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Effectiveness of Iron Fortification on Soy-Based Foods Using Ferrous Bisglycinate in the Presence of Phytic Acid

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Abstract

The major cause of iron deficiency in human body is the low intake of iron from foods. One of strategy to overcome the iron deficiency anemia (IDA) in Indonesia is iron fortification to soya-based food. Phytic acid is a compound that presents in soy-based food and inhibits iron adsorption in digestion thus increasing iron deficiency. Iron fortification is one method to increase iron content in food. In this research, $FeSO_4.7H_2O$ (**1**), ferrous bisglycinate (**2**) and Na-glycinate+$FeSO_4.7H_2O$ (**3**) were used as fortificants into three soy-based foods (i.e., soya milk, tempeh, and tofu. The addition of fortificant amount was performed based on the molar ratio of phytic acid contained to iron. The results shows that fortification using ferrous bisglycinate is the most effective compound compared to single compound $FeSO_4.7H_2O$ and the mixture of Na-glycine and $FeSO_47H_2O$. The effectiveness of fortification on soya milk of fortificant **1**, **2** and **3** are 48%, 55%, and 33% respectively, whereas on tempeh are 74%, 86%, and 56% respectively and for tofu are 51%, 55%, and 46% respectively. It indicates that ferrous bisglycinate has ability to prevent iron phytate formation due to its character as a chelating agent.

Abstrak

Efektivitas Fortifikasi Zat Besi pada Makanan Berbasis Kedelai Menggunakan Besi Bisglisinat dengan Kandungan Asam Fitat. Penyebab utama dari penyakit anemia adalah rendahnya asupan zat besi dari makanan. Salah satu strategi penanggulangan penyakit kekurangan zat besi di Indonesia adalah melalui fortifikasi zat besi pada makanan berbasis kedelai. Adanya asam fitat pada makanan berbasis kedelai dapat mengikat zat besi dan menurunkan nilai ketersediaan zat besi dalam darah. Pada penelitian ini digunakan tiga jenis fortifikan yang berbeda yaitu, $FeSO_4.7H_2O$ (**1**), besi-bisglisinat (**2**) dan campuran Na-glisinat dan $FeSO_4.7H_2O$ (**3**) untuk diberikan pada makanan berbasis kedelai yaitu susu kedelai, tempe dan tahu. Penambahan bahan fortifikasi didasarkan pada molar rasio kandungan asam fitat terhadap zat besi yang mampu diikatnya. Hasil penelitian menunjukkan bahwa fortifikasi zat besi menggunakan besi-bisglisinat merupakan fortifikasi yang paling efektif dibandingkan dengan fortifikasi mengunakan $FeSO_4.7H_2O$ dan campuran *in-situ* antara $FeSO_4.7H_2O$ dan Na-glisinat. Efektivitas fortifikasi pada susu kedelai menggunakan fortifikan **1**, **2** dan **3** berturut-turut adalah 48%, 55%, dan 33%, sedangkan pada tempe berturut-turut adalah 74%, 86%, dan 56%, fortifikasi pada tahu berturut-turut adalah 51%, 55%, dan 46%. Hasil penelitian menunjukkan bahwa penggunaan besi-bisglisinat sebagai agen pengkelat mampu menghalangi terbentuknya senyawa besi-fitat.

Keywords: ferrous bisglycinate, fortification, iron, phytic acid, soy-based foods

1. Introduction

Iron deficiency anemia (IDA) is one of the major health problems particularly in developing countries. IDA affects mostly to infants, children, and women of childbearing age. IDA has several major negative impacts on health, and contributes substantially to the risk of early death and disability [1]. The major cause of IDA is the low intake of iron from food. Iron fortification of food products is one way to prevent IDA. Food fortification concerns in the addition of micronutrients in processed food. Food fortification offers a cost-effective approach to provide additional iron to foodstuffs most population consumed.

In selecting a suitable iron fortificant, several objectives are needed to get the one with the highest bioavailability and cost-effective. Food fortificants can be broadly divided into three categories: water soluble, poorly water soluble but soluble in dilute acid, and water insoluble and poorly soluble in dilute acid [2]. Ferrous sulfate is a water soluble fortificant and ferrous sulfate is the most frequently used because it is the cheapest fortificant. Iron amino acid chelate such as ferrous bisglycinate is a compound formed by two glycine molecules bound to an iron atom resulting in a double heterocylic ring compound. It has been observed that ferrous bisglycinate is well adsorbed when it is added to foods with a predominance of inhibitors [3].

