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Effect of germination and eggshells nanocalcium fortification on characteristics of germinated mung bean drink (*Vigna radiata*.)

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ABSTRACT

Germinated mung bean drinks with eggshell nanocalcium fortification are an alternative beverage to replace milk. This study aimed to determine the effect of germination time and eggshells nanocalcium fortification on physicochemical characteristics of the germinated mung bean drink. This study used a Factorial Completely Randomized Design with two treatments factors. The first factor was germination time which consisted of three treatment levels (6, 12 and 18 hours) and the second factor was the type of eggshell nanocalcium which consisted of two type of treatment (chicken and duck eggshell). Each experiment was repeated three times. Observed parameters in this study were chemical characteristics (protein, calcium, vitamin C, and pH) and physical characteristics (viscosity and stability) of germinated mung bean drink. The results showed that the germination time treatment significantly affected the value of protein, calcium, vitamin C, pH, viscosity, and stability of the germinated mung bean drink. Germination increased vitamin C in mung beans with an average value of 8,21-14,66 mg/100 g. Eggshell nanocalcium fortification showed a significant effect on increasing calcium of germinated mung bean drink. The highest calcium content of germinated mung bean drink was found in drink produced from 6 hours of germination with fortification of duck nanocalcium, which was 24.82%.

Key words: physicochemical, chicken, duck, calcium, vitamin C

Introduction

To meet the calcium needs of the Indonesian people who generally suffer from lactose intolerance and digestive disorders after consuming milk, it is necessary to drink milk substitutes made from plant-based foods that contain high protein and calcium. Mung bean is the third highest source of vegetable protein after soybeans and peanuts with a content of protein around 20-25% (Purwono and Hartono, 2005; Hastuti et al., 2018). One of the superior varieties of mung beans is VIMA-1 (Vigna sinensis–Malang). VIMA-1 has a promising market potential due to its high protein content (28.02%), low fat (0.40%), and high starch content (67.62%) (Agency for Research and Development, 2019). However, mung beans have anti-nutritional compounds, compounds found naturally in various types of beans, which can prevent the absorption of nutrients in the body.

One process that can increase the nutrition in beans is germination. Germinated mung beans have improved nutritional, functional and biological properties by changing the content, nutritional composition and bioactive compounds, as well as eliminating anti-nutritional factors in beans (Liu et al., 2020). Germination is a condition in which dormant seeds begin to germinate and grow into seedlings under the right growing conditions. The growth of shoots in germinated seeds ranges from 2 mm to up to 5 mm (Munarko et al., 2019).

The germination process in mung bean seeds is able to increase the nutritional value by activating enzymes that can reduce or eliminate anti-nutritional factors (Ebert et al. 2017). Phytic acid, the main anti-nutritional substance found in green beans, can be reduced from 12.0 mg/g to 4.03 mg/g through the germination process (Faradilla et al., 2012). Furthermore, germination is able to reduce anti-nutritional substances in germinated bean flour by 66.70% (Nur et al., 2019), so the bioavailability of vitamins and minerals in the flour increase. This is because the germination process can release the bound form of vitamins and minerals into a freer form so that it is more easily digested and absorbed by the human digestive tract. Previous research explained that during germination there is a breakdown of complex molecules into simpler forms which causes germination of green beans to be more easily digested and absorbed by the body (Ghavidel and Prakash, 2007; Masood et al., 2014). In addition, the content of vitamin C increased with the increasing duration time of germination. Ebert et al. (2017) stated that the average vitamin C in germinated bean was 2.7 times higher than that without germination.

The eggshells of chickens and ducks are household waste that has not been utilized optimally due to the lack of public knowledge about the contents in it. Egshells consist of 95.1% salt, 3.3% organic matter, and 1.6% water. The main component of inorganic salts in eggshells is dominated by calcium carbonate (CaCO3) of 98.5% (Nurjayanti et al., 2012). The bioavailability of eggshells is ± 40% (Szeleszczuk et al., 2015) with calcium content in chicken and duck eggshells of 25.73% and 23.67%, respectively (Aminah and Meikawati, 2016).

Calcium contained in eggshells has the potential as an alternative source of calcium that can be fortified in germinated mung bean drinks as a substitute for milk. Calcium is generally available in micro size which is thought to be only able to absorb 50% of the total calcium consumed in its metabolism. The nano form of eggshell calcium can make it easier for calcium to be more soluble and optimally absorbed. According to Widyastuti and Kusuma (2017), nanocalcium is the manufacture of particles with a size of less than 100 nm by changing the nature or function of a material.

There are two methods in the manufacture of nanoparticles, namely top down (physical method) and bottom up (chemical method). The bottom up method is a method of making nanoparticles by arranging atoms or molecules to form nanometer-sized particles from a solution. In the bottom up method, the formation of nanoparticles has a high degree of cohesion so that it is able to produce a more uniform size. One of the bottom-up methods that is often used is precipitation because this method is very effective in the manufacture of nanoparticles, the process is simple, and requires low costs. The precipitation method is carried out by controlling the solubility of the material in the solution through changes in pH (Suptijah et al., 2012). Therefore, the objective of this study was to investigate the effect of mung bean germination time and nanocalcium fortification of eggshells on the characteristics of germinated mung bean drink (*Vigna radiata*).

Materials and Methods

The materials used in this study were VIMA-1 mung beans, eggshells (chicken and duck), 1N HCL, 3N NaOH, aquabidest, and other chemicals for analysis. The tools used were 100 mesh sieve, Beaker glass (Phyrex, Japan), blender (Philips HR 2116, China), Erlenmeyer (Iwaki, Japan), hot plate, muffle furnace (Barnstead FB1410M-26, USA), mortar, electric oven (Memmert, USA), measuring pipette (Iwaki, Japan), pH meter (Oakton do-35613, USA), Spectrophotometer (UV-VISAE S80, China), AAS (Thermo Scientific Nicolet^{TM}10, USA), analytical balance (Ohaus AR2140, USA), and a Brookfield viscometer (NDJ-8S, China).

Research Methods

This research was carried out at the Agricultural Product Chemistry Laboratory and Agricultural Product Processing Laboratory, Agricultural Technology Department, Faculty of Agriculture, Sriwijaya University. This research was conducted using a completely randomized factorial design with two treatment factors, germination time of mung bean (A) which consisted of 3 treatment levels (A1 = 6 hours; A2 = 12 hours; and A3 = 18 hours) and the type of eggshell nanocalcium (B) which consisted of 2 treatment (B1 = chicken eggshell, and B2 = duck eggshell). The treatment was repeated 3 times. Mung bean drink without germination and addition of eggshell nanocalcium was a control. Addition of eggshell nanocalcium to all treatments was 20% of the RDI for calcium, which was 1200 mg/day per serving size (240 mL).

Research Implementation

Eggshell micro powder preparation (Rahmawati and Nisa, 2015)

Preparation of eggshell micro powder was carried out by preparing 500 g for each chicken and duck eggshells and washed with running water until clean. The eggshells were boiled at 100^oC for 3 minutes to kill pathogenic microbes, and then the eggshells were drained. The eggshells were baked for 3 hours at 60^oC, and placed at room temperature. The cold eggshells were mashed using a blender at a speed of ±18.000 rpm for 3 minutes. The eggshell powder was then sieved through a 100 mesh sieve. The finished eggshell powder was put into OPP (Oriented Polystyrene) plastic and stored at 4^oC for analysis.

Eggshell nanocalcium powder preparation (Khoerunnisa, 2011)

Eggshell powder was soaked in 1N HCl (1:5) for 48 hours and extracted at 90^oC for 1 hour. The extracts were filtered using filter paper to obtain the filtrate and precipitate. The filtrate was precipitated with the addition of 3N NaOH and stirred, and then allowed to stand until a precipitate was formed. The precipitate was then neutralized using aquabidest until the pH was neutral. The solution is separated from the precipitate by pouring it slowly so that the precipitate does not get wasted. The precipitate was dried in oven for 3 hours at a temperature of 105^oC, followed by burning in muffle furnace at a temperature of 600^oC for 5 hours, and then crushed using a mortar to obtain nanocalcium powder. The nanocalcium powder was vacuum packed and stored at 4^oC.

