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The Effects of Isoflavone Soybean (*Glycine max* (L) Merill) Fermentation Results by *Lactobacillus bulgaricus* Towards In Vitro Osteoblast Cell Proliferation

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Abstract: Soybean have a high content of isoflavones as glycosides (genistin, daidzin, glycitin) to be used as an estrogen replacement therapy it needs to be converted into aglycone isoflavones (genistein, daidzein, glycitein). The purpose of this research to obtain soy isoflavone aglycone as genistein by fermentation process of soybean by probiotic bacterium *Lactobacillus bulgaricus* then this isoflavone aglycone will be testing proliferation activity to osteoblast cell as in vitro. Fermentation results in the extraction with ethyl acetate and the liquid extract was evaporated by a rotary evaporator and lyophilized by a freeze dryer. Extract 3mg in 10ml of 80% methanol levels were analyzed genistein on HPLC with standard of comparison that used genistein ≥ 98% at concentrations of 5, 10, 15, 20 and 25 ppm. Osteoblast proliferation testing prepared with MTT method, that's absorbance measured by ELISA reader and calculating percent of cell viability. Results on three replicate analyzes on HPLC demonstrate the average value of genistein4.99 % and the result of cell viability calculating occur highest percent at concentration 1 % is 30.3 %. The result indicates that genistein obtained as fermentation process of soybean by bacterium *Lactobacillus bulgaricus* is active to osteoblast cell proliferation.
Keywords :Isoflavone, genistein, osteoblast.

1. INTRODUCTION

Osteoporosis are major health problems in postmenopausal women, who experience a sharp decrease in estrogen concentration that leads to an increased rate of bone remodeling[1,2]. Hipoestrogenism can cause osteoporosis, impact of inhibition of paratiroid hormone (PTH) activity decreased, furthermore increasing of osteoclast activity[3].

Bone homeostasis requires balanced interactions between osteoblasts and osteoclasts. Osteoporosis is a reduction in skeletal mass due to an imbalance between bone resorption and bone formation[4], characterized by a reduction in bone density and strength to the extent that fractures occur after minimal trauma. Its well known that estrogen deficiency as in postmenopausal leads to acceleration of bone desorption and rapid bone loss, resulting in the development of osteoporosis[5].

Current therapies recommended for postmenopausal osteoporosis (PMO) treatment include supplementation with estrogen or hormone replacement therapies (ERT or HRT). Estrogen is the most potent inhibitor of bone desorption and the most widely recommended therapy to reduce the rate of postmenopausal bone loss. However, available evidence appears to suggest that the long-term use of HRT has numerous side effects[6,7].

Many plant foods contain small amounts of the diverse phytoestrogen molecules that have the potential to improve health. Food phytoestrogen molecules as isoflavone are found predominantly is soybeans (*Glycine max*). This molecules function as antioxidants in plants, but in mammalian tissues this natural products act as agonist, or partial agonist of estrogen[8].

Currently, natural alternatives with estrogen-like activities such as soy isoflavones are being investigated as possible alternatives for HRT[9,10,11]. Isoflavones are compounds in plant foods, particularly soybean, which are structurally and functionally similar to estrogens. They have recently received considerable attention for their potential use in the prevention of postmenopausal bone loss. Data from animal experiments provided evidence that soy protein can attenuate PMO and it was suggested that isoflavones in soy might be responsible for their protective effects on bone[12,13,14].

Epidemiological studies indicate that women who have high soy intake have a lower risk of osteoporosis than women who consume a typical Western diet[15,16].

The major isoflavones are based upon genistin, daidzin and glycitin, and occur in the soybean as glycosides, and hydrolyzed to genistein, daidzein and glycitein as aglycone by β-glycosidase occurs in the oralcavity and intestine, which allows absorption[17].

Probiotic microorganism also producing endogen β-glycosidase, that is important to change isoflavones profile during fermentation. The aglycones are already present in fermented products[17,18].

According to the text above, this research will used fermented microorganism, especially *Lactobacillus bulgaricus* to change isoflavone profile on soybean, from glycoside to aglycone. The extraction result of aglycone isoflavone soybean will continuing for trial effect to osteoblast cell proliferation in vitro.

2. MATERIALS AND METHOD

Chemical Materials.

Standard genistein G6649 which contains 5 mg was purchased from Sigma Aldrich Chemie GmbH, with purity ≥98%.

Sample Preparation.

Soybean 250 g which was remain in water and boiled, was blended with 2000 ml hot water, and the result was filtrated to omit residual.
Filtrate of soybean 50 ml added glucose 2%, check pH 6±0.2, pasteurization while 30 minutes at temperatures≤85^{o}C, and then inoculated with *Lactobacillus bulgaricus*, fermented as long as 24 hours at 37^{o}Cto make starter of probiotic.
Starter of probiotic inoculated into 450 ml filtrate of soybean which has pasteurization, and then fermented during 24 hours, check of pH at 4-5.

Extraction Procedure.

Fermented soybean as much as 500 ml was performed using 40-80% ethyl acetate 500 ml, the resulting mixture was heated at temperatures 30-40^{o}C for 1 hour and stirred constantly. Then the liquid extract was separated from insoluble fraction. The liquid extract then evaporated by rotary evaporator.

UFLC Analysis.

