Food Sci. Biotechnol. Vol. 17, No. 2, pp. 434 ~ 437 (2008)

RESEARCH NOTE

Food Science and Biotechnology

Potential Effect of *Monascus*-fermented Soybean Extracts on Alkaline Phosphatase Activity of Human Osteoblast-like Cells

Young-Hee Pyo*, Mi-Ja Kwon[1], and In-Ho Kim[2]

Department of Food and Nutrition, Sungshin Women's University, Seoul 136-742, Korea

[1]Department of Research and Development, Vitamin Bank, Seoul 135-090, Korea

[2]Food Processing and Marketing Research Division, Korea Food Research Institute, Seongnam, Gyeonggi 463-746, Korea

Abstract The aim of this study was to investigate whether *Monascus*-fermented soybean extracts (MFSE) containing natural estrogen-like compounds such as isoflavones and mevinolins has potential effects on human osteoblast-like SaOS2 cells using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and alkaline phophatase (ALP) assaies. MFSE exerted biphasic dose-dependent effect; stimulating osteoblastic activity at low concentrations and inhibiting SaOS2 cells viability at high concentrations. At 10^{-8}-10^{-4} mg /mL, MFSE is not only non-cytotoxic but also induced comparatively high ALP activity on SaOS2 cells. ALP activity (%) significantly increased (220.1%, $p<0.05$) when SaOS2 cells were treated with MFSE at a concentration of 10^{-5} mg/mL, whereas slowly increased (185.6%, $p<0.05$) in unfermented soybean extracts (UFSE) at 10^{-3} mg/mL. The potentially greater ALP activity of MFSE compared to the UFSE might partially be caused by its mevinolin, which was derived from the soybean during *Monascus*-fermentation. Our findings indicate that supplementation of MFSE may accelerate the speed of intracellular ALP synthesis by the bone cells when provided at optimal dosages.

Keywords: *Monascus*-fermentation, soybean, human osteoblast-like cell, alkaline phophatase activity, mevinolin, isoflavone

Introduction

Bone volume is maintained by two phases of bone remodeling: one is bone resorption by osteoclasts, and the other is bone formation by osteoblasts (1). An imbalance between bone formation and bone resorption leads to metabolic bone diseases. Osteoporosis, a chronic disease bone, characterized by deterioration of bone tissue, loss of bone mass, and risk of fracture must be due to a change in the balance between decreased activities of osteoblasts and increased activities of osteoclasts with advancing age (1,2). Phytoestrogens such as isoflavones are natural compounds that have the potential to maintain bone health and delay or prevent osteoporosis (3-8).

Soybean isoflavones, including genistein and daidzein, are known as phytoestroens because they are chemicals found in plants that bind to the estrogen receptor (3). Data from human studies provide convincing evidence for the potential role of soybean isoflavones in preventing osteoporosis (4,5). Genistein, in particular, has been shown to have a wide range of influences on bone-like cells such as proliferation and differentiation, and metabolism of osteoblasts, exhibiting contrasting estrogenic or antiestrogenic effects, as well as different dose-response on bone tissue and cells (6,7).

Recently, the statins, inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which controls the first step in the biosynthesis of cholesterol, have been shown to produce strong bone anabolic effects both *in vitro* and *in vivo* (9-12). These studies have demonstrated that statins mediate their effect on bone by enhancing the expression of the bone morphogenic proteins (BMPs), in particular of BMP2, which in turn leads to osteoblast differentiation and bone formation (9,10). Recent clinical data suggests that the administration of statins may reduce the risk of fracture in patients taking these drugs (10,11).

