Tempeh Extract Protects HepG2 Cells Against Oxidative Stress-Induced Cell Death

Reggie Surya* and Andreas Romulo
Food Technology Department, Faculty of Engineering, Bina Nusantara University, Jakarta 11480, Indonesia
reggie.surya@binus.edu

Abstract. Tempeh is an Indonesian fermented traditional food made from soybeans inoculated with \textit{Rhizopus oryzae} and/or \textit{Rhizopus oligosporus}. It exerts antioxidant activities mainly due to the presence of bioactive compounds known as isoflavones. We previously observed that tempeh ethanol extract increased the basal expression of some cellular antioxidant enzymes including catalase and superoxide dismutases in HepG2 cells in vitro. In this study, we showed that pretreatment with tempeh extract protected HepG2 cells from oxidative stress triggered by TBHP (tert-butyl hydroperoxide) by reducing cellular ROS (reactive oxygen species) generation, TBARS (thiobarbituric acid reactive species) levels, caspase-3 activity and eventually cell death. We hypothesize that the resistance of cells pretreated with tempeh extract towards the toxicity of TBHP was in part due to the upregulation of cellular antioxidant enzymes. Taken together, our findings highlight the antioxidative potentials of tempeh, particularly in an attempt to develop tempeh as a functional food offering health-related benefits to humans.

Keywords : tempeh, soybean, isoflavone, antioxidant, oxidative stress

1. Introduction
Tempeh is an Indonesian traditional food made from soybeans undergoing mold fermentation involving \textit{Rhizopus oryzae} and/or \textit{Rhizopus oligosporus}. It is commonly consumed as a plant-based protein source with healthful fats. Compared to unfermented soybeans, tempeh possesses more interesting nutritional values. Nutrients in tempeh are more bioavailable compared to unfermented soybeans due to mold enzymatic degradation of macronutrients (such as carbohydrates, fats and proteins) and antinutritional factors (such as hemagglutinins, saponins and phytic acid) during fermentation [1]. Tempeh also contains vitamin B12 that is generally found solely in animal-based foodstuff. Such a vitamin is formed \textit{de novo} through bacterial activities during tempeh fermentation [2]. Moreover, tempeh has been shown to exhibit higher antioxidant activities compared to unfermented soybeans [3-7].

Oxidative stress is defined as an imbalance between oxidative and antioxidative systems in cells. It is a result of the over production of free radicals and associated reactive oxygen species (ROS) [8]. High levels of ROS may lead to cellular damage, cell senescence, cell death, and, to a broader extent, diseases including chronic inflammation and cancer [9]. Antioxidants are compounds with functionalities to inhibit oxidation and reduce the cellular levels of ROS, thus minimizing oxidative stress. Tempeh is known to exert antioxidant activities, mainly due to the presence of bioactive compounds known as isoflavones, apart from other molecules such as short-chained peptides, free amino acids and 3-hydroxyanthranilic acid (HAA) [3, 10-11]. Isoflavones have been linked to the prevention of diseases including cancer, hypercholesterolemia, atherosclerosis, cardiovascular disease,
osteoporosis and relief of menopausal symptoms in certain women [12]. Owing to such phytochemicals, tempeh is believed to provide health-related benefits to humans [13]. In unfermented soybeans, isoflavones are mostly present in conjugated forms. During fermentation, enzyme beta-glucosidase secreted by molds catalyzes the hydrolysis of conjugated isoflavones, thus resulting in the formation of free isoflavones (known as aglycones) with higher bioavailability and antioxidant activities [14].

There are limited studies regarding antioxidant activities of tempeh extract and its cytoprotective effects in cellular culture. Nevertheless, in vitro approach is primordial to study the mechanisms and the potentials of a certain compound prior to performing further in vivo studies. In our laboratory, we previously observed a significant increase in the expression of cellular antioxidant enzymes including catalase and superoxide dismutases in HepG2 cells treated with 0.7%(w/v) tempeh extract for 12 h. Therefore, to follow-up these findings, we designed this study aiming to investigate whether a 12 h pretreatment with tempeh extract would protect HepG2 cells from oxidative stress generated by tert-butyl hydroperoxide (TBHP).