Isoflavone contents in sample was analysis using Ultra Fast Liquid Chromatography (UFLC) reversed phase C_{18} column (Shimadzu). Genistein standard diluted with methanol: water (8:2) with concentration 25 ppm, 20 ppm, 15 ppm, 10 ppm and 5 ppm. Therefore each of concentration performed 2 ml in UFLC to analysis. The sample injection volume was 10 µL. The mobile phase was acetonitrile : water (8:2), the temperature of the column was maintained at 40 ^{o}C, and the detection wavelength was set 255 nm, where absorbance peak areas were quantified. Extract fermented soybean as much as 3 mg in 10 ml methanol: water (8:2) performed in UFLC to analysis.
The identification of isoflavone was made by comparing the retention time with those of pure standards. Quantitative analysis was done by using regression linear. The standard curve were constructed individually by comparing the peak areas and concentration (y = a + bx). The result of sample extract analysis was plotting in to equation, it used to determine the concentration of isoflavone in the sample extract.

Cell Culture and Conditions.

The osteoblast cell from infant mouse[19] was maintained in sterile minimum essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), antibiotics (12.5 mg potassium penicillin G and 7.5 mg streptomycin sulfate) and 0,5625 g $NaHCO_3$, those supplements were homogenized in 250 ml MEM. Cell cultures were grown in flask at 37 ^{o}C in a humidified atmosphere with 5% CO_2 and subculture every 2-3 days[20].

Cell Treatment.

Well plates-96 (Iwaki) fill up cell and its medium, then incubated as long as 48 hour at 37 ^{o}C in a humidified atmosphere with 5% CO_2. In this assay, we used control, calcitonin and natrium flouride (NaF) as positive control (without FBS added), cell control (without sample and FBS added), medium control (without sample, FBS and cell added) and sample extract soybean treatment (1%, 0.5%, 0.25%, 0.125%) without FBS added.

MTT Cell Proliferation Assay.

Base on Zhang *et al*, 2007 with some modified; Cell viability was estimated according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, and Cell Proliferation Kit (MTT) (Roche) was used. All medium in well plates-96 was cleaned out, then added 50 µL MTT (5 mg /5 ml inphosfate buffer saline (PBS)) and incubated as long as 4 hours. MTT in well plates-96 was cleaned out and added 200 µL 0.01N HCl-isopropanol, homogenized. Cell proliferation was analyzed with ELISA reader at the wavelength 515 nm[20].

Statistical Analysis.

Every assay was repeated independently at least three times. All values are expressed as the mean ± standard deviation of three independent experiments. Difference between experimental groups was assessed by analysis of variance (ANOVA).

3. RESULT AND DISCUSSION

Extraction of Isoflavone Aglycone.

Isoflavone aglycone could be found if filtrate of soybean was fermented, because enzyme fermented from probiotic bacteria like a β-glycosidase will be solving glycoside binding. Soybean fermented result was extracted with ethyl acetate which modified using magnetic stirrer. We found extract in weight 344 mg.

Isoflavone Aglycone Analysis by UFLC.

Pure standard genistein was used as isoflavone aglycone to identification and calculating amount of genistein in sample extract soybean fermented. The result of linear regression, we found a, b and r value respectively: 201385, 96232.12 and 0.964.

Table 1. Genistein concentration and area from UFLC calculation

Sample	Concentration	Area (Y)
Genistein standard	25 ppm	2438419
	20 ppm	2195711
	15 ppm	1752267
	10 ppm	1414931
	5 ppm	423006

The genistein level of sample extract fermented soybean in the three replication is 50.61 µg/mg, 49.71 µg/mg and 49.40 µg/mg.

Table 2. Weight extract and level of genistein.

Weight extract (g)	Vol (ml)	Area (Y)	Level of genistein (µg/mg)	Avr (µg/mg)	% b/b
0,0032	10	1711386	50.61		
0,0032		1732603	49.71	49.90	4.99
0,0031		1723271	49.40		

Osteoblast Cell Proliferation.

Activity of cell proliferation could we see at the result of calculating viability cell. Sample extract in well plates-96 was measured of its absorbance by ELISA reader at wavelength 515 nm, then % viability calculated by equation:

$$\% \text{ Viability} = \frac{\text{Abs sample} - \text{Abs medium}}{\text{Abs cell} - \text{Abs medium}} \times 100\%$$

The result of calculating show that highest %viability at the 1% at value 30.3%, followed by concentration 0.5% and 0.25% at value 16.66%, and the last concentration 0.125% at value 12.12%.

Table 3. Absorbance of sample from ELISA reader.

Treatment	Abs1	Abs2	Abs3	Avr	% Viability
Cell	0.077	0.075	0.072	0.074	100
Naf	0.077	0.074	0.073	0.074	100
Calcitonin	0.075	0.074	0.072	0.073	95.45
1%	0.059	0.067	0.052	0.059	30.3
0.5%	0.053	0.057	0.059	0.056	16.66
0.25%	0.051	0.057	0.061	0.056	16.66
0.125%	0.057	0.057	0.052	0.055	12.12
Medium	0.043	0.057	0.056	0.055	0

Comparison standard was used has known could be increased osteoblast cell proliferation, that is NaF and Calcitonin which have % viability till 100%.

Percentage of cell viability as a sign of grade of cell proliferation. Increasingly of osteoblast cell proliferation, could be increasing bone mass, therefore as a preventive of osteoporosis. Basically isoflavone could be increased bone mass[21].

4. CONCLUSION

This research show that the level of genistein as isoflavone aglycone in extract fermented soybean by *Lactobacillus bulgaricus* average 4.99 % (b/b). The result of %viability calculation found the highest percentage at the 1% was 30.3% in value.

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