Purification of Active Component in Wasabi Leafstalk (*Wasabia japonica* MATSUM.) Extract in Stimulating Bone Calcification *in Vitro*

Toshihiro Suzuki[a] and Masayoshi Yamaguchi*[,b]

[a]*Functional Food Project Team, Shizuoka Industrial Research Institute of Shizuoka Prefecture, 2078 Makigaya, Shizuoka 421–1298, Japan and* [b]*Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan*

(Received May 18, 2004; Accepted June 4, 2004)

The active component in wasabi leafstalk (*Wasabia japonica* MATSUM.), which has a stimulatory effect on bone calcification in mouse calvaria tissue culture *in vitro*, was purified. Wasabi leafstalk extract was obtained from a homogenate with 20% ethanol. The active component, which was found in ethanol extraction, was purified by gel filtration chromatography with HiLoad 26/80 Superdex 30 pg column and reversed-phase chromatography (RPC) on a resource 15 RPC 3 ml column. The result of ESI mass spectra of the purified active component showed that its material had a molecular weight of 158. The absorption spectra of the material gave the maximum absorption at a wavelength of 221 nm. The material in wasabi leafstalk extract differed from genistein and 17β-estradiol, which can stimulate bone calcification *in vitro*, and had a comparatively lower molecular weight. This study demonstrates that the material with a low molecular weight of 158 is an active component in wasabi leafstalk which stimulates bone calcification.

Key words —— *Wasabia japonica*, wasabi leafstalk, bone calcification, osteoporosis

INTRODUCTION

Bone loss with increasing age induces osteoporosis,[1–3] a condition widely recognized as a major public health problem.[4] Bone loss may be due to increased bone resorption and decreased bone formation. A decrease in bone mass leads to bone fracture, and thus it is important to prevent bone loss with ageing.[5] The chemical compounds that prevent bone loss as nutrients in food, however, are poorly understood.

Recent studies have shown that isoflavones (including genistein and daidzein), which are contained in large quantities in soybean, have a stimulatory effect on osteoblastic bone formation[6] and an inhibitory effect on osteoclastic bone resorption,[7] thereby increasing bone mass.[8] Menaquinone-7, an analogue of vitamin K_2 which is essential for the γ-carboxylation of the osteocalcin of a bone matrix protein, is abundant in fermented soybean. It stimulates osteoblastic bone formation[9] and inhibits osteoclastic bone resorption[10] *in vitro*. The prolonged dietary preventive effect of isoflavone and menaquinone-7 on bone loss induced by ovariectomy in rats, which is an animal model for osteoporosis, has been demonstrated.[11,12] More recently, it has been demonstrated that β-cryptoxanthin, a carotenoid which is abundent in orange, has an anabolic effect on bone metabolism, stimulating bone formation and inhibiting bone resorption.[13,14] Thus, food factors play a role in bone health and may be important in the prevention of bone loss as humans age.

Other food factors have also been shown to have an anabolic effect on bone metabolism. More recently, it has been shown that among various marine algae, *Sargassum horneri* (*S. horneri*) has a unique anabolic effect on bone calcification *in vitro* and *in vivo*.[15–18] Moreover, wasabi leafstalk (*Wasabia japonica* MATSUM.) extract, among various plant extracts, has a unique stimulatory effect on bone

*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@u-shizuoka-ken.ac.jp

calcification *in vitro* and *in vivo*.[19–21] However, the active components with anabolic effects on bone in wasabi leafstalk extract have not been identified.

The present study was undertaken to determine this active component which can stimulate bone calcification *in vitro*. We found that it is a material with a molecular weight of 158.

MATERIALS AND METHODS

Chemicals —— Dulbecco's modified Eagle's medium (high glucose, 4.5 g/100 ml) and penicillin-streptomycin solution (5000 U/ml penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, N.Y., U.S.A.). Bovine serum albumin (fraction V) was obtained from Sigma Chemical (St. Louis, MO., U.S.A.). Other chemicals were reagent grade from Wako Pure Chemical Industries (Osaka, Japan).

Wasabi Leafstalk Extract —— Wasabi leafstalks (*Wasabia japonica* MATSUM.) were collected in May 2003 and used for the experiment. Fresh leafstalk (about 50 g) was homogenized for 3 min in 20% ethanol solution (150 ml), and the homogenate was centrifuged at 10000 g for 20 min.[19] The resulting supernatant was filtered through filtration paper, and the filtration solution was then extracted 3 times with diethyl ether (about 150 ml). The ether phase was removed, and the resulting aqueous phase was freeze-dried. The powder was dissolved in distilled water. Before wasabi leafstalk extract was used in bone culture experiments, the extract solution was aseptically filtered through a membrane filter (0.22 μm).

