

Full Length Research Paper

The effect of black soybean tempe and its ethanol extract on lymphocyte proliferation and IgA secretion in *Salmonella typhimurium* induced rat

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Children patient suffering from malnutrition and chronic diarrhea, who had been treated with tempe formula improved their health status, recovered from the diarrhea in a relatively shorter period, and gained weight. It is perceived that the quick recovery was due to improvement of the body's immune system. The aim of the research was to observe the effect of black soybean tempe and its ethanol extract on T and B cells proliferation and level of secretory IgA (sIgA) in rat induced with *Salmonella typhimurium*. A total of 48 rats were divided into four groups, 12 rats each assigned to standard diet, diet plus black soybean tempe flour, diet plus tempe ethanol extract, and diet plus mixture from tempe ethanol extract and flour of black soybean tempe. Body weights were recorded during 35 days of diet treatment, and index stimulation on T and B cells as well as level of secretory IgA were measured after *S. typhimurium* induction. Result indicated that diet with tempe, diet with ethanol extract and combination of both increased body weight better than that of control. Treatments with tempe and mixture of tempe with its ethanol extract were significantly different from the control in the proliferation of T cell; however, the treatment with tempe ethanol extract did not. The three treatments did not improved proliferation of B cell. Diet with tempe alone significantly increased secretory IgA in the intestinal tract fluid of rats, but the other treatments did not show significant difference with the control (without *Salmonella* induction).

Key words: Black soybean tempe, immune response, lymphocyte, secretory IgA.

INTRODUCTION

Tempe, an Indonesian traditional food, made from soybeans through fermentation with *Rhizopus* fungi, is respected as a high quality protein food derived from plant origin, excellent digestibility, low in antinutrition factors, such as phytic acid, oligosaccharides, and good source of complex of vitamin B (Suparmo and Markakis, 1987). The traditional food is regarded as health food due to its content of several antioxidants, such as genestein, daidzein and isoflavones. Tempe also modulates internal antioxidant super oxide dismutase (SOD), which improve

body capacity to defence against free radical attack (Astuti, 1997).

Due to the availability of soybeans in the world market and the imbalance of high consumption and low production of soybeans in the country, tempe in Indonesian markets are mostly processed from imported yellow soybeans. The black soybeans, native variety that re-gained its popularity locally, were proved to have more beneficial effect nutritionally. Xu and Chang (2007) indicated that black soybeans contained phenolic substances, tannins, isoflavones higher than the yellow variety and they also had higher antioxidant activity, as well. Astadi et al. (2009) found that anthocyanin in the black soy hull inhibited the oxidation of low density lipoprotein (LDL) in an *in vitro* test.

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In some Indonesian hospitals, tempe porridge is used by nutritionist experts as one of diarrhea therapy for children. Research conducted by Hermana et al. (1996) indicated that children suffering from malnutrition and chronic diarrhea that were given tempe formula had improved their nutritional status, gained weight, and healed from the diarrhea in a relatively shorter period. This is likely related to the recovery of the body immune system (immune) both systemically and locally in the digestive tract. Immune responses against antigens that enter the body are influenced by genetic factors, environment, and foods. Several researches had been conducted to study the effects of food on the immune system (Nurrahman et al., 1999; Tejasari, 2007; Zakaria-Rungkat et al., 2003). It is related to the active components of both nutritional and non-nutritional substances in foods that support formation of the body's defense system.

Xu and Chang (2007) indicated that phenolics, thanins and isoflavones, and antioxidant activity in black soybeans were higher than those of the yellow ones one. The superiority of the black soybeans and numerous benefits of tempe fermentation, demonstrated by researches in the past, have triggered the curiosity in studying the benefits, especially in nutritional and health aspects, of consuming tempe made of black soybeans. This research was aimed to study the effect of black soybean tempe and its antioxidant (ethanol) extract on T and B cells proliferation and content of secretory IgA in rats induced with *S. typhimurium*.