We recently found that soybean fermented with *Monascus pilosus* has a remarkable content of bioactive isoflavone aglycones (daidzein, glycitein, genistin) and natural statins (also known as mevinolin, lovastatin, monacolin K, or $C_{24}H_{36}O_5$) (13,14). It was also demonstrated that *Monascus*-fermented soybean extracts (MFSE) have not only the potential for strong free radical scavenging effects, but also the ability to inhibit angiotensin I-converting enzyme (15). Thus, we hypothesized that MFSE containing natural bone-enhancing compounds such as isoflavone aglycones (daidzein, genistein) and mevinolin (statins) may have an additive effects on stimulating bone formation when compared with the unfermented soybean extracts (UFSE), which has only higher content of isoflavone glucosides (daidzin, genistin) without mevinolin (14). There has been no study of the effect of *Monascal*-soy extracts supplementation on human osteoblast-like cells. Therefore, the objective of the present study was to compare the possible cytotoxic and bone anabolic effects of MFSE and UFSE on SaOS2 osteoblastic cells in culture.

Materials and Methods

Chemicals Dimethyl sulfoxide (DMSO), penicillin, streptomycin, RPMI-1640 medium, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), mevinolin, and authentic standards of daidzein, genistein were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and fetal bovine serum (FBS) was purchased

*Corresponding author: Tel: +82-2-920-7588; Fax: +82-2-920-2076
E-mail: rosapyo@sungshin.ac.kr; rosapyo@hotmail.com
Received August 6, 2007; accepted October 10, 2007

from JRH Bio Science (Lenexa, KS, USA). Daidzin, genistin, glycitin, and glycitein standards were obtained from Funakoshi Chemical Co. (Tokyo, Japan). Soy isoflavone (as a blend of aglycones) used as a positive control was purchased from Wako (Osaka, Japan). All other reagents were of the highest grade available unless otherwise indicated.

***Monascus*-fermented soybean extracts (MFSE) and unfermented soybean extracts (UFSE)** The preparations of MFSE and UFSE were carried out according to the method of Pyo and Lee (15). Briefly, 100 g of *Monascus*-fermented and unfermented soy powder was extracted in 1 L of 80% ethanol, respectively. Each extract was filtered through Whatman No. 4 filter paper and centrifuged at 12,000×g for 10 min. The supernatant was lyophilized for further assay.

High performance liquid chromatography (HPLC) analysis for isoflavones and mevinolin Quantitative data of isoflavone isomers and mevinolins were determined as described previously (14). Briefly, the mobile phase was composed of 0.1% phosphoric acid in acetonitrile (sovent A) and water (solvent B) for the analysis of isoflavones. The solvent flow rate was 1 mL/min and the eluted isoflavones were detected at 254 nm. A mixture of 0.1% phosphate buffer (pH 7.7) and acetonitrile (65 : 35, v/v) was used as the mobile phase with a flow rate of 0.8 mL/min and a detection wavelength of 238 nm for mevinolins analysis.

Cell culture The human osteoblast-like cell line (SaOS2) was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). SaOS2 cells were grown in RPMI-1640 medium containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained in a humidified incubator at 37°C and 5% CO_2 atmosphere. To maintain exponential growth, the cells were subcultured every 4 days.

Cell cytotoxicity The potential cytotoxic effects on the growth and viability of cells were evaluated using the MTT assay (16). Briefly, the cells were seeded in 96-well plates at a density of 2×10^4 cells/mL. Then, cells were treated with various concentrations (10^{-8} to 10^{-1} mg/mL) of samples in DMEM containing 1% FBS. After a certain period of time, 50 μL of MTT solution (0.5 mg/mL) was added to each well and then incubated for an additional 4 hr. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved in 150 μL of DMSO and the the optical density value was measured at 550 nm by a microplate reader (Spectra Max 340 PC; Molecular Devices, Sunnyvale, CA, USA). The control cells were grown under the same conditions without the addition of formaldehyde. Cell viability (% of control) was calculated relative to untreated control cells.

