Characterization of Active Component in Marine Alga Sargassum horneri Extract in Stimulating Bone Calcification *in Vitro*

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The marine alga *Sargassum horneri* (*S. horneri*) has an anabolic effect on bone metabolism (*J. Health Sci.*, **47**, 533–538, 2001). The effect of the fractionated extracts obtained from *S. horneri* on bone calcium content and osteoclast-like cell formation *in vitro* was investigated. *S. horneri* was gathered from various coasts (Shimoda, Iwate, or China). Rat femoral-diaphyseal and -metaphyseal tissues were cultured for 24 hr in a medium containing either vehicle or a water-solubilized extract ($25 \mu g/ml$ of medium) obtained from *S. horneri*. Diaphyseal and metaphyseal calcium contents were significantly increased in the presence of *S. horneri* extract from Shimoda, Iwate, or China. The active component of *S. horneri* extract in increasing calcium content in diaphyseal tissues was seen in the nearby fraction molecular weight (MW) of 1000. This effect completely disappeared upon heat treatment with 80°C for 30 min. Mouse marrow cells were cultured for 7 days in a medium containing 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (10⁻⁷ M) in the presence or absence of *S. horneri* extract (1.0 $\mu g/ml$ of medium). The 1,25(OH)₂D₃induced increase in osteoclast-like cell formation was markedly suppressed in the presence of the crude extract or a fraction more than MW 50000 of *S. horneri* extracts obtained from Iwate or China. This effect was also seen by heat treatment. The present study suggests that the active components of *S. horneri* extract in stimulating bone formation or suppressing osteoclastic bone resorption are different.

Key words —— Sargassum horneri, calcium, bone, osteoclast, osteoporosis

INTRODUCTION

Bone loss with increasing age induces osteoporosis.^{1–3)} 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.⁴⁾ Progression of this disease can result in bone fracture. Pharmacologic and nutritional factors can help to prevent bone loss with increasing age,⁵⁾ but these factors are poorly understood.

Recent studies have shown that isoflavones,⁶⁾ which are found in Leguminosae, vitamin K_2

(menaquinone-7), which is abundant in fermented soybean (*natto*),^{7,8)} and β -cryptoxanthin,^{9,10)} which is great in Satsuma mandarin (*Citrus unshiu* MARC.), all have an anabolic effect on bone metabolism in rats. These food factors have been shown to stimulate osteoblastic bone formation and inhibit osteoclastic bone resorption.^{11–16)} Thus, nutritional factors may be important in the prevention of bone loss with aging.

Further studies have shown that, among various marine algae, *Sargassum horneri* (*S. horneri*) extract has an anabolic effect on bone calcification in rat femoral tissues *in vivo* and *in vitro*.^{17–21} *S. horneri* extract has been demonstrated to stimulate osteoblastic bone formation and inhibit osteoclastic bone resorption *in vitro* using rat femoral-diaphyseal and -metaphyseal tissues.^{18,19} The intake of *S. horneri* extract has a preventive effect on bone loss in streptozotocin-diabetic rats *in vivo*.²¹ Thus the di-

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etary intake of *S. horneri* extract may have a preventive effect on osteoporosis with aging.

The present study was undertaken to determine the active component, which has an anabolic effect on bone metabolism, in *S. horneri* extract. *S. horneri* was gathered from various coasts to compare their effects. We found that the active components, which have a stimulating effect on bone calcification and a suppressing effect on osteoclastic bone resorption, are different.

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 μ g/ml) were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] was 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 S. horneri was seasonally gathered from the coast at Shimoda (Shizuoka prefecture, Japan), Miyako (Iwate prefecture, Japan), or Eisei (China), and was freeze-dried and powdered. The gathered fresh marine algae were homogenized in distilled water, are centrifuged at 5500 g in a refrigerated centrifuge for 10 min. The 5500 g supernatant fraction was pooled for freeze-drying. The powder of the water-solubilized extract was dissolved in ice-cold distilled water for use in the experiments. The water-solubilized extract from S. horneri was purified by the method of membrane fractionation with various molecular weights. In another experiment, the water-solubilized extract of S. horneri was treated at 80°C for 30 min in a water bath.

Animals — Male Wistar rats (conventional) weighing 100–200 g (4 weeks old) or male mice (ddY strain; 6 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 a room temperature of 25°C, with free access to distilled water.

