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Stimulating effect of fermented ginseng leaf saponin on the differentiation and mineralization of murine osteoblastic MC3T3-E1 cells

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Abstract

In this study, abundant ginseng leaf saponins were converted into minor ginsenosides that havemore pharmacological efficacy via fermentation process using recombinant β-glucosidase (bgp1). This fermented product was used to investigate the stimulatory effect on differentiation and mineralization of murine osteoblastic MC3T3-E1 cells. All major ginsenosides which areavailable in ginseng leaf were biotransformed into more pharmacologically active minor ginsenosides within a short time of incubation. The results showed that 100% of ginsenoside Rd, Rg1and Re were decomposed and transformed to Rg3, Rh1and Rg2, respectively within 03 (three) hours of incubation. Ginseng leaf saponin contains 17.1% Rg1, 44.9% Re, 10.8% Rd, 4.8% Rb1, 5.7% Rb2, 6.9% Rc,2.7% Rg2, and 6.8% F1 ginsenoside. But after fermentation, the products contain mostly pharmacological active minor ginsenosides including 42.2% Rg2, 13.7% Rg3, 8.8% Rh1, 4.9% F1 and 3.6% PPT ginsenosides. Moreover, we investigated and compared the effect of leaf saponins (LS) and fermented leaf saponins (FLS), on the differentiation and mineralization of pre-osteoblastic MC3T3-E1 cells. Treatment with FLS remarkably enhanced cell viability in a dose-dependent manner. FLS notably stimulated the ALP activity, Coll-I synthesis and mineralization ability of MC3T3-E1 cells. Based on the comparison between LS and FLS, it is clear that FLS has good effect on differentiation of osteoblastic MC3T3-E1 cells and bone formation. Therefore, bgp1-fermented ginseng leaf saponins could be a novel treatment for osteoporosis prevention.

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Introduction

Ginsenosides, the most pharmacological active compounds of *P. ginseng* also called ginseng saponin, has been reported to reveal a lot of pharmacological efficacy against different diseases like cancer (Chae *et al.,* 2009), aging (Cho *et al.,* 2006) and tumor (Wang *et al.,* 2008) etc, stimulate the central nervous system, improve the learning capability and increase memory, regulate immune system and contain anti-fatigue properties (Zhang *et al.,* 1988; Peña *et al.,* 2014). On the basis of sugar molecules, ginsenosides are three types: PPD (protopanaxadiol), PPT (protopanaxatriol) and oleanane type. In the aglycon PPD various sugar molecules are joined by glycosidic bonds to C-3 and C-20 positions and in the aglycon PPT they are attached with C-6 and C-20 positions (Fig. 1). Ginseng root takes 4-6 years to be adult from a seed that makes it expensive, but ginseng leaves can be harvested every year. Hence, ginseng leaves could be a possible alternative source of ginseng saponin compared to the roots that need long growth time. Six major ginsenosides are found in ginseng leaves, such as Re, Rg1,Rb1, Rc, Rb2, and Rd (Li *et al.,* 1996), among them Re, Rg1 and Rd are available. Pharmacologically more active ginsenosides (Rh1, Rg3, Rg2)

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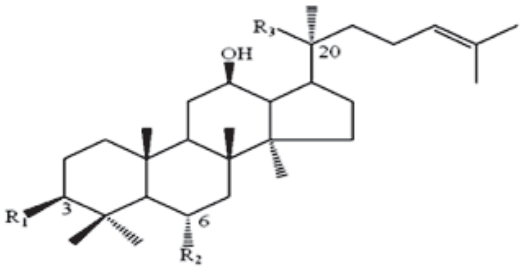
can be synthesized by hydrolyzing sugar molecules from available major ginsenosideslike, Rg1, Rb1, Rb2, Rc, Rd, and Re which consist ofaround 80% of the total ginsenoside content (Noh *et al.,* 2009). Intact major ginsenosides are poorly absorbed through the gastrointestinal (GI) tract and the oral bioavailability of these ginsenosides is very low (Xu *et al.,* 2003). Consequently, many scientists are trying to transform these intact ginsenosides into pharmacologically active minor forms (Chen *et al.,* 2008; Chi *et al.,* 2005; Han *et al.,* 2007).