Mung bean germination (Wea et al., 2014)

The mung beans were sorted and washed with running water until clean and then soaked for 6 hours. The mung beans are drained and placed on tray with a damp cloth. The green beans is placed in room temperature and germinated for 6, 12 and 18 hours, where every 6 hours watering is done with water. Germinated mung beans are separated from the skin and taken as much as 100 grams for further processing.

Germinated mung bean drinks preparation (Wea et al., 2014)

The germinated mung beans were blanched by boiling at 80°C for 2 minutes, peeled and then mashed with a blender for 3 minutes, added with water in a ratio of 1: 7.5. The germinated mung bean drinks is filtered and then cooked by adding nanocalcium eggshell and 7% sugar. Cooking is carried out until it boils and stirred for 5 minutes then cooled for 30 minutes.

Analysis

The analysis of the germinated mung bean drinks were chemical characteristics: protein content using Lowry method (Harjanto, 2017), calcium content was using AAS method (AOAC, 2005), vitamin C content (Sudarmadji et al. 2007), pH value (Sudarmadji et al., 2014), and physical characteristics: viscosity (Yuwono and Susanto, 1998) and stability (Wibowo et al., 2014).

Results

Analysis of the characteristics of germinated mung bean drink with nanocalcium fortification

The interaction between germination time and the type of eggshell nanocalcium gave no significant effect on all characteristics of germinated mung bean drink (Table 1). This was because addition of the nanocalcium (CaO) powder of chicken and duck eggshells to the germinated mung bean drinks in the same amount (20% of nutrition facts label for calcium). Moreover, the characteristics of the eggshell nanocalcium (CaO) powder were white color, very fine texture (particle size of CaO in chicken and duck eggshells is 41 .54 nm and 24.90 nm), and neutral pH so that it gave no significant effect on the value of viscosity, stability, protein, pH and vitamin C of germinated mung bean drink (Table 2).

Table 1. Physical and chemical characteristics of germinated mung bean drinks with nanocalcium fortification

Treatments	Parameter					
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)	Calcium (%)
A_1B_1	27,60±0,20[a]	87,00±1,00[a]	1,90±0,05[a]	6,81±0,02[a]	8,21±1,02[a]	17,82
A_1B_2	27,13±0,15[a]	85,67±1,53[a]	1,81±0,09[a]	6,77±0,04[a]	7,62±1,02[a]	24,82
A_2B_1	26,17±0,29[a]	73,00±1,00[a]	1,77±0,11[a]	6,74±0,04[a]	11,73±1,01[a]	16,45
A_2B_2	26,03±0,45[a]	71,67±1,53[a]	1,66±0,14[a]	6,69±0,04[a]	11,14±1,01[a]	23,66
A_3B_1	25,53±0,21[a]	65,67±0,58[a]	1,56±0,14[a]	6,63±0,04[a]	14,66±1,01[a]	15,66
A_3B_2	24,97±0,76[a]	64,67±1,53[a]	1,50±0,18[a]	6,62±0,01[a]	13,49±1,02[a]	22,49
Kontrol	28,80±0,01[a]	91,33±0,58[a]	2,01±0,16[a]	6,81±0,02[a]	5,28±1,76[b]	0,00

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
Control = germination time of green beans 0 hours without the addition of nanocalcium
A_1 = mung bean germination time for 6 hours B_1 = nanocalsium of chicken eggshell
A_2 = mung bean germination time for 12 hours B_2 = nanocalcium of duck eggshell
A_3 = mung bean germination time for 18 hours

Table 2. Effect of eggshell nanocalcium fortification on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH*	Vitamin C (mg/100g)
B_1	26,43±0,23[a]	75,22±0,86[a]	1,74±0,10[a]	6,73±0,03[a]	11,54±1,02[a]
B_2	26,04±0,46[a]	74,00±1,53[a]	1,66±0,13[a]	6,69±0,03 [a]	10,75±1,02[a]

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
B_1 = nanocalcium of chicken eggshell
B_2 = nanocalcium of duck eggshell

Germination time was significantly effect the characteristics of germinated mung been drinks (Table 3).

Table 3.The effect of germination time on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)
A_1	27,37±0,49^{a}	65,17±1,26^{a}	1,53±0,07^{a}	6,62±0,03^{a}	7,92±1,02^{a}
A_2	26,10±0,37^{b}	72,33±1,26^{b}	1,72±0,13^{ab}	6,72±0,04^{b}	11,44±1,01^{b}
A_3	25,25±0,18^{c}	86,33±1,05^{c}	1,85±0,16^{b}	6,79±0,02^{c}	14,08±1,02^{c}

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
A_1 = mung bean germination time for 6 hours
A_2 = mung bean germination time for 12 hours
A_3 = mung bean germination time for 18 hours

Discussion

Table 1 shows a significant increase in the vitamin C and calcium content of germinated mung bean drinks to the control. The increase in vitamin C in germinated mung bean drinks in all treatments in this study was due to the food reserves contained in the cotyledons in the form of starch hydrolyzed by the amylase enzyme into simple sugars which were then synthesized into vitamin C during the germination process. The results of this study were in line with the opinion of Ebert et al. (2017) which stated that the content of vitamin C in the germinated peanut seeds increased by 2.7 times compared to the ungerminated seed.

The calcium content of germinated mung bean drinks fortified with nanocalcium of duck eggshell was higher than the calcium content of germinated mung bean drinks fortified with nanocalcium of chicken eggshell. This was because the calcium content in duck eggshell nanocalcium (45.50%) was higher than the calcium level in chicken eggshell nanocalcium (35.50%).

Viscosity

Viscosity is a measure of the size of the friction that occurs in the fluid. The greater the viscosity of a food, the thicker the material, and vice versa (Srihidayati, 2017). The viscosity of germinated mung bean drinks in the control treatment (0 hour germination without nanocalcium fortification) was higher (28.80±0.01 m.Pa.s) compared to that of nanocalcium fortified germinated mung bean drinks for all treatments in this study. This is due to the fact that ungerminated mung bean starch has not been hydrolyzed so that it still has higher starch content than germinated mung bean starch. In the preparation of mung bean drinks, starch will undergo gelatinization in the presence of heat treatment which will cause the starch granules to break and the starch molecules to come out and be released from the granules and then enter the solution system. The results of this study are in line with the results of research conducted by Nurjanati et al. (2018), based on the hedonic test, ungerminated red bean milk (0 hours) has a higher viscosity than germinated red bean milk (8, 16 and 24 hours).

Table 3 shows that the viscosity of the green bean germination drink decreased significantly with the duration of germination. The decrease in the viscosity of the material during germination was caused by the hydrolysis of starch during the germination process by the amylase enzymes. The enzyme is formed at the beginning of germination by giberylic acid (Elobuike et al., 2021). Starch will be broken down into simple sugars in the form of glucose which is used as energy and needs for seed growth. Another factor that causes a decrease in the viscosity of mung bean germination drink is the increase in vitamin C during the germination process which results in a decrease in pH as shown in Table 3. A decrease in pH results in hydrolysis of glycosidic bonds which causes a decrease in viscosity. The results of this study are in line with the statement of Widjaja et al. (2019), that a solution will decrease its viscosity if its pH decreases.

Stability

The stability of the products can be seen by the presence or absence of precipitate in the product (Farikha et al., 2013). Germinated mung bean drinks in the control treatment (0 hour germination without the addition of nanocalcium) had a higher stability value (91%) compared to all treatments of germinated mung bean drinks fortified with nanocalcium in this study. This was because germinated mung bean seeds absorb more water than ungerminated mung bean seeds. During germination, bean seeds require optimal environmental conditions that are 50-80% RH (Yuwariah et al., 2015). Water absorption in the germination process is needed to maintain cell turgidity, including cell enlargement, photosynthetic reactions, salt solvents, gases and other substances that are transported between cells in tissues to maintain cell growth (Felania, 2017).

