DEPHYTINISATION OF CORN (*Zea mays* L) BY CITRIC ACID AND PHYTASE'S *Bacillus subtilis* HOLIWOOD GRESIK TO INCREASE THE IRON DIALYZABILITY

LENY YUANITA, SUZANA SURODJO

Chemistry Department, Faculty of Mathematics and Natural Sciences

State University of Surabaya

(yuanita@sby.dnet.net.id)

ABSTRACT

The research objective was to increase the iron dialyzability in corn defitinisation through variation of soaking, addition of citric acid and phytase. The stages of the research: 1) isolation of crude phytase extracts from *B.subtilis* Holiwood Gresik isolate by One shot case study design, 2) determining the variation of soaking time and concentration of citric acid that produced the lowest phytate by Factorial design, 3) soaking in citric acid and addition of phytase to increase the iron dialyzability by Post test control group design. Data were analyzed by two-way and one-way Anova (α = 5%). Results: 1) The crude phytase extracts activity: 0.254 U/ml. 2) 12-hour soaking in 9% citric acid yielded the decreasing phytate content of corn to the remaining 31.789% and if accompanied by the addition of 250 µl/50 ml phytase yielded the decreasing until the remaining 6.559% of the original. 3) The use of citric acid alone and the combination of citric acid and phytase increased the iron dialyzability of corn by 3.8 -and 5.7-fold respectively.

Keywords: *dephytinisation, phytase, B.substilis, Holiwood Gresik, iron dialyzability*

INTRODUCTION

Corn (*Zea mays* L) is one of people's staple food of in Indonesia, high in protein content (9,8%) but contains phytate which binds a variety of minerals, thereby reducing their bioavailability. Various treatments have been undertaken to degrade phytate (dephytinisation) through processing, the regulation of the medium pH, the addition of phytase enzyme, or the creation of transgenic plants. According to Hurrell (2002), through enzymatic technique is probably a way to completely degrade phytate of cereals and legumes; while Haros et al. (2001) indicated that the benefits in the use of phytase is to

improve nutrition through a reduction in phytate content and to increase the activity of endogenous α amylase.

Various compounds also have activities releasing phosphorus from the phytate, e.g. citric acid, acetate. The role of citric acid is due to one or more phosphate groups on phytic acid has a higher affinity for protons than Ca and Mg (Maenz, 2000). According to Attapattu and Nelligaswatta (2005), the possible mechanism is the formation of a bond between citric acid and mineral which reduces the formation of highly indigestible mineral-phytate complexes; or the controlling of the pH on the chemical bonds between phytic acid with fibre, protein and amino acids can be helpful to make them more accessible to endogenous enzymes. Proposed by Rice et al. (1997), phytase activity changes along the digestive tract, the efficient hydrolysis of phytate occurs in the stomach. An interaction of acid and phytase was obtained on average stomach pH. Phytase without acid will increase the pH of the stomach, but by adding acid and phytase in combination, the stomach pH would decrease. Baruah et al. (2005) found that the addition of 500 U/ kg microbial phytase and 3% citric acid in the feed *Labeo rohita* juveniles increased bone Fe contents by 40.7%, and the effects of phytase improved because of the addition of citric acid (3%).

It has been isolated bacteria which produce the highest activity mesophyll phytase from the soil on the west side of the Holiwood Gresik limestone mountain in East Java. Based on the test of physiology, biochemistry, and 16s-RNA gene analysis it is concluded that the bacterial species is *Bacillus subtilis* (*B.subtilis* Holiwood Gresik). The phytase which is the result of semipurification on *B.subtilis* Holiwood Gresik has an optimum pH of 6.5 – 7.0, an optimum temperature of 41^{o} C, Michaelis Menten constant (K_M) = 0.62 mM and V max = 0.393 µmol/ ml/ min (Yuanita et al., 2008).

In this study, phytase's *B.subtilis* Holiwood-Gresik was used for dephytinisation of corn through different variations of soaking, addition of citric acid and phytase to increase its iron dialyzability. Through this research we will learn about the condition of soaking, the concentration of citric acid and phytase for dephytinisation to produce 'free of phytate' corn flour that increase the bioavailability of minerals, especially iron.

MATERIAL AND METHODE

Research Design

The study was conducted through three stages. Stage 1: the isolation of crude phytase extracts from *B.subtilis* Holiwood Gresik isolate through One Shot Case Study Design. Stage 2: the determination of the variation of soaking time and concentration of citric acid that produces the lowest levels of phytate, through Factorial Design. The dependent variable is the phytate content of corn. Data were analyzed by two-way Anova (α = 5%). Stage 3: the use of soaking, citric acid and phytase to reduce phytate content and increase iron dialyzability through the Post Test Control Group Design. Data were analyzed by one-way Anova (α = 5%). Iron dialyzability analysis was performed by in-vitro at the gastrointestinal system conditions. Iron contents were analyzed by Atomic Absorption Spectrophotometer and one-way Anova (α = 5%). The sample of corn (hybrid corn varieties of Premier P 21) was obtained from PT Titan Multi Agro in Malang.

