Anti-oxidative and protective effect of soy tempeh on di(2-ethylhexyl)-phthalate (DEHP) injured FL83B mice liver cells

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Abstract. The interest on Tempeh, a well known *Rhizopus oligosporus*-fermented food from Indonesia, is keep increasing as it possess potential bioactivities. Previous research on co-inoculation of *Rhizopus oligosporus* together with lactic acid bacteria in tempeh making showed promising results as lactic acid bacteria did not inhibit the growth of *Rhizopus oligosporus.* The aims of this study are to analyze the bioactivities (antioxidative and hepatoprotective activities) of single- and co-inoculated tempeh compared to pre-fermented soybean. Experiments was conducted by observing the effect of different lactic acid bacteria (*Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*) on antioxidant capacities. Afterwards, one of lactic acid bacteria strain will be used to produce tempeh and the protective activity against di(2-ethylhexyl)-phthalate (DEHP)-injured FL83B cell was observed. The result showed that *Lactobacillus plantarum* co-inoculated tempeh had the highest ferrous ion chelating activity, while *Lactobacillus casei* co-inoculated tempeh had the highest radical scavenging acticity and reducing power. Moreover, genistein and daidzein, aglycone isoflavone, found highest in *Lactobacillus bulgaricus* co-inoculated tempeh. For hepatoprotective activity, *Lactobacillus plantarum* co-inoculated and single-inoculated tempeh had protective activities against DEHP-injured FL83B cell, while soybean has no significant protective effect.

Keywords: tempeh, antioxidant capacities, hepatoprotective activity, phenolic compounds

1. Introduction

Tempeh is mold fermented beans, cereals, or other food processing by-products bound together by the mycelium of a mold, which is mostly *Rhizopus* spp [1]. Soy-tempeh is originated in central and east Java of Indonesia and now is most popular soy-protein food in Indonesia [2]. Beside of its unique flavor, soy-tempeh is well-known for its health beneficial effects. Soybean, without fermentation, has been reported to have potential roles in the prevention and treatment of cancer, heart disease,

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osteoporosis and menopausal symptoms [3-5]. Tempeh also has been found to lower blood cholesterol levels and inhibit copper induced human blood LDL oxidation [6-7]. The potential bioactivities of soybean and its derivative products are mainly contributed from their isoflavone, bioactive peptide, and oligosaccharide compounds [8-10] and fermentation can generate higher number of peptides and conversion of isoflavone glycoside into aglycones due to enzyme and acid hydrolysis [11-12].

Lactic acid bacteria is gram positive bacteria that can convert carbohydrate into lactic acid that is commonly used for fermentation. Lactic acid bacteria exert positive effects through bioactive compounds production and acting as probiotic [13]. Even that *Rhizopus oligosporus* can prevent the growth of other microorganisms; it can grow well together with lactic acid bacteria in tempeh production [14]. During fermentation with *R. oligosporus* for 24 h at 35^0 C, *Lactobacillus plantarum* showed the highest growth, while *Lactobacillus reuteri* and *Lactobacillus lactis* showed significantly slower growth during 24 h incubation at 35^0 C [14]. Besides, lactic acid bacteria can produce lactic acid to decrease the pH of the media condition, which is preferable in tempeh making to prevent the growth of *Bacillus*. The combined inoculation between *Rhizopus* and lactic acid bacteria is expected to increase the bioactivities of tempeh produced. Therefore, this study aims to compare the antioxidant and hepatoprotective activities of tempeh made from single- and co-inoculation with lactic acid bacteria.

2.Materials and Methods

2.1. Materials

Tempeh inoculum, *Rhizopus oligosporus*, was purchased from PT Aneka Fermentasi Industry, Bandung, Indonesia. Australian non-GMO (genetically modified organism) soybean was purchased from Ming-Li, Fengsan, Taiwan. Cell used was mice liver FL83B cell (ATCC: CRL-2390). All chemical compounds used were analytical grade.