Alkaline phosphatase (ALP) activity assays ALP activities were determined using *p*-nitrophenyl phosphate as the substrate according to the method of Kang *et al.* (17). The ALP reagent was obtained from Thermo Electron (Louisville, CO, USA). The SaOS2 cells were cultured same as for the MTT test. After incubating at 37°C for 48 hr, the cells were washed with PBS and then lysed with 1% Triton X-100. The lysates were sonicated for 15 sec and centrifuged at 14,000×g for 20 min at 4°C. An aliquot of the supernatant was used for the determination of ALP activity by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at 405 nm using a microplate reader. Results are expressed as percentage of untreated control cells.

Statistics Data were expressed as mean±standard deviation (SD) from 3 independent parallel experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA).

Results and Discussion

Cytotoxicity The effect of incremental concentrations (10^{-8}-10^{-1} mg/mL) of MFSE and UFSE on cytotoxicity of SaOS2 osteoblast-like cells was investigated using MTT assay (16). Based on the results of the MTT assay, measures of mitochondrial succinate dehydrogenase activity in viable cells only, SaOS2 cells were unaffected by exposure down to the lower concentration (10^{-8} to 10^{-4} mg/mL) of MFSE compared to UFSE and pure isoflavone mixture (daidzein + genistein) used as a positive control (Fig. 1). However, treatment with MFSE at the range of 10^{-3}-10^{-1} mg/mL for 24 hr decreased cell viability by 51.9 %, suggesting that higher concentrations of MFSE containing natural estrogen-like compounds such as daidzein and genistein were cytotoxic in SaOS2 cells. As shown in Table 1, MFSE contained more isoflavone

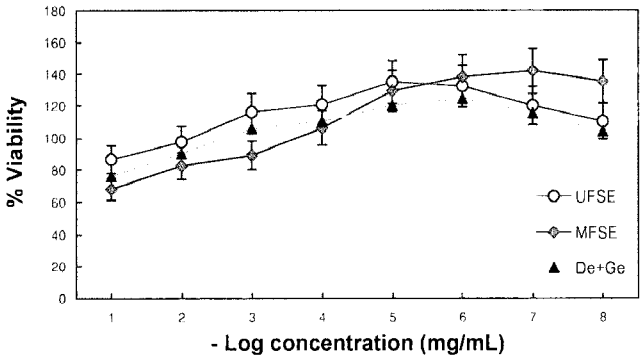

Fig. 1. Effects of the mixture of daidzein (De) and genistein (Ge), UFSE, and MFSE on SaOS2 cell viability. Data are expressed as percentage of vehicle-treated cells (control). Each value is the mean ±SD (n=3).

Table 1. Isomeric isoflavones and mevinolins concentrations of MFSE and UFSE[1)] Unit: μg/g d.w.

	MFSE	UFSE
Aglycone isoflavones[2)]	1,011.6±21.7[a]	71.3±11.2[b]
Glucoside isoflavones[3)]	397.8±4.2[c]	1,158.3±24.2[d]
Mevinolins	2,940±0.4	ND

[1)]Each value is the mean±SD (n=3); ND, not detected; different letters indicate significantly different values ($p<0.001$).
[2)]Aglycone isoflavones; daidzein + glycitein + genistein.
[3)]Glucoside isoflavones; daidzin + glycitin + genistin.

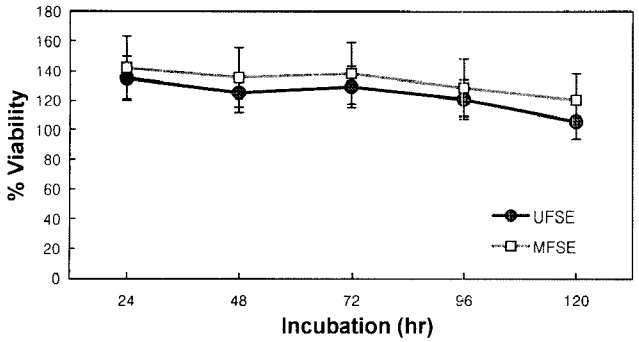

Fig. 2. Effects of exposure time to MFSE and UFSE on SaOS2 cell viability. Data are expressed as percentage of vehicle-treated cells (control). Each value is the mean±SD (n=3).

