Hemoglobin Regeneration and Distribution of Iron, Copper and Zinc in Hepatic Subcelluler Frantions of Rats Fed With Tempe Diets

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ABSTRACT

Hemoglobin regeneration efficiency was used to predict the iron availability of tempe diet by the method of depletion and repletion using rats for animal experiment. Hemoglobin regeneration efficiency of tempe showed the same effect with casein diet and unfermented soybean diet indicating that iron from soybeans are a good source of available iron. Iron, copper, and zinc from casein, unfermented soybean and tempe diets were distributed in hepatic subcelluler fractions in the same pattern. The highest level of iron and zinc were found in the cytosolic fraction and copper was found in the nuclear fraction. The different pattern among those trace minerals indicates that they have distinct roles in hemoglobin synthesis and metabolism.

INTRODUCTION

Iron is an important trace mineral for human life. It is present in all cells of the body and plays a key role in many biochemical reactions (Halleberg, 1984). Iron, as a component of hemoglobin, is able to bind oxygen in the lungs and transport it throughout the body.

In the body, iron has a close link with other trace minerals such as copper and zinc. Copper is a component of ceruloplasmin which has a key role in iron metabolism, and zinc is a component of methalloenzyme which involve in iron metabolism (Smith, 1988).

Nutritional anemia caused mainly by iron deficiency is the most recognized nutritional problem in both developing countries as well as affluent societies. It is particularly prevalent among infants, young children, pregnant women and lactating mothers (Halleberg, 1984).

The most important cause of iron deficiency in developing countries is poor absorption of iron or bio-unavailability of iron from the diets. Dietary iron that has low availability comes predominantly from cereals and vegetables (Layrisse *et al.*, 1969). Some plant foods are potentially rich sources of iron in the diet, however, they are poorly absorbed. Cereal grains and legumes contain relatively large amounts of iron but much of this iron is unavailable due to the high content of fiber. Another factor depressing the availability of iron from cereal grains and legumes is phytates (Jaffe, 1981; Wise, 1983; Platt and Clydesdale, 1984).

Mulyopawiro *et al.* (1987) reported that soybeans fermented with lactobacillus had a positive effect on iron bioavailability. Lactobacillus fermentation decreased the phytic acid in the bean due to phytase production by microorganism. Also, active phytase was demonstrated by *Rhizopus oligosporus* in tempe fermentation (Sudarmadji and Markakis, 1977) and by yeast in the baking process (Erdman and Forber, 1981).

Tempe, a traditional fermented soybean is consumed in significant quantities in Indonesia and now gaining popular among vegetarians throughout the world. Consideration must be given as to how this change will affect the iron status of the population if soybean tempe is used increasingly as a meat substitute. Even though soybean protein has been considered to be a good source of available iron by some researchers, others have found a contradictory result.

The specific objectives of this study were: to evaluate the iron availability of soybean tempe, and to study the distribution of iron, copper and zinc in hepatic subcelluler fractions. Hemoglobin regeneration efficiency was used to predict the iron availability by rats.

MATERIALS AND METHODS

Preparation of tempe samples

Tempe was prepared from Wilis variety soybeans grown in Yogyakarta, Indonesia. Clean whole soybeans were washed and boiled in distilled deionized water (DD water) for 30 min. The beans were then soaked for 24 hrs at room temperature, in the ratio 3 parts of DD water to 1 part of beans. Following soaking, the DD water was discarded, the beans were dehulled by hand then soaked again for 24 hrs at room temperature. After overnight soaking the DD water was discarded and the beans were boiled for 60 min. Following boiling, the excess DD water following boiling, was drained off. As the temperature of cooked bean reached 30°C, they were inoculated with tempe inoculum powder 0.2 g/100 g dried beans. The inoculated beans were packed in Petri dishes and incubated at 30°C for 0 and 48 hh, then steamed for 5 min, and subsequently minced, freeze-dried, ground and filtered in a 35 mesh sieve. The product was designed as the unfermented soybean and tempe powder for animals diets.