2.2. Tempeh making

Tempeh was made by acid cooking method. About 200 g of soybean was soaked for 4 h and dehulled. After removing the hull, soybean was added into boiling water containing 1% lactic acid for 30 min. It was cooled down and added with 10^6 CFU/gram commercial *Rhizopus oligosporus* starter and 10^8 CFU/g lactic acid bacteria, which were *Lactobacillus plantarum* (*Lp*), *Lactobacillus fermentum* (*Lf*), *Lactobacillus acidophilus* (*La*), *Lactobacillus bulgaricus* (*Lb*), and *Lactobacillus casei* (*Lc*). Afterwards, soybean mixed with the starter and lactic acid bacteria were added to a perforated plastic bag and incubated at 30ºC for 2 days. After incubation, the tempehs were then freeze-dried, ground into powder and kept at -20ºC prior analysis.

2.3 Antioxidant capacities

Freeze-dried tempehs were first extracted with 1: 10 ratio of tempeh powder: double distilled water. ABTS or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity was carried out according to Re et al. [15]. Radical scavenging activity (%) was counted using the following equation: (1-(Absorbance of sample/Absorbance of control)) x 100%. EC_{50} value denotes the effective concentration required to induce 50% of radical scavenging ability. Reducing power was carried out by following the method described by Canabady-Rochelle et al. [16]. EC_{50} of reducing power denotes minimum concentration to reach the absorbance of 0.5. The ferrous ion chelating ability was determined according to the method of Huang et al. [17]. The capability of the sample to chelate the ferrous iron was calculated from: Chelating effect (%)=(1-(Absorbance of sample/ Absorbance of control))×100. EC_{50} value denotes the effective concentration required to induce 50% of chelating ability.

2.4. Isoflavone compounds determination

Freeze-dried tempeh was extracted with hexane thrice to remove the oil and further extracted with 80% methanol thrice to extract the isoflavone compounds. The HPLC system used (Hitachi, Tokyo, Japan) consisted of a Mightysil RP-18 GP (4.6 x 250 mm) column (Kanto Chemical Co. Inc., Tokyo, Japan), a Chromaster 5210 autosampler, Chromaster 5110 pump, and a Chromaster 5430 diode array detector, and set at 40ºC. The contents of isoflavones were determined by high performance liquid chromatography (HPLC) as previously described [18].

2.5. Hepatoprotective capacity

For hepatoprotective activity, DEHP, di(2-ethylhexyl)-phthalate, is used as agent to induce liver injury. DEHP is the phthalate representative with the highest toxicity, which dose-related adverse effects of DEHP were found in liver, kidney, thyroid gland, male development and reproductive tissues [19]. Human can be easily exposed to DEHP though plastic application on product for daily purposes and through environment. Cell culture approach was used to observe the impact of the tempeh isoflavone as protective agent against DEHP injury in FL83B liver cell.

Freeze-dried tempeh was extracted with hexane thrice to remove the oil and further extracted with 80% methanol thrice to extract the isoflavone compounds. The extract was then freeze dried. FL83B cells were seeded in a 96 well-plate at concentration of 1×10^4 cells/well and incubated at 37°C for 24 h before treatment to let the cell adhere to the well-plate. Afterwards, cells were treated with various concentrations of isoflavone extract (0-100 mg/ml) and 24 h later, the medium was subsequently washed out and replaced with a fresh medium (for non-injured treatment) and 536 µM DEHP containing medium that is expected to cause cell injury to 50% reduction in cell viability after 6 h exposure [20]. MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to assess the cell viability. Media-removed cells were added with 10 µL of MTT solution (5 mg/ml) and 100 µL medium. The reaction between cell and MTT was conducted at 37°C for 4 h. The crystal formed was then dissolved in 100 µL of DMSO for 10 min. Microplate spectrophotometer (Bio-Tek, VT, USA) was used to measure the absorbance of the lysate in each well at 570 nm.