Table 3 shows that the stability of the germinated mung bean drinks decreased significantly as the duration of germination increased. This was due to an increase in the water content of germinated mung beans along with the length of germination due to the watering the mung bean seeds every 6 hours during germination time. The water content of germinated mung bean with a time of 0, 6, 12 and 18 hours were 4.32%, 4.99%, 5.42% and 7.22%, respectively. The increase in water content in germinated mung beans resulting germinated mung bean drinks have more water content in turns it affects the stability value of the drinks. In line with the viscosity value of the germinated mung bean drinks, the average value of stability of each treatment decreased with the duration of germination. The decrease in viscosity at germinated mung bean drinks correlated to that in stability.

Protein Content

The protein of germinated mung bean drinks in treatment A2 (12 hours of germination time) was not significantly different from that of treatment A1 (6 hours of germination time) and treatment A3 (12 hours of germination time). The decreasing of protein content in germinated mung bean drinks was due to the hydrolysis of protein into amino acids that are used for embryo growth. The results of this study were in line with the statement of Masood et al. (2014) which stated a decrease in protein content along with an increase in amino acid content during germination of green beans. This is due to an increase in the activity of the protease enzyme which is an endogenous enzyme that is active during the imbibition process, when green beans are in contact with water. Proteases hydrolyze proteins into peptides and amino acids (Ferdiawan et al., 2019). Free amino acids along with glutamic and aspartic acids (in the form of amides) will be translocated to the embryo in the formation of new structures in line with the ongoing germination stage (Pertiwi et al., 2013).

pH value

pH is a parameter used to determine changes in the acidity level of a food product (Widowati et al., 2020). Table 3 shows that the longer the germination time, the lower the pH of the germinated mung bean drinks significantly. According to Wea et al. (2014), in germinated seed plants there was an increase in vitamin synthesis, especially vitamin C (Wea et al., 2014). This situation is in accordance with the vitamin C content in the germinated mung bean drinks in this study (Table 3) so that the pH of the germinated mung bean drinka is low. Based on Indonesian National Standard 01-3830-1995, the pH standard for bean drinks is 6.5-7.0. The pH of germinated mung bean drinks with nanocalcium fortification in this study has a value of 6.62-6.79; it has met the pH standard of bean drinks.

Vitamin C

Table 3 shows that the vitamin C of the germinated mung bean drinks increased significantly with increasing of germination time. The increase in vitamin C along with germination time is caused by several enzyme systems being active during germination. This happens because of the accumulation of ascorbic acid as a result of biosynthesis. Germination causes reactivation of the enzyme L-Galactono-γ-lactone dehydrogenase which is involved in the oxidation of L-galactono-1,4-lactone to ascorbic acid. The activity of this enzyme increased in parallel to the biosynthesis of ascorbic acid during the seed germination process. Differences in the level of ascorbic acid biosynthesis in germination of mung bean seeds can also be influenced by legume varieties, maturity, climate, light, harvesting and storage methods (Masood et al., 2014). Increasing the content of vitamin C in germination of mung beans can help the solubility of nanocalcium fortification in the germinated mung bean drinks. This is in line with the statement of Yonata et al. (2017), that pH can affect the solubility of minerals. Acidic conditions and small particle size can increase the speed of nanocalcium dissolution from both duck and chicken eggshells, so that the germinated mung bean drinks with eggshells nanocalcium fortification has good digestibility.

Calcium Content

Based on the results of the analysis, calcium content of nanocalcium in chicken and duck eggshells was 35.50% and 45.50%, respectively. Calcium content in eggshells is influenced by the thickness and type of eggshell. Duck eggshells have an average thickness of 0.36 - 0.46 mm (Septiana et al., 2015), while the average thickness of chicken eggshells was 0.33 - 0.35 mm (Azizah et al., 2015). Differences in eggshell thickness can affect the amount of mineral content and organic salts, especially calcium carbonate ($CaCO_3$) which acts as a source of calcium in eggshells.

Fortification is a process of increasing the content of essential micronutrients in the form of vitamins and minerals into food, aiming to increase nutrients that do not yet exist or to enrich existing nutrients (Valentina et al., 2014). According to the Indonesian Food and Drug Supervisory Agency (2019), the minimum amount of micronutrient fortification in foodstuffs is 10% of the RDI per serving. In this study, the amount of nanocalcium fortification of chicken and duck eggshells in germinated mung bean drink was 20% of the RDI for calcium per 250 mL serving.

Calcium content of nongerminated mung bean drinks with nanocalcium fortification of chicken and duck eggshells was 18.97% and 25%, respectively. This showed that the calcium content of germinated mung bean drinks both fortified with nanocalcium chicken and duck eggshells decreased with the increasing of germination time (Table 1). This was in accordance with the statement of Oghbaei and Prakash (2020) that the calcium content decreased significantly in germinated and peeled bean seeds. Peeling can reduce some of the minerals present in the skin of green beans. However, this germination and skin peeling treatment had an impact on increasing the bioavailability of mung beans which contributed to stimulating the reduction of anti-nutritional substances. The percentage of bioavailability in the seeds before germination was 14.91–17.19% and in the germinated beans which were peeled 22.96–25.27%.

In addition, the decrease in calcium content of germinated mung bean drinks in this study was thought to be due to the treatment with germination time less than 24 hours so that the phytase enzyme had not worked optimally, causing the complex phytic acid that binds to minerals such as calcium could not be completely released. Based on the results of research by Ghavidel and Davoodi (2011), enzyme activity (phytase, amylase and protease) increased significantly at 24, 48, and 72 hours of germination in several types of legumes, one of which was green beans. The maximum enzymatic activity occurred at 72 hours of germination. Although the calcium of germinated mung bean drinks decreased in the 6, 12 and 18 hour germination treatment, but with an increase in vitamin C at the same germination time treatment it could increase the calcium absorption efficiency of the germinated mung bean drinks.

Conclusion

Nanocalcium fortification of chicken and duck eggshells by 20% of the RDI for calcium per 250 mL serving had no significant effect on the physical (viscosity and stability) and chemical (protein, pH, and vitamin C) characteristics of germinated mung bean drinks. Germination of mung beans at different times was significantly increased value of pH, vitamin C, protein, and stability; but significantly decreased value of viscosity. Germinated mung bean drinks are a good carrier for chicken and duck eggshells nanocalcium fortification.

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Table 1. Physical and chemical characteristics of germinated mung bean drinks with nanocalcium fortification

Treatments	Parameter					
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)	Calcium (%)
A_1B_1	27,60±0,20[a]	87,00±1,00[a]	1,90±0,05[a]	6,81±0,02[a]	8,21±1,02[a]	17,82
A_1B_2	27,13±0,15[a]	85,67±1,53[a]	1,81±0,09[a]	6,77±0,04[a]	7,62±1,02[a]	24,82
A_2B_1	26,17±0,29[a]	73,00±1,00[a]	1,77±0,11[a]	6,74±0,04[a]	11,73±1,01[a]	16,45
A_2B_2	26,03±0,45[a]	71,67±1,53[a]	1,66±0,14[a]	6,69±0,04[a]	11,14±1,01[a]	23,66
A_3B_1	25,53±0,21[a]	65,67±0,58[a]	1,56±0,14[a]	6,63±0,04[a]	14,66±1,01[a]	15,66
A_3B_2	24,97±0,76[a]	64,67±1,53[a]	1,50±0,18[a]	6,62±0,01[a]	13,49±1,02[a]	22,49
Kontrol	28,80±0,01[a]	91,33±0,58[a]	2,01±0,16[a]	6,81±0,02[a]	5,28±1,76[b]	0,00

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
Control = germination time of green beans 0 hours without the addition of nanocalcium
A_1 = mung bean germination time for 6 hours
A_2 = mung bean germination time for 12 hours
A_3 = mung bean germination time for 18 hours
B_1 = nanocalsium of chicken eggshell
B_2 = nanocalcium of duck eggshell