Soybean (Glycine max L) is one source of protein with a protein content as much as 35%, even in high-yielding varieties the protein content can reach 30-40 % [4]. Soybeans also have nutrients that are needed in the human body including minerals such as iron. In 1 gram of soybeans contained as much as 3.55 mg of iron [5]. Soy-based foods such as soya milk, tempeh and tofu are commonly consumed in Indonesia. Soya milk and tofu are produced by traditional nonfermented food, while tempeh is made through fermentation process using *Rhizopus oligosporus L 41* inoculum. In addition to containing essential nutrients that the body needs, soybeans also contain phytic acid, a compound that inhibits the bioavailability of iron.

Phytic acid (inositol hexaphosphoric acid) presents in cereals, legumes, oil seeds, and nuts. This compound is not digestable to humans or animals and known as anti-nutritive due to its nature as an inhibitor of iron adsorption. Phytic acid and iron form insoluble complexes that are not available for adsorption under the pH conditions of the small intestine [6]. When iron chelated to phytic acid, it becomes insoluble and will be non-adsorbable in the intestines. This chelating effect system contributes to a deficiency of iron intake. The amount of phytic acid could be decreased using phytase. Some food processes such as sprouting, soaking, and fermenting raw grains allows this enzyme becomes active so that reduces the phytic acid.

In this work, ferrous bisglycinate **1** and mixture of Na-glycinate and $FeSO_4.7H_2O$ **2** were added separately into three kinds of soy-based foods (i.e., soy milk, tempeh, and tofu) in order to increase the amount of iron in the presence of phytic acid. This fortification method is perfomed to obtain the optimum amount of fortificant to be added to soy-based foods. These fortificant addition should consider the amount of phytic acid content of each sample. Furthermore, higher effectiveness fortification using ferrous bisglycinate was expected due to the effect of chelating.

Herein we report a new fortification process of iron addition to soya based foods such as tempeh, tofu and soya milk. To the best of our knowledge the previous iron fortifications were done for noodles, flours, cereal [7-8] no information has been reported for iron fortification of soya based food.

2. Experiment

Materials. $FeSO_4.7H_20$, glycine, citric acid, ammonium thiocyanate, HNO_3, $FeCl_3.6H_2O$, and amyl alcohol were all purchased from Merck. Sodium-glycinate and sodium-phytate were supplied by Sigma. Soybeans (Glycine max L), *Rhizopus oligosporus* L 41 inoculum and acetic acid food grade were purchased from the local market (Pasar Kemiri, Depok-West Java). Double distilled water used throughout this study.

Preparation of samples. Preparation of samples soya milk and tofu were following the method from Radiyati *et al*. (1992) [9], and sample tempeh was following the method from Pangastuti *et al*. (1996) [10]. The fermentation process of tempeh was done by using of *Rhizopus oligosporus* L 41 inoculum. Each sample was made of 30 g soybeans source. The samples of tofu and tempeh were dried in the oven with a temperature 70 °C for 6 hours.

Preparation of ferrous bisglycinate chelate (2). Ferrous bisglycinate was prepared by modified method of reference [11]. Compound **2** was synthesized by mixing 23 g ferrous iron ($FeSO_4.7H_20$), 60 g glycine, 17 g citric acid, and 150 ml water. The mixture was stirred for 24 hours in 50 °C under nitrogen atmosphere. Once the reaction was complete, the mixture was cooled down for crystallization. Further re-crystallization process was needed to remove sulfate ion completely. The crystal result was dried in oven before characterized using FT-IR.