2.6. Statistical analysis

Values were expressed as mean ± standard deviation (SD) from three replications. Statistical analysis was done by one way analysis of variance (ANOVA) followed by post hoc testing (Duncan's test) at $p<0.05$ by using SPSS (Statistical Package for Social Sciences) version 21.

3. Results and Discussion

This study used five different strains of lactic acid bacteria to be co-inoculated with *Rhizopus*, which were *L. plantarum, L. fermentum, L. acidophilus, L. bulgaricus, L. casei*. After 2 days of incubation at 30ºC, soy tempeh fully grown with mold were collected. The appearances of the soy tempeh is shown in figure 1. There was no differences in the appearance of single or co-inoculated soy tempeh, which indicated that lactic acid bacteria co-inoculation did not prevent the *Rhizopus* growth, which is the main microorganism to make tempeh. These soy tempeh were further freeze dried and ground into powder for extraction and analysis.



Figure 1. The appearance of single- and co-inoculated soy tempeh. *R* refers to *Rhizopus*, *Lp* refers to *Lactobacillus plantarum*; *Lf* refers to *Lactobacillus fermentum*; *La* refers to *Lactobacillus acidophilus*; *Lb* refers to *Lactobacillus bulgaricus*; and *Lc* refers to *Lactobacillus casei.*

The impact of lactic acid bacteria on co-inoculated soy tempeh was observed through antioxidant analyses. Antioxidant analyses conducted were ABTS radical scavenging activity, reducing power and ferrous ion chelating activity and the results of antioxidant analyses were represented as EC_{50} values (table 1). EC_{50} represents the concentration needed to reach 50% of the total antioxidant activities. The lower the EC_{50} value, the higher the antioxidant capacity. The results showed that ABTS radical scavenging activity and reducing power had almost the same tendency, but ferrous ion chelating activity was different. Pre-fermented soybean showed the lowest antioxidant capacity among all the antioxidant analyses, which resulted the EC_{50} values of pre-fermented soybean to be too high for determination. In other words, the results showed that *Rhizopus* fermentation caused a high increase in antioxidant activity of soybean. All strains of lactic acid bacteria used showed to increase the antioxidant capacity of *Rhizopus* only fermented tempeh. *L. casei* co-inoculated soy tempeh had the highest antioxidant capacity in ABTS radical scavenging activity (35.038±1.060 mg/ml) and reducing power (11.800±0.341 mg/ml). Moreover, *L. plantarum* co-inoculated soy tempeh had the highest antioxidant capacity in ferrous ion chelating activity (3.715±0.094 mg/ml)

Table 1. EC_{50} (mg/ml) of antioxidant capacities of single- and co-inoculated soy tempeh compared to pre-fermented soybean.

Sample*	EC_{50} of ABTS radical scavenging activity	EC_{50} of reducing power[2]	EC_{50} of ferrous ion chelating activity
Pre-fermented soybean	>250	>50	9.97 ± 0.56^{a}
R tempeh	49.80 ± 3.16^{ab}	13.22 ± 0.12^{c}	9.38 ± 0.34^{b}
R+Lp tempeh	54.12 ± 9.77^{a}	14.11 ± 0.97^{bc}	3.72 ± 0.09^{g}
R+Lf tempeh	43.65 ± 1.01^{b}	17.71 ± 0.36^{a}	4.60 ± 0.08^{f}
R+La tempeh	45.24 ± 2.64^{b}	14.85 ± 0.40^{b}	6.51 ± 0.04^{d}
R+Lb tempeh	41.63 ± 1.07^{bc}	14.42 ± 0.54^{b}	5.25 ± 0.08^{e}
R+Lc tempeh	35.04 ± 1.06^{c}	11.80 ± 0.34^{d}	7.35 ± 0.09^{c}

**R* refer to *Rhizopus*; *Lp* refers to *Lactobacillus plantarum*; *Lf* refers to *Lactobacillus fermentum*; *La* refers to *Lactobacillus acidophilus*; *Lb* refers to *Lactobacillus bulgaricus*; and *Lc* refers to *Lactobacillus casei*
[a-g]Means in each column with the same letter are not significantly different ($p>0.05$)

