Anabolic Effect of Soybean Saponin on Bone Components in the Femoral Tissues of Rats

Rie Ono[a,b] **and Masayoshi Yamaguchi**[*,a]

[a]*Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan and* [b]*Department of Research and Development, Marumiya K.K, 2211 Uchida, Kikusui-machi, Tamana Gunn, Kumamoto 865–0104, Japan*

(Received June 28, 1999; Accepted July 25, 1999)

The effect of soybean saponin on bone components in the femoral tissues of rats was investigated. Rats were orally administered saponin (100 μg/ml/100 g body weight) for 10 d. The administration caused a significant increase in calcium content, alkaline phosphatase activity, and deoxyribonucleic acid (DNA) content in the diaphyseal and metaphyseal tissues of the femur in the animals. When bone tissues were cultured for 24 h in a medium containing either vehicle or saponin (10 μg/ml of medium), the presence of saponin caused a significant rise in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and metaphyseal tissues. The effect of saponin in increasing bone components was completely prevented by the presence of cycloheximide (10^{-6} M), suggesting that the saponin effect is partly based on a newly synthesized protein component. This study demonstrates that soybean saponin has an anabolic effect on bone components in rats, suggesting its role as a nutritional factor in the prevention of osteoporosis.

Key words —— saponin, soybean, bone metabolism, bone formation, osteoporosis

INTRODUCTION

Bone mass decreases with increasing age.[1–3] This decrease may be due to increased bone resorption and to decreased bone formation. Osteoporosis with decrease of bone mass is widely recognized as a major public health problem.[4] Pharmacological and nutritional factors have the potential to prevent bone loss with increasing age, however, these factors are not yet fully understood.

Isoflavones including daidzin, daidzein, genistin, and genistein are present in soybean with a comparatively higher concentration. More recently, it has been demonstrated that isoflavones have an anabolic effect on bone metabolism in rats,[5–9] suggesting their role in the prevention of osteoporosis. Genistin and daidzin are hydrolyzed to genistein and daidzein by β-glucuronidase in gastric juice, respectively. Genistein and daidzein have been shown to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption,[6,7,10] so that bone mass is increased.

Soybeans contain great quantities of saponin besides isoflavones.[11] Whether saponin has an anabolic effect on bone metabolism, however, remains unknown. Therefore, this study was undertaken to determine the effect of saponin on bone metabolism *in vivo* and *in vitro*. We found an anabolic effect of saponin on bone components in the femoral tissues of rats.

*To whom correspondence should be addressed: Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan. Tel. (Fax): +81-54-264-5580; E-mail: yamaguch@fns1.u-shizuoka-ken.ac.jp

MATERIALS AND METHODS

Chemicals —— Dulbecco's modified Eagle's medium (MEM) (high glucose) and a penicillin-streptomycin solution (5000 units/mg penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, N.Y., U.S.A.). Bovine serum albumin and cycloheximide were obtained from Sigma Chemical (St. Louis, MO., U.S.A.). Saponin, from soybeans, and all other chemicals were reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled.

Animals —— Male Wistar rats (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The

animals were fed commercial laboratory chow (solid) containing 57.4% carbohydrate, 1.1% Ca, and 1.1% P at a room temperature of 25℃, and were given distilled water freely.

Administration Procedures —— Saponin was dissolved in 10% ethanol solution (diluted with distilled water) to give a concentration of 50, 100, and 150 μg per ml. Saponin solution (1 ml/100 g body weight) or 10% ethanol solution was orally administered to rats (weighing 95—100 g) with a stomach tube once a day for 5 and 10 d, and 24 h after the last adminstration the animals were sacrificed by cardiac puncture under light anethesia with ether, and the blood and femur were removed immediately.

Bone Culture —— The femurs were removed aseptically after bleeding and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-diaphyseal and metaphyseal tissues were cut into small pieces. Femoral-diaphyseal or metaphyseal fragments were cultured for 24 h in a 35-mm dish in 2.0 ml medium consisting of Dulbecco's MEM (high glucose, 4.5 g/dl) supplemented with 0.25% bovine serum albumin plus antibiotics (100 units of penicillin and 100 μg of streptomycin/ml of medium).[5] In experiments, bone tissues were cultured for 24 h in a medium containing either vehicle (including 0.1% ethanol) or saponin (diluted with 0.1% ethanol). Cultures were maintained at 37℃ in a water-saturated atmosphere containing 5% CO_2 and 95% air.