Research Procedure

On the first stage, crude phytase extracts were isolated from *B. subtilis* HG cultures (Greiner et al., 2001). On the second stage, each 100 grams of corn grains (20 mesh sieve) were treated with different variation of citric acid levels (0, 1.5, 3, and 4.5%) and lengths of soaking time (0/without soaking, 2, 4, and 6 hours). The citric acid volume was 50 ml, the soaking was at room temperature. From phytate content analysis (AOAC, 1990), it was then determined the treatment that produced the lowest phytate (such as Px). On the third stage, six groups of sample grains of corn were randomly prepared. After the Px treatment of each group, the corn grains were rinsed and drained. Each with the addition of phytase as follows: P1 (Px treatment), P2 (Px treatment + 50 µL/50 ml phytase), P3 (Px treatment + 100 µL/ 50 ml phytase), P4 (Px treatment + 150 µL/ 50 ml phytase), P5 (Px treatment + 200 µL/ 50 ml phytase), P6 (Px treatment + 250 µL/ 50 ml phytase) (Haros, et al., 2001); and it was then determined the treatment that produced the lowest phytate (such as Py). The iron dialyzability tests were performed on the results of the treatment of P0, Px and Py by Miller et al. (1981) procedure.

RESULTS AND DISCUSSION

Through of growth on liquid screening media, containing sodium phytate substrate, 175 rpm, 37°C and 20-hour incubation, phytase crude extracts was obtained. Phytase activity was determined by measuring the phosphate content generated during the enzymatic reaction, changes in phytic acid ($C_6H_{18}O_{24}P_6$) to become mioinositol (pentakis, tetrakis, tris, bis and monophosphate) and inorganic phosphate (Boyce et al., 2004). Inorganic phosphate reacts with ammonium molybdate reagent (Ferrous sulfate ammonium phosphomolybdate complex) to produce blue-green ammonium phosphomolybdate (λ = 660 nm). The test resulted on phytase activity of crude extracts of 0.254 U / ml.

The phytate contents of corn that had been treated with various concentrations of citric acid (0, 3, 6 and 9%) and soaking time (0, 4, 8, 12 hours) were presented in Table 1. They were determined based on the formation of Fe (III) phytate, and the results of the reduction were detected with o-phenantroline form purple-red complex (λ = 510 nm).

Table 1. The effect of citric acid concentration (% w/v) and soaking time (hours) on phytate content of corn (% dry weight of corn)[†]

Soaking time	Concentration of citric acid (%)				F,p
	0	3	6	9	
0 hours	1,252[a]	0,565[b]	0,583[c]	0,499[d]	F= 10,432 p= 0,000
4 hours	0,624[e]	0,649[f]	0,612[g]	0,437[h]	
8 hours	0,592[i]	0,628[j]	0,563[k]	0,421[l]	
12 hours	0,579[m]	0,593[n]	0,526[o]	0,398[p]	
	F= 22,983; p= 0,000				F= 8,641 p= 0,009

[†] Values in each column or row not followed by the same superscript letter are significantly different at $p \leq 0.05$

Table 1 shows that through soaking in 9% citric acid during 12 hours yielded a reducing level of phytate to 31.789% (from 1.252% to 0.398%). Increasing the soaking time and the citric acid concentration yielded a significant decrease in phytate content of corn ($p<0.05$). This is because citric acid is able to release phosphate groups on phytate (Maenz, 2000). According to Attapattu and Nelligaswatta (2005), with the presence of citric acid, the bonding between citric acid and minerals will take place, which reduces the mineral-phytate complex formation. Mineral-phytate complexes are indigestible in the intestines, so that phosphorus and inositol that are obtained can not be used by the body.

Phytase is widespread in plants, animal tissues or intestines, and several species of fungi and bacteria. The foodstuff that contain phytase are corn, rice and their subsequently generated products. However, phytases are not stable in food or have low activities, so they can not be expected to serve as enzyme sources. The distribution of phytase in plants is inconsistent with their phytate content and there is a possibility that phytase activities are inhibited by high phytate content, so exogenous phytases must be added.

The corn grains that are treated with soaking in 9% citric acid for 12 hours, then rinsed and drained, were organolepticly acceptable; therefore the soaking treatment in 9% citric acid for 12 hours (as Px) was used in analyzing the effect of phytase concentration on phytate content in corn. The effect of variations of phytase concentration added on the phytate content in corn were presented in Table 2.