2. Materials and Methods

2.1. Sample Preparation

Tempeh was produced as previously described [15] with some modifications. Briefly, boiled soybeans (var Anjasmoro, harvest age 85 days, produced by UD Sumber Makmur, Nganjuk, Indonesia) were acidified through the addition of white vinegar (5 ppm) and inoculated with tempeh starter containing a spore mixture of R. oryzae and R. oligosporus (brand Ungkul, produced by PD Sukma Jaya, Tegal, Indonesia, 5g/kg soybean). Afterwards, the inoculated soybeans were wrapped in holed plastic bags and left fermented at room temperature (25°C) for 36 h to produce raw tempeh. To make tempeh ethanol extract, tempeh was cut into small cubes (2x2x2 cm³) and mixed with ethanol 95% with a ratio of 1:3 (w/v) using a blender, resulting in puree. The puree was then filtered with a cheese cloth to separate the pulp from the filtrate. The filtrate was collected and kept in a freezer (-20°C) for further analyses.

2.2. Cell Culture and Treatments

HepG2 cells purchased from American Type Culture Collection (ATCC, USA) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B at 37°C in a humidified incubator with 5% CO₂. During the actual experiments using tempeh extract, dilution was done in supplemented DMEM but without FBS to prevent any potential interaction with the tested molecules. Tempeh extract was diluted in supplemented DMEM with a ratio of 1:50 (final dilution 150x) before being exposed to cells. Our preliminary toxicity experiments showed that 2% ethanol was not toxic for HepG2 cells even after a 24 h of exposure. Tert-butyl hydroperoxide (TBHP, Sigma Aldrich, 500 µM), was used to provoke cellular oxidative stress by inducing the formation of ROS (reactive oxygen species). Prior to treatments, cells were incubated in medium containing tempeh extract for 12 h if the tested solution contained tempeh extract.

2.3. Cellular ROS, Cellular TBARS, Caspase-3 Activity and Cell Viability Assay

All assays were performed by fluorimetry according to the instructions of their respective manufacturer using a fluorescence spectrometer (VersaFluor, Bio-Rad Laboratories, CA, USA). Cellular ROS were quantified with a fluorogenic dye DCFDA/H2DCFDA-Cellular ROS detection assay kit (Abcam). Cellular TBARS (thiobarbituric acid reactive substances) were analyzed with OxiSelect TBARS assay kit (Cell Biolabs). The analysis of caspase-3 activity was done using caspase-3 activity assay kit containing a fluorogenic substrate Ac-DEVD-AMC (Cell Signaling Technology). Cell viability was quantified with resazurin cell viability kit (Cell Signaling Technology). The percentage of viable cells was determined by dividing the RFU (relative fluorescence unit) of the corresponding sample with the RFU of control (untreated cells at t=0 h).
2.4. Statistical Analysis
Data (n=3) were analyzed by one-way ANOVA followed by Dunnett’s test in case of significant differences (p<0.05) using software Systat 10 software for Windows. All data were reported as mean±SD.

3. Results and Discussion
In this study, we assessed in vitro potentials of tempeh extract for attenuating detrimental effects of oxidative stress induced by TBHP, an oxidant mediating oxidative stress by generating ROS and causing eventually cell death [16]. The concentration of TBHP applied to HepG2 cells in this study was 500 µM according to previous study conducted using the same cell line [17]. HepG2 cell systems are considered as a good tool for studying cytoprotective mechanisms of antioxidants and phytochemicals in vitro without using laboratory animals since the cells secrete many phase I, II and other antioxidant enzymes [18]. We extracted tempeh using ethanol 95% since it was previously reported that tempeh ethanol extract exhibited higher antioxidant activities compared to tempeh extracts in other nonpolar solvents such as ether, petroleum ether and hexane [4].

Cellular oxidative stress was quantified by analyzing cellular ROS generation and thiobarbituric acid reactive species (TBARS) levels. TBARS are the end products resulting from the oxidation of unsaturated fatty acids (lipid peroxidation), mostly in the form of malondialdehydes (MDA). Higher cellular TBARS levels correspond to higher oxidative stress [19]. Cell death as the end point of cellular responses towards damage caused by oxidative stress was evaluated. As presented in Figure 1, exposure to TBHP led to a sharp increase in cellular ROS (Figure 1A) that contributed to high cellular levels of TBARS (Figure 1B) as final products of lipid peroxidation. Interestingly, such a rise in cellular ROS and TBARS ensued to a much lesser extent when cells had been pretreated with tempeh extract for 12 h prior to exposure to TBHP, thus highlighting the protective potentials of tempeh extract with regard to TBHP-induced oxidative stress. We hypothesized two mechanisms to explain such potentials. Firstly, antioxidants present in tempeh extract could directly scavenge ROS generated by TBHP. Secondly, we previously observed the upregulation of several cellular antioxidant enzymes including catalase and superoxide dismutases in HepG2 cells treated with tempeh extract. The higher levels of these antioxidant enzymes would provide the cells with a stronger capacity to suppress TBHP-induced oxidative stress.
Apoptosis is a programmed cell death that is considered as a vital component in normal cell turnover, immune system, embryonic development and cellular responses following exposures towards chemicals [20]. It is characterized by the activation of caspase-3, a protease responsible for executing apoptotic signaling by catalyzing the specific cleavage of DNA and many key cellular proteins [21]. Exposure to TBHP has been reported to induce apoptotic cell death in HepG2 cells [17, 22], as confirmed in Figure 2A showing a drastic increase in caspase-3 activity upon exposure to TBHP. Pretreatment with tempeh extract for 12 h prior to exposure to TBHP reduced the activation of caspase-3 by approximately a half in a significant way. In accordance with the caspase-3 activity in Figure 2A, Figure 2B shows that cells pretreated with tempeh extract exhibited higher survival upon exposure to TBHP compared to those without pretreatment with tempeh extract.
Figure 2. Relative caspase-3 activity (A) and cell viability (B) recorded over time following exposure to TBHP (500 μM) in HepG2 cells pretreated or not with tempeh extract (dilution 150x). Data (n=3) are reported as mean±SD. *) Significant difference with cells treated with TBHP (p<0.05).