Table 2. Effect of eggshell nanocalcium fortification on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH*	Vitamin C (mg/100g)
B_1	26,43±0,23[a]	75,22±0,86[a]	1,74±0,10[a]	6,73±0,03[a]	11,54±1,02[a]
B_2	26,04±0,46[a]	74,00±1,53[a]	1,66±0,13[a]	6,69±0,03 [a]	10,75±1,02[a]

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
B_1 = nanocalcium of chicken eggshell
B_2 = nanocalcium of duck eggshell

Germination time was significantly effect the characteristics of germinated mung been drinks

Table 3.The effect of germination time on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)
A_1	27,37±0,49[a]	65,17±1,26[a]	1,53±0,07[a]	6,62±0,03[a]	7,92±1,02[a]
A_2	26,10±0,37[b]	72,33±1,26[b]	1,72±0,13[ab]	6,72±0,04[b]	11,44±1,01[b]
A_3	25,25±0,18[c]	86,33±1,05[c]	1,85±0,16[b]	6,79±0,02[c]	14,08±1,02[c]

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)

Data = mean ± standard deviation

A_1 = mung bean germination time for 6 hours

A_2 = mung bean germination time for 12 hours

A_3 = mung bean germination time for 18 hours

2. Bukti Review dari Reviewer Pertama dan ke Dua

(22 Desember 2022)

Effect of germination and eggshells nanocalcium fortification on characteristics of germinated mungbean drink (*Vigna radiata*.)

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ABSTRACT

Germinated mung bean drinks with eggshell nanocalcium fortification are an alternativebeverageto replace milk. This study aimed to determine the effect of germination time and eggshells nanocalcium fortificationon physicochemical characteristics of the germinated mung bean drink.
This study used a Factorial Completely Randomized Design with two treatmentsfactors. The first factor was germination timewhich consisted of three treatment levels (6,12 and 18 hours) and the second factor was the type of eggshell nanocalcium which consisted of two type of treatment (chicken and duck eggshell), plus the control treatment as mung bean drink without germination and addition of eggshell nanocalcium. Each experiment was repeated three times.Observed parameters in this study were chemical characteristics (protein, calcium, vitamin C, and pH) and physical characteristics(viscosityand stability) of germinated mung beandrink. The results showed that the germination time treatment significantly affected the value of protein, calcium, vitamin C, pH, viscosity, and stability of the germinated mung beandrink. Germination increasedvitamin Cin mung beans with an average value of 8,21-14,66 mg/100 g. Eggshell nanocalcium fortification showed a significant effect on increasing calcium of germinated mung bean drink. The highest calcium content of germinated mung bean drink was found in drink produced from 6 hoursof germination with fortification of duck nanocalcium, which was 24.82%.

Keywords: physicochemical, chicken, duck, calcium, vitamin C

Introduction

To meet the calcium needs of the Indonesian people who generally suffer from lactose intolerance and digestive disorders after consuming milk, it is necessary to drink milk substitutes made from plant-based foods that contain high protein and calcium. Mung bean is the third highest source of vegetable protein after soybeans and peanuts with a content of protein around 20-25% (Purwono and Hartono, 2005; Hastuti et al., 2018). One of the superior varieties of mung beans is VIMA-1 (Vigna sinensis–Malang). VIMA-1 has a promising market potential due to its high protein content (28.02%), low fat (0.40%), and high starch content (67.62%) (Agency for Research and Development, 2019). However, mungbeans have anti-nutritional compounds, compounds found naturally in various types of beans, which can prevent the absorption of nutrients in the body.

One process that can increase the nutrition in beans is germination. Germinated mung beans have improved nutritional, functional and biological properties by changing the content, nutritional composition and bioactive compounds, as well as eliminating anti-nutritional factors in beans (Liu et al., 2020). Germination is a condition in which dormant seeds begin to germinate and grow into seedlings under the right growing conditions. The growth of shoots in germinated seeds ranges from 2 mm to up to 5 mm (Munarko et al., 2019).

The germination process in mung bean seeds is able to increase the nutritional value by activating enzymes that can reduce or eliminate anti-nutritional factors (Ebert et al. 2017). Phytic acid, the main anti-nutritional substance found in green beans, can be reduced from 12.0 mg/g to 4.03 mg/g through the germination process (Faradilla et al., 2012). Furthermore, germination is able to reduce anti-nutritional substances in germinated bean flour by 66.70% (Nur et al., 2019), so the bioavailability of vitamins and minerals in the flour increase. This is because the germination process can release the bound form of vitamins and minerals into a freer form so that it is more easily digested and absorbed by the human digestive tract. Previous research explained that during germination there is a breakdown of complex molecules into simpler forms which causes germination of green beans to be more easily digested and absorbed by the human body (Ghavidel and Prakash, 2007; Masood et al., 2014).In addition, the content of vitamin C increased with the increasing duration time of germination. Ebert et al. (2017) stated that the average vitamin C in germinated bean was 2.7 times higher than that without germination.

The eggshells of chickens and ducks are household waste that has not been utilized optimally due to the lack of public knowledge about the contents in it.Egshells consist of 95.1% salt, 3.3% organic matter, and 1.6% water. The main component of inorganic salts in eggshells is dominated by calcium carbonate (CaCO3) of 98.5% (Nurjayanti et al., 2012). The bioavailability of eggshells is ± 40% (Szeleszczuk et al., 2015) with calcium content in chicken and duck eggshells of 25.73% and 23.67%, respectively (Aminah and Meikawati, 2016).

Calcium contained in eggshells has the potential as an alternative source of calcium that can be fortified in germinated mung bean drinks as a substitute for milk. Calcium is generally available in micro size which is thought to be only able to absorb 50% of the total calcium consumed in its metabolism. The nano form of eggshell calcium can make it easier for calcium to be more soluble and optimally absorbed. According to Widyastuti and Kusuma (2017), nanocalcium is the manufacture of particles with a size of less than 100 nm by changing the nature or function of a material.

There are two methods in the manufacture of nanoparticles, namely top down (physical method) and bottom up (chemical method). The bottom up method is a method of making nanoparticles by arranging atoms or molecules to form nanometer-sized particles from a solution. In the bottom up method, the formation of nanoparticles has a high degree of cohesion so that it is able to produce a more uniform size. One of the bottom-up methods that is often used is precipitation because this method is very effective in the manufacture of nanoparticles, the process is simple, and requires low costs. The precipitation method is carried out by controlling the solubility of the material in the solution through changes in pH (Suptijah et al., 2012). Therefore, the objective of this study was to investigate the effect of mung bean germination time and nanocalcium fortification of eggshells on the characteristics of germinated mung bean drink (*Vigna radiata*).

What were the hypothesis of the research?

Material and Methods

The materials used in this study were VIMA-1 mung beans, eggshells (chicken and duck), 1N HCL, 3N NaOH, aquabidest, and other chemicals for analysis. The tools used were 100 mesh sieve, Beaker glass (Phyrex, Japan), blender (Philips HR 2116, China), Erlenmeyer (Iwaki, Japan), hot plate, muffle furnace (Barnstead FB1410M-26, USA), mortar, electric oven (Memmert, USA), measuring pipette (Iwaki, Japan), pH meter (Oakton do-35613, USA), Spectrophotometer (UV-VISAE S80, China), AAS (Thermo Scientific Nicolet^{TM}10, USA), analytical balance (Ohaus AR2140, USA), and a Brookfield viscometer (NDJ-8S, China).

Research Methods

This research was carried out at the Agricultural Product Chemistry Laboratory and Agricultural Product Processing Laboratory, Faculty of Agriculture, Sriwijaya University, Indonesia(-3.2196118175204167, 104.64789811916464). This research was conducted using a completely randomized factorial design with two treatment factors, germination time of mung bean (A) which consisted of 3 treatment levels (A1 = 6 hours; A2 = 12 hours; and A3 = 18 hours) and the type of eggshell nanocalcium (B) which consisted of 2 treatment (B1 = chicken eggshell, and B2 = duck eggshell). The treatment was repeated 3 times. Mung bean drink without germination and addition of eggshell nanocalcium was a control. Addition of eggshell nanocalcium to all treatments was 20% of the RDI for calcium, which was 1200 mg/day per serving size (240 mL).