Analytical Procedures —— Blood samples were centrifuged for 30 min after collection, and the serum was separated and analyzed immediately. Serum calcium was determined by the method of Willis.[12] Serum inorganic phosphorus was measured by the method of Taussky and Shon.[13]

The diaphyseal and metaphyseal tissues were dried for 16 h at 110℃ and weighed. Bone tissues were digested for 24 h at 110℃. Calcium was determined by atomic absorption spectrophotometry.[14] Calcium content in bone tissues was expressed as mg per g dry bone.

To assay alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized with a Physcotron homogenizer, and disrupted for 60 s with an ultrasonic device. The supernatant centrifuged at 600 × g for 5 min was used to measure enzyme activity. Enzyme assay was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt.[15] Enzyme activity was expressed as μmol of p-nitrophenol liberated per min per mg protein. Protein concentration was determined by the method of Lowry *et al.*[16]

To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 h after the homogenization of the bone tissues.[17] After alkali extraction, the samples were centrifuged at 1000 × g for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti[18] and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

Statistical Analysis —— The significance of the difference between values was estimated by Student's t-test. p-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Effect of Saponin Administration on Bone Component in Rats *in Vivo*

Saponin (100 μg/ 100 g body weight) was orally administered to rats for 5 and 10 d. Body weight of animals was not significantly altered by the administration of saponin for 10 d as compared with that of control rats (data not shown). Control rats received an oral administration of 10% ethanol solution (1 ml/ 100 g body weight). Saponin administration also did not cause a significant alteration in calcium and

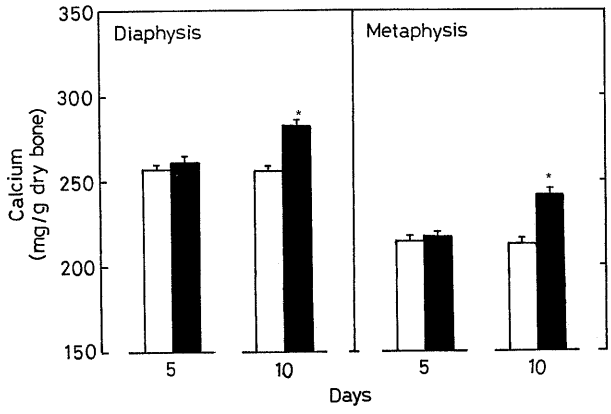

Fig. 1. Alteration in Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissues of Rats Orally Administered Saponin

Rats were orally administered saponin (100 μg/ml/100 g body weight) for 5 and 10 d, and 24 h after the last administration the animals were sacrificed by bleeding. Each value is the mean ± S.E. M. of six rats. *$p<0.01$, compared with the control value. □, Control ; ■, saponin.

inorganic phosphorus concentrations in the serum of rats (data not shown). Thus, the dose of saponin used may not have a toxic effect on rats.

The administration of saponin (100 μg/ 100 g) for 10 d caused a significant increase in calcium content (Fig. 1), alkaline phosphatase activity (Fig. 2), and DNA content (Fig. 3) in the femoral-diaphyseal and metaphyseal tissues. Femoral-diaphyseal and metaphyseal DNA content was significantly elevated by the administration of saponin (100 μg/ 100 g) for 5 d (Fig. 3). Such a rise was not seen by saponin administration at 50 μg/ 100 g for 5 d. At a greater dose (150 μg/ 100 g), the effect of saponin in raising bone DNA content was saturated (data not shown).

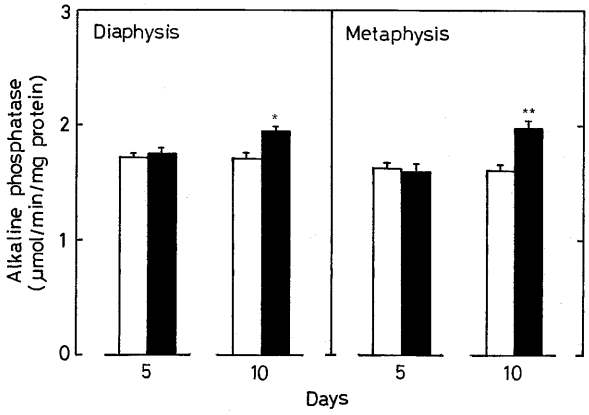

Fig. 2. Alteration in Alkaline Phosphatase Activity in the Femoral-Diaphyseal and Metaphyseal Tissues of Rats Orally Administered Saponin

Rats were orally administered saponin (100 μg/ml/100 g body weight) for 5 and 10 d, and 24 h after the last administration the animals were sacrificed by bleeding. Each value is the mean ± S.E. M. of six rats. $*p<0.05$ and $**p<0.01$, compared with the control value. □, Control ; ■, saponin.

