

# Effect of Marine Algae Extract on Bone Calcification in the Femoral-metaphyseal Tissues of Rats: Anabolic Effect of *Sargassum horneri*

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The effect of various algae on bone calcification in the femoral-metaphyseal tissues of rats was investigated. *Undaria pinnatifida*, *Sargassum horneri*, *Eisenia bicyclis*, *Cryptonemia scmitziana*, *Gelidium amansii*, and *Ulva pertusa* Kjellman, which were gathered seasonally, were used. Water suspensions (5%) of marine algae powder were orally administered once daily for 7 days. Bone calcium content was significantly increased by the administration of *U. pinnatifida*, *S. horneri*, *E. bicyclis*, or *C. scmitziana*. Bone alkaline phosphatase activity, which is an enzyme for calcification, was significantly enhanced by the administration of *S. horneri* or *G. amansii*. Femoral-metaphyseal tissues were cultured for 48 hr in Dulbecco's modified Eagle's medium containing either vehicle or water-solubilized extract (25 and 50 µg/ml) obtained from *U. pinnatifida*, *S. horneri*, *E. bicyclis*, and *C. scmitziana*. The bone calcium content was significantly elevated in the presence of *S. horneri* extract (25 and 50 µg/ml). No effect was seen with other extracts. The effect of *S. horneri* extract in increasing bone calcium content was completely inhibited in the presence of cycloheximide (10<sup>-6</sup> M), an inhibitor of protein synthesis. The present study demonstrates that *S. horneri* extract has an anabolic effect on bone calcification *in vivo* and *in vitro*. The anabolic effect of *S. horneri* extract may be based on a newly synthesized protein component.

**Key words** — marine algae, *Sargassum horneri*, bone metabolism, osteoporosis, rat femur

## INTRODUCTION

Bone loss with increasing age induces osteoporosis.<sup>1-3</sup> This loss may be due to increased bone resorption and decreased bone formation. Osteoporosis with a decrease in bone mass is widely recognized as a major public health problem.<sup>4</sup> The most dramatic expression of this disease is represented by fractures of the proximal femur. Nutritional factors can help to prevent bone loss with increasing age,<sup>5</sup> but these factors are poorly understood.

Recent studies have shown that isoflavone found in Leguminosae has an anabolic effect on bone metabolism in rats.<sup>6-8</sup> Soybean contains large quantities of isoflavones including genistein, genistin, daidzein, and daidzin. Isoflavones have been demonstrated to stimulate osteoblastic bone formation<sup>9,10</sup>

and inhibit osteoclastic bone resorption,<sup>11-13</sup> thereby increasing bone mass. Saponin in addition to isoflavones is contained in soybean, and it has been shown to have an anabolic effect on bone components.<sup>14</sup> Vitamin K<sub>2</sub> is suggested to play a role in preventing age-related bone loss. Vitamin K<sub>2</sub> is essential for the  $\gamma$ -carboxylation of osteocalcin, a bone matrix protein containing  $\gamma$ -carboxyglutamic acids, which is synthesized in osteoblasts of bone tissues.<sup>15</sup> Menaquinone-7, an analogue of vitamin K<sub>2</sub>, is abundant in fermented soybean (*natto*).<sup>16</sup> It was recently demonstrated that the prolonged dietary intake of menaquinone-7 has a preventive effect on bone loss induced by ovariectomy in rats.<sup>16,17</sup> Thus there is growing evidence that nutritional factors may be important in the prevention of bone loss with increasing age.

The effect of marine algae on bone metabolism, however, has not yet been clarified. The present study was undertaken to determine the effect of various marine algae on bone calcification in the femoral-metaphyseal tissue of rats *in vivo* and *in vitro*. Ma-

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rine algae (*Undaria pinnatifida*, *Sargassum horneri*, *Eisenia bicyclis*, *Cryptonemia scmitziana*, *Gelidium amansii*, and *Ulva pertusa* Kjellman), which are utilized in food, were used in this study. We found that *S. horneri* extract has an anabolic effect on bone calcification.