This study also determined the content of daidzin (daidzein 7-O-glucoside), daidzein (7,4'-dihydroxyisoflavone), genistin (genistein 7-O-glucoside), genistein (5,7,4'-trihydroxyisoflavone), the most common isoflavone found in soybean. There was significant increase in daidzein and genistein, followed by significant decrease in daidzin and genistin compared to pre-fermented soybean (table 2). Lactic acid bacteria co-inoculated tempeh also showed higher aglycone (daidzein and genistein) compared to single-inoculated tempeh (*R* tempeh). The highest increase of daidzein and genistein was found in *L. bulgaricus* co-inoculated tempeh. This finding is supported by Murakami et al. [21] results which found that the major isoflavones in tempeh were genistein and daidzein, produced by digesting genistein and daidzein, respectively, with β-glucosidase during *R. oligospous* fermentation. In addition, lactic acid bacteria also can produce β-glucosidase which cause an increase in aglycone isoflavones of co-inoculated tempeh [22]. Genistein is the most biologically active isoflavone in the soy diet and it can be taken up by cells with-out prior metabolic activation to exert its effects [23]. Combination of daidzein and genistein has been reported to cause synergistic preventive effects against prostate cancer [24]. Through fermentation, the amount of genistein and daidzein increase, which increase the bioactivities of soybean.

Tabel 2. Isoflavone profile of single- and co-inoculated soy tempeh compared to pre-fermented soybean.

Sample*	Glycosides content (µg/g dried sample)		Aglycones (µg/g dried sample)	
	Daidzin	Genistin	Daidzein	Genistein
Pre-fermented soybean	349.19±8.00^{a}	684.16±3.86^{a}	392.95±2.09^{c}	465.44±1.88^{d}
R tempeh	43.21±3.95^{c}	184.22±15.99^{c}	612.16±9.98^{b}	735.11±11.13^{c}
R+*Lp* tempeh	60.25±1.36^{b}	199.86±2.70^{b}	613.84±2.04^{b}	735.38±7.07^{c}
R+*Lf* tempeh	55.10±1.00^{b}	200.00±2.15^{b}	636.55±11.68^{a}	770.86±12.42^{a}
R+*La* tempeh	45.09±1.51^{c}	176.57±1.57^{c}	634.01±3.18^{a}	756.14±8.18^{a,b}
R+*Lb* tempeh	47.81±2.46^{c}	183.06±6.66^{c}	642.89±12.24^{a}	773.76±8.38^{a}
R+*Lc* tempeh	60.03±2.51^{c}	208.46±8.15^{b}	638.52±16.79^{a}	750.15±15.39^{b,c}

**R* refer to *Rhizopus*; *Lp* refers to *Lactobacillus plantarum*; *Lf* refers to *Lactobacillus fermentum*; *La* refers to *Lactobacillus acidophilus*; *Lb* refers to *Lactobacillus bulgaricus*; and *Lc* refers to *Lactobacillus casei*
[a-g]Means in each column with the same letter are not significantly different (p>0.05)

For hepatoprotective activity, only *L. plantarum* was chosen for observing its protective activity against DEHP injury in FL83B cell. Pre-fermented soybean, single-inoculated soy tempeh and *L. plantarum* co-inoculated soy tempeh did not cause any decrease in the cell viability of FL83B cells (figure 2). DEHP caused a decrease in cell viability of FL83B cells; however the cell viability increased on the cells pretreated with single-inoculated soy tempeh and co-inoculated soy tempeh, but not with pre-fermented soybean. It represents that single- and co-inoculated soy tempeh possessed hepatoprotective activity, which indicated that fermentation resulted in the hepatoprotective activity of the soybean. The highest protective activity was found in *L. plantarum* co-inoculated soy tempeh, but its difference with single-inoculated tempeh was not significant. In conclusion, even there are significant differences between antioxidative capacities of co-inoculated tempeh to single-inoculated tempeh, their effect on hepatoprotective against DEHP injury showed no significant difference. This result is supported from previous results reported some antioxidant compounds have showed no hepatoprotective activity against DEHP induced liver injury [25-27]. Based on the findings, this study reveals the potential of tempeh as a promising functional foods.