Determination of iron-phytic acid contained in samples. Phytic acid content was determined using Davies and Reid method [12]. Samples for analysis were obtained by suspending 1 g sample of tempeh and tofu (1 g soybean) or 10 ml of liquid soya milk (1 g soybean), in 50 ml of 0.5 M HNO_3 solution. The suspension was stirred for 2 hours at room temperature, and then filtered. 0.5 ml filtrate obtained were added with 0.9 ml of 0.5 M HNO_3 and 1 ml $FeCl_3.6H_2O$ (Fe^{3+} concentration of 50 mg/ml), and then soaked in boiling water for 20 minutes. Once the solution was cooled, then wad added 5 ml of amyl alcohol and 1 ml of ammonium thiocyanate and then was sentrifuged at speed of 1500 rpm for 10 minutes. Two layers were formed and the upper layer of amyl alcohol was measured the content of $Fe(H_2O)_5SCN^{2-}$ using a UV-Vis spectrophotometer at a wavelength of 465 nm.

Standard curve of Na-phytate concentration against absorbance value of $Fe(H_2O)_5SCN^{2-}$ was made by using a variation of solution concentration Na-phytate 0.02 mM, 0.04 mM, 0.06 mM, 0.08 mM, and 0.12 mM.

Fortification of samples using $FeSO_4.7H_2O$, ferrous bisglycinate, and Na-glycinate+$FeSO_4.7H_2O$. Each fortificant was added into each prepared. The amount of soya beans for the preparation is 30 g for each tofu and tempeh, while 200 ml (30 g soybeans result in 200 ml soya milk) of soya milk was used for every fortificant. The addition of fortificant was added based on the amount of phytic acid in the sample.

Determination of non-fitate iron total. 1 g of each tempeh and tofu, 10 ml soya milk were suspended in 0.5 M HNO_3 50 ml. Each suspension was stirred for 2 hours and then filtered. Filtrate obtained was analyzed using AAS.

3. Results and Discussion

Ferrous bisglycinate is an amino acid chelate with iron surrounded by amino acid. The chelate structure formed is small enough to be easily adsorbed in the body, and the iron bond is protected. Ferrous bisglycinate consists of one molecule of iron that is bound to two molecules of glycine. The iron bound to the carboxyl groups on the bonding anionic glycine and the amino group is coordinated covalent bonds forming two heterocyclic rings [11].

Synthesis of ferrous bisglycinate was carried out by reaction between iron (II) with two molar excess of glycine in water and the nitrogen atmosphere, the pH is controlled by the addition of citric acid. Through this condition, metal forming coordinate covalent complexes with carboxylic acid of glycine, and a coordinate covalent bond with the amino group of glycine. Ferrous bisglycinate complex is more favor formed due to its complex stability constant higher than carboxylic complex.

The ferrous bisglycinate chelate crystal was characterized by FT-IR to determine the structure of the molecule by evaluating the energy transmitted in a frequency range. A spectrum of the ferrous bisglycinate was obtained and the peaks representing the bonding energies of interest were identified (Figure 1). There are two bonding of particular interest in the chelation of glycine to an iron by coordination bond between Fe-OC (carboxylic group) and Fe-N (amine group). The spectral of the Fe-N bonding at the frequency between 3000-3200/cm shows the broadband stretch indicating amine bond to Fe. The Fe-OC (carboxylic group) peaks were observed at 511/cm as the carbon participated in the chelate bond.

Phytic acid content in each soy-based food sample is different from each other. The lowest amount of phytic acid in tofu was caused by the heating and disposal of the solution in synthesis process. The lower amount of phytic acid in tempeh compared with soya milk is caused by the presence of an active enzyme phytase after the fermentation process with the inoculum. Figure 2 shows the phytic acid content of each sample. These levels were measured by extrapolating absorbance data into calibration curve of phytic acid.

Method used to determine the levels of phytic acid based on iron bound to phytic acid (Fe^{3+}-phytate) that is insoluble in water [13]. Fe^{3+}-phytate will be drawn into the nonpolar solvent, in this study amyl alcohol is used.

The amount of phytic acid was determined by measuring the formation of iron complexes. Source of iron used in this study was iron (III) chloride ($FeCl_3.6H_2O$). After the addition of a ammonium thiocyanate solution has formed red brick $[Fe(H_2O)_5SCN]^{2+}$ complex which is explained by the equation:

$$[Fe(H_2O)_6]^{3+}(aq) + SCN^-(aq) \rightarrow [Fe(H_2O)_5SCN]^{2+}(aq) + H_2O \quad (1)$$

This $[Fe(H_2O)_5SCN]^{2+}$ complex ion is easily extracted with ether or amyl alcohol. The existence of amyl alcohol causes the formation of two mutually insoluble phases. Amyl alcohol layer which is then analyzed using UV-Vis spectrophotometer to obtain the levels of iron ions that are not bound to phytate.