Rats

Male Wistar strain rats, 21 days old, were obtained from the Japan CLEA company, Tokyo. The rats were housed in individual stainless cages. Temperature and humidity were maintained at 20°C and 60 percent, respectively. Lighting was regulated to provide 12 hh of light and 12 hh of darkness.

Diets Preparation

The composition of the diets is listed in Table 1.

Table 1. **The composition of diets (%)**

Ingredients	C	US	T	FeD
Casein	22	–	–	22
Unfermented soybean	–	35	–	–
Tempe	–	–	35	–
Starch, corn	29	25	24	29
Oil, soybeans	8	0	1	8
Sucrose	30	30	30	30
Mineral mix 1	4	4	4	4
Vitamin mix 2	2	2	2	2
Iron, ppm	54	33	34	11

1. The mineral mixture was formulated according to A.E. Harper (J. Nutr. 68, 1959) and contained the following mineral g/100 g of mixture: KH_2PO_4, 34.3 g; $CaCO_3$, 29.29 g; NaCl, 25.06 g; $MgSO_47H_2O$, 9.98 g; $Fe(C_6H_5O_7)$ $6H_2O$, 0.623 g; $CaHPO_42H_2O$, 0.43 g; $CuSO_45H_2O$, 0.156 g; $MnSO_4H_2O$, 0.121 g; $ZnCl_2$, 0.020 g; KI, 0.0005 g; (NH_4) $6MO_7O_44H_2O$, 0.0025 g. Only in the diet of group C the mineral mixture was composed with Ferric citrate.

2. The vitamin mixture was obtained from Toyo Roshi Co., Ltd., Tokyo, Japan, and contained the following vitamin per g of mixture retinyl palmitate, 2500 IU; calciferol, 200 IU; thiamin nitrate, 1 mg; riboflavin, 1.5 mg; nicotinamide, 10 mg; pyridoxine HCl, 1 mg; folic acid, 0.05 mg; CaD-pantothenate, 5 mg; cyanocobalamin, 1 μg; ascorbic acid 37.5 mg; DL-tocopheryl acetate, 1 mg.

Design of experiment

The depletion and repletion method was used to evaluate hemoglobin regeneration efficiency. Twenty one male weaning rats weighing 40 – 50 were used. The rats were rendered moderately anemic by feeding iron deficient diets containing 11 ppm Fe (Table 1) for 14 days. At the end of depletion period, the body weights were recorded and the blood was taken from the tail vein for hemoglobin analysis.

The anemic rats were randomly assigned to three groups of seven. During 11 days repletion period, group C (control group) were fed the casein diet containing 54 ppm Fe; group US (unfermented soybean) were fed the soybean diet containing 33 ppm Fe and group T (tempe) were fed the tempe diet containing 34 ppm Fe (Table 1). Diets and deionized distilled water were given *ad libitum,* any spilled food was weighed and recorded to determine the net food consumption and used for determining the nutrient intake. The weight gain of the rats were recorded daily. At the end of the experiment, the blood was taken from tail vein of rats and was analyzed for hemoglobin. The rats were then killed, the abdomens were opened, and the liver was perfused in situ by portal vein with cold 0.9% NaCl. The liver was removed and weighed. Two gram of liver was fractionated to isolate the nucleus, mitochondrial, cytosol and microsomal fractions and were analyzed for iron, copper and zinc.