Research Implementation

Eggshell micro powder preparation (Rahmawati and Nisa, 2015)

Preparation of eggshell micro powder was carried out by preparing 500 g for each chicken and duck eggshells and washed with running water until clean. The eggshells were boiled at 100°C for 3 minutes to kill pathogenic microbes, and then the eggshells were drained. The eggshells were baked for 3 hours at 60°C, and placed at room temperature. The cold eggshells were mashed using a blender at a speed of ±18.000 rpm for 3 minutes. The eggshell powder was then sieved through a 100 mesh sieve. The finished eggshell powder was put into OPP (Oriented Polystyrene) plastic and stored at 4°C for analysis.

Eggshell nanocalcium powder preparation (Khoerunnisa, 2011)

Eggshell powder was soaked in 1N HCl (1:5) for 48 hours and extracted at 90°C for 1 hour. The extracts were filtered using filter paper to obtain the filtrate and precipitate. The filtrate was precipitated with the addition of 3N NaOH and stirred, and then allowed to stand until a precipitate was formed. The precipitate was then neutralized using aquabidest until the pH was neutral. The solution is separated from the precipitate by pouring it slowly so that the precipitate does not get wasted. The precipitate was driedin oven for 3 hours at a temperature of 105°C, followed by burning in muffle furnace at a temperature of 600°C for 5 hours, and thencrushed using a mortar to obtain nanocalcium powder. The nanocalcium powder was vacuum packed and stored at 4°C.

Mung bean germination (Wea et al., 2014)

The mung beans were sorted and washed with running water until clean and then soaked for 6 hours. The mung beans are drained and placed on tray with a damp cloth. The green beans wereplaced in room temperature and germinated for 6, 12 and 18 hours, where every 6 hourswatering is done with water. Germinated mung beans are separated from the skin and taken as much as 100 grams for further processing.

Germinated mungbean drinks preparation (Wea et al., 2014)

The germinated mung beans were blanched by boiling at 80°C for 2 minutes, peeled and then mashed with a blender for 3 minutes, added with water in a ratio of 1: 7.5. The germinated mung bean drinkswasfiltered and then cooked by adding nanocalcium eggshell and 7% sugar. Cooking wascarried out until it boils and stirred for 5 minutes and finallycooled for 30 minutes.

Analysis

The analysis of the germinated mung bean drinkswere chemical characteristics: protein content using Lowry method (Harjanto, 2017), calcium content was usingAAS method (AOAC, 2005), vitamin C content (Sudarmadji et al. 2007), pH value (Sudarmadji et al., 2014), and physical characteristics: viscosity (Yuwono and Susanto, 1998) and stability (Wibowo et al., 2014).

How was done the statistical analysis? Was done test Dunnett to compare treatments with control?

Results

Analysis of the characteristics of germinated mung bean drink with nanocalcium fortification

The interaction between germination time and the type of eggshell nanocalcium wasno significant effect on all characteristics of germinated mung bean drink, excepted for Vitamin C and calcium that were higer in all treatments compared to control (Table 1).The non-significanceamong the treatments was attributed toaddition of the nanocalcium (CaO) powder of chicken and duck eggshells to the germinated mung bean drinks in the same amount (20% of nutrition facts label for calcium). Moreover, the characteristics of the eggshell nanocalcium (CaO) powder were white color, very fine texture (particle size of CaO in chicken and duck eggshells is41.54 nm and 24.90 nm, respectivelly), and neutral pH so that it take to theno significant effect on the value of viscosity, stability, protein, pH and vitamin C of germinated mung bean drink (Table 2).

Table 1. Physical and chemical characteristics of germinated mung bean drinks with nanocalcium fortification

Treatments	Parameter					
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)	Calcium (%)
A_1B_1	27,60±0,20^{a}	87,00±1,00^{a}	1,90±0,05^{a}	6,81±0,02^{a}	8,21±1,02^{a}	17,82
A_1B_2	27,13±0,15^{a}	85,67±1,53^{a}	1,81±0,09^{a}	6,77±0,04^{a}	7,62±1,02^{a}	24,82
A_2B_1	26,17±0,29^{a}	73,00±1,00^{a}	1,77±0,11^{a}	6,74±0,04^{a}	11,73±1,01^{a}	16,45
A_2B_2	26,03±0,45^{a}	71,67±1,53^{a}	1,66±0,14^{a}	6,69±0,04^{a}	11,14±1,01^{a}	23,66
A_3B_1	25,53±0,21^{a}	65,67±0,58^{a}	1,56±0,14^{a}	6,63±0,04^{a}	14,66±1,01^{a}	15,66
A_3B_2	24,97±0,76^{a}	64,67±1,53^{a}	1,50±0,18^{a}	6,62±0,01^{a}	13,49±1,02^{a}	22,49
Control	28,80±0,01^{a}	91,33±0,58^{a}	2,01±0,16^{a}	6,81±0,02^{a}	5,28±1,76^{b}	0,00

Note:Numbers marked with the same letter notation in the same column indicate not significantly different (5%) write the significante letter in the calcium column.
Data = mean ± standard deviation
Control = germination time of green beans 0 hours without the addition of nanocalcium
A_1 = mung bean germination time for 6 hours B_1 = nanocalsium of chicken eggshell
A_2 = mung bean germination time for 12 hours B_2 = nanocalcium of duck eggshell
A_3 = mung bean germination time for 18 hours

Table 2.Effect of eggshell nanocalcium fortification on the characteristics of germinated mung bean drinks Why the data of calcium is no showing in this Table?

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH*	Vitamin C (mg/100g)
B_1	26,43±0,23^{a}	75,22±0,86^{a}	1,74±0,10^{a}	6,73±0,03^{a}	11,54±1,02^{a}
B_2	26,04±0,46^{a}	74,00±1,53^{a}	1,66±0,13^{a}	6,69±0,03^{a}	10,75±1,02^{a}

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation

B_1 = nanocalcium of chicken eggshell
B_2 = nanocalcium of duck eggshell

Germination time had significantly effect on the characteristics of germinated mung been drinks (Table 3). Write the results of the characterisitics analysed (Viscosity, Stability, Protein, pH and Vitamin C).

Table 3.The effect of germination time on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)
A_1	27,37±0,49^{a}	65,17±1,26^{a}	1,53±0,07^{a}	6,62±0,03^{a}	7,92±1,02^{a}
A_2	26,10±0,37^{b}	72,33±1,26^{b}	1,72±0,13^{ab}	6,72±0,04^{b}	11,44±1,01^{b}
A_3	25,25±0,18^{c}	86,33±1,05^{c}	1,85±0,16^{b}	6,79±0,02^{c}	14,08±1,02^{c}

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
A_1 = mung bean germination time for 6 hours
A_2 = mung bean germination time for 12 hours
A_3 = mung bean germination time for 18 hours

Discussion

Table 1 shows a significant increase in the vitamin C and calcium content of germinated mung bean drinks to the control. The increase in vitamin C in germinated mung bean drinks in all treatments in this study was due to the food reserves contained in the cotyledons in the form of starch hydrolyzed by the amylase enzyme into simple sugars which were then synthesized into vitamin C during the germination process. The results of this study were in line with the opinion of Ebert et al. (2017)

which stated that the content of vitamin C in the germinated peanut seeds increased by 2.7 times compared to the ungerminated seed.

The calcium content of germinated mung bean drinks fortified with nanocalcium of duck eggshell was higher than the calcium content of germinated mung bean drinks fortified with nanocalcium of chicken eggshell (Show this result in the Table 2). This was because the calcium content in duck eggshell nanocalcium (45.50%) was higher than the calcium level in chicken eggshell nanocalcium (35.50%).