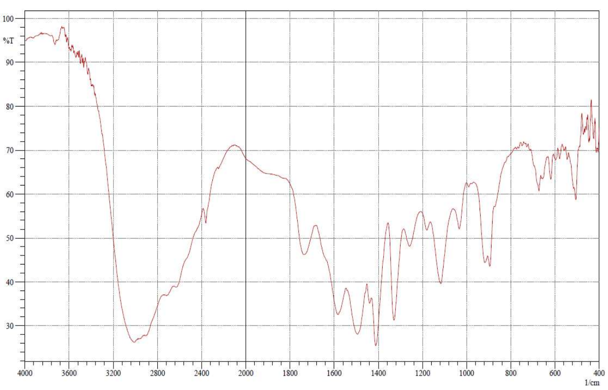


Figure 1. The FTIR of Ferrous Bisglycinate

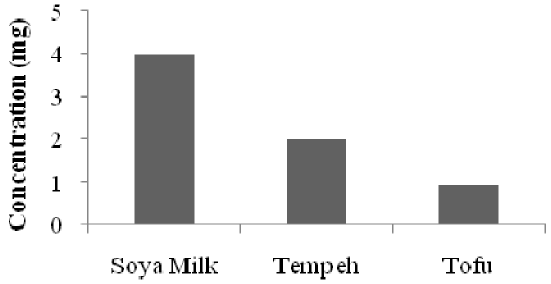


Figure 2. The Concentration of Phytic Acid on Sample Soya Milk, Tempeh, and Tofu. The Analysis of 10 ml of Soya Milk, 1 g of Tempeh, and 1 g of Tofu

Fe^{3+} ions bound to the ligan SCN^- and phytate is derived from the same source $FeCl_3$. When the phytic acid is added more, the reaction will shift to the right so that the Fe^{3+}-phytate formed more and complex $Fe(SCN)^{2+}$ is reduced. Therefore the calibration curve of phytic acid (Figure 3) produced a curve that decreases with the increasing phytic acid concentration.

The addition of iron fortificant into samples was done on the sample preparation. This addition was based on the amount of molar ratio between phytic acid contained and iron, a method that used for determining bioavaibility of mineral in human body [15-16]. In this research the molar ratio of phytic acid:Fe is 1:3.

The amount variations of $FeSO_4.7H_2O$ that widely used as fortificant were conducted to determine the effectiveness of the fortificant. The addition of this fortificant was also aimed as a comparison for two other fortificant.

Figure 4 (a) shows the curve of the addition of fortificant **1** with the highest effectiveness is occurred when the addition of **1** for soya milk, tempeh and tofu as much as 100 mg, 50 mg, and 25 mg, respectively. Our observation reveal that the more amount of fortificant is added, the effectiveness would be decreased, this can be caused by the more addition of iron will increasingly provide more opportunities of phytic acid to bind iron ions. The maximum effectiveness percentage for tofu and tempeh a was occurred at the addition of 50 mg of **1** as high 51%, and 74% respectively. Surprisingly the maximum effectiveness of soya milk will reach 48% after addition of 100 mg of **1**.

Sulfate ion with iron has a stable bond in aqueous solution. When the bonds are formed, iron ions will not be freely bind into phytate. Without the presence of sulfate ions, iron ions can bind two phytate anions, whereas with the presence of sulfate allowed only able to bind one phytate anion [14].

The use of chelating agent ferrous bisglycinate as fortificant has been widely applied because this compund have a high level of bioavailbility as well as a