Microbacterium esteraromaticum is a soil borne bacteria which contains a strong β-glucosidase gene, Bgp1. Bgp1 was cloned from *M. esteraromaticum* and overexpressed in *E. coli* competent cell (BL21, DE3). The length of bgp1 gene is 2,496 bp encoding total 831 amino acids (Quan *et al.,* 2012).



Ginsenoside (PPD)	R1(C-3)	R3(C-20)
Rb1	Glc (1-2) Glc	Glc (1-6) Glc
Rb2	Glc (1-2) Glc	Arap (1-6) Glc
Rc	Glc (1-2) Glc	Araf (1-6) Glc
Rd	Glc (1-2) Glc	Glc
F2	Glc	Glc
Rg3	Glc (1-2) Glc	H
C-K	H	Glc
Rh2	Glc	H
Ginsenoside (PPT)	**R2(C-6)**	**R3(C-20)**
Re	Glc (2-1) Rha	Glc
Rf	Glc (2-1) Glc	H
Rg1	Glc	Glc
Rg2	Glc (2-1) Rha	H
F1	H	Glc
Rh1	Glc	H

Fig. 1. Chemical structures of PPT and PPD ginsenosides. Arap, *α*-L-arabinopyranosyl; Glc, *β*-D-glucopyranosyl; Rha, *α*-L-rhamnopyranosyl and Araf, *α*-L-arabinofuranosyl

Bone is anextremely organized tissue of the body, plays anextraordinary role for maintaining skeletal system via remodeling, which is strongly involved with bone formation as well as bone resorption (Roodman 1996; Rodan and Martin 2000). The integrity of bone remodeling is fully dependent on the actual function of osteoblasts and osteoclasts. Any abnormalities in the functions of that cell leading to bone diseases like osteoporosis (Manolagas and Jilka 1995; Ducy *et al.,* 2000; Parfitt 1987). Osteoporosis is involved with significant changes in bone remodeling, as a result reducesthe bone formation and enhances the bone resorption. Osteoporosis creates micro-architectural deterioration of bone mass, fragility of bone and consequently leads to bone fracture (Isomura *et al.,* 2004). There are many kinds of drugs and therapies have been used to solve osteoporosis, like HR (hormone replacement) therapy, raloxifene, bisphosphonate, denosumab etc. But thetherapies are too much expensive and alsolong term use of these drugs maylead to many undesired side effects. Hence, researchers are trying to develop alternative therapies, specially using herbal sources, which would be cost effective without any side effects.In our present study, bgp1 enzyme was used for fermentation of ginseng leaf saponins and investigated their effect onpre-osteoblastic MC3T3-E1cell line. This is the first report on the treatment of osteoblastic cells using this fermented ginseng leaf saponin by bgp1.

Materials and methods

Materials

Standard ginsenosides and ginseng leaf saponins were collected from Ginseng Genetic Resource Bank (Global campus, Kyung Hee University, Yongin, South Korea). All other compounds and reagents used in this study were commercial products available in the market.

Preparation of bgp1recombinant enzyme

The recombinant pMAL-bgp1 vector was transformed into *Escherichiacoli* BL21 (DE3) and grown in LB(Luria–Bertani)–ampicillin medium at 37°C until the OD (optical density) reached 0.4 at 600 nm. To induce the protein expression, 0.5 mM IPTG (isopropyl-β-D- thiogalacto pyranoside) was added and then, the culture broth was incubated at 28°C for extra 9 h. Cells were collected by centrifugation (20 min, 6,000 rpm at 4°C). The cells were washed by sodium phosphate buffer (20mm, pH 7.0) and resuspended the cells using same buffer.Sonication was carried out to lyse the cells and supernatant was collected by centrifugation at 9000 rpm for 20 min at 4°C). The collected supernatant was used as bgp1 enzyme and stored at 4°C (Quan *et al.,* 2012).

Ginseng leaf saponin transformation by bgp1 enzyme

50 ml screw-cap tube was used to carry out the transformation procedure. 05 (Five)mlcrude bgp1 enzyme

and 05 (five) ml crude ginseng leaf saponin (2.0 mg/ml in 20 mM sodium phosphate buffer, pH 7) were mixed. Then the mixture was incubated into a shaking incubator (160 rpm)at 37°C. Every 03 (three) hours ofintervals, samples were withdrawn and equal volume of water-saturated n-butanol was added to each sample to stop the reaction and mixed properly. Afterward, the butanol fraction was collected and evaporated completely and finally dissolved by methanol to analyze TLC and HPLC.