Taken together, our findings conveyed the potentials of tempeh in tackling cellular oxidative stress. Indeed, oxidative stress has been related to various diseases including cardiovascular diseases, chronic obstructive pulmonary disease, chronic kidney disease, neurodegenerative diseases and even cancer [23]. Thus, these findings might support the development of tempeh as a functional food offering beneficial properties to human health. With regard to the antioxidative properties of tempeh, previous in vivo studies have shown that tempeh consumption could reduce blood pressure in spontaneously hypertensive rats [24], protect against alcohol-induced liver damage in mice [25], prevent atherosclerosis in rats when combined with carrot [26] and improve cognitive functions in senescence-accelerated rats [27]. The mechanisms by which antioxidants in tempeh can exert health-related beneficial properties are not fully understood. However, such mechanisms are likely to involve nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant response since this response can be modulated by isoflavones [28]. In vivo, hippocampal Nrf2 levels were shown to be upregulated in tempeh-fed rats through the reduction of p-p38 and p-JNK expression [27].

4. Conclusions
To conclude, our results show that pretreatment with tempeh ethanol extract strongly protects HepG2 cells against TBHP-induced oxidative stress by reducing ROS generation, TBARS formation, caspase-3 activity and, eventually, cell death. We suggest that such protective effects would be due to the upregulation of cellular antioxidant enzymes mediated by tempeh extract during pretreatment, as observed in our previous study. As a whole, our findings provide a novel insight into developing tempeh as a functional food offering potential health-related benefits to humans beyond nutritional benefits.

References
[1] Astuti M, Meliala A, Dalais F S and Wahlqvist M L 2000 Tempe, a nutritious and healthy food from Indonesia Asia Pacific Journal of Clinical Nutrition 9(4) 322-325
[2] Handoyo T and Morita N 2006 Structural and functional properties of fermented soybean (tempeh) by using Rhizopus oligosporus International Journal of Food Properties 9(2) 347-355
[3] Watanabe N, Fujimoto K and Aoki H 2007 Antioxidant activities of the water-soluble fraction in tempeh-like fermented soybean (GABA-tempeh) International Journal of Food Sciences and Nutrition 58(8) 577-587

[4] Chang C T, Hsu C K, Chou S T, Chen Y C, Huang F S and Chung Y C 2009 Effect of fermentation time on the antioxidative activities of tempeh prepared from fermented soybean using Rhizopus oligosporus International Journal of Food Science and Technology 44(4) 799-806

[5] Nakajima N, Nozaki N, Ishihara K, Ishikawa A and Tsuji H 2005 Analysis of isoflavone content in tempeh, a fermented soybean, and preparation of a new isoflavone-enriched tempeh Journal of Bioscience and Bioengineering 100(6) 685-687

[6] Kuligowski M, Pawłowska K, Jasinska-Kuligowska I and Nowak J 2017 Isoflavone composition, polyphenols content and antioxidative activity of soybean seeds during tempeh fermentation CyTA-Journal of Food 15(1) 27-33

[7] Lo D, Rawendra R D, Huang C S, Khatri-Chhetri R, Wang Y T and Wu M C 2018 Antioxidative and protective effect of soy tempeh on di (2-ethylhexyl)-phthalate (DEHP) injured FL83B mice liver cells, Jakarta, Indonesia IOP Conference Series: Earth and Environmental Science 195 012058

[8] Newsholme P, Cruzat V F, Keane K N, Carlessi R and de Bittencourt Jr P I H 2016 Molecular mechanisms of ROS production and oxidative stress in diabetes Biochemical Journal 473(24) 4527-4550