## MATERIALS AND METHODS

**Chemicals** — Dulbecco's modified Eagle's medium (MEM) (high glucose, 4.5 g/dl) and a penicillin-streptomycin solution (penicillin 5000 U/mg; streptomycin 5000 µg/ml) were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). Bovine serum albumin (fraction V) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan).

**Marine Algae Extracts** — Marine algae (*U. pinnatifida*, *S. horneri*, *E. bicyclis*, *C. scmitziana*, *G. amansii*, and *U. pertusa* Kjellman) were seasonally gathered from the coast at Shimoda or Haibara (Shizuoka prefecture, Japan), and it freeze-dried and powdered. The powder of marine alga was suspended in distilled water as a concentration of 5.0%. In another experiment, the gathered fresh marine algae were homogenized in distilled water or 20% ethanol solution with a Phycotron homogenizer, and the homogenate was centrifuged at 5500 *g* in a refrigerated centrifuge for 10 min. The 5500 *g* supernatant fraction was pooled for freeze-drying. The powder of water-solubilized extract or 20% ethanol-solubilized extract was dissolved in ice-cold distilled water or 20% ethanol to use in experiments, respectively.

**Animals** — Male Wistar rats (conventional) weighing 100–120 g (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium, and 1.1% phosphorus at room temperature of 25°C, with free access to distilled water.

**Administration Procedures** — The water suspension (5%, 1.0 ml/100 g body weight) of marine algae powder was orally administered to rats through a stomach tube once daily for 7 days. Control rats received distilled water (1.0 ml/100 g body weight) orally. The animals were killed 24 hr after the last administration by cardiac puncture under light ether anesthesia, and the blood and femur were removed immediately.

In other experiments, rats were orally administered the water suspension (20 mg/ml/100 g body weight) of a freeze-dried powder prepared from water-solubilized extract of *S. horneri* through a stomach tube once daily for 7 days. Animals were killed 24 hr after the last administration, and the blood and femur were removed.

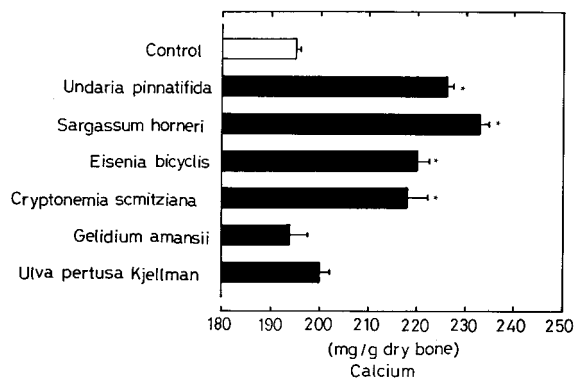
**Bone Culture** — The femurs were removed aseptically after bleeding and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-metaphyseal tissues were cut into small pieces. Femoral-metaphyseal fragments were cultured for 24 hr in a 35-mm dish in 2.0 ml of medium consisting of Dulbecco's MEM (high glucose, 4.5 g/dl) supplemented with 0.25% bovine serum albumin plus antibiotics (penicillin 100 units and streptomycin 100 µg/ml of medium).<sup>18)</sup> In experiments, bone tissues were cultured for 48 hr in a medium containing either vehicle or water-solubilized marine algae extract. The concentration of calcium in the marine algae extracts was in the range of 0.05 to 1.0 µg/ml of medium. In other experiments, culture medium was contained as a 5–50 µg/ml of 20% ethanol-solubilized extract. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO<sub>2</sub> 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.<sup>19)</sup> Serum inorganic phosphorus was measured by the method of Taussky and Shon.<sup>20)</sup>

The metaphyseal tissues were dried for 16 hr at 110°C. Calcium was determined by atomic absorption spectrophotometry.<sup>18)</sup> Calcium content in bone tissues was expressed as milligrams per gram of dry bone.