TLC and HPLC analysis for bioconversion of ginsenosides

TLC analysis was carried out using silica gel plateswith a solvent system of $CHCl_3$:CH_3OH:H_2O (65:35:10 v/v/v). The TLC plates were sprayed with 10% (v/v) H_2SO_4 and heated at 110°C for 10 min before detection.

For HPLC analysis, a C18 column was used with acetonitrile (solvent A) and distilled water (solvent B) as the mobile phases with 85% B for 5 min, 79% B for 20 min, 42% B for 55 min, 10% B for 12 min, and 85% B for 18 min, flow rate 1 ml/min. The sample was detected at UV 203 nm.

Cell cultures and differentiation

The MC3T3-E1 (RCB1126) cell line was gained from the RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured in α-MEM (alpha modification of Eagle's Medium) with 10% heat-inactivated FBS, 100 mg/mL streptomycin, and100 U/mL penicillin, and then incubated in an incubator with 5% CO2at 37°C. Cells were plated onto a 6 or 12-well plate to induce differentiation and allowed to grow 80 to 90% confluence. At confluence (day 0), cells were moved to α-MEM that comprises 10% FBS, 1% penicillin-streptomycin, 10mM β-glycerophosphate and 100 μg/mL ascorbic acid along with differentiation medium (DM) for extra 6 to 24 days.

Cell viability assay

The MC3T3-E1 cells were seeded in 96-well micro-plate (density, 5 x 10^3 cells/well)and suspended in α-MEM carrying1% (v/v) P/S and 10% (v/v) FBS.Then the plate was incubated in incubator with5% CO_2 at 37°Cfor 24 h. 24 h later,the cells were washed by PBS buffer, and then, the medium waschanged with serumfree medium that contains different concentrations (100 μg/ml, 10 μg/ml and 1 μg/ml) of both Leaf Saponin (LS) and Fermented Leaf Saponin (FLS). Cell viability was measured after 72 hof incubation. 10μL MTT solutions (5mg/mL) were added to each well, and the plates were incubated for 4 h. Then, MTT solution was removed and 100 μLDMSO (dimethyl sulfoxide) was added for dissolving formazan products (Quarles *et al.,* 1992). Finally, the plates were shaken for 5min and OD was taken by ELISA reader at 570nm.

Alkaline phosphatase (ALP) activity

For checking ALP activity, MC3T3-E1 cells were cultured in a 24-well micro-plate using same medium and conditions which were used for cell viability assay. After 24 h of incubation, the medium was changedby DM with or without FLS and incubated for additional 14 days. Every2 days after, the medium was changed by new one. After 12 days of treatment, the alkaline phosphatase activity was calculated. The cells were washed by PBS buffer and then suspended in Tris/HClbuffer (10 mM, pH 7.5) supplemented with 0.1% Triton X-100, 2 mM MgSO4 and incubated for 2 hat 37°C. Absorbance was taken at 405 nm and ALP activity was measured using *p*-Nitrophenol phosphate as a substrate (Siddiqi *et al.,* 2015).

Type-I collagen measurement

Collagen contentwas measured through the protocol described by (Siddiqi *et al.,* 2015). In brief, the cells were culturedwith same reagents as explained in MTT assay. After 12 days of treatment, Sirius Red-based colorimetric assay was used to measure the Coll-1 content.

Alizarin red S staining

MC3T3-E1 cells were platedas explained above. After 24 h of incubation, the medium was exchanged with DM, supplemented with or without fermented leaf saponin (FLS) and incubated for 24 days. Every 2 days after, the medium was exchanged by new one.Then the cells were washed with buffer (PBS) for twice and fixed by 70% EtOH and incubated for additional 1 h at 37°C in 5 % CO_2 atmosphere. Then, the cells were stained by Alizarin Red S (40 mM) for 30 min using mild shaker and absorbance was taken at 570 nm (Siddiqi *et al.,* 2015).

Statistical analysis

Three independent experiments were carried out and the results wereshowed as the mean ± standard deviation (SD). Comparisons with control were performed using ANOVA and Dunnett's multiple comparison test in the Prism program (Graphpad software Inc. La Jolla USA). Differences of $*P<0.05$ were considered statistically significant.