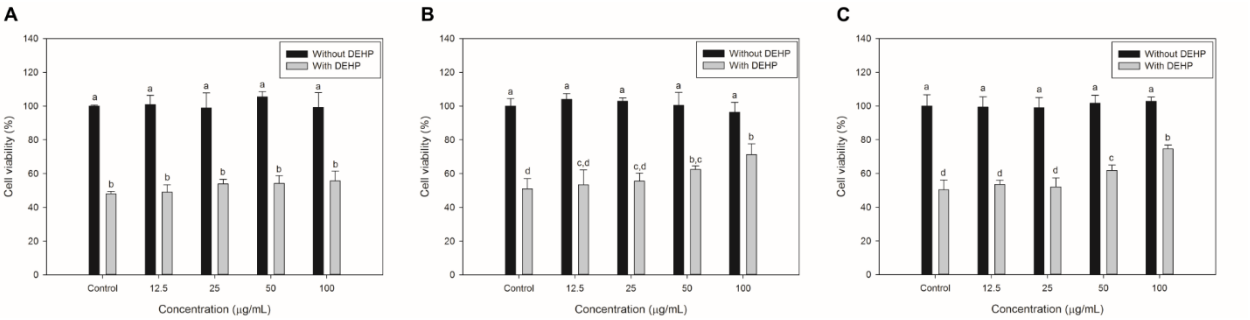

Figure 2. FL83B cell viability after 24 h incubation of pre-fermented soybean (A), single-inoculated soy tempeh (B), and *Lactobacillus plantarum* co-inoculated soy tempeh (C) followed by DEHP exposure for 6 h. [a-d]Data with different letters was significantly different at $p<0.05$ (n=3) analyzed by analysis of variance (ANOVA) with Duncan's post-hoc.

References

[1] Nout M J R and Kiers J L 2005 Tempe fermentation, innovation and functionality: Update into the third millenium *J. Appl. Microbiol.* **98**(4) 789-805
[2] Astuti M, Meliala A, Dalais F S and Wahlqvist M L 2000 Tempe, a nutritious and healthy food from Indonesia *Asia Pac. J. Clin. Nutr.* **9** 322-325
[3] Kennedy A R 1995 The evidence for soybean products as cancer preventive agents *J. Nutr.* **125** 733S–743S
[4] Messina M 1995 Modern applications for an ancient bean: soybeans and the prevention and treatment of chronic disease *J. Nutr.* **125**(suppl_3) 567S-569S
[5] Liu K 2000 Expanding soybean food utilization *Food Technol.* **54** 46–58
[6] Guermani L, Villaume C, Bau H M, Nicolas J P and Mejean L 1993. Modification of soyprotein hypocholesterolemic effect after fermentation by *Rhizopus oligosporus* spT3. *Sci. Aliment.* **13** 317–324
[7] Karyadi D and Lukito W 1996 Beneficial effects of tempeh in disease prevention and treatment *Nutr. Rev.* **54** S94–S98
[8] Messina 2006 *Soy application in Food* ed M N Riaz (Boca Raton: CRC press) p 23-38
[9] Wang W and Mejia E G 2005 A new frontier in soy bioactive peptides that may prevent age-related chronic diseases *Compr. Rev. Food Sci. F.* **4** 63-78
[10] Espinosa-Martos I and Rupérez P 2006. Soybean oligosaccharides: Potential as new ingredients in functional food. *Nutr. Hosp.* **21**(1) 92-96
[11] Okamoto A, Hanagata H, Kawamura Y and Yanagida F 1995. Anti-hypertensive substances in fermented soybean, natto. *Plant Food Hum. Nutr.* **47** 39–47
[12] McCue P and Shetty K 2003 Role of carbohydrate-cleaving enzymes in phenolic antioxidant mobilization from whole soybean fermented with *Rhizopus oligosporus Food Biotechnol.* **17** 27–37
[13] Teneva-Angelova T, Hristova I, Pavlov A and Beshkova D 2018 *Advances in Biotechnology for Food Industry* ed A M Holban and A M Grumezescu (London: Academic Press) p 91-133
[14] Feng X, Erikson A R B and Schnurer J 2005 Growth of lactic acid bacteria and Rhizopus oligosporus during barley tempeh fermentation *Int. J. Food Microbiol.* **104** 249– 256
[15] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C 1999 Antioxidant activity applying an improved ABTS radical cation decolorization assay *Free Radic. Biol. Med.* **26**(9) 1231-1237
[16] Canabady-Rochelle L L, Harscoat-Schiavo C, Kessler V, Aymes A, Fournier F and Girardet J M 2015 Determination of reducing power and metal chelating ability of antioxidant peptides: Revisited methods *Food Chem.* **183** 129-135
[17] Huang G J, Chiu C S, Wu C H, Huang S S, Hou W C, Amegaya S, Sheu M J, Liao J C and Lin Y H 2010 Redox status of Bowman-Birk inhibitor from soybean influence its in vitro antioxidant activities *Bot. Stud.* **51** 431-437
[18] Yang S, Wang L, Yan Q, Jiang Z and Li L 2009 Hydrolysis of soybean isoflavone glycosides by a thermostable β-glucosidase from *Paecilomyces thermophila Food Chem.* **115**(4) 1247-1252
[19] Heudorf U, Mersch-Sundermann V and Angerer J 2007 Phthalates: Toxicology and Exposure. *Int J. Hyg. Envir. Heal.* **210** 623-634