HPLC Chromatography of Wasabi Leafstalk Extract —— A high pressure liquid chromatography (HPLC) system (Amersham Pharmacia Biotechnology, U.S.A.) was used in this study. Wasabi leafstalk extract was put on a Hiload 26/60 Superdex 30 pg column (Amersham Pharmacia Biotechnology) equilibrated with 0.1 N ammonium acetate at a flow rate of 1.5 ml/min. Elution was monitored at 280 nm and 245 nm, and the extract was fractionated into six fractions. Each fraction was lyophilized, and the frozen powder was dissolved in a small amount of distilled water to use in bone culture experiments.

The active component-containing fraction, which was obtained from gel filtration of the extract, was lyophilized and the lyophilized powder was dissolved in water. The solution was put on a Resource 15 reverse phase chromatography (RPC) 3 ml column (6.4 × 100 mm, Amersham Pharmacia Biotechnology) equilibrated with distilled water containing 0.65% trifluoroacetic acid (TFA), and was eluted with a linear gradient of 5–80% acetonitrile containing 0.65% trifluoroacetic acid at a flow rate of 2 ml/min. Elution was monitored at 280 nm and 254 nm. Each fraction was lyophilized. These frozen powders were dissolved in a small amount of distilled water to use in bone culture experiments.

LC/MS Assay —— The liquid chromatography/mass spectrometer (LC/MS) system used consisted of an electrospray ionization-mass spectrometer (Applied Biosystems, API3000), coupled to the HPLC system (Hewlett-Packard, Agilent 1100). Active component samples obtained from RPC were injected onto a Resource 15 RPC 3 ml column (6.4 × 100 mm, Amersham Pharmacia Biotechnology), and eluted with a linear gradient of 5–80% acetonitrile containing 0.65% TFA at a flow rate of 2 ml/min. Mass spectrometer conditions were: mode, positive; mass range, 100–1000; ion source, electrospray ionization; nebulizer gas temperature, 400°C; ion spray voltage, 1000 V; decrustering potential, 30 V; focusing potential, 300 V; entrance potential, 10 V.

Bone Culture —— Male ddy mice (4 weeks old) were obtained from Japan SLC Inc. (Hamamatsu, Japan). These animals were fed commercial laboratory chow (solid; Oriental Yeast Co. Ltd., Tokyo, Japan) containing 57.4% carbohydrate, 1.1% Ca, and 1.1% P at a room temperature of 25°C, and were given distilled water freely until sacrifice. Mice were sacrificed by decapitation under light anesthesia with diethyl ether. Calvaria tissues were removed aseptically, and cut along the sagittal suture into left and right halves. One-half of each calvarium served as a control for its paired, treated half. Calvaria fragments were cultured for 48 hr in a 35-mm dish in 2.0 ml of Dulbecco's modified Eagle's medium (high glucose, 4.5 g/100 ml) supplemented with 0.25% bovine serum albumin plus antibiotics (100 U penicillin and 100 μg streptomycin/ml of medium).[22] The experimental medium contained either vehicle or the active component, obtained from the wasabi leafstalk extract. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air.

Analytical Procedures —— Calvaria tissues cultured were dried for 16 hr at 110°C, weighed, and then digested with nitric acid (2.0 ml) for 12 hr at 120°C. Calcium was determined by atomic absorption spectrophotometry,[22] and the content was cal-

