

Characterization of the Active Component in Bee Pollen *Cistus ladaniferus* Extract in Stimulating Bone Calcification and in Inhibiting Bone Resorption *in Vitro*

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The bee pollen *Cistus ladaniferus* (*C. ladaniferus*) extract has an anabolic effect on bone metabolism. The effects of the fractionated extracts obtained from bee pollen on bone calcium content and osteoclast-like cell formation *in vitro* were investigated. Rat femoral-diaphyseal and -metaphyseal tissues were cultured for 48 hr in a medium containing either vehicle or a water-solubilized extract with the membrane fractions obtained from bee pollen. The active component of bee pollen in increasing calcium content in diaphyseal tissues was seen in the fraction of molecular weight (MW) of less than 1000, and it was not observed in fractions of greater than MW 1000. Culture with the fractionated bee pollen extract (25 or 50 $\mu\text{g}/\text{ml}$ of medium) of less than MW 1000 caused a significant increase in calcium content in the diaphyseal or metaphyseal tissues. The parathyroid hormone (PTH; 10^{-6} M)-induced decrease in diaphyseal calcium content was significantly prevented in the presence of the fractionated bee pollen extracts (10 $\mu\text{g}/\text{ml}$) of less than MW 1000 or greater than MW 1000. Mouse marrow cells were cultured for 7 days in a medium containing PTH (10^{-6} M) in the presence or absence of the fractionated bee pollen extract (10 or 50 $\mu\text{g}/\text{ml}$). The PTH-induced increase in osteoclast-like cell formation was markedly suppressed in the presence of extracts of less than MW 1000 as compared with that in the presence of fractions of greater than MW 1000. The effects of the fractionated bee pollen extracts of less than MW 1000 in increasing diaphyseal calcium content and in inhibiting PTH-induced osteoclastic cell formation were significantly decreased upon heat treatment for 20 and 60 min at 80°C. This study demonstrates that the active component of bee pollen *C. ladaniferus* extract, which stimulates bone formation and inhibits osteoclastic bone resorption, is the fraction with MW less than 1000.

Key words — *Cistus ladaniferus*, calcium, bone formation, bone resorption, osteoporosis

INTRODUCTION

Bone loss with aging induces osteoporosis, which is widely recognized as a major public health problem.¹⁻³ A decrease in bone mass leads to bone fracture. Bone loss may be due to decreased bone formation and increased bone resorption. The prevention of bone loss caused by increasing age is important to maintain bone health.⁴ Food chemical and nutritional factors may help to prevent bone loss with increasing age.⁵

Recent studies have shown that isoflavones, which are contained in soybeans,⁵⁻⁸ menaquinone-7, an analogue of vitamin K₂ abundant in fermented soybeans,⁹⁻¹¹ and β -cryptoxanthin, a carotenoid abundant in Satsuma mandarin oranges (*Citrus unshiu* MARC)¹²⁻¹⁵ have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption, thereby increasing bone mass. The intake of dietary factors has been shown to have a preventive effect on bone loss in animal models of osteoporosis.¹⁶⁻¹⁸ Thus food factors may be useful in the prevention of osteoporosis.¹⁹⁻²¹

We demonstrate that the water-solubilized extract of bee pollen *Cistus ladaniferus* (*C. ladaniferus*) has an anabolic effect on bone components in rats *in vitro* and *in vivo*.²² The extract of bee pollen has a stimulatory effect on bone formation and an inhibi-

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tory effect on bone resorption *in vitro*.^{22,23} The extract of bee pollen stimulates bone calcification as potently as propolis.²² Royal jelly does not have an inhibitory effect on osteoclastic cell formation.²⁴ The effect of bee pollen on bone metabolism may thus be unique among bee-related products.

This study was undertaken to characterize the active components in bee pollen *C. ladaniferus* extract in stimulating bone calcification and in inhibiting bone resorption *in vitro*.

MATERIALS AND METHODS

Chemicals — Dulbecco's modified Eagle's medium (MEM) (high glucose, 4.5 g/dl) and a penicillin-streptomycin solution (penicillin 5000 unit/mg; streptomycin 5000 µg/ml) were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.). Parathyroid hormone(1-34) (human; PTH) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of reagent grade from Sigma and Wako Pure Chemical Industries (Osaka, Japan).