[20] Lo D, Wang Y T and Wu M C 2014 Hepatoprotective effect of silymarin on di (2-ethylhexyl) phthalate (DEHP) induced injury in liver FL83B cells. *Environ. Toxicol. Pharmacol.* **38**(1) 112-118
[21] Murakami H, Asakawa T, Terao J and Matsushita S 1984 Antioxidative stability of tempeh and liberation of isoflavones by fermentation. *Agric. Biol. Chem.* **48**(12) 2971-2975
[22] Chien H-L, Huang H-Y, and Chou C-C 2006 Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. *Food Microbiol.* **23**(8) 772-778
[23] Takimoto C H, Glover K, Huang X., Hayes S A, Gallot L, Quinn M, Jovanovic B D, Shapiro A, Hernandez L, Goetz A, Llorens V, Lieberman R, Crowell J A, Poisson B A and Bergan R C 2003 Phase I pharmacokinetic and pharmacodynamic analysis of unconjugated soy isoflavones administered to individuals with cancer *Cancer Epidemiol. Biomarkers Prev.*. **12**(11) 1213-1221
[24] Dong X, Xu W, Sikes R A and Wu C 2013 Combination of low dose of genistein and daidzein has synergistic preventive effects on isogenic human prostate cancer cells when compared with individual soy isoflavone *Food Chem.* **141**(3) 1923-1933
[25] Oishi S 1994 Prevention of di(2-ethylhexyl)phthalate-induced testicular atrophy in rats by co-administration of the vitamin B12 derivative adenosyl cobalamin. *Arch. Environ. Contam.Toxicol.* **26**(4) 497–503
[26] Botelho G G, Bufalo A C, Boareto A C, Muller J C, Morais R N, Martino-Andrade A J, Lemos K R and Dalsenter P R 2009 Vitamin C and resveratrol supplementation to rat dams treated with di(2-ethylhexyl)phthalate: Impact on reproductive and oxidative stress end points in male offspring *Arch. Environ. Contam. Toxicol.* **57**(4) 785–793
[27] Abeer A K M, Nermine N, Mahmoud S K and Mona K T 2007 Histological and electron microscopic study of the postulatedprotective role of green tea against DEHP liver toxicity in mice *Afr. J. Health Sci.* **14** 19–36