# Prolonged Intake of Dietary Fermented Isoflavone-Rich Soybean Reinforced with Zinc Affects Circulating Bone Biochemical Markers in Aged Individuals

**Masayoshi Yamaguchi,*[,a] Aki Igarashi,[a] Masakazu Sakai,[b] Hideyuki Degawa,[c] and Yoichi Ozawa[c]**

[a]*Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan,* [b]*Division of Internal Medicine, Nishi-Kumamoto Hospital, 1012 Koga, Tomiai, Kumamoto 861–4157, Japan, and* [c]*Food Science Laboratory, Taishi Foods Inc., 25–1 Shimomaegawara Osaka, Towada, Aomori 034–0041, Japan*

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Changes in circulating biochemical markers of bone metabolism in aged individuals with the intake of fermented soybean (*natto*), which was made from isoflavone-rich soybean, supplemented with zinc were investigated. Sixty-three volunteers (31 men and 32 women) were divided into four groups of 15 or 16 male volunteers and 16 or 16 female volunteers, and each group was sequentially given *natto* (40-g pack) containing two different levels of zinc once a day for 4 or 8 weeks as follows: either regular *natto* with naturally occuring isoflavone 35.0 mg, zinc 0.8 mg and calcium 51.4 mg or supplemented *natto* containing isoflavone 35.0 mg, zinc 3.6 mg, and calcium 60.0 mg. As serum bone markers, bone-specific alkaline phosphatase, $\gamma$-carboxylated osteocalcin, bone tartrate-resistant acid phosphatase (TRAP), and *N*-telopeptide of type I collagen were assayed. The intake of regular *natto* for 4 or 8 weeks in men or women persons caused a significant increase in $\gamma$-carboxylated osteocalcin, a marker of bone formation, and a significant decrease in serum bone *N*-telopeptide of type I collagen, a marker of bone resorption, as compared with the value before intake. Moreover, the intake of zinc-supplemented *natto* for 8 weeks in men or women caused a significant increase in serum bone-specific alkaline phosphatase activity and $\gamma$-carboxylated osteocalcin concentration and a significant decrease in serum bone TRAP activity and *N*-telopeptide of type I collagen, as compared with the values with the intake of regular *natto*. This study suggests that the intake of regular *natto* with isoflavone-rich soybean has a stimulatory effect on bone formation and an inhibitory effect on bone resorption in aged individuals, and that the effect is enhanced by supplementation with zinc.

**Key words** —— zinc, isoflavone, bone alkaline phosphatase, $\gamma$-carboxylated osteocalcin, bone tartrate-resistant acid phosphatase, type I collagen

## INTRODUCTION

Bone loss with aging induces osteoporosis, which is widely recognized as a major public health problem. A decrease in bone mass leads to bone fracture. Bone loss may be due to decreased bone formation and increased bone resorption. Pharmacologic and nutritional supplements can prevent bone loss caused by increasing age.[1)] Food chemical factors may also help to prevent bone loss with increasing age.

Recent studies have shown that isoflavones (including genistein and daidzen), which are contained in soybeans,[2–6)] and menaquinone-7, an analogue of vitamin $K_2$, which is abundant in fermented soybeans,[7–10)] have a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption *in vitro*, thereby increasing bone mass.

Isoflavone glycosides (daidzin, genistin, and glycitin) and aglycone isoflavones (daidzein, genistein, and glycitein) are present in soybeans at comparatively high concentrations. Daidzin, genistin, and glycitin are hydrolyzed to daidzein, genistein, and glycitein, respectively, by $\beta$-glucosidase in the intestine. Genistein and daidzein have

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

been shown to have an anabolic effect on bone metabolism in rats.[11)]

Zinc, an essential trace element, has been demonstrated to have a potent stimulatory effect on bone formation and an inhibitory effect on bone resorption.[12–16)] Zinc can stimulate protein synthesis in osteoblastic cells *in vivo* by activating aminoacyl-tRNA synthetase.[17)] The oral administration of a zinc compound can prevent bone loss in an animal model of osteoporosis.[18,19)]

Whether the combination of nutritional factors reveals an additive or synergistic effect on bone components has not been fully determined. This knowledge may be important in the prevention of bone loss with increasing age. Recently, it has been shown that the combination of genistein and zinc can have a synergistic effect on bone components in femoral tissue from elderly female rats.[2,20,21)] Moreover, it has been demonstrated that the preventive effect of dietary fermented soybean on bone loss is enhanced by supplementation of isoflavone and zinc in ovariecotomized rats, an animal model of osteoporosis.[22)] The intake of fermented soybean (*natto*), which is made from isoflavone-rich soybeans, supplemented with zinc and calcium has been shown to be effective in preventing the decrease in bone density in postmenopausal women,[23)] although the effects of zinc supplementation are uncertain.