Fractionation of liver cells

Fractionation of the liver cells was done by a mechanical method based on their density as follows: Two grams of liver tissue was cut with scissor into small pieces and kept in a strong glass tube, suspended with

6 mL 5mM – Tris HCl buffer pH 7.4, containing 0.25 M sucrose and 0.1 mM EDTA. The cell membrane was ruptured at 0 – 2°C in a Tomy homogenizer. The glass tube, fitted with a plastic plunger and kept in an ice bucket, was given a single run upward and down against the plunger. The resulting homogenates were centrifugated at 600 × G for 15 min at 0°C. The sediment was observed as a nuclear fraction which was composed of nucleus, plasma membrane and unbroken cells. The resulting supernatant was centrifuged at 9,000 × G for 20 min at 0°C. The sediment was mixed with 10 mL of 5 mM Tris HCl buffer pH 7.4 containing 0.25 M sucrose and 0.1 mM EDTA, and then was discrupted in a Tomy ultrasonic disrupter for 10 sec. This mitochondrial fraction was composed of mitochondria and lysosome. The resulted supernantant of 9,000 × G centrifugation was made up to 20 mL with 5 mM Tris HCl buffer pH 7.4 containing 0.25 M sucrose and 0.1 mM EDTA and then was centrifug at 105,000 × G for 60 min at 0°C. The sediment was the microsomal fraction composed of endoplasmic reticulum, golgi apparatus and ribosomes. The supernatant was the soluble fraction or cytosol fraction.

Determination of hemoglobin

The blood taken from the tail vein of each rats at the end of depletion and repletion period were analyzed for hemoglobin using a cyanmethemoglobin method (Crosby *et al.*, 1954). Twenty microliters of whole blood were delivered into exactly 5.0 mL Drabkin's solution (1.0 g sodium bicarbonate, 0.2 g potassium ferricyanide, and 0.05 g potassium cyanide in 1.000 mL of double distilled water). The blood solution were mixed in a vortex mixer and the solution was allowed to stand for 15 minute. The absorbance was then read at 540 nm in a Shimadzu Spectrophotometer model VV-110-02. Hemoglobin was calculated using a standard Hb 16.0 g/100 ml provided from Nippon Shoji.

Hemoglobin regeneration

Body weight and hemoglobin levels were then used to calculate the initial and final hemoglobin iron contents on the assumption that the total blood volume of the rats is 7.5% (Whittaker *et al.*, 1984) of body weight and iron content of hemoglobin is 3.55 mg/g (Zhang *et al.*, 1985).

$$\text{mg Hb iron} = \text{g body weight} \times \frac{0.075 \text{ mL blood}}{\text{g body weight}} \times \frac{\text{g hemoglobin}}{\text{mL blood}} \times \frac{3.35 \text{ mg Fe}}{\text{g hemoglobin}}$$

Hemoglobin regeneration efficiency was calculated for each rat as follows:

$$\text{Efficiency} = \frac{\text{mg HbFe (final)-mg HbFe (initial)}}{\text{mg iron consumed}^*} \times 100\%$$

* mg iron consumed was calculated as food intake × iron content in the diets.

Determination of total iron

The total iron in the diet was determined using the dry ashing and then the total iron was analyzed using atomic absorption spectrophotometry.

Approximately 1 gram of each sample was digested by ashing in a muffle furnace at 500°C for 40 hours. The white ash was then extracted with HCl 1 : 4 and made up the volume to 50 mL by using distilled deionized water. Iron was determined in spectrophotometer model AA 640.13 (Shimadzu Atomic Absorption/Flame Emission, Japan). Standard solution of iron was prepared from certified iron reference solution (Fisher Scientific Co.).

Determination of iron, copper and zinc in subcelluler fractions liver

Approximately 1 – 2 mL of sample of each fractions was wet digested by using 1 – 2 mL of concentrated HNO_3 solution in a glass tube and heated at 80°C for 1 hh, then increased to 120°C. The dry ash was extracted (2 mL of 0.1 N HCl) then 1 mL of solution was mixed well with 4 mL deionized water. Iron, copper and zinc content were determined in a Shimadzu Atomic Absorption/Flame Emission Spectrophotometer AA 640.14. Standard solutions of iron, copper and zinc were prepared from Certified iron, copper and zinc solution (Fisher Scientific Co.).

Statistical analysis

The means and standard error were computed. Analysis of variance was used to evaluate the diffe-

rences among the groups. The Duncan's Multiple Range Test (DMRT) was used to determine the significant differences between group and the level was set at 5% (Gomez and Gomez, 1983).