Results and discussion

TLC and HPLCanalysis of ginsenosides

Six major ginsenosides were identified in ginseng leaf such as, Rd, Rc, Rb1, Rb2,Rg1, and Re. Rg1, Re and Rd are the most abundant among all of these. Minor ginsenoside F1 also found in ginseng leaf. Ginsenosides Rd,Rg1 and Re were biotransformed into Rg3, Rh1, and Rg2 respectively, by hydrolysis of a glucose moleculefrom C-20 position of the ginsenosideaglycone. Ginsenoside Rg3 was synthesized from Rb1 by hydrolysis of two glucose moleculesfrom C-20 position. Ginsenoside F1 was incompletely transformed into PPT ginsenoside by hydrolysis of a glucose molecule from same place of aglycone. All abundant ginsenosides Rg1, Re and Rd found in ginseng leaf were fully transformed into more pharmacologically active ginsenosides Rh1, Rg2 and Rg3, respectively by bgp1 enzyme within three hours of incubation. From TLC analysis, it was observed that there was no variation in the concentrations for 3 h, 6 h, 9 h and 12 h treated products, which indicates that fermentation time does not have effect on stability of metabolites (Fig. 4).

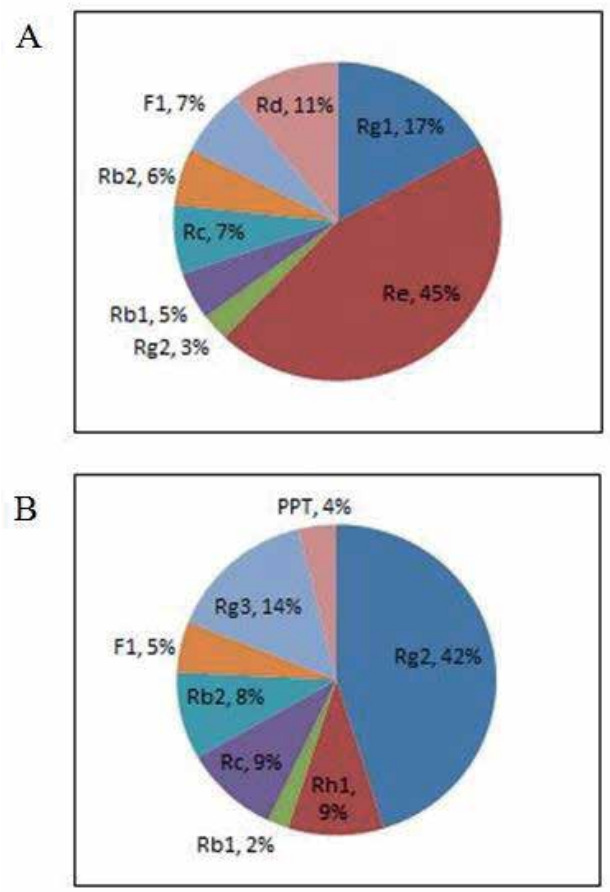


Fig. 2. Quantitative HPLC analysis of bioconversion of crude ginseng leaf saponin by bgp1. (A) Ginseng leaf saponin, (B) fermented ginseng leaf saponin. From the data it is clear that the dominant major ginseng leaf saponins Re, Rg1 and Rd are fully biotransformed into more pharmacologically active minor ginsenosides by bgp1

Quantitative HPLC was used to confirm the transformation of ginseng leaf saponin (Fig. 2). Fig. 2A has shown that ginseng leaf saponin contains 17.1% Rg1, 44.9% Re, 10.8% Rd,4.8% Rb1, 5.7% Rb2, 6.9% Rc, 2.7% Rg2 and 6.8% F1 ginsenoside. But after fermentation, the products contain mostly pharmacological active minor ginsenosides including 42.2% Rg2, 13.7% Rg3,8.8% Rh1, 4.9% F1 and 3.6% PPT ginsenosides (Fig. 2B).

Effect of LS and FLS saponin concentration on viability of osteoblastic MC3T3-E1 cells

In order to study cellular toxicity effect of fresh leaf saponin (LS) and fermented leaf saponin (FLS) on differentiation and mineralization of preosteoblastic MC3T3-E1 cells. Cell viability assay was first carried outusing various concentrations of fresh and fermented ginseng leaf saponin (100 µg/ml, 10 µg/ml and 1 µg/ml) for 48 hours. Both LS and FLS did not show the cellular toxicity up to 100 µg/mL compared to control. Therefore, concentrations from 1 to 100 µg/mL of both LS and FLS were usedfor further experiment (Fig. 3A).