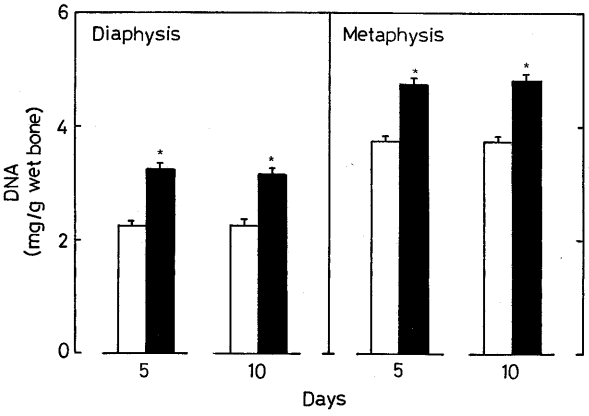

Fig. 3. Alteration in DNA Content in the Femoral-Diaphyseal and Metaphyseal Tissues of Rats Orally Administered Saponin

Rats were orally administered saponin (100 μg/ml/100 g body weight) for 5 and 10 d, and 24 h after the last administration the animals were sacrificed by bleeding. Each value is the mean ± S.E. M. of six rats. $*p<0.01$, compared with the control value. □, Control ; ■, saponin.

Effect of Saponin on Bone Component in Tissue Culture in Vitro

Femoral-diaphyseal and metaphyseal fragments were cultured for 24 h in a medium containing either vehicle (containing 0.1% ethanol) or saponin (1, 5, and 10 μg/ml of medium). The presence of saponin (10 μg/ml) caused a significant increase in calcium content (Fig. 4), alkaline phosphatase activity (Fig. 5), and DNA

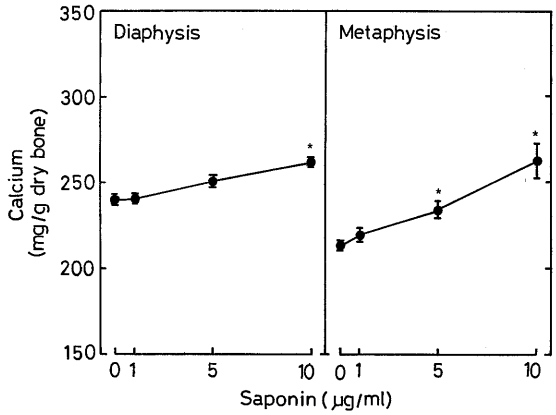

Fig. 4. Effect of Saponin on Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissues Obtained from Rats *in Vitro*

Bone tissues were cultured for 24 h in a medium containing either vehicle or saponin (1, 5, and 10 μg/ml of medium). Each value is the mean ± S.E.M. of six bone tissues obtained from separate rats. $*p<0.01$, compared with the control (none) value.

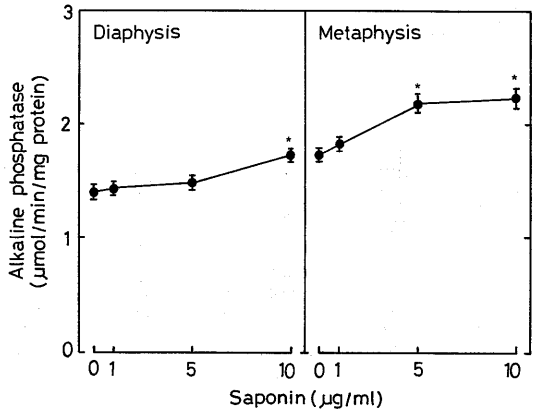

Fig. 5. Effect of Saponin on Alkaline Phoaphatase Activity in the Femoral-Diaphyseal and Metaphyseal Tissues Obtained from Rats *in Vitro*

Bone tissues were cultured for 24 h in a medium containing either vehicle or saponin (1, 5, and 10 μg/ml of medium). Each velue is the mean ± S.E.M. of six bone tissues obtained from separate rats. $*p<0.01$, compared with the control (none) value.

content (Fig. 6) in the femoral-diaphyseal tissues as compared with the value obtained from control culture with vehicle solution. Such an elevation was also seen in the femoral-metaphyseal tissues with the saponin concentration of 5 μg/ml.