To assay alkaline phosphatase activity, the metaphyseal tissues were immersed in 3.0 ml of ice-cold barbital buffer 6.6 mM (pH 7.4), cut into small pieces, and disrupted for 60 sec 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.<sup>21)</sup> Enzyme activity was expressed as micromol of *p*-nitrophenol liberated per minute per milligram of protein. Protein concentration was determined by the method of Lowry *et al.*<sup>22)</sup>

**Statistical Analysis** — The significance of dif-



**Fig. 1.** Effect of Various Marine Algae on Calcium Content in the Femoral-Metaphyseal Tissues of Rats

Rats were orally administered either vehicle (distilled water) or marine alga (1.0 ml of 5% suspension/100 g body weight) once daily for 7 days. Animals were killed 24 hr after the last administration. Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control value.

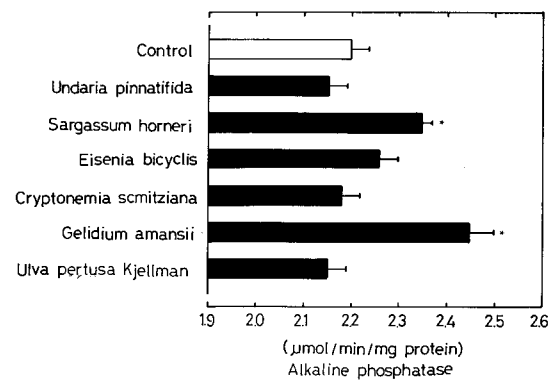
ference between values was estimated by Student's *t*-test. *p* values of less than 0.05 were considered to indicate statistically significant differences. We also used a multiple analysis of variance (ANOVA) and the Tukey-Kramer multiple-comparison test to compare the treatment groups.

## RESULTS

### Effect of Administration Various Marine Algae on Bone Components in Rats

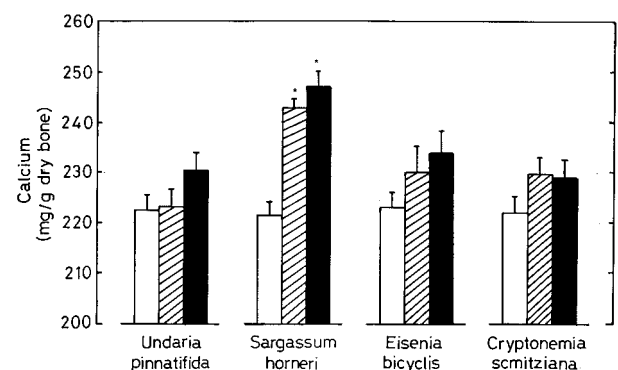
The body weight of animals was not significantly altered by the administration of various marine algae (data not shown). Serum calcium and inorganic phosphorus concentrations were also not significantly altered by marine algae administration in rats (data not shown). Calcium content in the femoral-metaphyseal tissues of rats was significantly increased by the administration of water suspension obtained from *U. pinnatifida*, *S. horneri*, *E. bicyclis*, and *C. scmitziana* (Fig. 1). The administration of *G. amansii* or *U. pertusa* Kjellman did not cause a significant alteration in bone calcium content.

Changes in alkaline phosphatase activity in the femoral-metaphyseal tissues of rats orally administered various marine algae are shown in Fig. 2. The enzyme activity was significantly enhanced by the administration of water suspensions of *S. horneri* or *G. amansii* to rats. The administration of water suspensions of *U. pinnatifida*, *E. bicyclis*, *C. scmitziana*, and *U. pertusa* Kjellman to rats did not affect on the enzyme activity.



**Fig. 2.** Effect of Various Marine Algae on Alkaline Phosphatase Activity in the Femoral-Metaphyseal Tissues of Rats

Rats were orally administered either vehicle (distilled water) or marine alga (1.0 ml of 5% suspension/100 g body weight) once daily for 7 days. Animals were killed 24 hr after the last administration. Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control value.