Bone Culture — The femurs were removed aseptically after bleeding, and were then 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. Diaphyseal or 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 antibiotics (penicillin 100 units and streptomycin 100 μ g/ml of medium.²²⁾ In our experiments, bone tissues were cultured in a medium containing either vehicle or water-solubilized *S. horneri* extract. The concentration of calcium in the extracts was in the range of 0.05 to 1.0 μ g/ml of medium. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂ and 95% air.

Bone Calcium — The diaphyseal and metaphyseal tissues were dried for 16 hr at 110°C. Calcium was determined by atomic absorption spectrophotometry.^{22,23)} Calcium content in bone tissues was expressed as milligrams per gram of dry bone.

Marrow Culture —— Bone marrow cells were isolated from mice, as reported elsewhere.^{24,25)} Briefly, bone ends of the femur were cut off, and the marrow cavity was flushed with 1 ml of α -MEM. The marrow cells were washed with α -MEM and cultured in the same medium containing 10% heatinactivated fetal bovine serum at 1.0×10^7 cells/ml in 24-well plates (0.5 ml/well) in a water-saturated atmosphere containing 5% CO₂ and 95% air at 37°C. The cells were cultured for 3 days, then 0.2 ml of the old medium was replaced with fresh medium, and the cultures were maintained for an additional 4 days. The extract of S. horneri was added to the culture medium containing either vehicle or $1.25(OH)_2D_3$ (10^{-7} M), with an effective concentration at the beginning of the cultures and at the time of medium change.

Enzyme Histochemistry — After being cultured for 7 days, cells with adherence to the 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts.^{26,27)} Briefly, cells were washed with Hank's balanced salt solution and fixed with 10% neutralized formalinphosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied according to the method of Burstone.²⁶⁾ The fixed cells were incubated for 12 min at room temperature (25°C) in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma Chemical Co.) as a substrate, and red violet LB salt (Sigma Chemical Co.) as a stain for the reaction product, in the presence of 10 mM sodium tartrate. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells.



Fig. 1. Effect of S. horneri on Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissue in Vitro

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 24 hr in a medium containing either vehicle or watersolubilized extract (25 μ g/ml of medium) of *S. horneri*. Each value is the mean ± SEM of six rats. *p < 0.01 compared with the control (none) value. White bars, control (none); black bars, *S. horneri* from Shimoda; double hatched bars, *S. horneri* from Iwate; hatched bars, *S. horneri* from China.

Statistical Analysis — The significance of 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 Fractionated *S. horneri* Extracts on Bone Calcium Content *in Vitro*

Rat femoral-diaphyseal and -metaphyseal tissues were cultured for 24 hr in a medium containing either vehicle or S. horneri extract (25 µg/ml of medium) obtained from Shimoda, Miyako, or Eisei in vitro. Diaphyseal or metaphyseal calcium content was significantly increased in the presence of S. horneri extract obtained from the various coasts (Fig. 1). S. horneri extracts were fractionated for molecular weight (MW). The effect of S. horneri extract in increasing calcium content in rat femoraldiaphyseal tissues was seen in fractions of less than MW 1000 or more than MW 1000 in the extracts obtained from Iwate (Fig. 2). Also, the fraction of less than MW 3000 obtained from China had a significant stimulating effect on the diaphyseal calcium content (Fig. 2).

Effect of Fractionated S. *horneri* Extracts on Osteoclast-Like Cell Formation *in Vitro*

Mouse marrow cells were cultured for 7 days in a medium containing $1,25(OH)_2D_3$ (10^{-7} M) in the presence or absence of *S. horneri* extract ($1.0 \ \mu g/ml$ of medium). The $1,25(OH)_2D_3$ -induced increase in osteoclast-like formation was markedly suppressed in the presence of *S. horneri* extract (crude) obtained





Femoral-diaphyseal tissues obtained from normal rats were cultured for 24 hr in a medium containing either vehicle or *S. horneri* (25 µg/ml of medium) obtained from Iwate or China. *S. horneri* extracts were fractionated to less than 1000 of MW, more than MW 1000, between MW 1000 and 3000, less than MW 3000, or more than MW 3000, respectively. Each value is the mean ± SEM of six rats. *p < 0.05 or #p < 0.01 compared with the control (none) value.

from the coast of Iwate or China (Fig. 3). The fractions of less than MW 50000 of the extracts obtained from Iwate or China did not have a potent suppressive effect on osteoclast-like cell formation. However, a remarkable inhibitory effect on osteoclastlike cell formation was seen in the fraction of more than MW 50000 (Fig. 3).