Fermented leaf saponin (FLS) stimulates ALP activity in MC3T3-E1 cells

ALP activity analysis is one of the most important phenotypic markers for the differentiation and maturation of osteoblast. Therefore, the stimulatory effect of LS and FLS on the differentiation and mineralization of MC3T3-E1 cells was investigated by measuring ALP activity. FLS treated cells showed significantly high ALP activity compared to those of treated by LS and non-treated cells, as indicated in Fig. 3B.

Fermented leaf saponin (FLS) enhances intracellular Coll-1 production in MC3T3-E1 cells

Collagen is the most important protein in the extracellular bone matrix which is essential for matrix maturation and osteoblast function. Hence, we examined the effect of both fermented and fresh leaf saponin on collagen synthesis. The effect of LS and FLS on collagen synthesis in preosteoblastic MC3T3-E1 cells is shown in Fig.3C. The collagen content was significantly increased in MC3T3-E1 cells by the treatment of 1 to 100 µg/mL FLS compared to LS.

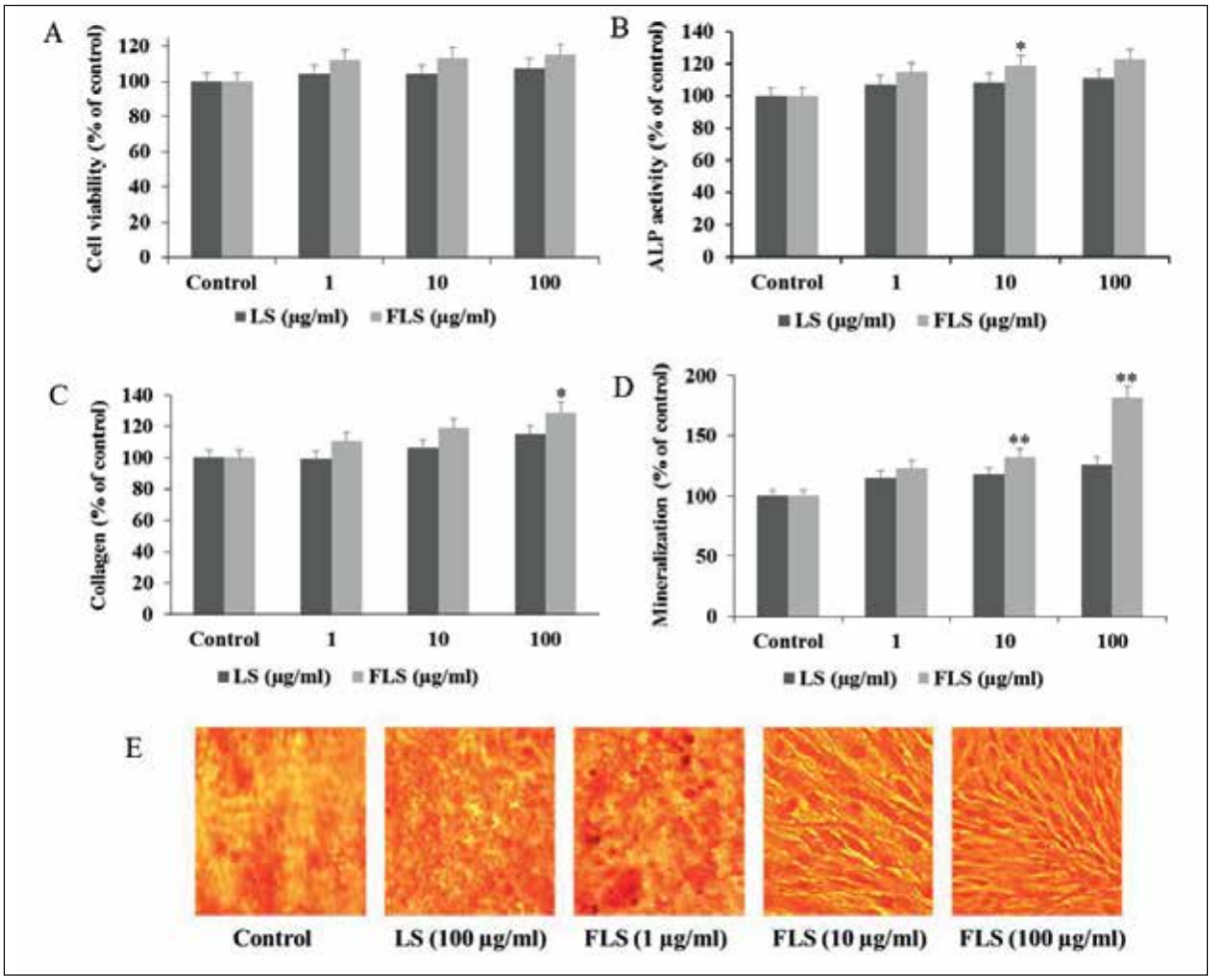


Fig. 3. Stimulatory effect of fermented leaf saponin on the growth of MC3T3-E1 cells (A), ALP activity (B), Coll-I activity (C), mineralization and calcium deposition of MC3T3-E1 cells (D) and visualized by Alizarin red S staining (E). Data were expressed as a percentage of control. *P <0.05, is consider to be significant.LS, leaf saponin; FLS, fermented leaf saponin

Fermented leaf saponin (FLS) enhances mineralization of osteoblastic MC3T3-E1 cells