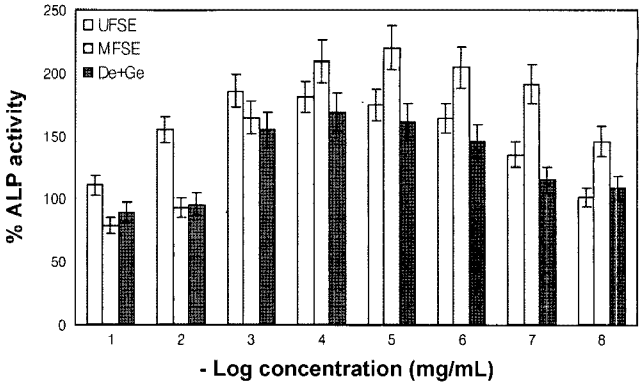

Fig. 3. Effects of the mixture of daidzein (De) and genistein (Ge), UFSE, and MFSE on ALP activity (%). Data are expressed as percentage of vehicle-treated cells (control). Each value is the mean±SD (n=3).

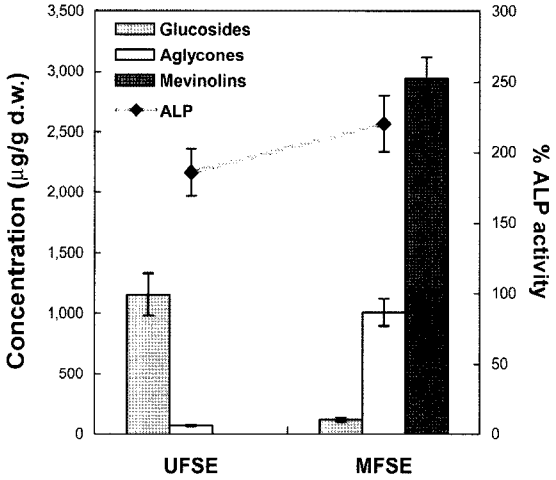

Fig. 4. Effects of MFSE and UFSE on ALP activity (%) and their isomeric isoflavones and mevinolins concentrations (μg/g d.w.). Each value is the mean±SD (n=3).

aglycones (1,011.6 μg/g d.w.) as well as mevinolin (2.94 mg/g d.w.) when compared with UFSE (71.3 μg/g d.w., mevinolin was not detected). UFSE and pure isoflavone mixture also exhibited cytotoxicity when their concentrations were higher than 10^{-2} mg/mL. These results are consistent with previous reports where soybean isoflavones have less positive effects on bone cells at lower and higher doses than the optimal dose range and thereby display a biphasic effect (18,19). In general, cell studies of genistein suggest that at high doses it contributes to the loss of normal cell functions (18-20), whereas daidzein at the same concentration has a beneficial effect on osteoblast-like cells (21). In a further series of experiments, SaOS2 cell lines were exposed to the lower concentrations of 10^{-8} to 10^{-4} mg/mL, by incrementing once 24 hr, to examine the effects of longer-term exposure to MFSE and UFSE on cell viability. Both MFSE (120.4%) and UFSE (105.7%) had no cytotoxic effect on SaOS2 cells after 120 hr at 10^{-4} mg/mL as determined by the MTT assays (Fig. 2). Our data demonstrate an apparent biphasic effect of MFSE on viability of SaOS2 cells: a stimulation in optimal range of concentrations (10^{-8}-10^{-4} mg/mL), but an inhibition at a higher concentration (10^{-3}-10^{-1} mg/mL). To ascertain whether MFSE and UFSE increase osteoblastic activity in SaOS2 cells, we examined the changes in ALP activity.