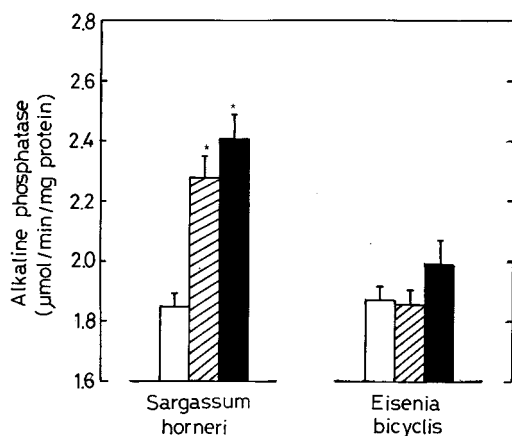
**Fig. 3.** Effect of Various Marine Algae on Calcium Content in the Femoral-Metaphyseal Tissues *in Vitro*

Femoral-metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or water-solubilized extract (25 or 50  $\mu\text{g/ml}$  of medium) of marine algae. Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control (none) value. White bars, control (none); hatched bars, marine algae extract (25  $\mu\text{g/ml}$ ); black bars, marine algae extract (50  $\mu\text{g/ml}$ ).

### Effect of Various Marine Algae on Bone Components *in Vitro*

Bone calcium content was significantly increased in the presence of *S. horneri* extract (25 and 50  $\mu\text{g/ml}$  of medium) (Fig. 3). No significant effect was seen after administration of *U. pinnatifida*, *E. bicyclis*, or *C. scmitziana*.

Changes in alkaline phosphatase activity in the femoral-metaphyseal tissues cultured with water-solubilized extracts of *S. horneri* or *E. bicyclis* are shown in Fig. 4. Bone alkaline phosphatase activity was significantly raised in the presence of *S. horneri* extract (25 or 50  $\mu\text{g/ml}$  of medium). No effect was



**Fig. 4.** Effect of Various Marine Algae on Alkaline Phosphatase Activity in Rat Femoral-Metaphyseal Tissues *in Vitro*

Femoral-metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or water-solubilized extract (25 or 50 µg/ml of medium) of *S. horneri* or *E. bicyclis*. Each value is the mean ± S.E.M. of six rats. \* $p < 0.01$  compared with the control (none) value. White bars, control (none); hatched bars, marine algae extract (25 µg/ml); black bars, marine algae extract (50 µg/ml).

seen in the presence of *E. bicyclis* (25 or 50 µg/ml), however.

The effects of cycloheximide, an inhibitor of protein synthesis, on *S. horneri* extract-enhanced alkaline phosphatase activity in the femoral-metaphyseal tissues are shown in Fig. 5. The effect of water-solubilized extract of *S. horneri* in increasing bone alkaline phosphatase activity was completely inhibited in the presence of cycloheximide ( $10^{-6}$  M).

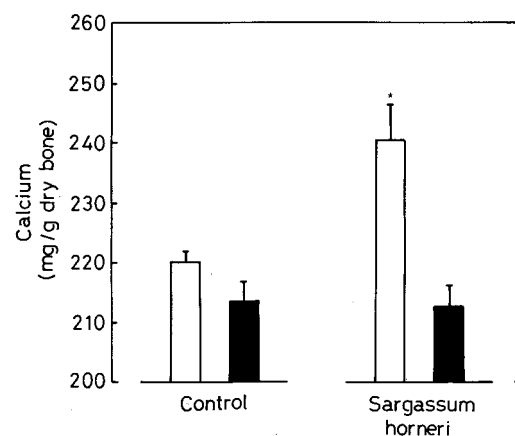
Bone calcium content and alkaline phosphatase activity were not significantly altered in the presence of 20% ethanol-solubilized extracts obtained from various marine algae (data not shown).

#### Effect of Water-Solubilized Extract of *S. horneri* on Bone Components *in Vivo*

Calcium content (Fig. 6A) and alkaline phosphatase activity (Fig. 6B) in the femoral-metaphyseal tissues were significantly increased by the administration of water-solubilized *S. horneri* extract to rats. The administration did not cause a significant alteration in the body weight of animals, or in serum calcium and inorganic phosphorus concentrations (data not shown).