MATERIALS AND METHODS

The materials used in this research were black soybean tempe (Mallika, breed from native variety), antioxidant (ethanolic) extract of the tempe, culture of *S. typhimurium* FNCC0135, rats (wistar, male, 6 to 8 weeks old of 44.2 to 54.9 g in weight), and some reagents such as Roswell Park Memorial Institute (RPMI) 1640 (RPMI-1640, Sigma, USA), IgA Rat Kit (Immunology Consultants Laboratory, Inc, USA), Phytohemagglutinin (PHA) (PHA, Sigma, USA), Lipopolysaccharides (LPS) (LPS, Sigma, USA), Vitamin mix AIN 93 and Mineral mix AIN 93.

Tempe starter preparation

Rice and water in a ratio 1:1 by weight were put in an erlenmeyer flask, and then sterilized at 121°C for 15 min. The rice was allowed to cool at room temperature and then inoculated with 1 ml suspension of *Rhizopus stolonifer* DUCC204 (source of *Rhizopus* Diponegoro University Culture Collection, Indonesia) having 10^6 cell. The inoculated rice was spread in containers and covered with aluminium foil. Tiny holes were made on the aluminium cover to provide micro aeration. The mixture was incubated at room temperature for 3 days, dried, and then pulverized to fine tempe starter powder.

Processing of black soybean tempe

The tempe preparation adopted in this research was the regular way of tempe making in Indonesia with modification in the inoculum

(pure culture of *R. stolonifer* DUCC204) and way of hull separation (10% of the hull was remixed with the cotyledone). Black soybean were cleaned to remove dirt and foreign objects, washed with water, soaked for 30 min, and then boiled with water for 30 min. Boiled soybeans were dehulled by hand and 10% of the hull was put back with the cotyledone, soaked for 36 h, thoroughly drained, and then steamed for 1 h. The steamed dehulled soybeans were allowed to cool for 30 min prior to inoculation with *R. stolonifer* DUCC204 starter. The ratio of inoculum to soybeans was 2 g of starter for every kg of soybeans. The inoculated soybeans were packed in plastic bags (with tiny holes in it) and incubated at room temperature (25 to 27°C) for 36 h. The resulted tempe was then dried at temperature of 40 to 45°C for 24 h and ground to tempe powder (60 mesh).

Antioxidant extraction of tempe powder with ethanol

Antioxidant extractions were conducted upon black soybean tempe powders according to the method of Xu and Chang (2007). Tempe powders (100 g) were put in erlenmeyer flasks, mixed with 200 ml of ethanol 70% and let to stay for 24 h. The extracts and the remaining mass were separated by way of filtration. The mass was mixed again with 100 ml of 70% ethanol and then refiltered. The two filtrates were combined and the solvent was evaporated to obtained dry extract.

Care of rats

Ethical clearance (approval) was obtained from Ethical Committee of Research in Medical Health of Faculty of Medicine Gadjah Mada University for the study for lymphocyte and IgA study. A total of 48 male Wistar rats, age 6 to 8 weeks, obtained from Gadjah Mada University Animal Laboratory were divided into four groups, 12 rats each. Each group was assigned to different treatment diet (Table 1). The rats were placed in individual cages at room temperature of 25 to 27°C and acclimated on standard diet for 5 days prior to feeding treatment. The animals were then kept on treatment diets for 28 days. Both diet and water were provided *ad libitum*. Body weight during feeding treatment was monitored.

Preparation of *Salmonella typhimurium* culture

The preparation of *Salmonella* culture was conducted by inoculating 300 ml of nutrient broth with 3 ml of the bacteria suspension. After incubation at 37°C for 20 to 24 h the optical density of the suspension was measured at 600 nm. At absorbance of 0.5 the population is 10^8 cfu/ml (by standard curve previously prepared). The suspension was centrifuge at 3500 rpm for 15 min, the pellet was resuspension in 30 ml NaCl 0.85%, the suspension concentration became 10^9 cfu/ml.

Preparation of intestinal fluid before induction with *Salmonella typhimurium*

A total of 24 rats (6 from each group) were sacrificed and their small intestine were separated. Extraction of intestinal fluid was conducted by injecting 5 ml phosphate buffer saline (PBS) through the tube of the intestine, after which the fluid that passed the intestine were collected.