Bee Pollen Extracts — Bee pollen was obtained from *C. ladaniferus*. The powder of bee pollen (5 g) was suspended in distilled water (20 ml) and mixed vigorously, and the suspension was centrifuged at 10000 *g* in a refrigerated centrifuge for 20 min. The 10000 *g* supernatant fraction was collected and filtered, and the filtrate was freeze-dried. The powder of the water-solubilized extract was dissolved in ice-cold distilled water for use in experiments. The water-solubilized extract from bee pollen was purified using the membrane fractionation method with various molecular weights (MW): less than MW 1000 (fraction A), from MW 1000 to MW 10000 (fraction B), or greater than MW 10000 (fraction C). In another experiment, fraction A obtained from the water-solubilized extract was treated at 80°C for 20 and 60 min in a water bath.

Animals — Male Wistar rats (conventional) weighing 90–100 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 exsanguination, and then soaked in ice-cold sucrose 0.25 M solution. The femurs were 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 48 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 µg/ml of medium).²⁵ In our experiments, bone tissues were cultured in a medium containing either vehicle or water-solubilized bee pollen extract. The concentration of calcium in the extracts was negligible. 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 content was determined with atomic absorption spectrophotometry.²⁵ 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.^{26,27} Briefly, the 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% heat-inactivated 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, and 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 bee pollen was added to the culture medium containing either vehicle or PTH (10^{-6} 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 adherency to the 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts.^{26,27} Briefly, the cells were washed with Hank's balanced salt solution and fixed with 10% neutralized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied according to the method of Burstone.²⁸ 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.

Statistical Analysis — Data are expressed as mean \pm SEM. Statistical differences were analyzed using Student's *t*-test. *p*-Values of < 0.05 were considered to indicate statistically significant differences. The analysis of variance (ANOVA) multiple-comparison test was used to compare treatment groups.

RESULTS

Effects of Fractionated Bee Pollen Extracts on Bone Calcium Content *in Vitro*

Rat femoral-diaphyseal tissues were cultured for 48 hr in a medium containing either vehicle or fractionated bee pollen *C. ladaniferus* extract (50 or 100 $\mu\text{g}/\text{ml}$ of medium). Diaphyseal calcium content was significantly increased in the presence of the fraction (50 $\mu\text{g}/\text{ml}$) of less than MW 1000 (A) (Fig. 1). The fractions of from MW 1000 to MW 10000 (B) or greater than MW 10000 did not have a significant stimulatory effect on the diaphyseal calcium content. Culture with fraction A (25 or 50 $\mu\text{g}/\text{ml}$ of medium) caused a significant increase in calcium content in the femoral-diaphyseal and -metaphyseal tissues (Fig. 2).

Effects of Fractionated Bee Pollen Extracts on PTH-Induced Bone Resorption *in Vitro*

Rat femoral-diaphyseal tissues were cultured for 48 hr in a medium containing either vehicle or PTH (10^{-6} M). The diaphyseal calcium content was significantly decreased in the presence of PTH (Fig. 3A). This decrease was significantly prevented in the presence of the fractionated bee pollen *C. ladaniferus* extracts (10 $\mu\text{g}/\text{ml}$ of medium) of less than MW 1000 (A), from MW 1000 to MW 10000 (B), or greater than MW 10000 (C) (Fig. 3B).

Mouse marrow cells were cultured for 7 days in a medium containing PTH (10^{-6} M) in the presence or absence of the fractionated bee pollen extracts (10 or 50 $\mu\text{g}/\text{ml}$ of medium). Culture with PTH caused a marked increase in osteoclast-like cell formation (Fig. 4A). This increase was markedly suppressed in the presence of the fractionated bee pollen extracts (10 or 50 $\mu\text{g}/\text{ml}$ of medium) with fraction A (less than MW 1000) as compared with fraction B (from MW 1000 to 10000) or fraction C (greater than MW 10000) (Fig. 4B).