To examine the effects of fermented leaf saponins on mineralization, MC3T3-E1 cells were treated with various concentrations of both original and fermented ginseng leaf saponins for 24 days, and the calcium deposition was determined by Alizarin Red S staining. For mineralized nodule formation extracellular matrix Calcium deposition, the pre-osteoblastic MC3T3-E1 cells were stained with Alizarin red S dye, which combines with calcium ions. Moreover, our results showed that the mineralized nodules increased in MC3T3-E1 cellswhen treated with fermented leaf saponin (FLS) as compared to original leaf saponin treatment (Fig. 3D). It is also indicated that mineralized nodules were more bright red in color by Alizarin red S staining (Fig.3E). It can be seen from Fig. 3D and 3E that the FLS might have improved mineralization and bone formation in MC3T3-E1 pre-osteoblastic cells compared to LS.

Ginseng leaf is a good source of ginseng saponin which carries six major ginsenosides: Re, Rg1, Rd, Rc, Rb2 and Rb1. However, minor ginsenosides are pharmacologically more active than that of intact major ginsenosides (Huq *et al.,* 2016a). The de-glycosylated ginsenosides Rg3 has strong anti-metastatic (Mochizuki *et al.,* 1995), anticancer (Park *et al.,* 2014), hepatoprotective (Lee *et al.,* 2005), neuroprotective (Tian *et al.,* 2005) and vasodilating effects

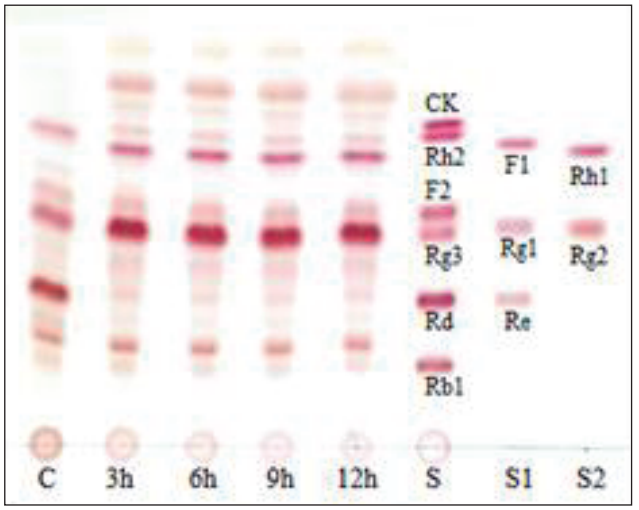


Fig. 4. Time course TLC analysis of transformation of crude ginseng leaf saponin by bgp 1.C, control; S, S1 and S2, saponin standards