RESULTS AND DISCUSSION

Hemoglobin regeneration

Hemoglobin regeneration efficiency were used to estimate iron availability of casein, unfermented soybean and tempe diets in moderate anemic rats. Body weight and blood hemoglobin levels were used to calculate initial and final hemoglobin iron contents. Table 2 displays the values asserted in establishing the efficiency of hemoglobin regeneration.

Table 2. **Hemoglobin Regeneration**

Items	Diets		
	Control (C)	Unfermented soybean (US)	Tempe (T)
Food intake, g	188.22 ± 2.36a	198.74 ± 3.85a	204.24 ± 5.68a
Iron intake, mg	9.17 ± 0.13b	6.03 ± 0.12c	6.33 ± 0.19c
Initial B.W., g	112.19 ± 2.16d	111.37 ± 2.72d	115.22 ± 1.44d
Final B.W., g	184.57 ± 2.73e	181.35 ± 3.91e	182.03 ± 2.13e
B.W. gain, g	72.38 ± 2.31f	68.92 ± 1.23f	66.76 ± 1.85f
Initial Hb, g/dL	9.03 ± 0.19g	9.04 ± 0.11g	9.03 ± 0.19g
Final Hb, g/dL	14.69 ± 0.19h	11.76 ± 0.36i	12.04 ± 0.24i
Hb gain, g/dL	5.65 ± 0.19j	2.68 ± 0.36k	2.99 ± 0.25k
Initial FeHb, mg	2.27 ± 0.04l	2.26 ± 0.05l	2.33 ± 0.13l
Final FeHb, mg	6.08 ± 0.14m	4.77 ± 0.13m	4.92 ± 0.16m
FeHb gain, mg	3.81 ± 0.13n	2.51 ± 0.11n	2.59 ± 0.14n
Efficiency, %	45.64 ± 1.66o	44.76 ± 0.89o	44.79 ± 2.07o

B.W. = body weight Hb = hemoglobin
Values are means ± SE of 7 rats per group. Values not labeled with the same superscript letter indicate significant differences ($P < 0,05$, ANOVA and DMRT).

As illustrated in Table 2, the high iron intake of rats in the casein group was due to the higher iron content in the diet of casein group (Table 1) than that in the unfermented soybean and tempe groups. Iron intake was determined from food intake times iron content in the diets. Food intake during repletion period was 188.22, 198.74, and 204.24 g, for casein, unfermented soybean and tempe groups respectively and there were no significant different among these three groups of rats.

Hemoglobin levels at the end of repletion period of rats fed with casein, unfermented soybeans and tempe diets was 14.16, 11.76 and 12.04 g/dL, respectively. The highest hemoglobin level in casein group due to the higher iron intake. The hemoglobin level of rats fed with tempe diet show a normal level. This, indicates that tempe contains a good nutrient for hemoglobin synthesis.

The iron content in hemoglobin at the end depletion period of rats fed with iron deficient diet was 2.27 mg; 2.26 mg and 2.33 mg, for casein, unfermented soybeans and tempe groups respectively. The iron content in hemoglobin at the end of repletion period of rats fed with casein, unfermented soybeans and tempe diets was 6.08 mg, 4.77 mg and 4.92 mg, respectively. Statistic evaluation showed no significant different among these three groups. Hemoglobin iron gain for casein, unfermented soybean and tempe groups were 3.81, 2.51 and 2.59 mg. Ranger and Neale (1984) reported that anemic rats (initial hemoglobin level was 7.07 g/dL) fed with soy concentrate diet for 7 days gained hemoglobin iron of 0.54 mg compared to 2.51 mg in extrusion-textured soy concentrate and 2.75 mg in standard diet (25 ppm Fe as $FeSO_4$)

The efficiency of iron for hemoglobin regeration of the three groups of the rats were 45.64, 44.76, and 44.79 percent, for casein, unfermented soybean and tempe groups respectively. Eventhough the hemoglobin level of casein group was significantly different with unfermented soybean and tempe groups but the efficiency for hemoglobin regeneration for the three groups of rats statistically was not significant different at $P < 0.05$. This results indicate that iron from tempe and unfermented soybean is a good source of available iron.