Bone tissues were cultured for 24 h in a medium containing either vehicle or saponin (10 μg/ml of medium) in the absence or presence of cycloheximide (10^{-6} M). The effect of saponin in increasing calcium content in the femoral-diaphyseal and metaphyseal tissues was completely prevented by the presence of cycloheximide, an inhibitor of protein synthesis (Fig. 7). In the presence of this inhibitor, saponin (10 μg/ml) did not have a significant effect on alkaline phosphatase activity or DNA content in these tissues (data not shown).

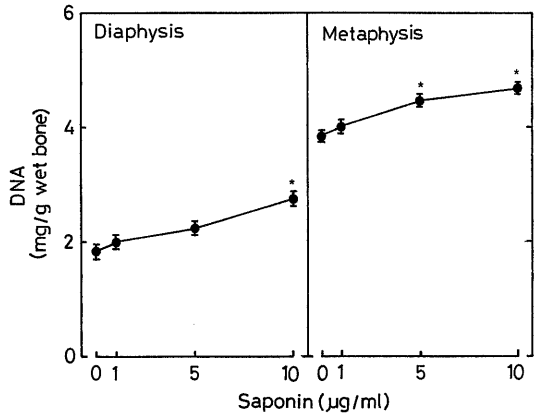

Fig. 6. Effect of Saponin on DNA Content in the Femoral-Diaphyseal and Metaphyseal Tissues Obtained from Rats *in Vitro*

Bone tissues were cultured for 24 h in a medium containing either vehicle or saponin (1, 5, 10 μg/ml of medium). Each value is the ± S.E.M. of six bone tissues obtained from separate rats. *$p <$ 0.01, compared with the control (none) value.

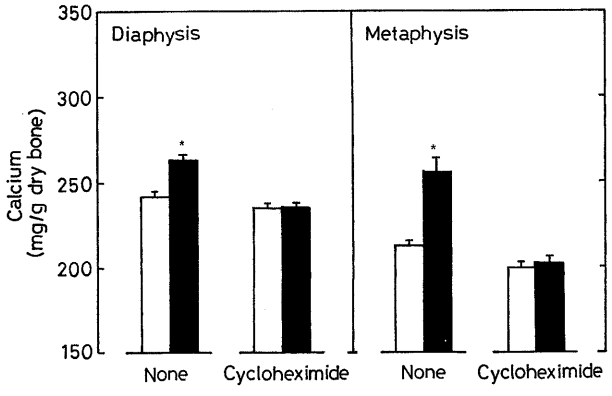

Fig. 7. Effect of Cycloheximide on Saponin-Increased Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissues Obtained from Rats *in Vitro*

Bone tissues were cultured for 24 h in a medium containing either vehicle or saponin (10 μg/ml of medium) in the absence or presence of cycloheximide (10^{-6}M). Each value is the mean ± S.E.M. of six bone tissues obtained from separate rats. *$p < 0.01$, compared with the control (none) value. □, Control ; ■, cycloheximide.

DISCUSSION

Osteoporosis is widely recognized as a major public health problem. The most dramatic expression of this disease is represented by fractures of the proximal femur.[19,20] Malnutrition or undernutrition is often observed in the elderly, and it appears to be more severe in patients with hip fracture than in the general ageing population.[4] Deficiency in both micronutrients and macronutrients appears to be strongly implicated in the pathogenesis and the consequences of hip fracture in osteoporotic elderly.[4] Nutritional factors may be important to prevent bone loss with increasing age. More recently, it has been reported that genistein and daidzein, a soybean isoflavone, can stimulate bone formation and bone resorption, suggesting their role in the prevention of this condition.[5-10] Soybean also contains a large quantity of saponin.[11] Whether saponin has an anabolic effect on bone metabolism is unknown so far. This study is the first to clearly demonstrate that saponin has an anabolic effect on bone components *in vivo* and *in vitro*.