(Kim *et al.,* 2003). Minor ginsenosides Rh1 and Rg2 are absent in ginseng root which have high pharmacological efficacy against different diseases, such as anti-inflammatory, anti-allergicactivities, and inhibition of glutamate-induced neurotoxicity in PC12 cells (Li *et al.,* 2007; Park *et al.,* 2004). There are several reports on the bioconversion of major ginsenoside into the minor forms using microorganisms, including the crude enzymes from *Lactobacillus rossiae* (Huq *et al.,* 2014), *Paenibacillus ginsengiterrae* (Huq et al. 2015a), *Weissellahellenica* (Huq *et al.,* 2015b, 2016b), *Fusarium sacchari* (Han *et al.,* 2007) and *Acremoniumstrictum* (Chen *et al.,* 2008). However, these microorganisms are able to convert single ginsenoside to minor form but unable to transform crude ginseng saponin. In this study, we converted the total ginseng leaf saponin into highly active minor ginsenosides using bgp1 enzyme and investigated their efficacy on the differentiation and mineralization of murine pre-osteoblastic MC3T3-E1 Cells. Bgp1 enzyme transformed all dominant major ginsenosides available in ginseng leaf into high active minor ginsenosides within three hours of incubation (i.e., 100% Rd, Re, and Rg1 were decomposed and transformed into Rg3, Rg2, and Rh1, respectively) by hydrolysis of one glucose molecule at the C-20 position of the ginsenosideaglycone.GinsenosideRg3 was synthesized from Rb1 by deglycosylation of two glucose molecules, and F1 was partially transformed into PPT by deglycosylation of a glucose molecule at the C-20 position of the ginsenosideaglycone. The bgp1 enzyme hydrolyzed all glucose molecules attached to the C-20 position of the ginsenosides Re, Rg1, Rd, Rb1, and F1. The optimum pH and temperature of bgp1 enzyme were reported to be 7 and 37°C, respectively (Quan *et al.,* 2012).

Ginseng crude extracts also have been traditionally used for the treatment of various diseases such as hypertension, hyperlipidemia, arteriosclerosis, obesity, diabetes mellitus etc. (Han *et al.,* 1998; Inoue *et al.,* 1999; Kim *et al.,* 2005). However, very few studies have been carried out on their antiosteoporotic activities.In recent time, various*in vivo*and *in vitro* studies have demonstrated that ginsenosides probably possess the beneficial effects to prevent osteoporosis. For example, the minor ginsenosides Rh1 and Rh2 (S) have the ability to induce differentiation and mineralization of MC3T3-E1 cells (Siddiqi *et al.,* 2014; Kim *et al.,* 2011). Thus in the present study, we checked and compared the efficacy of both leaf saponin and fermented leaf saponin on differentiation and mineralization of osteoblastic MC3T3-E1 cells. For cell viability assay, we used three different concentrations (100 µg/ml,10 µg/ml and 1 µg/ml) of original and fermented ginseng leaf saponin for 48 hours. Compared with the control and LS, FLS displayed significantly stimulatory effects on preosteoblastic MC3T3-E1 cell viability at all the tested doses (Fig. 3A). Thus, FLS did not show any cytotoxicity on the proliferation of osteoblastic MC3T3-E1 cells at the given dosages. Furthermore,we examined several widely used biological markers for the growth and differentiation of osteoblasts included type I collagen content, ALP activity, calcium deposition. MC3T3-E1 cells treated with different concentrations of FLS showed in a noticeable increase in ALP activity compared to LS (Fig. 3B). The collagen-I production of MC3T3-E1 cells was also remarkably increased in a dose dependent manner with the treatment of FLS compared with those of LS treatment (Fig. 3C). FLS revealed a significant stimulatory effect on mineralization compared to that of LS (Fig. 3D). As shows in Fig. 3E, FLS-treated cells dramatically increased the mineralized area. The mineralized calcium deposition nodules seemed bright orange red in colour by Alizarin Red S staining (Fig. 3E). Significant improvement in mineralization occurred up to 100 µg/ml of FLS.

In conclusion, this study demonstrated that recombinant β-glucosidase (bgp1) enzyme transformed all dominant major ginsenosides available in ginseng leaf (*Panax ginseng*) into more pharmacologically active minor ginsenosides. The *in vitro* analysis clearly showed that fermented leafsaponin-treated cells significantly stimulate the differentiation and mineralization of MC3T3-E1 osteoblastic

cells compared to original leaf saponin. These data provide evidence that bgp1-fermented ginseng leaf saponins may be a novel treatment for osteoporosis prevention.

Acknowledgement

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