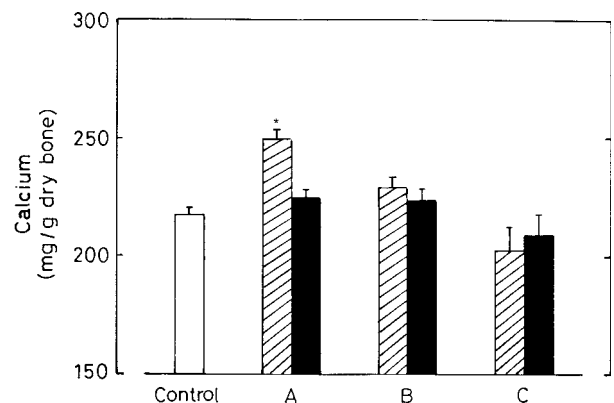


Fig. 1. Effects of Bee Pollen *C. ladaniferus* Extract Fractions with Different Molecular Weights on Calcium Content in Rat Femoral-Diaphyseal Tissues *in Vitro*

Femoral-diaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or bee pollen extracts (50 or 100 $\mu\text{g}/\text{ml}$ of medium). Bee pollen extracts were fractionated to less than MW 1000 (A), from MW 1000 to MW 10000 (B), or greater than MW 10000 (C), respectively. Each value is the mean \pm SEM of six rats. **p* < 0.01 compared with the control (none) value. Hatched bars, 50 $\mu\text{g}/\text{ml}$; black bars, 100 $\mu\text{g}/\text{ml}$.

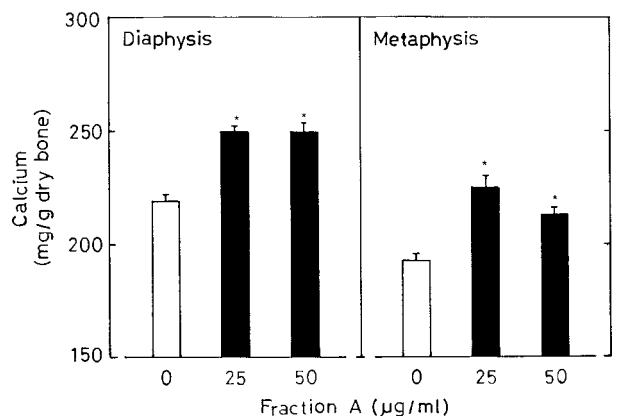


Fig. 2. Effects of Bee Pollen *C. ladaniferus* Extract Fractions of Molecular Weight Less than 1000 on Calcium Content in Rat Femoral-Diaphyseal and -Metaphyseal Tissues *in Vitro*

Femoral-diaphyseal or -metaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or bee pollen extracts (25 or 50 $\mu\text{g}/\text{ml}$ of medium) with the fraction of MW less than 1000. Each value is the mean \pm SEM of six rats. **p* < 0.01 compared with the control (none) value.

Effects of Fractionated Bee Pollen Extracts with Heat Treatment *in Vitro*

Rat femoral-diaphyseal tissues were cultured for 48 hr in a medium containing either vehicle or the fractionated bee pollen *C. ladaniferus* extracts (less than MW 1000; 25 or 50 $\mu\text{g}/\text{ml}$ of medium). The effect of the fractionated bee pollen extracts in in-

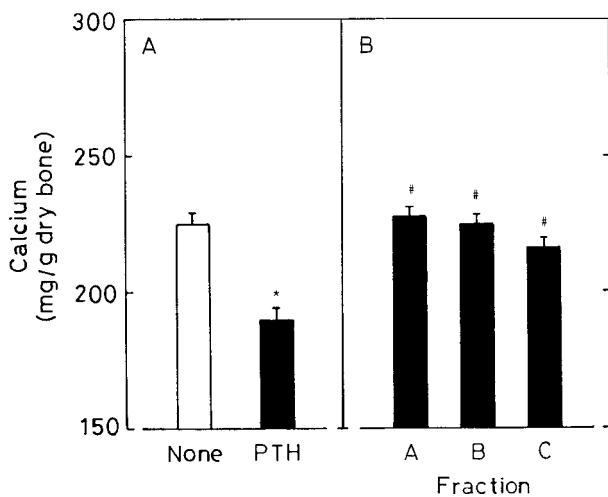


Fig. 3. Effects of Bee Pollen *C. ladaniferus* Extract Fractions with Different Molecular Weights on the PTH-Induced Decrease in Calcium Content in Rat Femoral-Diaphyseal Tissues *in Vitro*

Rat femoral-diaphyseal tissues were cultured for 48 hr in medium containing either vehicle or PTH (10^{-6} M) (A), or containing bee pollen extracts ($10 \mu\text{g}/\text{ml}$ of medium) fractionated to MW less than 1000 (A), MW from 1000 to 10000 (B), or MW more than 10000 (C) in the presence of PTH (10^{-6} M) (B). Each value is the mean \pm SEM of six rats. * $p < 0.01$ compared with the control (none) value. # $p < 0.01$ compared with the value obtained with PTH alone.