ALP activity ALP is the most widely recognized biochemical marker for osteoblastic activity (22). ALP activity, one of the osteoblastic phenotype markers, was measured to investigate the beneficial effect of MFSE on SaOS2 cells. As shown in Fig. 3, ALP activity of osteoblasts induced by MFSE increased in a dose-dependent manner at 10^{-8} to 10^{-5} mg/mL. The highest ALP activities of the SaOS2 cells to MFSE and UFSE were 220.1 and 185.6% at the concentrations of 10^{-5} and 10^{-3} mg/mL, respectively (Fig. 3). The average ALP activities of MFSE were a 17.3% higher than those of UFSE ($p<0.05$) in the range of 10^{-8} to 10^{-3} mg/mL. While, at concentration of 10^{-1} mg/mL, pure isoflavones mixture and MFSE had no ALP activity on SaOS2 cells. These results are same as those of Dang and Lowik (19) who reported that phytoestrogens such as isoflavones exert biphasic dose-dependent effects on osteoblasts and osteoprogenitor cells, stimulating osteogenesis at low concentrations, whereas inhibiting osteogenesis at high concentrations. Biphasic dose-dependent stimulatory and inhibitory effects of isoflavones on osteogenesis in preosteoblast MC3T3-E1 cells have also been reported (7,18,19). In this study MFSE at 10^{-8} to 10^{-4} mg/mL concurrently activated both viability and ALP activity of human osteoblast-like cell. Interestingly, ALP activities of MFSE and UFSE were greater than the effect of pure isoflavones mixture used as a positive control. In particular, ALP activity was significantly increased (220.1%, $p<0.05$) when SaOS2 cells were treated with MFSE at 10^{-5} mg/mL, whereas slowly increased (185.6%, $p<0.05$) in UFSE at a concentration of 10^{-3} mg/mL. These results indicate that MFSE may act primarily as a phytoestrogen in a low-estrogen environment such as postmenopausal bone loss.

In the present study, high ALP activity of both soy extracts may be mostly related to its concentration of isoflavones. However, the potentially high ALP activity of MFSE might partially be caused by its mevinolin, which was derived from the *Monascus*-fermented soybean. As shown in Fig. 4, MFSE composed primarily of an extract with high concentrations of mevinolin (2.94 mg/g d.w.) and isoflavone aglycones (1,011.6 μg/g d.w.) exerted significantly high ALP activity (average, 212.4% at 10^{-6}-

10^{-5} mg /mL; $p<0.05$) than UFSE (183.3%). Therefore, higher ALP activity of MFSE might be accounted for the combined effect of isoflavones and mevinolin rather than being only by isoflavones. These results are consistent with previous findings, where red yeast rice containing lovastatin (natural statins) showed strong anabolic effect both *in vitro* and *in vivo* (12). Recently, Maeda *et al.* (10) and Tanriverdi *et al.* (11) reported that statins stimulate the expression of bone anabolic factors and promote osteoblastic differentiation and mineralization in MC3T3-E1 cells. Further study is required to determine the effect of mevinolin of MFSE on the stimulation of osteoblast cells. However, the present study clearly shows that *Monascus*-fermented soybean is a more potent inducer of osteogenesis compared with the unfermented soybean.

Thus, the conclusion of this study is that MFSE at optimal dosages might be an effective dietary protective factor against a physiological disorder caused by osteoporosis.

Acknowledgments

This work was supported by the Sungshin Women's University Research Grant.