Distribution of iron, copper and zinc in hepatic

Subcelluler fractions

Iron, copper and zinc distribution in hepatic subcelluler fractions were listed in Fig 1, 2, and 3. As shown in those figures, the three groups of rats display the same distribution pattern of the trace minerals. On the other hand, among those minerals showed a different distribution pattern. This is related to the function of the organellas in the subcelluler fraction and the role of those minerals in each fraction.

As illustrated in those figures, the highest iron levels of casein, unfermented soybean and tempe groups was found in cytosol fraction followed by the mitochondrial, nuclear and microsomal fractions, in that order. However, the highest copper level was found in the nuclear fraction and the decrease orderly to the cytosol, motochondrial and microsomal frac-

tions. Meanwhile, the level of zinc increased orderly from the microsomal fraction to mitochondrial, nuclear and cytosol fractions. The lowest levels of iron, copper and zinc for three groups of rats were found in microsomal fractions.

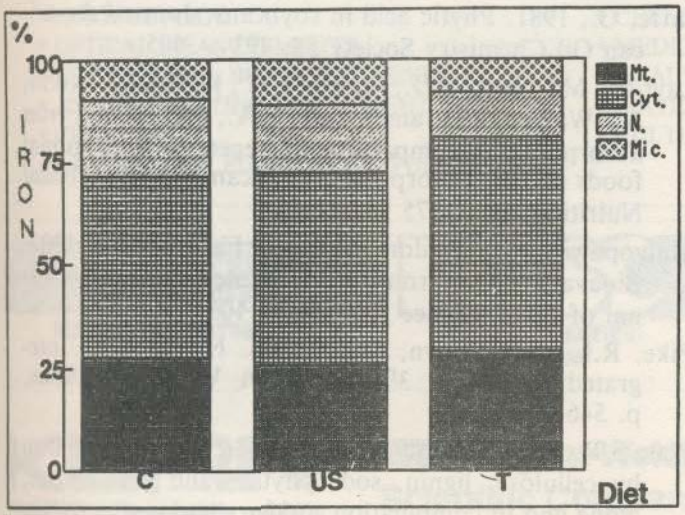

Figure 1. **Distributin of iron in hepatic subcelluler fractions Mt = mitochondria fraction; Cyt = cytosol fraction N = nuclear fraction; Mic = microsomal fraction.**

The high iron level in cytosol fraction, the copper content in nuclear fraction and zinc level in cytosol fraction seem to be correlated to hemoglobin synthesis. During depletion period, all of the rats were fed with iron deficient diets (11 ppm Fe) resulted in the low levels of hemoglobin. In the repletion period, new hemoglobin was synthetized. For hemoglobin synthesis, many kinds of nutrient are needed including minerals, amino acids, coenzymes and also vitamins (Pike and Brown, 1984). Iron for hemoglobin synthesis not only comes from the diet but also from iron stored. The transfer of iron from ferritin appears to take place in the cytoplasma. This is one reason why the highest level of iron is found in cytosol fraction. This result supports the previous suggestion about the place of iron from ferritin is released (Spik *et al.*, 1985). They suggested that iron from ferritin is released in the cytosol. The amount of iron in mitochondrial fraction was smaller than that in the cytosol fraction. This possibly related to the function of mitochondria in that fraction.

In the synthesis of hemoglobin in the liver, the code information in the gene is needed. The gene information is found in the nucleus of the cell. The genetic material for protein synthesis is DNA. Copper in nuclear fraction appear to be bound to chromatin. Both protein and DNA are required for binding copper to chromatin. The function of copper in the nucleus suggested to be related with the process of genetic information (Bloomer and Lee, 1978). Therefore as a results of new synthesis of protein and hemoglobin, the copper level in nuclear fraction increased. In the normal condition, the highest copper level is found in cytosol fraction (Bloomer and Lee, 1978). In this experiment, copper in cytosol fraction possibly moved to nuclear fraction.