Viscosity

Viscosity is a measure of the size of the friction that occurs in the fluid. The greater the viscosity of a food, the thicker the material, and vice versa (What is better in this case? High or low viscosity? (Srihidayati, 2017). The viscosity of germinated mung bean drinks in the control treatment (0 hour germination without nanocalcium fortification) was higher (28.80±0.01 m.Pa.s) compared to that of nanocalcium fortified germinated mung bean drinks for all treatments in this study. This is due to the fact that ungerminated mung bean starch has not been hydrolyzed so that it still has higher starch content than germinated mung bean starch. In the preparation of mung bean drinks, starch will undergo gelatinization in the presence of heat treatment which will cause the starch granules to break and the starch molecules to come out and be released from the granules and then enter the solution system. The results of this study are in line with the results of research conducted by Nurjanati et al. (2018), based on the hedonic test, ungerminated red bean milk (0 hours) has a higher viscosity than germinated red bean milk (8, 16 and 24 hours).

Table 3 shows that the viscosity of the green bean germination drink decreased significantly with the duration of germination. The decrease in the viscosity of the material during germination was caused by the hydrolysis of starch during the germination process by theamylase enzymes. The enzyme is formed at the beginning of germination by giberylic acid (Elobuike et al., 2021). Starch will be broken down into simple sugars in the form of glucose which is used as energy and needs for seed growth. Another factor that causes a decrease in the viscosity of mung bean germination drink is the increase in vitamin C during the germination process which results in a decrease in pH as shown in Table 3. A decrease in pH results in hydrolysis of glycosidic bonds which causes a decrease in viscosity. The results of this study are in line with the statement of Widjaja et al. (2019), that a solution will decrease its viscosity if its pH decreases.

Stability

The stability of the products can be seen by the presence or absence of precipitate in the product (Farikha et al., 2013). What means high or low values of stability? Is high value better or no for mung beasn drink? Germinated mung bean drinks in the control treatment (0 hour germination without the addition of nanocalcium) had a higher stability value (91%) compared to all treatments of germinated mung bean drinks fortified with nanocalcium in this study. This was because germinated mung bean seeds absorb more water than ungerminated mung bean seeds. During germination, bean seeds require optimal environmental conditions that are 50-80% RH (Yuwariah et al., 2015). Water absorption in the germination process is needed to maintain cell turgidity, including cell enlargement, photosynthetic reactions, salt solvents, gases and other substances that are transported between cells in tissues to maintain cell growth (Felania, 2017).

Table 3 shows that the stability of the germinated mung bean drinks decreased significantly as the duration of germination increased. This was due to an increase in the water content of germinated mung beans along with the length of germination due to the watering the mung bean seeds every 6 hours during germination time. The water content of germinated mung bean with a time of 0, 6, 12 and 18 hours were 4.32%, 4.99%, 5.42% and 7.22%, respectively. The increase in water content in germinated mung beans resulting germinated mung bean drinks have more water content in turns it affects the stability value of the drinks. In line with the viscosity value of the germinated mung bean drinks, the average value of stability of each treatment decreased with the duration of germination. The decrease in viscosity at germinated mung bean drinks correlated negativelyto that in stability.

Protein Content

The protein of germinated mung bean drinks in treatment A3(18hours of germination time) was significantly higherfrom that of treatment A1 (6 hours of germination time) but without differ from thatA2(12 hours of germination time). The decreasing of protein content in germinated mung bean drinks was due to the hydrolysis of protein into amino acids that are used for embryo growth. The results of this study were in line with the statement of Masood et al. (2014) which stated a decrease in protein content along with an increase in amino acid content during germination of green beans. This is due to an increase in the activity of the protease enzyme which is an endogenous enzyme that is active during the imbibition process, when green beans are in contact with water.Proteases hydrolyze proteins into peptides and amino acids (Ferdiawan et al., 2019). Free amino acids along with glutamic and aspartic acids (in the form of amides) will be translocated to the embryo in the formation of new structures in line with the ongoing germination stage (Pertiwi et al., 2013).

pH value

pH is a parameter used to determine changes in the acidity level of a food product What is the adequate pH for mung beans drink? (Widowati et al., 2020). Table 3 shows that the longer the germination time, the lower the pH of the germinated mung bean drinkswas recorded significantly. According to Wea et al. (2014), in germinated seed plants there was an increase in vitamin synthesis, especially vitamin C (Wea et al., 2014). This situation is in accordance with the vitamin C content in the germinated mung bean drinks in this study (Table 3) so that the pH of the germinated mung bean drinka is low.Based on Indonesian National Standard 01-3830-1995, the pH standard for bean drinks is 6.5-7.0. The pH of germinated mung bean drinks with nanocalcium fortification in this study has a value of 6.62-6.79; it has met the pH standard of bean drinks.

Vitamin C

Table 3 shows that the vitamin C of the germinated mung bean drinks increased significantly with increasing of germination time. The increase in vitamin C along with germination time is caused by several enzyme systems being active during germination. This happens because of the accumulation of ascorbic acid as a result of biosynthesis. Germination causes reactivation of the enzyme L-Galactono-γ-lactone dehydrogenase which is involved in the oxidation of L-galactono-1,4-lactone to ascorbic acid. The activity of this enzyme increased in parallel to the biosynthesis of ascorbic acid during the seed germination process (citation). Differences in the level of ascorbic acid biosynthesis in germination of mung bean seeds can also be influenced by legume varieties, maturity, climate, light, harvesting and storage methods (Masood et al., 2014). Increasing the content of vitamin C in germination of mung beans can help the solubility of nanocalcium fortification in the germinated mung bean drinks. This is in line with the statement of Yonata et al. (2017), that pH can affect the solubility of minerals. Acidic conditions and small particle size can increase the speed of nanocalcium dissolution from both duck and chicken eggshells, so that the germinated mung bean drinks with eggshells nanocalcium fortification has good digestibility.

Calcium Content

Based on the results of the analysis, calcium content of nanocalcium in chicken and duck eggshells was 35.50% and 45.50%, respectively. Calcium content in eggshells is influenced by the thickness and type of eggshell. Duck eggshells have an average thickness of 0.36 - 0.46 mm (Septiana et al., 2015), while the average thickness of chicken eggshells was 0.33 - 0.35 mm (Azizah et al., 2015). Differences in eggshell thickness can affect the amount of mineral content and organic salts, especially calcium carbonate (CaCO3) which acts as a source of calcium in eggshells.

Fortification is a process of increasing the content of essential micronutrients in the form of vitamins and minerals into food, aiming to increase nutrients that do not yet exist or to enrich existing nutrients (Valentina et al., 2014). According to the Indonesian Food and Drug Supervisory Agency (2019), the minimum amount of micronutrient fortification in foodstuffs is 10% of the RDI per serving. In this study, the amount of nanocalcium fortification of chicken and duck eggshells in germinatedmung bean drink was 20% of the RDI for calciumper 250 mL serving.

Calcium content of nongerminated mung bean drinks with nanocalcium fortification of chicken and duck eggshells was 18.97% and 25%, respectively. This showed that the calcium content of germinated mung bean drinks both fortified with nanocalcium chicken and duck eggshells decreased with the increasing of germination time (Table 1). This was in accordance with the statement of Oghbaei and Prakash (2020) that the calcium content decreased significantly in germinated peeled bean seeds. Peeling can reduce some of the minerals present in the skin of green beans. However, this germination and skin peeling treatment had an impact on increasing the bioavailability of mung beans which contributed to stimulating the reduction of anti-nutritional substances. The percentage of bioavailability in the seeds before germination was 14.91–17.19% and in the germinated beans which were peeled 22.96–25.27%.

In addition, the decrease in calcium content of germinated mung bean drinks in this study was thought to be due to the treatment with germination time less than 24 hours so that the phytase enzyme had not worked optimally, causing the complex phytic acid that binds to minerals such as calcium could not be completely released. Based on the results of research by Ghavidel and Davoodi (2011), enzyme activity (phytase, amylase and protease) increased significantly at 24, 48, and 72 hours of germination in several types of legumes, one of which was green beans. The maximum enzymatic activity occurred at 72 hours of germination. Although the calcium of germinated mung bean drinks decreased in the 6, 12 and 18 hour germination treatment, but with an increase in vitamin C at the same germination time treatment it could increase the calcium absorption efficiency of the germinated mung bean drinks.