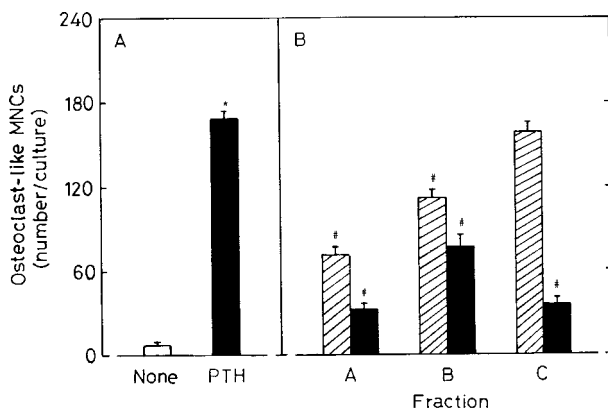


Fig. 4. Effects of Bee Pollen *C. ladaniferus* Extract Fractions with Different Molecular Weights on PTH-Induced Osteoclast-Like Cell Formation in Mouse Marrow Culture *in Vitro*

Mouse marrow cells were cultured for 7 days in medium containing either vehicle or PTH (10^{-6} M) (Figure A), or containing bee pollen extracts (10 or $50 \mu\text{g}/\text{ml}$ of medium) fractions of MW less than 1000 (A), MW from 1000 to 10000 (B), or MW more than 10000 (C) in the presence of PTH (10^{-6} M) (B). Each value is the mean \pm SEM of six mice. * $p < 0.01$ compared with control (none) value. # $p < 0.01$ compared with the value obtained with PTH alone. In B, hatched bars, $10 \mu\text{g}/\text{ml}$; black bars, $50 \mu\text{g}/\text{ml}$.

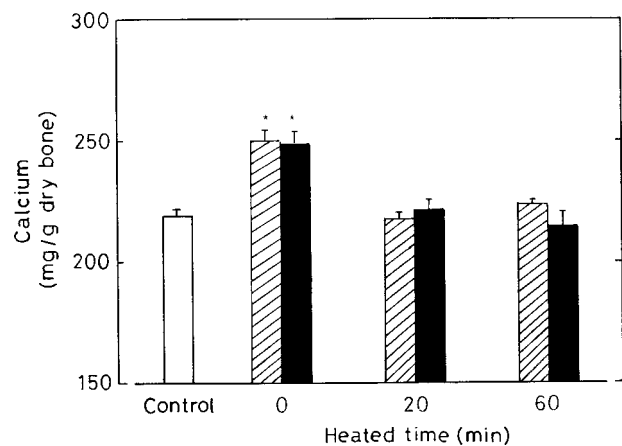


Fig. 5. Effects of Heat-Treated Bee Pollen *C. ladaniferus* Extracts Fraction with Molecular Weights Less than 1000 on Calcium Content in Rat Femoral-Diaphyseal Tissues *in Vitro*

Femoral-diaphyseal tissues obtained from normal rats were cultured for 48 hr in medium containing either vehicle or bee pollen fraction with MW less than 1000 (25 or $50 \mu\text{g}/\text{ml}$ of medium) after heat treatment for 0, 20, or 60 min at 80°C . Each value is the mean \pm SEM of six rats. * $p < 0.01$ compared with the control (none) value. Hatched bars, $25 \mu\text{g}/\text{ml}$; black bars, $50 \mu\text{g}/\text{ml}$.

creasing diaphyseal calcium content was not seen with culture of the extracts with heat treatment for 20 or 60 min at 80°C (Fig. 5).

Mouse marrow cells were cultured for 7 days in a medium containing PTH (10^{-6} M) in the presence or absence of the fractionated bee pollen extracts (less than MW 1000; 10 or $50 \mu\text{g}/\text{ml}$ of medium). The inhibitory effects of the fractionated bee pollen on the PTH-induced increase in osteoclast-like cell formation were significantly decreased with heat treatment for 20 or 60 min at 80°C (Fig. 6).