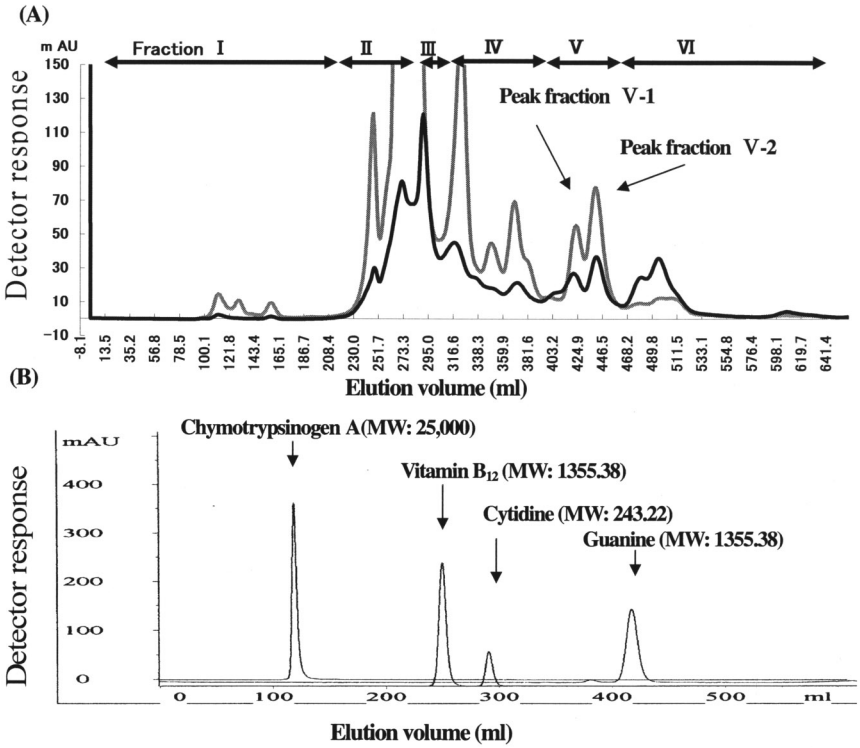

Fig. 1. Filtration Chromatography of Wasabi Leafstalk Extract (A) and Standard Materials (B) on HiLoad 26/60 Superdex 30 PG Column

Wasabi leafstalk extract was applied to a HiLoad 26/60 Superdex 30 pg column that had been equilibrated with 0.1 N ammonium acetate solution. The flow rate was 1.5 ml/min, and 2 ml fractions were collected. Absorbance was measured at 280 nm and 254 nm.

culated as milligrams (mg) per gram (g) of dry bone. Data were represented as the percentage (%) of control (none) value.

Statistical Analysis —— The significance of difference between values was estimated by Student's *t*-test. *p*-Value of less than 0.05 was considered to show statistically significant differences. We also used a multi-way analysis of variance (ANOVA) with a Duncan's post hoc test to compare the treatment groups.

RESULTS

The active components prepared from a 20% ethanol extract of wasabi leafstalk homogenate, were subjected to gel filtration chromatography with HiLoad 26/60 Superdex 30 pg column (Fig. 1A). The eluate was monitored at 280 nm and 254 nm, 2 ml was collected from each fraction (fractions I–VI), and then each fraction was lyophilized. The frozen powders were dissolved in a small amount of distilled water and were used for bone culture experiments. The component from fraction I, II, III, VI,

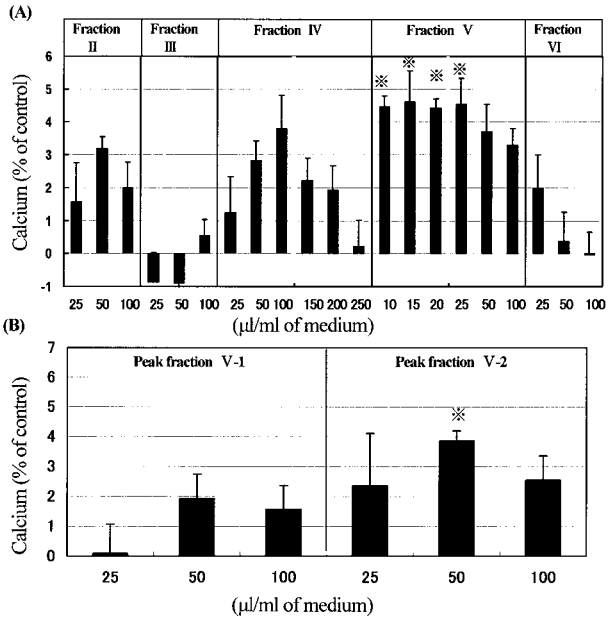

Fig. 2. Stimulatory Activity of Chromatography Fractions Obtained from Wasabi Leafstalk Extract on Calcification in Mouse Calvaria Tissues *in Vitro*

Mouse calvaria tissues were cultured for 48 hr in the medium containing either vehicle or chromatography fractions (as shown in Fig. 1) obtained from wasabi leafstalk extract (10, 15, 25, 50, 100, 200, and 250 μl/ml). Each value represents the mean ± S.E.M. of five bone tissues from different animals. *$p < 0.01$ compared with the control value.