References

1. Parfitt AM. Bone remodeling and bone loss-understanding the pathophysiology of osteoporosis. Clin. Obstet. Gynecol. 30: 789-811 (1987)
2. Rodan GA. Introduction to bone biology. Bone 13: S3-S6 (1992)
3. Anderson JJB, Garner SC. The effects of phytoestrogens on bone. Nutr. Res. 17: 1617-1632 (1997)
4. Greendale GA, FitzGerald G, Huang MH, Sternfeld B, Gold E, Seeman T, Sherman S, Sowers M. Dietary soy isoflavones and bone mineral density: Results from the study of women's health across the nation. Am. J. Epidemiol. 155: 746-754 (2002)
5. Alekel DL, St Germain A, Perterson CT, Hanson KB, Stewart JW, Toda T. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. Am. J. Clin. Nutr. 72: 679-680 (2000)
6. Gao YH, Yamaguchi M. Suppressive effects of genistein on rat bone osteoclasts: Involvement of protein kinase inhibition and protein tyrosine phosphatase activation. J. Mol. Med. 5: 261-267 (2000)
7. Sugimoto E, Yamaguchi M. Anabolic effect of genistein in osteoblastic MC3T3-E1 cells. Int. J. Mol. Med. 5: 515-520 (2000)
8. Kim D-H, Lee J-H, Ahn E-M, Lee Y-H, Back N-I, Kim I-H. Phenolic glycosides isolated from safflower (*Carthamus tinctorius* L.) seeds increase the alkaline phosphatase (ALP) activity of human osteoblast-like cells. Food Sci. Biotechnol. 15: 781-785 (2006)
9. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossigni G, Boyce B, Zhao M, Gutierrez G. Stimulation of bone formation *in vitro* and in rodents by statins. Science 286: 1946-1949 (1999)
10. Maeda T, Kawane T, Horiuchi N. Statins augument vascular endothelial growth factor expression in osteoblastic cells via inhibition of protein prenylation. Endocrinology 144: 681-692 (2003)
11. Tanriverdi HA, Barut A, Sarikaya S. Statins have additive effects to vertebral bone mineral density in combination with risedronate in hypercholesterolemic postmenopausal women. Eur. J. Obstet. Gyn. R. B. 120: 63-68 (2005)
12. Gutierrez GE, Mundy B, Rossini G, Garrett R, Chen ST, Mundy GR. Red yeast rice stimulates bone formation in rats. Nutr. Res. 26: 124-129 (2006)
13. Pyo Y-H, Lee Y-C. Mevinolin production by *Monascus pilosus* KFRI-1140 in solid state fermentation using soymeal. Food Sci. Biotechnol. 15: 647-649 (2006)
14. Pyo Y-H. Production of a high value-added soybean containing bioactive mevinolins and isoflavones. J. Food Sci. Nutr. 12: 29-34 (2007)
15. Pyo Y-H, Lee T-C. The potential antioxidant capacity and angiotensin I- converting enzyme inhibitory activity of *Monascus*-fermented soybean extracts: Evaluation of *Monascus*-fermented soybean extracts as multifunctional food additives. J. Food Sci. 72: S708-S713 (2007)
16. Mossman T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Method 65: 55-63 (1983)
17. Kang S-W, Cho Y-W, Park E-H, Ahn K-S, Lim C-J. Cloning and expression of alkaline phophatase gene from *Schizosaccharomyces pombe*. J. Biochem. Mol. Biol. 34: 262-267 (2001)
18. Hwang C-S, Kwak H-S, Lim H-J, Lee S-H, Kang Y-S, Choe T-B, Hur H-G, Han K-O. Isoflavone metabolites and their *in vitro* dual functions: They can act as an estrogenic agonist or antagonist depending on the estrogen concentration. J. Steroid Biochem. 101: 246-253 (2006)
19. Dang ZC, Lowik C. Dose-dependent effects of phytoestrogens on bone. Trends Endocrin. Met. 16: 207-213 (2005)
20. Anderson JJ, Ambrose WW, Garner SC. Biphasic effects of genistein on bone tissue in the ovariectomized, lactating rat model. P. Soc. Exp. Biol. Med. 217: 345-350 (1998)
21. Sugimoto E, Yamaguchi M. Stimulatory effect of daidzein in osteoblastic MC3T3-E1 cells. Biochem. Pharmacol. 59: 471-475 (2000)
22. Delmas PD. Clinical use of biochemical markers of bone remodeling osteoporosis. Bone 13: S17-S21 (1992)