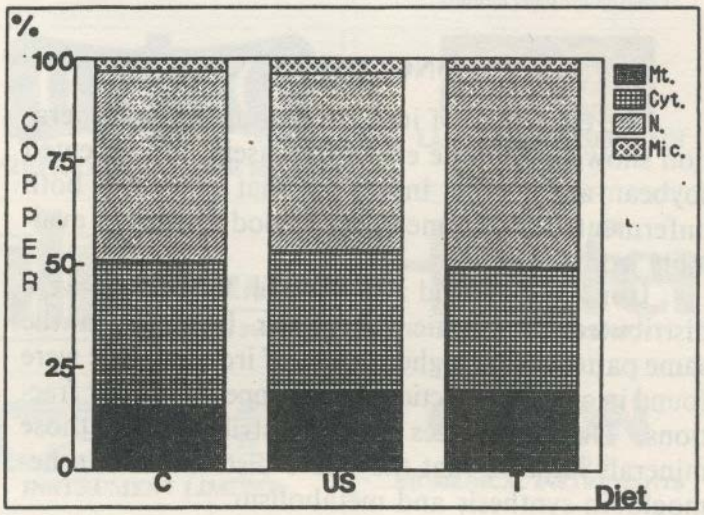

Figure 2. **Distribution of copper in hepatic subcelluler fraction Mt = mitochondria fraction; Cyt = cytosol fraction N = nuclear fraction; Mic = microsomal fraction**

The distribution on zinc in subcelluler fraction was thought to be related with the role of zinc in biological proceses. Zinc as a component of many kinds of enzymes involves in protein synthesis which primarily occur in cytosol fraction synthesis protein in the nuclear fraction. Therefore, the highest level of zinc was found in cytosol and followed in nuclear fraction. In mitochondrial fraction zinc have a role in heme synthesis and another enzymatic reaction.

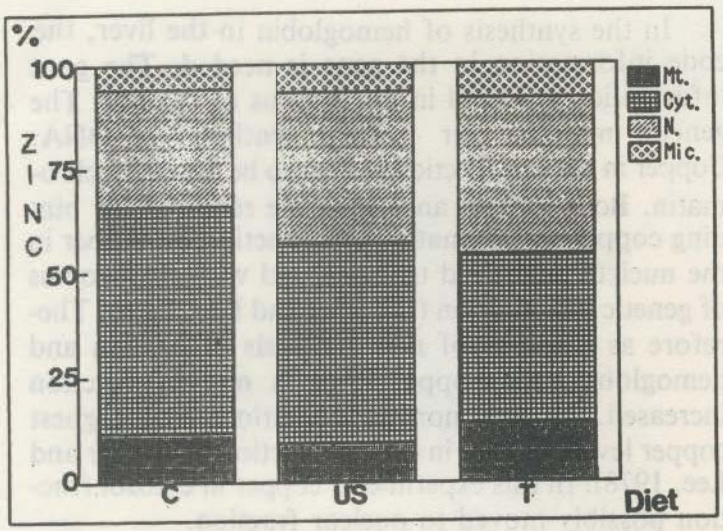

Figure 3. **Distribution of zinc in hepatic subcelluler fraction Mt = mitochondria fraction; Cyt = cytosol fraction N = nuclear fraction; Mic = microsomal fraction**

CONCLUSIONS

The efficiency of iron for hemoglobin regeneration showed the same effect for casein, unfermented soybean and tempe, indicating that soybeans, both unfermented and fermented are good sources of available iron.

Iron, copper, and zinc from all tested diets were distributed in hepatic subcelluler fractions in the same pattern. The highest levels of iron and zinc were found in cytosolic fractions, and copper in nuclear fractions. The differences in the distribution of those minerals indicate that they have distinct roles in hemoglobin synthesis and metabolism.

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