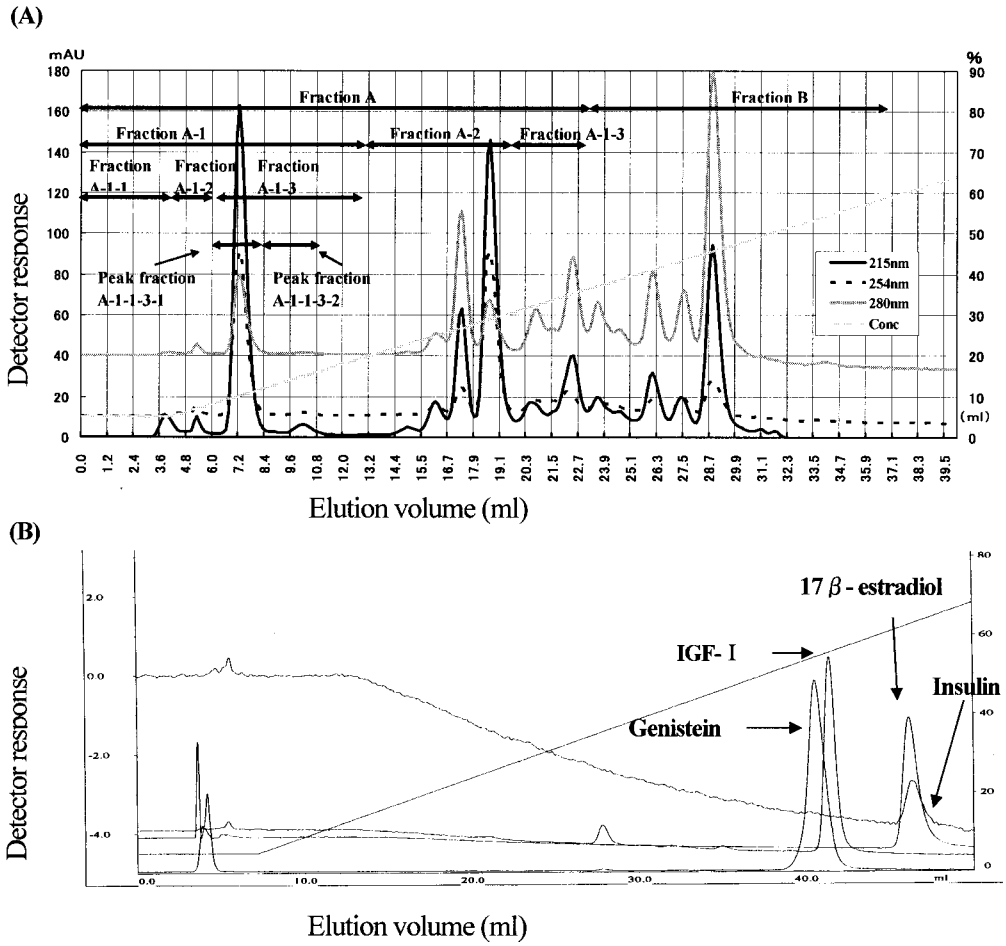

Fig. 3. Reversed-Phase Chromatography of Peak Fraction V-2 Obtained from Gel Filtration of Wasabi Leafstalk Extract on Resource 15 RPC 3 ml Column

Peak fraction V-2 (A), which was obtained from gel filtration of wasabi leafstalk extract, or standard materials (B) was applied to RPC on a resource 15 RPC 3 ml column. The flow rate was 1.5 ml/min, and fractions (2.0 ml) were collected. Absorbance was measured at 215 nm, 254 nm, and 280 nm.

and VI did not cause a significant alteration in bone calcium content (Fig. 2A). However, the addition of the component of fraction V to the culture medium caused a significant increase in the content (Fig. 2A). Fraction V contained two peak components of fraction V-1 and fraction V-2 (Fig. 1A). The active components, which caused a significant increase in bone calcium content, were found in fraction V-2 but not fraction V-1 (Fig. 2B). The active components in fraction V-2 differed from chymotrypsinogen A [25000 of molecular weight (MW)], vitamin B_{12} (MW; 1355.4), cytidine (MW; 243.2), and guanine (MW; 151.3), as compared by gel filtration chromatography for fraction V-2 (Fig. 1B).