Preparation of lymphocyte and intestinal fluid after induction with *Salmonella typhimurium*

The remaining 24 rats were inoculated with 1 ml of *S. typhimurium*

Table 1. Composition of diet (g/ kg).

Composition	Diet of standard	Diet of tempe	Diet of tempe ethanol extract	Diet of tempe + tempe ethanol extract
Casein	140	-	140	70
Corn starch	620.7	561.66	620.7	591.18
Tempe flour	-	278.5	-	139,25
Antioksidant extract	-	-	28	14
Soybean oil	42.29	-	42.29	21.145
Sucrose	100	100	100	100
CMC	50	35.4	50	42.7
Vitamin mix AIN 93	10	10	10	10
Mineral mix AIN 93	35	35	35	35
L-cystin	1.8	1.8	1.8	1.8
Kholin bitatrat	2.5	2.5	2.5	2.5

suspension containing 10^9 cfu/ml per oral. After 1 weeks on corresponding treatment diets, the rats were sacrificed to obtain their small intestines, Peyer's patches, and spleens. The small intestine were treated with PBS to obtain the fluid for the determination of IgA after induction. The spleens and Peyer's patches were individually put in Petri dish containing RPMI1640 media for the observation of lymphocyte proliferation and IgA with indirect ELISA method, respectively.

Isolation of lymphocyte from spleen and Peyer's patch

The spleen and Peyer's patch were washed with RPMI media three times, extracted by injecting 10 ml of RPMI media into the spleen by a syringe. Suspension came out from the spleen or Peyer's patches consisting the media and the extracted lymphocyte. The suspensions were collected into 15 ml centrifuge tubes, centrifuged at 3000 rpm for 5 min, the filtrates were removed, the pellets were resuspended in 10 ml of RPMI, recentrifuged in the same manner, filtrates were removed, and the remaining pellets were resuspended again using complete media consisting RPMI and fetal bovine serum (FBS) 10%.

Measurement of lymphocyte proliferation

The number of lymphocyte suspension from spleen were counted using haemocytometer and then diluted to the concentration of 1.5×10^6 cells/ml. Lymphocyte suspension from control and the three feeding treatments were filled into the 96 wells microplate, 200 μ l each. Lymphocyte suspension taken from a single rat was assigned to 9 wells, so a total of 9×24 wells were used (3 plates). The 9 wells of lymphocyte suspension from single rat were divided into three treatments, 3 wells were added with 10 μ l containing 5 μ g of PHA solution, another 3 were added with 10 μ l solution containing 1 μ g of LPS, and the last 3 were added with 10 μ l of complete media. Lymphocyte suspensions in the plates were incubated under 95% humidity, 5% CO₂ atmosphere, and temperature of 37°C. After 72 h of incubation, 10 μ l of MTT solution of 5 mg/ml were added into each well using micro pipette. The incubation was continued for another 4 h. Living cells reacted with MTT to form a purple color. Reaction with MTT was stopped by adding 100 μ l of stopper reagents containing 10% sodium dodecyl sulfate (SDS) solution in 0.01 N hydrochloric acid. The absorbances were read using microplate reader on 550 nm wavelength. Index of stimulation indicating the lymphocyte proliferation was calculated as follows:

$$IS = \frac{\text{Absorbance of mitogen treatment}}{\text{Absorbance of control}}$$

Measurement of immunoglobulin (IgA) with indirect ELISA method

The measurement of immunoglobulin were conducted according to IgA rat kit Bioscience manufacturer's procedure. Samples were intestinal fluid and isolated lymphocyte from Peyer's patch. Sample (5 ml) from intestinal fluid was centrifuged at 3000 rpm for 5 min and the filtrate was ready for IgA analysis. Sample from isolated lymphocyte from Peyer's patch was adjusted to obtain 3×10^5 cells/ml. An amount of 200 μ l of sample was put in 96-well microplate and incubated for 72 h. The lymphocyte suspension from Peyer's patch was ready to be used as sample for IgA analysis.