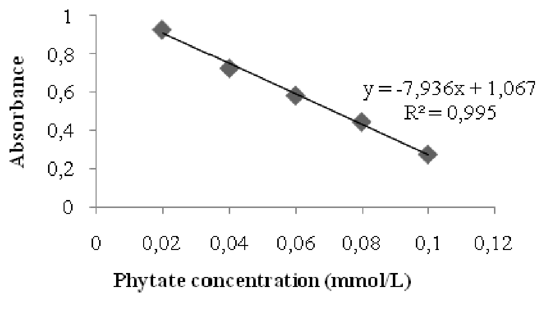
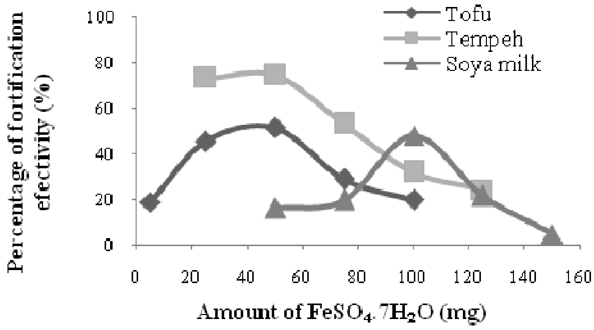
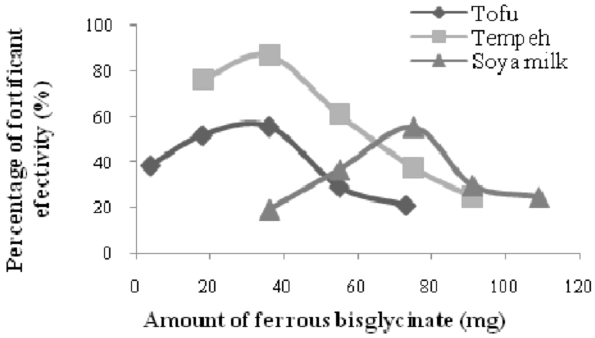


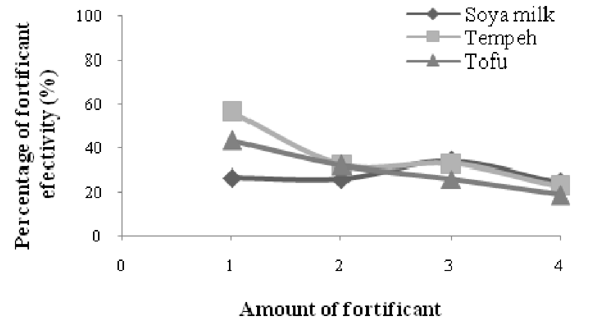
Figure 3. Calibration Curve of Phytic Acid



(a)



(b)



Group	$FeSO_4.7H_2O$ (mg)	Na-glycine (mg)
1	50	43
2	74	65
3	99	87
4	124	108

(c)

Figure 4. Percentage of Fortificant Efectivity with Fortificant Variants: (a) $FeSO_4.7H_2O$, (b) Ferrous Bisglycinate, (c) Na-Glycine+$FeSO_4.7H_2O$

stable source of iron [17-20]. The stability of ferrous bisglycinate related to pH condition and temperature stability up to 220 °C over the application in foods. This compound is stable at between pH 3-10, out of this pH range the chelate bond will break [11]. The figure 4(b) reveals that the maximum effectiveness percentage for tofu, and tempeh was occurred at the addition of 36 mg of **2** as high 55% and 86%, respectively, whereas maximum effectiveness of soya milk will reach 55% after addition of 75 mg of **2**.

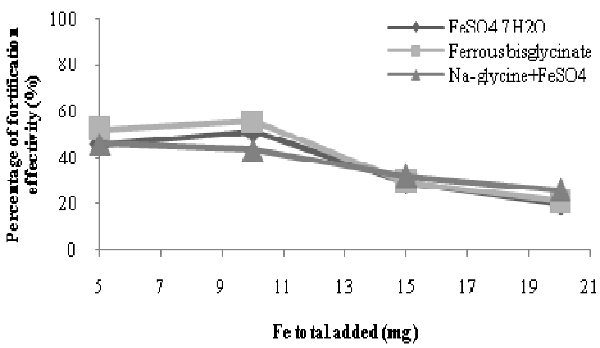
The result of this study in the addition of ferrous bisglycinate chelate in the process of making tofu and tempeh (soybeans source of 30 g each) showed that the highest percent effectiveness fortificant to tofu and tempeh is at the amount of 36 mg ferrous bisglycinate (Figure 4(b)).

Ferrous bisglycinate prevent iron to bind with phytate due to its natural character as a chelating agent. This chelate is formed from two molecules of glycine that bind with iron cation resulting in double heterocyclic ring. This configuration is protect iron from acid inhibitors such as phytic acid.