Fraction V-2 was applied to RPC on a resource 15 RPC 3 ml column (Fig. 3). The eluate was monitored at 280 nm, 254 nm, and 215 nm, and 2 ml was collected from each fraction (fractions A and B). Each fraction was the lyophilized and the frozen powders were dissolved in a small amount of distilled water and used for bone culture experiments. Fraction A caused a significant increase in bone calcium content (Fig. 3A), however, fraction B had no effect on bone calcium content. Fraction A contained three major peaks (fraction A-1, A-2, or A-3). The active components which increase the content were found in fraction A-1 (Fig. 4B). The active components in fraction A-1 differed from genistein, insulin-like growth factor-I (IGF-I), 17β-estradial, and insulin, which have a stimulatory effect on bone calcification (Fig. 3B). Moreover, fraction A-1 was distinguishable in fraction A-1-1, A-1-2, or A-1-3 (Fig. 3A). Fraction A-1-3 contained the active components for bone calcium content (Fig. 5A). Fraction A-1-3 composed a major peak (A-1-3-1) and minor peak (A-1-3-2). The stimulatory effect on bone calcium content was found in fraction A-1-3-1 but not fraction A-1-3-2 (Fig. 5B).

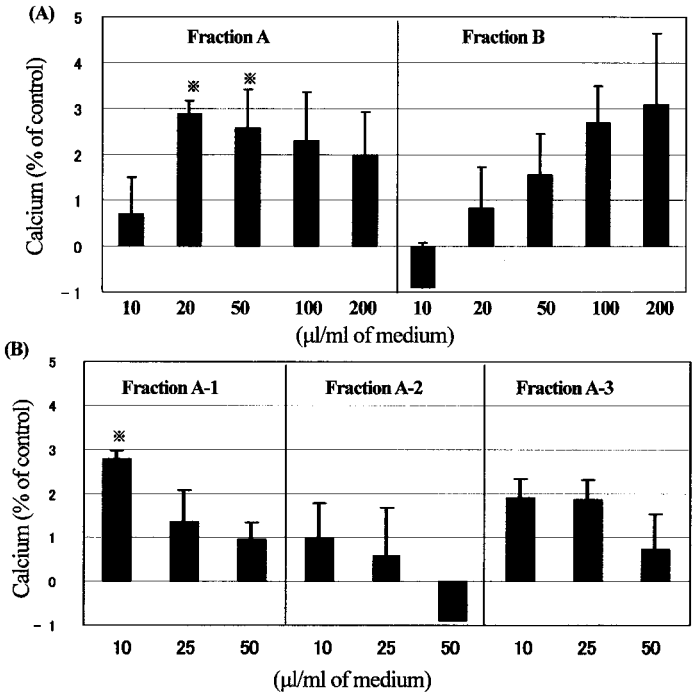

Fig. 4. Stimulatory Activity of Chromatography Fractions Obtained from RPC of V-2 Fraction on Calcification in Mouse Calvaria Tissues *in Vitro*

Mouse calvaria tissues were cultured for 48 hr in a medium containing either vehicle or chromatography fractions (10, 20, 25, 50, 100, and 200 μl/ml of medium) obtained from RPC. Each value represents the mean ± S.E.M. of five bone tissues from different animals. *$p < 0.01$ compared with the control value. (A); Stimulatory activity of fractions A and B from RPC. (B); Stimulatory activity of fractions A-1, A-2, and A-3 from RPC.

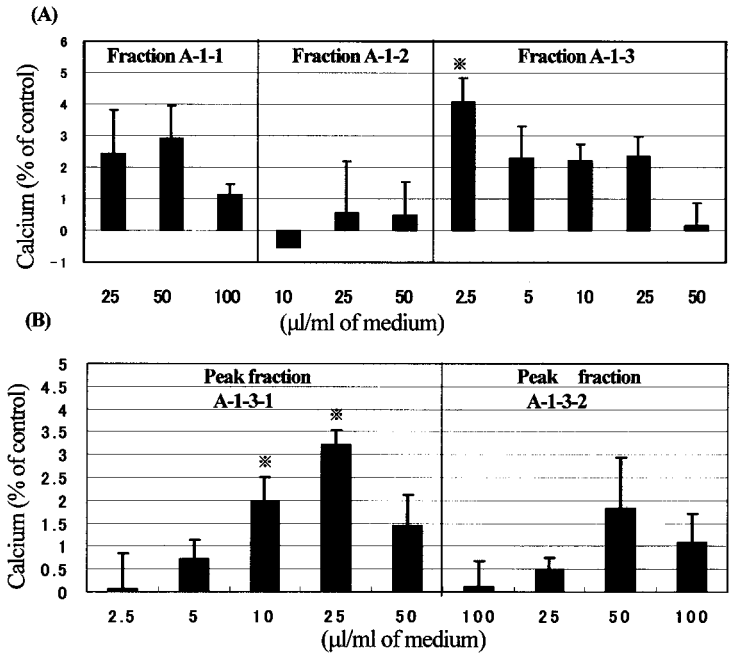

Fig. 5. Stimulatory Activity of Chromatography Fractions Obtained from Reverse-Phase Chromatography of Peak V-2 Fraction on Calcification in Mouse Calvaria Tissues *in Vitro*