Standards (rat IgA calibrators) and samples (from fluid intestine and lymphocyte culture) were taken, 100 μ l each, and put into different labelled wells, which already contain antigen. The plate were incubated at room temperature 60 min, content of wells were discarded, washed with washing solution three times. An amount of 100 μ l of antibody enzyme conjugate were added into each wells and incubated at room temperature for 30 min in a dark room. Wells were washed again as previously done. Tetramethylbenzidine substrate (TMB, Immunology Consultants Laboratory, Inc, USA), 100 μ l, were added into each of wells, incubated at room temperature in a dark room for 10 min, and then mixed with 100 μ l stop solution (0.3 M H₂SO₄). The micro plates were read using microplate reader (Bio_Rad, Model 680 XR, USA) at a wavelength of 450 nm.

RESULTS AND DISCUSSION

Growth of rats during experiment

Body weight of rats at the beginning of the trial was were between 44.2 to 54.9 g. Changes in body weight during maintenance with diet treatment are presented in Figure 1. All of rats experienced growth during the experiment ($p < 0.05$). It showed that rats under treatment diets

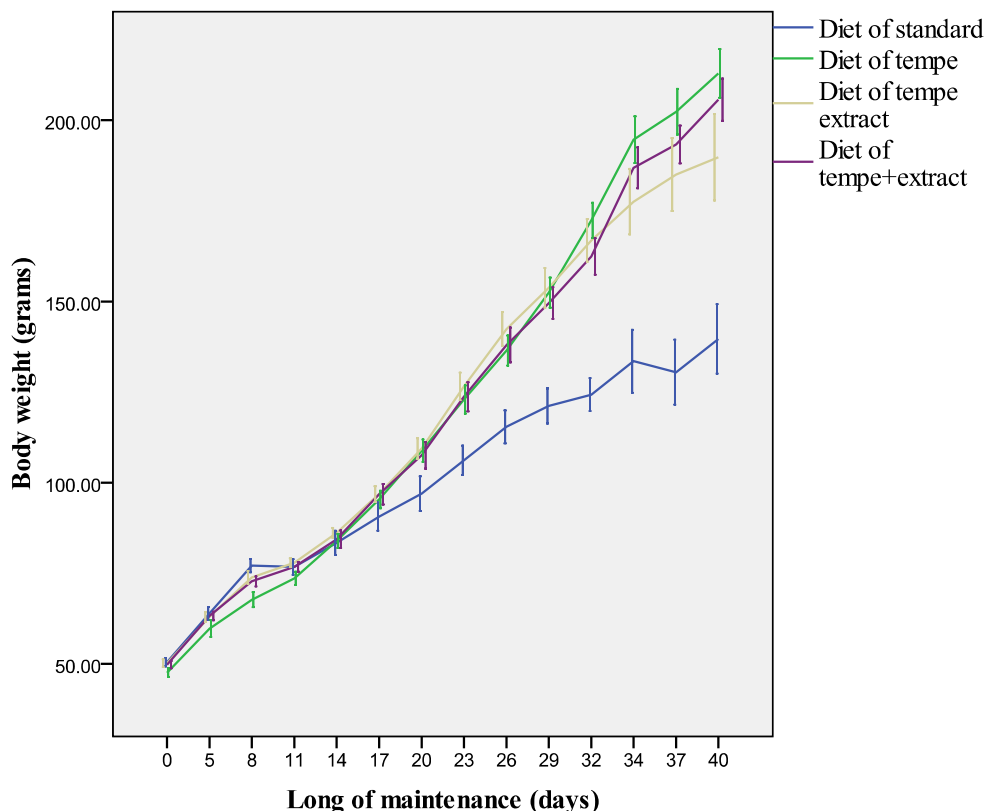


Figure 1. Graphic effect of diet and long of maintenance against growth of body weight of rats.

significantly gained more weight than those on the standard diet ($p < 0.05$), while there were no significant difference among treatments. Rats fed on tempe and ethanol extracts of tempe formulas experienced higher growth than the standard one. This shows that the nutrients contained in tempe played a role in the growth of rats. The findings were consistent with the result of Hermana et al. (1996) that consuming diet of tempe formula affect weight gain faster in children suffering from malnutrition.