The present study was undertaken to determine whether the supplementation of zinc in *natto* made from isoflavone-rich soybeans has an effect on bone metabolism in individuals. We found that the intake of dietary zinc-supplemented *natto* made from isoflavone-rich soybeans has a stimulatory effect on serum bone formation markers and a suppressive effect on serum bone resorption markers in aged men and women.

## MATERIALS AND METHODS

**Materials** —— Regular *natto* was supplied by Taishi Food Inc. (Towada, Japan), of which the zinc content was 0.8 mg per 40-g pack (wet weight). Supplemented *natto* with increased zinc content was made using zinc-fortified baker's yeast. The content of zinc in the supplemented *natto* was 3.6 mg per 40-g pack (wet weight). The nutritional content of the regular and supplemented *natto* per 40-g pack is shown in Table 1. The *natto* used in experiments was isoflavone rich (containing 35.0 mg of isoflavone per 40 g pack). Commercial *natto* contains 22.4 mg of isoflavone per 40-g pack.

**Table 1.** Nutritional Content of the Regular and Supplemented *natto* (per 40-g Pack)

| Ingredients | Regular *natto* | Supplemented *natto* |
|---|---|---|
| Protein (g) | 5.9 | 5.8 |
| Lipid (g) | 3.5 | 3.5 |
| Sugar (g) | 2.2 | 2.2 |
| Dietary fiber (g) | 2.8 | 2.8 |
| Ash (g) | 0.8 | 0.8 |
| Isoflavone (mg) | 35.0 | 35.0 |
| Zinc (mg) | 0.8 | 3.6 |
| Magnesium (mg) | 35.9 | 35.9 |
| Calcium (mg) | 51.4 | 60.0 |

**Experimental Procedures** —— Sixty-three adults (31 men and 32 women) aged 68–72 years men and 58–68 years women who were judged to be healthy with no abnormal liver or kidney functions and diabetes as assessed by standard biochemical data were enrolled as volunteers in this study. Informed consent was obtained from all. The period for washout or intake of each type of *natto* was 2 weeks or 4 and 8 weeks, respectively. The 63 volunteers were divided into four groups of 15 or 16 men and 16 or 16 women. Each group was sequentially given 40 g (wet weight) of fermented soybean (regular *natto* or supplemented *natto*) containing either 0.8 or 3.0 mg of zinc per 40-g pack once daily for 8 weeks. The *natto* was eaten between 06:00 and 08:00. Blood samples were collected at 10:00 and 12:00 on the day prior to intake, or 4 and 8 weeks after intake. Serum zinc, calcium, inorganic phosphorus, and various bone markers were measured using analytical methods.

**Analytical Procedures** —— Serum calcium, inorganic phosphorus, and zinc levels were determined using a kit (Wako Pure Chemicals, Osaka, Japan). The serum $\gamma$-carboxylated osteocalcin concentration was assayed using a Gla-type Osteocalcin (Gla-OC) EIA kit (Takara Shuzou, Shiga, Japan).[24)] Serum bone-specific alkaline phosphatase activity was assayed using a Metra BAP EIA kit (Quidel, San Diego, CA, U.S.A.).[25)] Serum bone tartrate-resistant acid phosphatase (TRAP) activity was assayed using a Bone TRAP Assay EIA kit (SBA Sciences, Turku, Finland).[26)] Serum bone *N*-telopeptide of type I collagen was measured using Osteomark NTx Serum EIA kit (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan).[27)]

**Statistical Analysis** —— Differences in values before and after the intake of each type of *natto* were

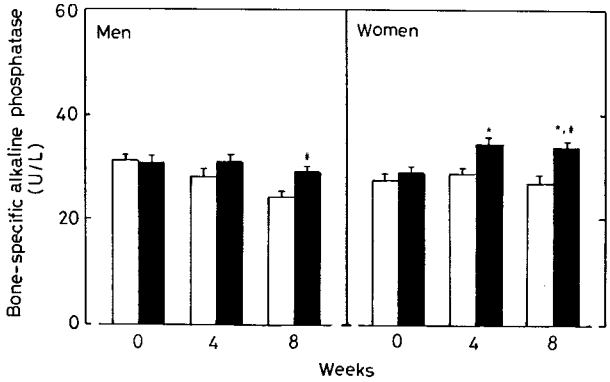


**Fig. 1.** Changes in Serum Bone-Specific Alkaline Phosphatase Activity Following Intake of Fermented Soybean (*natto*) Supplemented with Zinc

Dietary fermented soybean supplemented with zinc was given to volunteers for 4 or 8 weeks. Sixty-three persons (before intake) were divided into four groups; 15 men or 16 women for the intake of regular *natto* and 16 men and 16 women for the zinc-supplemented *natto*. Each value is the mean ± SEM of 15 or 16 persons. $*p < 0.05$ compared with the value before intake. $\#p < 0.05$ compared with the value obtained with regular *natto* intake. White bars, intake of regular *natto*; black bars, zinc-supplemented *natto*.