Mouse calvaria tissues were cultured for 48 hr in the medium containing either vehicle or chromatography fractions (2.5, 5, 10, 20, 25, 50, and 100 μg/ml of medium) obtained from RPC. Each value represents the mean ± S.E.M. of five bone tissues from different animals. *$p < 0.01$ compared with the control value.

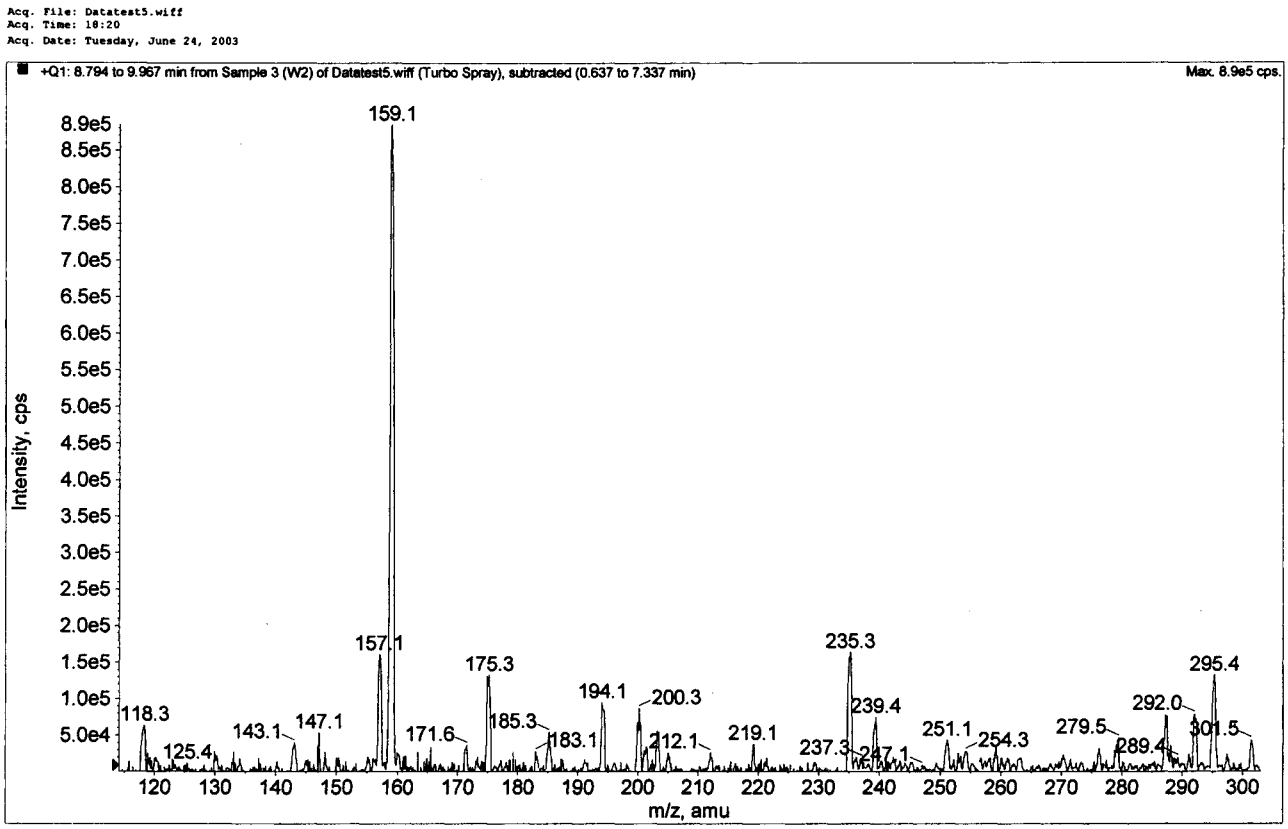

Fig. 6. ESI Mass Spectra of an Active Component in Wasabi Leafstalk Extract Which Stimulates Bone Calcification

Wasabi leafstalk extract was applied to gel filtration on Hiload 26/60 Superdex 30 pg column and RPC on a resource 15 RPC 3 ml column. Fractions with stimulatory activity on bone calcification were collected, lyophilized and dissolved in distilled water. The active component (A-1–3-1 fraction) shown in Figs. 3 and 5 was applied on ESI mass spectra.