Conclusion

Nanocalcium fortification of chicken and duck eggshells by 20% of the RDI for calcium per 250 mL servinghad no significant effect on the physical (viscosity and stability) and chemical (protein, pH, and vitamin C) characteristics of germinated mung bean drinks. Germination of mung beans at different times was significantly increased value of pH, vitamin C, protein, and stability; but significantly decreased value of viscosity. Germinated mung bean drinksare a good carrier for chicken and duck eggshells nanocalcium fortification.

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Effect of germination and eggshells nanocalcium fortification on characteristics of germinated mung bean drink (*Vigna radiata.*)

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ABSTRACT

Germinated mung bean drinks with eggshell nanocalcium fortification are an alternative beverage to replace milk. This study aimed to determine the effect of germination time and eggshells nanocalcium fortification on physicochemical characteristics of the germinated mung bean drink. This study used a Factorial Completely Randomized Design with two treatments factors. The first factor was germination time which consisted of three treatment levels (6, 12 and 18 hours) and the second factor was the type of eggshell nanocalcium which consisted of two type of treatment (chicken and duck eggshell), plus the control treatment as mung bean drink without germination and addition of eggshell nanocalcium. Each experiment was repeated three times. Observed parameters in this study were chemical characteristics (protein, calcium, vitamin C, and pH) and physical characteristics (viscosity and stability) of germinated mung bean drink. The results showed that the germination time treatment significantly affected the value of protein, calcium, vitamin C, pH, viscosity, and stability of the germinated mung bean drink. Germination increased vitamin C in mung beans with an average value of 8,21-14,66 mg/100 g. Eggshell nanocalcium fortification showed a significant effect on increasing calcium of germinated mung bean drink. Germinated mung bean drink produced from 6 hours of germination and fortification of duck nanocalcium had the highest calcium content with value of 24.82%.

Key words: Physicochemical, Chicken, Duck, Calcium, Vitamin C

INTRODUCTION

To meet the calcium needs of the Indonesian people who generally suffer from lactose intolerance and digestive disorders after consuming milk, it is necessary to drink milk substitutes made from plant-based foods that contain high protein and calcium. Mung bean is the third highest source of vegetable protein after soybeans and peanuts with a content of protein around 20-25% (Purwono and Hartono, 2005; Hastuti et al., 2018). One of the superior varieties of mung beans is VIMA-1 (Vigna sinensis–Malang). VIMA-1 has a promising market potential due to its high protein content (28.02%), low fat (0.40%), and high starch content (67.62%) (Agency for Research and Development, 2019). However, mung beans have anti-nutritional compounds, compounds found naturally in various types of beans, which can prevent the absorption of nutrients in the body.

One process that can increase the nutrition in beans is germination. Germinated mung beans have improved nutritional, functional and biological properties by changing the content, nutritional composition and bioactive compounds, as well as eliminating anti-nutritional factors in beans (Liu et al., 2020). Germination is a condition in which dormant seeds begin to germinate and grow into seedlings under the right growing conditions. Germination in brown rice refers to the point where the growth of a radicle ranges from 2 mm to 5 mm, and pre-germination brown rice is the stage with an expanded a radicle exposed approximately 0.5–1 mm (Watanabe et al., 2004). Moreover, Palmiano and Juliano (1972) stated that the optimal germination period was 18 to 24 hours.

The germination process in mung bean seeds is able to increase the nutritional value by activating enzymes that can reduce or eliminate anti-nutritional factors (Ebert et al. 2017). Phytic acid, the main anti-nutritional substance

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found in green beans, can be reduced from 12.0 mg/g to 4.03 mg/g through the germination process (Faradilla et al., 2012). Furthermore, germination is able to reduce anti-nutritional substances in germinated bean flour by 66.70% (Nur et al., 2019), so the bioavailability of vitamins and minerals in the flour increase. This is because the germination process can release the bound form of vitamins and minerals into a freer form so that it is more easily digested and absorbed by the human digestive tract. Previous research explained that during germination there is a breakdown of complex molecules into simpler forms which causes germination of green beans to be more easily digested and absorbed by the human body (Ghavidel and Prakash, 2007; Masood et al., 2014). In addition, the content of vitamin C increased with the increasing duration time of germination. Ebert et al. (2017) stated that the average vitamin C in germinated bean was 2.7 times higher than that without germination.

The eggshells of chickens and ducks are household waste that has not been utilized optimally due to the lack of public knowledge about the contents in it. Egshells consist of 95.1% salt, 3.3% organic matter, and 1.6% water. The main component of inorganic salts in eggshells is dominated by calcium carbonate (CaCO3) of 98.5% (Nurjayanti et al., 2012). The bioavailability of eggshells is ± 40% (Szeleszczuk et al., 2015) with calcium content in chicken and duck eggshells of 25.73% and 23.67%, respectively (Aminah and Meikawati, 2016).

Calcium contained in eggshells has the potential as an alternative source of calcium that can be fortified in germinated mung bean drinks as a substitute for milk. Calcium is generally available in micro size which is thought to be only able to absorb 50% of the total calcium consumed in its metabolism. The nano form of eggshell calcium can make it easier for calcium to be more soluble and optimally absorbed. According to Widyastuti and Kusuma (2017), nanocalcium is the manufacture of particles with a size of less than 100 nm by changing the nature or function of a material.

There are two methods in the manufacture of nanoparticles, namely top down (physical method) and bottom up (chemical method). The bottom up method is a method of making nanoparticles by arranging atoms or molecules to form nanometer-sized particles from a solution. In the bottom up method, the formation of nanoparticles has a high degree of cohesion so that it is able to produce a more uniform size. One of the bottom-up methods that is often used is precipitation because this method is very effective in the manufacture of nanoparticles, the process is simple, and requires low costs. The precipitation method is carried out by controlling the solubility of the material in the solution through changes in pH (Suptijah et al., 2012). Therefore, the objective of this study was to investigate the effect of mung bean germination time and nanocalcium fortification of eggshells on the characteristics of germinated mung bean drink (*Vigna radiata*).

The hypothesis of this study was it is suspected that germination time and type of eggshell nanocalcium fortification has a significant effect on the physico-chemical characteristics of the resulting germinated mung bean drink.

MATERIAL AND METHODS

The materials used in this study were VIMA-1 mung beans, eggshells (chicken and duck), 1N HCL, 3N NaOH, aquabidest, and other chemicals for analysis. The tools used were 100 mesh sieve, Beaker glass (Phyrex, Japan), blender (Philips HR 2116, China), Erlenmeyer (Iwaki, Japan), hot plate, muffle furnace (Barnstead FB1410M-26, USA), mortar, electric oven (Memmert, USA), measuring pipette (Iwaki, Japan), pH meter (Oakton do-35613, USA), Spectrophotometer (UV-VISAE S80, China), AAS (Thermo Scientific NicoletTM10, USA), analytical balance (Ohaus AR2140, USA), and a Brookfield viscometer (NDJ-8S, China).

RESEARCH METHODS

This research was carried out at the Agricultural Product Chemistry Laboratory and Agricultural Product Processing Laboratory, Faculty of Agriculture, Sriwijaya University, South Sumatera, Indonesia (-3.2196118175204167, 104.64789811916464). This research was conducted using a completely randomized factorial design with two treatment factors, germination time of mung bean (A) which consisted of 3 treatment levels (A1 = 6 hours; A2 = 12 hours; and A3 = 18 hours) and the type of eggshell nanocalcium (B) which consisted of 2 treatment (B1 = chicken eggshell, and B2 = duck eggshell). The treatment was repeated 3 times. Mung bean drink without germination and addition of eggshell nanocalcium was a control. Addition of eggshell nanocalcium to all treatments was 20% of the Recommended Dietary Intake for calcium, which was 1200 mg/day per serving size (240 mL).