## DISCUSSION

Osteoporosis with a decrease in bone mass is widely recognized as a major public health problem.<sup>4)</sup> Food and nutritional factors may play a role



**Fig. 5.** Effect of Cycloheximide on *S. horneri*-Increased Calcium Content in Rat Femoral-Metaphyseal Tissues *in Vitro*

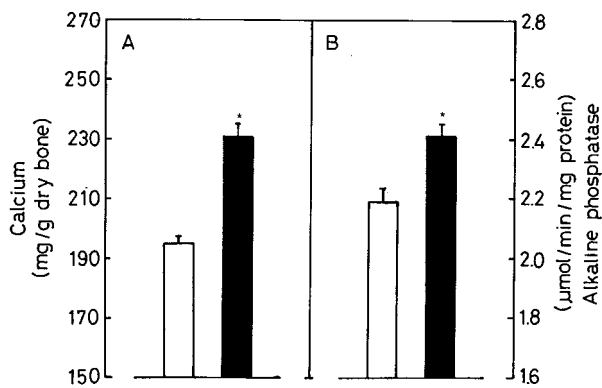
Femoral-metaphyseal tissues obtained from normal rats were cultured for 48 hr in a medium containing either vehicle or water-solubilized extract (50 µg/ml of medium) of *S. horneri* in the absence or presence of cycloheximide ( $10^{-6}$  M). Each value is the mean ± S.E.M. of six rats. \* $p < 0.01$ , compared with the control (none) value. White bars, control (none); black bars, cycloheximide. \* $p < 0.01$  compared with the control (none) value.

in the prevention of bone loss with increasing age. *S. horneri* extract was found to have a unique anabolic effect on bone calcification *in vivo* and *in vitro* for the first time in this study.

Of the marine algae studied, *S. horneri* extract had a potent anabolic effect on bone calcification in the femoral-metaphyseal tissues of rats. Water-solubilized extract of *S. horneri* significantly increased bone calcium content and alkaline phosphatase activity, and the bone calcium enhancement was completely inhibited in the presence of cycloheximide, an inhibitor of protein synthesis.<sup>23)</sup> This result suggests that the anabolic effect of *S. horneri* extract is based on newly synthesized protein components in osteoblastic cells of bone tissues.

Water-solubilized extract of *S. horneri* had an anabolic effect on bone calcification but 20% ethanol-solubilized extract did not have any effect. Components solubilized by water contain an active factor that stimulates bone calcification. Only small amounts of calcium were found in water-solubilized extract (25 and 50 µg/ml of medium) added to bone culture medium; this concentration was less than 0.1 µg/ml of medium. This indicates that calcium in water-solubilized extract of *S. horneri* does not play a role in the stimulation of bone calcification. The identification of active components in water-solubilized extract of *S. horneri* is required.

The oral administration of water-solubilized ex-



**Fig. 6.** Change in Calcium Content (A) and Alkaline Phosphatase Activity (B) in the Femoral-Metaphyseal Tissues of Rats Orally Administered Water-solubilized Extract of *S. Horneri* *in Vivo*

Rats were orally administered water-solubilized extract (20 mg/ml/100 g body weight) once daily for 7 days, and the animals were killed 24 hr after the last administration. Each value is the mean  $\pm$  S.E.M. of six rats. \* $p < 0.01$  compared with the control (none) value. White bars, control; black bars, marine algae extract.

tract (20 mg/ml/100 g body weight) of *S. horneri* to rats had a stimulatory effect on calcium content and alkaline phosphatase activity in the femoral-metaphyseal tissues of rats. This anabolic effect with comparatively low doses of *S. horneri* extract may suggest the existence of potentially active component in stimulating bone formation and calcification.

Prolonged intake of *S. horneri* extract may play a role in the prevention of bone loss with increasing age. This remains to be elucidated in animal models of osteoporosis.

In conclusion, it has been demonstrated that *S. horneri* extract has an anabolic effect on bone calcification *in vivo* and *in vitro*, suggesting its role in the prevention of osteoporosis.

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