DISCUSSION

Royal jelly, propolis, and bee pollen products are commercially available. Bee pollen *C. ladaniferus* extract has a stimulatory effect on bone calcification²²⁾ and an inhibitory effect on bone resorption²³⁾ *in vitro* and *in vivo*. The effect of propolis extract in stimulating bone calcification is weak.²²⁾ Royal jelly does not have an inhibitory effect on osteoclastic bone resorption *in vitro*, although it had a stimulatory effect on bone calcification *in vitro*.²⁴⁾ Of various bee-related products, bee pollen extract has a potent anabolic effect on bone components.

The active component was present in a water-

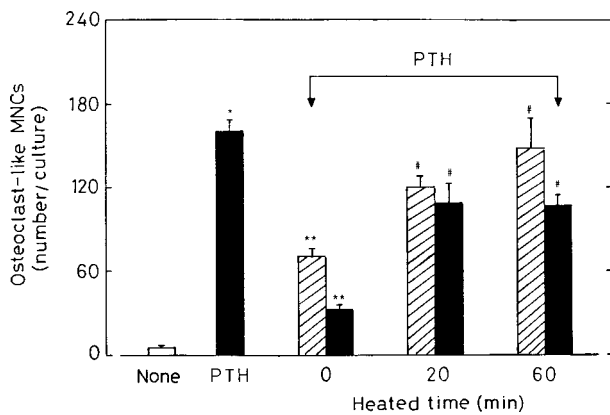


Fig. 6. Effects of Heat-Treated Bee Pollen *C. ladaniferus* Extracts Fraction with Molecular Weight Less than 1000 on PTH-Induced Osteoclast-Like Cell Formation in Mouse Marrow Culture *in Vitro*

Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-6} M), or bee pollen extract fraction of MW less than 1000, which was treated with heat for 0, 20, or 60 min at 80°C , in the presence of PTH (10^{-6} M). Each value is the mean \pm SEM of six mice. * $p < 0.01$ compared with control (none) value. ** $p < 0.01$ compared with the value with PTH alone. # $p < 0.01$ compared with the value with bee pollen extracts fraction of MW 1000 without heat treatment. Hatched bars, 10 $\mu\text{g}/\text{ml}$; black bars, 50 $\mu\text{g}/\text{ml}$.

solubilized extract obtained from bee pollen *C. ladaniferus*.²²⁾ The component stimulating bone calcification was found in the fraction of less than MW 1000 in the water-solubilized extract obtained from bee pollen *C. ladaniferus*. The anabolic effect on bone calcification was not seen in the fraction of greater than MW 1000.

PTH stimulates osteoclastogenesis and osteoclastic bone resorption.^{29,30)} Culture with PTH caused a significant decrease in bone calcium content and a marked increase in osteoclastic cell formation *in vitro*. These effects of PTH were inhibited in the presence of crude bee pollen *C. ladaniferus*.²³⁾ The fraction of less than MW 1000 had a potent inhibitory effect on PTH-induced osteoclastic bone resorption as compared with that of the fractions of from MW 1000 to 10000 or greater than MW10000.

Whether the active component in the fraction of less than MW 1000 obtained from a water-solubilized bee pollen *C. ladaniferus* in stimulating bone calcification and in inhibiting bone resorption is identical is unknown. The fraction of less than MW 1000 was treated with heat for 20 and 60 min at 80°C . Heat treatment eliminated the effects of the active component that stimulates bone calcification and inhibits bone resorption. It is speculated that the active component stimulating bone calcification and

inhibiting bone resorption is identical. This remains to be elucidated.

The oral administration of the water-solubilized extract obtained from the bee pollen of *C. ladaniferus* to rats caused a significant increase in bone components *in vivo*.²²⁾ If the active component is transported in the intestine, it may not be a high MW compound.²²⁾ This was supported by the present finding that the active component was less than MW 1000.

In conclusion, it has been demonstrated that the active component in a water-solubilized extract of bee pollen *C. ladaniferus*, which stimulates bone calcification and inhibits bone resorption *in vitro*, is a molecule of less than MW 1000.

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