Proliferation of T and B cells

The T cell stimulation indexes of rats spleen after 35 days of diet treatments is presented in Table 2. T cell stimulation index of rats spleen after treatment ranged from 0.888 to 1.301. Rats that consumed tempe and tempe combined with its ethanol extract had higher indexes than those of standard ($p < 0.05$), however, treatment with ethanol extract alone did not. This may be due to insufficient amount of extracted substances to create the difference. It also indicates that substance present in tempe other than those extracted with ethanol may poses the activity of increasing the index. Dixon and Ferreira (2002) found that, in *in vitro* experiment,

genestein, an isoflavone contained in soy and soy products (tempe), was able to bind to estrogen receptors, while Zao et al. (2005), based on *in vivo* experiment, mentioned that components in soy products may poses the ability to interact with receptors on the surface of the T cell to increase its proliferation. Ramprasath et al. (2005) and Rimbach et al. (2008), reported that nutrition nutrients in feed improved wellbeing, resulted in higher weight gain and may improve immune system of the animal.

The B cell proliferation indexes showed that rats which consumed feed with black soybean tempe flour as a substitution for casein, tempe ethanol extract and their combination with ethanol extracts produced stimulation higher than standard diet, however, there were not statistically different ($p > 0.05$).

Content of Secretory IgA

IgA content of intestinal fluid in rats treated with tempe diet and its combination with ethanol extracts looked higher than those of standard. This occurred in rats both before and after induction with *S. typhimurium*, which has the same pattern (Table 2). However, statistical analysis with ANOVA methods indicated that diet treatment's

Table 2. Effect of diet treatment on rats against index of stimulation and secretory IgA.

Parameters	Type of diet			
	Standard	Tempe	Tempe extract	Tempe + extract
Index of stimulation from T cells	0.936 ± 0.030	1.089 ± 0.106	1.034 ± 0.057	1.047 ± 0.082
Index of stimulation from B cells	0.986 ± 0.093	1.416 ± 0.349	1.161 ± 0.247	1.274 ± 0.368
Secretory IgA of intestine fluid (ng/ml) ^a	48.600 ± 26.445	73.900 ± 18.499	74.8 00 ± 26.530	55.133 ± 25.533
Secretory IgA of intestine fluid (ng/ml) ^b	50.533 ± 23.322	81.533 ± 17.319	76.367 ± 21.520	56.800 ± 26.977
Secretory IgA of lymphocytes from peyer's patches (ng/ml)	120.467 ± 43.602	130.667 ± 41.221	94.4667 ± 49.270	70.017 ± 30.216

^abefore induced by *Salmonella typhimurium*, ^bafter induced by *Salmonella typhimurium*.

effect were not significantly different ($p > 0.05$). Analyzing the difference using t test between secretory IgA before and after *S. typhimurium* showed that the bacterial induction increased the secretory for rats on tempe diet ($p < 0.05$), however, this increase were not the case for other treatments ($p > 0.05$).

Analysis of secretory IgA from the culture supernatant of lymphocytes from peyer's patches showed that rats which were treated with tempe diet had higher secretory IgA content than the other treatments. However, statistically the differences in contents of IgA from the four treatment groups were not significant ($p > 0.05$). Thus, tempe diet and its ethanol extract had no effect on the production of secretory IgA which is produced by lymphocytes of rats peyer's patches after induction with *S. typhimurium*.

The effect of all treatment that did not improve secretory of IgA was in-line with the effect on B cell proliferation. This is true since the B cell is the one that produce the IgA.

Conclusion

Diet with tempe, tempe ethanol extract, and combination of tempe and its ethanol extract of tempe improved weight gain of rats as compared to those on standard diet. Those diets also responded to improve T cell proliferation in the rat's spleen but not B cell proliferation and it's subsequence secretory IgA from intestinal fluid and Peyer's patch, respectively. Diet with tempe alone significantly increased secretory IgA in the intestinal tract fluid of rats, but the other treatments did not show significant difference with the control (without *Salmonella* induction).

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