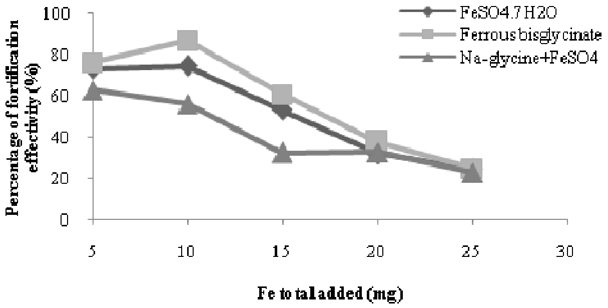
Iron fortification was also done with addition of **3**. The **3** is the in-situ mixture between Na-glycine and $FeSO_4.7H_2O$ to the sample matrix. Figure 4(c) shows that the percentage of fortification effectivity is decreased with higher amount of iron addition. The Figure 4(c) reveals that the maximum effectiveness percentage for tofu, and tempeh was occurred at the addition of 50 mg of $FeSO_4.7H_2O$ as high 46% and 56%, respectively, whereas maximum effectiveness of soya milk will reach 33% after addition of 100 mg of $FeSO_4.7H_2O$. The addition of this mixture is performed to compare the effectivity of the Fe ions in between the in-situ iron complexes and single compound 1.

Fortification using ferrous bisglycinate showed more efective result for each sample. Figure 5 (a-c) shows that the addition of ferrous bisglycinate fortificant (**2**) provide higher efficacy than the fortificant **1** and **3**. Fe ions in the form of ferrous bisglycinate are more stable because Fe ions are in the form of chelates that bind to the glycine. This causes the phytic acid is more difficult to attack Fe ions. Whereas using the in-situ mixture of Na-glycine and $FeSO_4.7H_2O$ as fortificant **3** might possible that not all the Fe ions in $FeSO_4.7H_2O$ bind with glycine to form a stable bisglycinate complex.

In all sample matrix used in this study showed that tempeh showed more effective for iron fortification than of tofu and soya milk. This is caused that during the fermentation of tempeh using yeast; the pytic acid was also fermented. This can be seen by the phytic acid content in the sample matrix was significant different. The phytic acid content in tofu, tempeh and soya milk are 4.0 mg, 0.7 mg and 4.0 mg, respectively.



(a)



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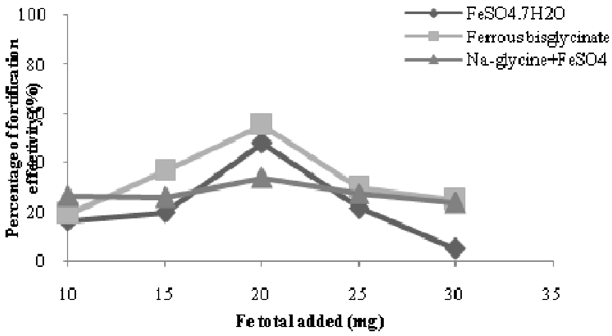

(c)

Figure 5. Fortificant Effectivity Comparison for Each Sample. (a) Tofu, (b) Tempeh, (c) Soya Milk

4. Conclusions

Iron fortification using $FeSO_4.7H_2O$, ferrous bisglycinate, and Na-glycine+$FeSO_4.7H_2O$ into three samples of soy-based foods, tofu, tempeh, and soya milk was done.The chelate agent ferrous bisglycinate was succesfully synthesized in this research with FTIR spectrum of the nitrogen bonding at the frequency between 3000-3200 cm^{-1} with broadband stretch indicates amine bond to Fe and the carbon peaks at 511 cm^{-1} as the carbon participated in the chelate bond. Phytic acid content in each sample was determined and resulted in the highest phytic acid level in soya milk with 988 mg/200 ml soya milk.

Iron fortification using ferrous bisglycinate resulted in highest effectiveness of fortification for the three samples compared to $FeSO_4.7H_2O$ and Na-glycine+$FeSO_47H_2O$. Ferrous bisglycinate prevent iron to bind with phytate due to its character as a chelating agent. The more the amount of fortificant is added, fortification effectiveness decreased as the result of more opportunities of phytic acid to bind iron ions.

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