Saponin is converted to sapogenin by β-glucosidase in gastric juice. Its oral administration to rats caused a significant rise in calcium content, alkaline phosphatase activity, and DNA content in the femoral tissues. The effect of saponin administration may be partly related to sapogenin. However, saponin has been demonstrated to have an anabolic effect on bone components in a culture system using femoral-diaphyseal and metaphyseal tissues *in vitro*, indicating that saponin has a direct effect on bone metabolism.

Saponin-increased bone calcium-content was completely prevented by the presence of cycloheximide, *in vitro*. Likewise, the effect of saponin in raising bone alkaline phosphatase activity and DNA content was also completely blocked when cycloheximide was present (data not shown). These results suggest that an anabolic effect of saponin is partly based on a newly synthesized protein component. Thus, saponin may stimulate

bone formation and calcification.

Bone cells including osteoblasts, osteocytes and osteoclasts are present in bone tissues, and bone formation and calcification are related to osteoblastic function. Alkaline phosphatase is a marker enzyme of osteoblasts, and the enzyme participates in bone mineralization.[21] DNA content is an index of bone growth and the number of bone cells.[22] Saponin has been shown to increase bone alkaline phosphatase activity and DNA content, indicating that it acts on osteoblastic cells. The cellular mechanism of saponin in osteoblasts, however, remains to be elucidated.

In conclusion, it has been demonstrated that the intake of soybean saponin has an anabolic effect on bone metabolism. Dietary saponin may be useful in the prevention of osteoporosis with increasing age.

REFERENCES

1) Nishimoto S.K., Chang C.-H., Gendler E., Stryker W.F., Nimni M.E., *Calcif. Tissue Int.*, **37**, 617—624 (1985).
2) Schapira C., Linn S., Sarid M., Mokadi S., Kabala A., Silbermann M., *Bone*, **16**, 575—582 (1995).
3) Wild R.A., Buchaman J.R., Myers C., Demers L. M., *Proc. Soc. Exp. Biol. Med.*, **186**, 355—360 (1987).
4) Bonjour J.-P., Schurch M.-A., Rizzoli R., *Bone*, **18**, 1395—1445 (1986).
5) Yamaguchi M., Gao Y.H., *Res. Exp. Med.*, **197**, 101—107 (1997).
6) Yamaguchi M., Gao Y.H., *Mol. Cell. Biochem.*, **178**, 377—382 (1998).
7) Yamaguchi M., Gao Y.H., *Biochem. Pharmacol.*, **55**, 71—76 (1998).
8) Gao Y.H., Yamaguchi M., *Gen. Pharmac.*, **31**, 199—202 (1998).
9) Gao Y.H., Yamaguchi M., *Mol. Cell. Biochem.*, **194**, 93—98 (1999).
10) Gao Y.H., Yamaguchi M., *Biochem. Pharmacol.*, **58**, 767—772 (1999).
11) Ono R., Yamaguchi M., *J. Health Sci.*, **42**, 66—69 (1999).
12) Willis J.B., *Nature* (London), **186**, 249—250 (1960).
13) Taussky H.H., Shon E., *J. Biol. Chem.*, **202**, 675—685 (1953).
14) Yamaguchi M., Ozaki K., Hoshi T., *Res. Exp. Med.*, **189**, 9—14 (1989).
15) Walter K., Schutt C., "Methods of Enzymatic Analysis," Vol.1—2, Bermeger H.V. (ed.), Academic Press, New York, 856—860 (1974).
16) Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., *J. Biol. Chem.*, **193**, 265—273 (1951).
17) Flanagan B., Nichols G. Jr., *J. Biol. Chem.*, **237**, 3786—3789 (1962).
18) Ceriotti G., *J. Biol. Chem.*, **214**, 39—77 (1955).
19) Cooper C., Melton J. III., *Trends Endocrinol. Metab.*, **3**, 224—229 (1992).
20) Cummings S.R., Kelsey J.L., Nevitt M.C., O' Dowd K.J., *Epidemiol. Rev.*, **7**, 178—199 (1985).
21) Canalis E., *Endocr. Rev.*, **4**, 62—77 (1983).
22) Hickman J., McElduff A., *Endocrinology*, **124**, 701—706 (1989).