Table 2. The effect of phytase concentration (µl/50ml) on corn phytate content (% dry weight of corn) †

Treatment	Phytase concentration (µl)	Phytate content (% w/w)	F,p
P1	0	0,398[a]	F= 14,219
P2	50	0,386[b]	p= 0,000
P3	100	0,344[c]	
P4	150	0,287[d]	
P5	200	0,161[e]	
P6	250	0,082[f]	

† Values in each column not followed by the same superscript letter are significantly different at $p \leq 0.05$

Table 2 shows that the phytate content experienced a decrease in elevated concentration of phytase (50-250µl/50ml) ($p < 0.05$). The addition of 250 µl/50ml phytase indicated the lowest content of phytate in comparison to other concentrations.

According to Miller et al. (1981), the iron availability or bioavailability for its absorption can be tested in vitro on the condition of the gastrointestinal system. The soluble iron compound with low relative molecular weights (MW) was obtained from protein digestion by the pepsin-pancreatic enzyme, and which diffuse through a semipermeable membrane MWCO 6000-8000 is known as iron dialyzability; and it is used to estimate iron bioavailability. In this research, iron dialyzability was measured from 20 ml corn suspension of 0.289 gram/ml. The results of iron dialyzability analysis were presented in Table 3

Table 3. The effect of soaking in citric acid and the addition of phytase on the iron dialyzability[†]

Treatment	Phytate content (%)	Iron dialyzability (ppm)	F,p
Without treatment	1.252	0.059[a]	F= 495.454 p=0.000
9% citric acid, 12-hours soaking time	0.398	0.224[b]	
9% citric acid, 12-hours soaking time, 250µl/50ml enzyme	0.082	0.338[c]	

[†] Values in each column not followed by the same superscript letter are significantly different at $p \leq 0.05$

Table 3 shows that there is a significant increase in iron dialyzability at the addition of 9% citric acid and 12-hour soaking time (3.8x of the original), the addition of 9% citric acid, 12- hour soaking time and the addition of phytase 250µl/50ml (5.7x of the original). The increase of iron dialyzability of 3.8 and 5.7 fold accompanied by a decrease of phytate content until 31.789% and 6.559% respectively. The effects of adding phytase decreased object to 20.603%, and increased iron dialyzability 1.5x on the suspension of corn that

have been soaked in 9% citric acid for 12 hours. This phenomenon indicates that the decrease in phytate levels was accompanied by the increase of the iron dialyzability. The use of a combination of citric acid and phytase decreased phytate content until 6.559% (or decreased 93.441% of the phytate content) and increased iron dialyzability at 5.7 times. The former research results showed that the wheat phytase content decreased 85% through the use of phytase and citric acid to enhance iron dialyzability until 15 times (Porres et al., 2001). According to Hurrell (2002), the use of phytase enzyme caused a complete degradation, thus significantly increases its bioavailability.

There are two kinds of iron (Fe) in the body, i.e haem Fe and non-haem Fe. Haem Fe is more easily absorbed (15-35%) than non-haem Fe (2-20%). The absorption velocity of haem Fe by the body is higher than non-haem Fe, but the majority of foodstuffs containing non-haem Fe. Non-haem Fe absorption from corn is influenced by an inhibitor and an activator factor. Organic acids, including citric acid is a non-haem Fe absorption activator, whereas phytate is an inhibitor. The role of citric acid is to form bonds between citric acid and minerals, furthermore, they release the phosphate groups on phytate. According to Clydesdale (1988), citric acid can act as an activator or an inhibitor of Fe absorption, it depends on the pH and the ratio of Fe/citrate. The high ratio of citrate/Fe will form ferricitrate complex and the alkaline conditions (duodenum) to form a soluble chelate $[FeCit_2]^{5-}$.

The ability of phytic acid to chelate mineral ions will be lost when a phosphate group is hydrolyzed by the phytase enzyme. The degradation by phytase is through the hydrolysis of phytate to lower inositol phosphates, which can occur during fermentation, food processing, and digestion in humans. So it will lower the binding capacity of mineral or a more soluble compound is formed, therefore it will reduce the negative effects on the mineral absorption or bioavailability of Fe will increase.

CONCLUSION

1. The isolation of crude enzyme extracts from *B.subtilis* Holiwood Gresik isolate produced phytase with the enzyme activity of 0.254 U/ml.

2. The variation of 12-hour soaking in 9% citric acid yielded the decreasing phytate content of corn to 31.789% and if accompanied by the addition of 250 µl/50 ml phytase yielded the decreasing to 6.559% of the original.

3. The use of citric acid alone and the combination of citric acid and phytase increased the iron dialyzability of corn by 3.8 -and 5.7-fold respectively of the original.

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