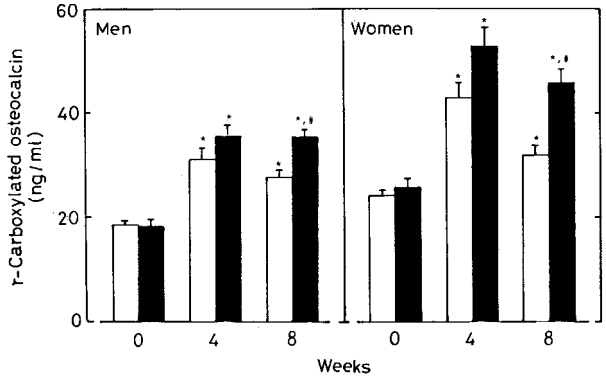


**Fig. 2.** Changes in Serum γ-Carboxylated Osteocalcin Levels Following Intake of Fermented Soybean (*natto*) Supplemented with Zinc

The procedure for the intake of *natto* supplemented with zinc is described in Fig. 1. Each value is the mean ± SEM of 15 or 16 persons. $*p < 0.01$ compared with the value before intake. $\#p < 0.01$ compared with the value obtained from regular *natto* intake. White bars, intake of regular *natto*; black bars, zinc-supplemented *natto*.

estimated using Student's *t*-test. A paired *t*-test was used for the difference in values before and after the intake of each type of *natto* or between the two groups after each intake period. *p*-Values of less than 0.05 were considered statistically significant.

## RESULTS

The change in serum biochemical markers of bone metabolism in aged individuals following the start of intake of regular *natto* made from isoflavone-rich soybeans, supplemented with zinc was examined. The period of intake for each type *natto* was 4 or 8 weeks. Serum calcium, inorganic phosphorus, and zinc concentrations did not change significantly with the intake of regular *natto* or the zinc-supplemented *natto* for 4 or 8 weeks (data not shown).

Serum bone-specific alkaline phosphatase activity in men and women did not change significantly with the intake of regular *natto* for 4 or 8 weeks (Fig. 1). However, the enzyme activity in men was significantly increased with the intake of the zinc-supplemented *natto* for 8 weeks as compared with that with regular *natto* intake. In women, the enzyme activity was significantly increased with the intake of the zinc-supplemented *natto* for 4 or 8 weeks as compared with the value before intake. The intake of the zinc-supplemented *natto* for 8 weeks resulted in a significant increase in the enzyme activity as compared with that with regular *natto* (Fig. 1).

The serum γ-carboxylated osteocalcin level in men and women was significantly increased with the intake of regular *natto* or the zinc-supplemented *natto* for 4 or 8 weeks (Fig. 2). The intake of zinc-supplemented *natto* for 8 weeks caused a significant increase in serum γ-carboxylated osteocalcin levels in men and women as compared with the levels with the intake of regular *natto* (Fig. 2).

Serum bone TRAP activity in men and women was significantly decreased with the intake of zinc-supplemented *natto* for 4 or 8 weeks as compared with the value obtained with the intake of regular *natto* (Fig. 3).

Serum levels of the *N*-telopeptide of type I collagen in men and women was significantly decreased with the intake of regular *natto* for 4 or 8 weeks as compared with the value before intake (Fig. 4). The intake of zinc-supplemented *natto* for 4 weeks caused a significant decrease in the serum concentration of the *N*-telopeptide of type I collagen in men and women. This effect of the zinc-supplemented *natto* was also seen in women after intake for 8 weeks (Fig. 4).