The extracts of *S. horneri* were heated with 80°C for 30 min. Femoral-diaphyseal tissues were cultured for 24 hr in the presence of heat-treated *S. horneri*





Fig. 3. Effect of *S. horneri* Extract on 1,25-Dihydroxyvitamin D₃-Induced Osteoclast-Like Cell Formation in Mouse Marrow Culture *in Vitro*

Mouse marrow cells were cultured for 7 days in a medium containing either vehicle, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (10⁻⁷ M) or 1,25(OH)₂D₃ (10⁻⁷ M) plus *S. horneri* extract (1.0 μ g/ml of medium) obtained from Iwate or China. *S. horneri* extract from Iwate were fractionated to crude, 1000–3000 of MW, MW 3000–10000, MW 10000–50000, or more than MW 50000, and the extracts from China were fractionated to crude, less than MW 1000, MW 1000–50000, or more than MW 50000, and the extract from China were fractionated to crude, less than MW 1000, MW 1000–50000, or more than MW 50000. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. **p* < 0.01 compared with the control (none) value. #*p* < 0.01 compared with the value for 1,25(OH)₂D₃ alone.



Fig. 4. Effect of Heat-Treated *S. horneri* Extract on Calcium Content in Rat Femoral-Diaphyseal Tissues and 1,25-Dihydroxyrivitamin D₃-Induced Osteoclast-Like Cell Formation in Mouse Marrow Culture *in Vitro*

A. Femoral-diaphyseal tissues obtained from normal rats were cultured for 24 hr in a medium containing either vehicle or *S. horneri* (crude; 25 μ g/ml of medium) from Iwate. B. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (10⁻⁷ M) or 1,25(OH)₂D₃ (10⁻⁷ M) plus *S. horneri* extract (1.0 μ g/ml of medium) either crude or more than 50000 of MW from Iwate or China, respectively. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. **p* < 0.01 compared with the control (none) value. #*p* < 0.01 compared with the value for 1.25(OH)₂D₃ alone.

extract (25 μ g/ml of medium) obtained from Iwate. The effect of *S. horneri* extract in increasing calcium content in the diaphyseal tissues completely disappeared (Fig. 4A).

Also, fractions of more than MW 50000 of *S*. *horneri* extract obtained from Iwate or China were heated at 80°C for 30 min. The formation of osteo-

clast-like cell formation from mouse marrow cells cultured in a medium containing $1,25(OH)_2D_3$ (10^{-7} M) was markedly suppressed in the presence of the crude $(1.0 \ \mu\text{g/ml})$ of medium) or fractionated extract $(1.0 \ \mu\text{g/ml})$ of more than MW 50000 of *S*. *horneri* extract (Fig. 4B).

DISCUSSION

Among various marine algae, *S. horneri* extract has an anabolic effect on bone calcification in rat femoral tissues *in vivo* and *in vitro*.^{17–21)} *S. horneri* extract has been shown to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption *in vitro*. This study demonstrates that the active components of *S. horneri* extract on bone formation and bone resorption are different. Those active components were found to be present in *S. horneri* obtained from various coasts in Japan and China.

The active component of *S. horneri* extract in stimulating bone calcification seems to be nearby MW 1000. The effect of this component disappeared by heat treatment of the extract. Meanwhile, the active component of *S. horneri* extract in inhibiting osteoclastic cell formation is at more than MW 50000. This component was stable under heat treatment. It is speculated that the active component in stimulating bone formation is a peptide, and that the compound in inhibiting bone resorption is a polysaccharide. These active components obtained from the coasts of Iwate or China showed an identical molecular weight. Thus, the active components were found in *S. horneri* extracts obtained from different coasts.

The oral administration of *S. horneri* extract has been shown to have a preventive effect on bone loss in streptozotocin-induced diabetic rats *in vivo*.²¹⁾ Moreover, its administration caused a significant suppression of streptozotocin-induced hyperglycemia and hyperlipidemia *in vivo*. Whether the active component of *S. horneri* extract in preventing a streptozotocin-induced diabetic state is identical to components which can prevent bone loss in diabetic rats *in vivo* is unknown. This remains to be elucidated.

In conclusion, it has been demonstrated that the active components of *S. horneri* extract in stimulating bone calcification and inhibiting osteoclastic cell formation are different.

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