ESI mass spectra for the active component (fraction A-1-3-1) are shown in Fig. 6, and a molecular weight of this component was 158. Absorption spectra of this material showed the maximum absorption at a wavelength of 221 nm (Fig. 7).

DISCUSSION

Among various plant and food extracts, wasabi leafstalk (*Wasabia japonica* MATSUM.) has a stimulatory effect on the calcification in mouse calvaria tissues *in vitro*.[19–21] The wasabi leafstalk extract-induced increase in bone calcium content is suggested to result from newly synthesized protein components.[19] However, it has been shown that this extract does not have an inhibitory effect on bone resorption induced by a bone-resorbing hormone (parathyroid hormone).[23] The active component in the extract may be a material with lower molecular weight than genistein (MW; 270),[23] which has a stimulatory effect on bone formation *in vitro*.[6,8]

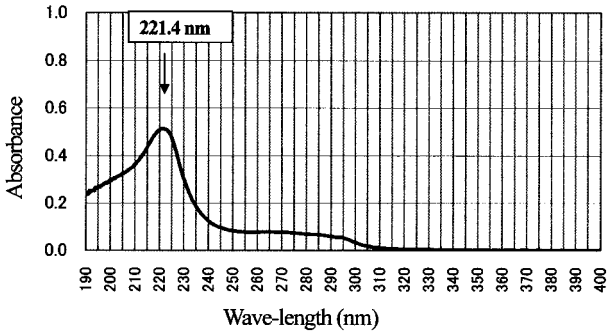

Fig. 7. Absorption Spectra of Component (A-1–3-1 fraction) in Wasabi Leafstalk Extract that Stimulates Bone Calcification

Wasabi leafstalk extract was applied to gel filtration on Hiload 26/60 Superdex 30 pg column and reversed-phase chromatography on a resource 15 RPC 3 ml column. Fractions with stimulatory activity on bone calcification were collected, lyophilized and dissolved in distilled water. The absorption spectra of the active component (A-1–3-1 fraction) were measured.

In the present study, the active component was isolated which stimulates bone calcification *in vitro*. It was purified by gel filtration chromatography with HiLoad 26/80 Superdex 30 pg column and RPC on a resource 15 RPC column and was isolated as a single material. The result of ESI mass spectra showed that the material has a molecular weight of 158. The absorbance spectra of the material gave the maximum absorption at a wavelength of 221 nm. Further studies to identify the chemical structure are in progress.

The oral administration of wasabi leafstalk extract to young and aged rats has been shown to have an anabolic effect on bone components,[21,24] suggesting that intake of the extract tends to prevent bone loss with increasing age. Thus this extract in the diet appears to have a role in preventing of osteoporosis.

In conclusion, it has been demonstrated that the active component in wasabi leafstalk extract which has a stimulatory effect on bone calcification is a material with a molecular weight of 158.