## DISCUSSION

Zinc has been shown to have a stimulatory effect on bone formation and an inhibitory effect on

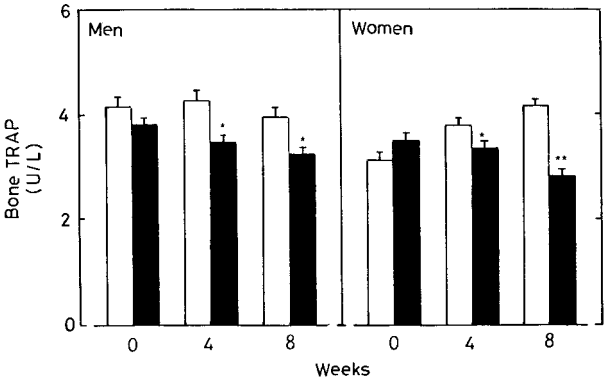


**Fig. 3.** Changes in Serum Bone TRAP Activity Following Intake of Fermented Soybean (*natto*) Supplemented with Zinc

The procedure for the intake of *natto* supplemented with zinc is described in Fig. 1. Each value is the mean ± SEM of 15 or 16 persons. $*p < 0.05$ or $**p < 0.01$ compared with the value obtained from regular *natto* intake. White bars, intake of regular *natto*; black bars, zinc-supplemented *natto*.

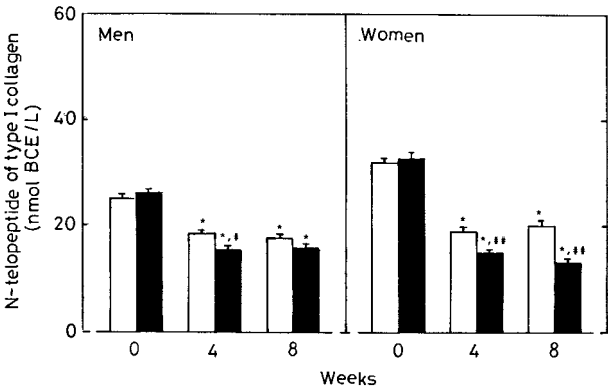


**Fig. 4.** Changes in Serum Levels of *N*-Telopeptide of Type I Collagen Following Intake of Fermented Soybean (*natto*) Supplemented with Zinc

The procedure for the intake of *natto* supplemented with zinc is described in Fig. 1. Each value is the mean ± SEM of 1 or 16 persons. $*p < 0.01$ compared with the value before intake. $\#p < 0.05$ or $\#\#p < 0.01$ compared with the value obtained from regular *natto* intake. White bars, intake of regular *natto*, black bars, zinc-supplemented *natto*.

bone resorption in rats,[28] suggesting that dietary zinc has a role in preventing osteoporosis. This study was undertaken to determine whether the intake of dietary zinc has an effect on specific serum biochemical markers of bone metabolism in aged individuals. We found that the prolonged intake of dietary fermented isoflavone-rich soybean (*natto*) supplemented with zinc causes a significant increase in serum markers of bone formation and a corresponding decrease in serum markers of bone resorption in aged men and women, suggesting that the intake of zinc-supplemented *natto* has a preventive effect on osteoporosis.

Serum bone-specific alkaline phosphatase and $\gamma$-carboxylated osteocalcin are expressed in osteoblastic cells,[29,30] which stimulate bone formation. Serum bone TRAP is a specific marker enzyme in osteoclasts,[26] and the *N*-telopeptide of type I collagen is specifically formed following the stimulation of bone resorption.[27]

The intake of *natto* made from isoflavone-rich soybeans caused a significant increase in serum $\gamma$-carboxylated osteocalcin levels and a significant decrease in serum levels of the *N*-telopeptide of type I collagen in aged men and women, as compared with the value before intake. This finding suggests that the intake of dietary isoflavone-rich *natto* has a stimulatory effect on bone formation and an inhibitory effect on bone resorption in aged individuals. Isoflavone has been shown to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption *in vitro*.[2–6]

The intake of isoflavone-rich *natto* supplemented with zinc caused a significant increase in serum bone-specific alkaline phosphatase activity and $\gamma$-carboxylated osteocalcin level and a significant decrease in serum bone TRAP activity and serum level of the *N*-telopeptide of type I collagen in aged individuals. Thus the effects of isoflavone-rich *natto* intake were significantly enhanced by the supplementation of zinc. Zinc has been demonstrated to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption in rats.[28] The effects of genistein or daidzein in stimulating bone formation *in vitro* and *in vivo* are significantly enhanced by the combination with zinc in rats.[20–22] Presumably, the anabolic effect on bone metabolism with the intake of zinc-supplemented isoflavone-rich *natto* result from the direct effect of zinc and isoflavone (including genistein and daidzein) on osteoblasts and osteoclasts in the bone tissues of aged individuals.

In conclusion, it has been shown that the intake of zinc-supplemented *natto*, which contains more zinc than regular *natto*, has a potent stimulatory effect on bone formation and an inhibitory effect on bone resorption in aged individuals. The prolonged intake of dietary isoflavone-rich *natto* supplemented with zinc may be useful for bone health and the prevention of bone loss caused by increasing age.

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