesterol absorption and/or influence cholesterol metabolism as an intact or a derivative form after absorption. Generally the hypocholesterolemic effect of dietary vegetable oils has accounted for their fatty acid composition (21) and their sterol contents (22), but this effect may be affected by their specific triterpene alcohol contents, also.

It is known that rice bran oil, corn oil and wheat germ oil have a greater hypocholesterolemic effect than other dietary vegetable oils, partly because they have a large amount of sterol, but interestingly the principal triterpene alcohols in these oils are cycloartenol and its derivatives (23).

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