REFERENCES

1) Nishimoto, S. K., Chang, C.-H., Gendler, E., Stryker, W. F. and Nimni, M. E. (1985) The effect of aging on bone formation capacity. *Calcif. Tissue Int*., **37**, 617–624.
2) Schapira, C., Slinn, S., Sarid, M., Mokadi, S., Kabala, A. and Silbermann, M. (1995) Calcium and vitamin D enriched diets increase and preserve vertebral mineral content in aging laboratory rats. *Bone*, **16**, 575–582.
3) Wild, R. A., Buchamain, J. R., Myers, C. and Demers, L. M. (1987) Declining adrenal androgen; an association with bone loss in aging women. *Proc. Soc. Exp. Biol. Med*., **186**, 335–360.
4) Cooper, C. and Melton, J., III (1992) Epidemiology of osteoporosis. *Trends Endocrinol. Metab*., **3**, 224–229.
5) Bonjour, J.-P., Schurch, M.-A. and Rozzori, R. (1996) Nutritional aspects of hip fractures. *Bone*, **18**, 1395–1445.
6) Sugimoto, E. and Yamaguchi, M. (2000) Anabolic effect of genistein in osteoblastic MC3T3-E1 cells. *Int. J. Mol. Med*., **5**, 515–520.
7) Yamaguchi, M. and Gao, Y. H. (1998) Inhibitory effect of genistein on bone resorption in tissue culture. *Biochem. Pharmacol*., **55**, 71–76.
8) Yamaguchi, M. (2002) Isoflavone and bone metabolism: Its cellular mechanism and preventive role in bone loss. *J. Health Sci*., **48**, 209–222.
9) Yamaguchi, M., Sugimoto, E. and Hachiya, S. (2001) Stimulatory effect of menaquinone-7 (vitamin K_2) on osteoblastic bone formation *in vitro*. *Mol. Cell. Biochem*., **223**, 131–137.
10) Yamaguchi, M. and Ma, Z. J. (2001) Inhibitory effect of menaquinone-7 (vitamin K_2) on osteoclast-like cell formation and osteoclastic bone resorption in rat bone tissues *in vitro*. *Mol. Cell. Biochem*., **228**, 39–47.
11) Ma, Z. J., Shimanuki, S., Igarashi, A., Kawasaki, Y. and Yamaguchi, M. (2000) Preventive effect of dietary fermented soybean on bone loss in ovariectomized rats: Enhancement with isoflavone and zinc supplementation. *J. Health Sci*., **46**, 263–268.
12) Yamaguchi, M., Kakuda, H., Gao, Y. H. and Tsukamoto, Y. (2000) Prolonged intake of fermented soybean (*natto*) diets containing vitamin K_2 (menaquinone-7) prevents bone loss in ovariectomized rats. *J. Bone Miner. Metab*., **18**, 71–76.
13) Yamaguchi, M. and Uchiyama, S. (2004) β-Cryptoxanthin stimulates bone formation and inhibits bone resorption in tissue culture *in vitro*. *Mol. Cell. Biochem*., **258**, 137–144.
14) Uchiyama, S. and Yamaguchi, M. (2004) Inhibitory effect of β-cryptoxanthin on osteoclast-like cell formation in mouse marrow cultures. *Biochem. Pharmacol*., **67**, 1297–1305.
15) Yamaguchi, M., Hachiya, S., Hiratuka, S. and Suzuki, T. (2001) Effect of marine algae extract on bone calcification in the femoral-metaphyseal tissues of rats: Anabolic effect of *Sargassum horneri*. *J. Health Sci*., **47**, 533–538.
16) Uchiyama, S. and Yamaguchi, M. (2002) Stimulatory effect of *Sargassum horneri* extract on bone formation in rat femoral-diaphyseal and -metaphyseal tissues *in vitro*. *J. Health Sci*., **48**, 148–153.
17) Uchiyama, S. and Yamaguchi, M. (2002) Inhibitory effect of marine algae *Sargassum horneri* extract on bone resorption in tissue culture *in vitro*. *J. Health Sci*., **48**, 154–160.
18) Uchiyama, S. and Yamaguchi, M. (2003) Preventive effect of marine alga *Sargassum horneri* extract on bone loss in streptozotocin-diabetic rats *in vivo*. *J. Health Sci*., **49**, 149–155.
19) Suzuki, T., Nakayama, H. and Yamaguchi, M. (1997) Effect of wasabi leafstalk (*Wasabia japonica* MATSUM.) extract on bone metabolism in mouse calvaria tissue culture. *Food Sci. Technol. Int*., **3**, 366–369.
20) Suzuki, T. and Yamaguchi, M. (1999) Characterization of stimulatory effect of wasabi leafstalk (*Wasabia japonica* MATSUM.) extract on bone calcification in mouse calvaria tissue culture. *Food Sci. Technol. Res*., **5**, 304–307.

21) Yamaguchi, M., Ma, Z. J. and Suzuki, T. (2003) Anabolic effect of wasabi leafstalk (*Wasabia japonica* MATSUM.) extract on bone components in the femoral-diaphyseal and -metaphyseal tissues of aged female rats *in vitro* and *in vivo*. *J. Health Sci.*, **49**, 123–128.
22) Yamaguchi, M., Oishi, H. and Suketa, Y. (1987) Stimulatory effect of zinc on bone formation in tissue culture. *Biochem. Pharmacol.*, **36**, 4007–4012.
23) Suzuki, T. and Yamaguchi, M. (1999) Characterization of stimulatory effect of wasabi leafstalk (*Wasabia japonica* MATSUM.) extract on bone calcification in mouse calvaria tissue culture. *Food Sci. Technol. Res.*, **5**, 304–307.
24) Suzuki, T. and Yamaguchi, M. (1999) Anabolic effect of wasabi leafstalk (*Wasabia japonica* MATSUM.) extract on bone metabolism in rats. *Food Sci. Technol. Res.*, **5**, 61–63.