[9] Kowaltowski A J, de Souza-Pinto N C, Castilho R F and Vercesi A E 2009 Mitochondria and reactive oxygen species Free Radical Biology and Medicine 47(4) 333-343

[10] Esaki H, Onozaki H, Kawakishi S and Osawa T 1996 New antioxidant isolated from tempeh Journal of Agricultural and Food Chemistry 44(3) 696-700

[11] Matsuo M, Nakamura N, Shidoji Y, Muto Y, Esaki H and Osawa T 1997 Antioxidative mechanism and apoptosis induction by 3-hydroxyanthranilic acid, an antioxidant in Indonesian food Tempeh, in the human hepatoma-derived cell line, HuH-7 Journal of Nutritional Science and Vitaminology 43(2) 249-259

[12] Messina M 2016 Soy and health update: evaluation of the clinical and epidemiologic literature Nutrients 8(12) 754

[13] Karyadi D and Lukito W 1996 Beneficial effects of tempeh in disease prevention and treatment Nutrition Reviews 54(11) S94-S98

[14] Izumi T, Piskula M K, Osawa S, Obata A, Tobe K, Saito M and Kikuchi M 2000 Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans The Journal of Nutrition 130(7) 1695-1699

[15] Surya R and Rahayu W P 2012 Production and characteristics of canned tempe extract Asian Journal of Food and Agro-Industry 5(4) 299-306

[16] Anand T and Khanum F 2018 Attenuation of cytotoxicity induced by tbhp in h9c2 cells by bacopa monniera and bacoside a Pathophysiology 25(2) 143-149

[17] Yoon J, Ham H, Sung J, Kim Y, Choi Y, Lee J S and Kim D 2014 Black rice extract protected HepG2 cells from oxidative stress-induced cell death via ERK1/2 and Akt activation Nutrition Research and Practice 8(2) 125-131

[18] Mersch-Sundermann V, Knasmüller S, Wu X J, Darroudi F and Kassie F 2004 Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents Toxicology 198(1-3) 329-340

[19] Ghani M A, Barril C, Bedgood Jr D R and Prenzler P D 2017 Measurement of antioxidant activity with the thiobarbituric acid reactive substances assay Food Chemistry 230 195-207

[20] Elmore S 2007 Apoptosis: a review of programmed cell death Toxicologic Pathology 35(4) 495-516

[21] Porter A G and Jänicke R U 1999 Emerging roles of caspase-3 in apoptosis Cell Death and Differentiation 6(2) 99-104
[22] Piret J P, Arnould T, Fuks B, Chatelain P, Remacle J and Michiels C 2004 Mitochondria permeability transition-dependent tert-butyl hydroperoxide-induced apoptosis in hepatoma HepG2 cells Biochemical Pharmacology 67(4) 611-620

[23] Liguori I, Russo G, Curcio F, Bulli G, Aran L, Della-Morte D and Abete P 2018 Oxidative stress, aging, and diseases Clinical Interventions in Aging 13 757

[24] Aoki H, Furuya Y, Endo Y and Fujimoto K 2003 Effect of γ-aminobutyric acid-enriched tempeh-like fermented soybean (GABA-tempeh) on the blood pressure of spontaneously hypertensive rats Bioscience, Biotechnology, and Biochemistry 67(8) 1806-1808

[25] Yusof M H, Ali N M, Yeap S K, Ho W Y, Beh B K, Koh S P, Long K, Aziz S A and Alitheen N B 2013 Hepatoprotective effect of fermented soybean (nutrient enriched soybean tempeh) against alcohol-induced liver damage in mice Evidence-Based Complementary and Alternative Medicine 2013 1-8.

[26] Ari-Agung I G A, Suryadhi N T, Mantik N A, Suter I K and Partama I B G 2013 Combination of tempeh and carrot prevent atherosclerosis wistar rat: indicated by increase of HDL and total antioxidant, decrease LDL, F2-isoprostan, and IL-6 Indonesia Journal of Biomedical Science 7(1) 30-36

[27] Chan Y C, Lee I T, Wang M F, Yeh W C and Liang B C 2018 Tempeh attenuates cognitive deficit, antioxidant imbalance, and amyloid β of senescence-accelerated mice by modulating Nrf2 expression via MAPK pathway Journal of Functional Foods 50 112-119

[28] Liang F, Cao W, Huang Y, Fang Y, Cheng Y, Pan S and Xu X 2019 Isoflavone biochanin A, a novel nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element activator, protects against oxidative damage in HepG2 cells Biofactors 45(4) 563-574