RESEARCH IMPLEMENTATION

Eggshell micro powder preparation (Rahmawati and Nisa, 2015)

Preparation of eggshell micro powder was carried out by preparing 500 g for each chicken and duck eggshells and washed with running water until clean. The eggshells were boiled at 100°C for 3 minutes to kill pathogenic microbes, and then the eggshells were drained. The eggshells were baked for 3 hours at 60°C, and placed at room temperature. The cold eggshells were mashed using a blender at a speed of ±18.000 rpm for 3 minutes. The eggshell powder was then sieved through a 100 mesh sieve. The finished eggshell powder was put into OPP (Oriented Polystyrene) plastic and stored at 4°C for analysis.

Eggshell nanocalcium powder preparation (Khoerunnisa, 2011)

Eggshell powder was soaked in 1N HCl (1:5) for 48 hours and extracted at 90°C for 1 hour. The extracts were filtered using filter paper to obtain the filtrate and precipitate. The filtrate was precipitated with the addition of 3N NaOH and stirred, and then allowed to stand until a precipitate was formed. The precipitate was then neutralized using aquabidest until the pH was neutral. The solution is separated from the precipitate by pouring it slowly so that the precipitate does not get wasted. The precipitate was dried in oven for 3 hours at a temperature of 105°C, followed by burning in muffle furnace at a temperature of 600°C for 5 hours, and then crushed using a mortar to obtain nanocalcium powder. The nanocalcium powder was vacuum packed and stored at 4o°C.

Mung bean germination (Wea et al., 2014)

The mung beans were sorted and washed with running water until clean and then soaked for 6 hours. The mung beans are drained and placed on tray with a damp cloth. The green beans is placed in room temperature and germinated for 6, 12 and 18 hours, where every 6 hours watering is done with water. Germinated mung beans are separated from the skin and taken as much as 100 grams for further processing.

Germinated mung bean drinks preparation (Wea et al., 2014)

The germinated mung beans were blanched by boiling at 80°C for 2 minutes, peeled and then mashed with a blender for 3 minutes, added with water in a ratio of 1: 7.5. The germinated mung bean drinks is filtered and then cooked by adding nanocalcium eggshell and 7% sugar. Cooking is carried out until it boils and stirred for 5 minutes then cooled for 30 minutes.

Analysis

The analysis of the germinated mung bean drinks were chemical characteristics: protein content using Lowry method (Harjanto, 2017), calcium content was using AAS method (AOAC, 2005), vitamin C content (Sudarmadji et al. 2007), pH value (Sudarmadji et al., 2014), and physical characteristics: viscosity (Yuwono and Susanto, 1998) and stability (Wibowo et al., 2014).

Statistical analysis

All analyses were performed in triplicates. The data were subjected to the analysis of variance followed by Fisher's least significant difference (LSD) test to compare among treatment means and control. Differences were considered at a significant level of 95% (p <0.05) by using SPSS v.19 software.

RESULTS

Analysis of the characteristics of germinated mung bean drink with nanocalcium fortification

The interaction between germination time and the type of eggshell nanocalcium no significant effect on all characteristics of germinated mung bean drink, excepted for Vitamin C and calcium that were higer in all treatments compared to control (Table 1). The non-significance among the treatments was attributed to addition of the nanocalcium (CaO) powder of chicken and duck eggshells to the germinated mung bean drinks in the same amount (20% of nutrition facts label for calcium). Moreover, the characteristics of the eggshell nanocalcium (CaO) powder were white color, very fine texture (particle size of CaO in chicken and duck eggshells is 41.54 nm and 24.90 nm respectivelly), and neutral pH so that it take to the no significant effect on the value of viscosity, stability, protein, pH and vitamin C of germinated mung bean drink (Table 2).

Germination time was significantly effect the characteristics of germinated mung been drinks (Table 3).

DISCUSSION

Table 1 shows a significant increase in the vitamin C and calcium content of germinated mung bean drinks to the control. The increase in vitamin C in germinated mung bean drinks in all treatments in this study was due to the food reserves contained in the cotyledons in the form of starch hydrolyzed by the amylase enzyme into simple sugars which were then synthesized into vitamin C during the germination process. The results of this study were in line with the opinion of Ebert et al. (2017) which stated that the content of vitamin C in the germinated peanut seeds increased by 2.7 times compared to the ungerminated seed.

The calcium content of germinated mung bean drinks fortified with nanocalcium of duck eggshell was higher than the calcium content of germinated mung bean drinks fortified with nanocalcium of chicken eggshell. This was

Table 1: Physical and chemical characteristics of germinated mung bean drinks with nanocalcium fortification

Treatments	Parameter					
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)	Calcium (%)
A_1B_1	27.60 ± 0.20^a	87.00 ± 1.00^a	1.90 ± 0.05^a	6.81 ± 0.02^a	8.21 ± 1.02^a	17.82
A_1B_2	27.13 ± 0.15^a	85.67 ± 1.53^a	1.81 ± 0.09^a	6.77 ± 0.04^a	7.62 ± 1.02^a	24.82
A_2B_1	26.17 ± 0.29^a	73.00 ± 1.00^a	1.77 ± 0.11^a	6.74 ± 0.04^a	11.73 ± 1.01^a	16.45
A_2B_2	26.03 ± 0.45^a	71.67 ± 1.53^a	1.66 ± 0.14^a	6.69 ± 0.04^a	11.14 ± 1.01^a	23.66
A_3B_1	25.53 ± 0.21^a	65.67 ± 0.58^a	1.56 ± 0.14^a	6.63 ± 0.04^a	14.66 ± 1.01^a	15.66
A_3B_2	24.97 ± 0.76^a	64.67 ± 1.53^a	1.50 ± 0.18^a	6.62 ± 0.01^a	13.49 ± 1.02^a	22.49
Control	28.80 ± 0.01^a	91.33 ± 0.58^a	2.01 ± 0.16^a	6.81 ± 0.02^a	5.28 ± 1.76^b	nd

Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
Control = germination time of green beans 0 hours without the addition of nanocalcium
A_1 = mung bean germination time for 6 hours B_1 = nanocalsium of chicken eggshell
A_2 = mung bean germination time for 12 hours B_2 = nanocalcium of duck eggshell
A_3 = mung bean germination time for 18 hours

Table 2: Effect of eggshell nanocalcium fortification on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH*	Vitamin C (mg/100g)
B_1	26.43 ± 0.23^a	75.22 ± 0.86^a	1.74 ± 0.10^a	6.73 ± 0.03^a	11.54 ± 1.02^a
B_2	26.04 ± 0.46^a	74.00 ± 1.53^a	1.66 ± 0.13^a	6.69 ± 0.03^a	10.75 ± 1.02^a

Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
B_1 = nanocalcium of chicken eggshell
B_2 = nanocalcium of duck eggshell

Table 3: The effect of germination time on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)
A_1	27.37 ± 0.49^a	86.33 ± 1.05^c	1.85 ± 0.16^b	6.79 ± 0.02^c	7.92 ± 1.02^a
A_2	26.10 ± 0.37^b	72.33 ± 1.26^b	1.72 ± 0.13^{ab}	6.72 ± 0.04^b	11.44 ± 1.01^b
A_3	25.25 ± 0.18^c	65.17 ± 1.25^a	1.53 ± 0.07^a	6.62 ± 0.03^a	14.08 ± 1.02^c

Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
A_1 = mung bean germination time for 6 hours
A_2 = mung bean germination time for 12 hours
A_3 = mung bean germination time for 18 hours

because the calcium content in duck eggshell nanocalcium (45.50%) was higher than the calcium level in chicken eggshell nanocalcium (35.50%).

VISCOSITY

Viscosity is a measure of the size of the friction that occurs in the fluid. The greater the viscosity of a food, the thicker the material, and vice versa (Srihidayati, 2017). The viscosity of germinated mung bean drinks in the control treatment (0 hour germination without nanocalcium fortification) was higher (28.80±0.01 m.Pa.s) compared to that of nanocalcium fortified germinated mung bean drinks for all treatments in this study. This is due to the fact that ungerminated mung bean starch has not been hydrolyzed so that it still has higher starch content than germinated mung bean starch. In the preparation of mung bean drinks, starch will undergo gelatinization in the presence of heat treatment which will cause the starch granules to break and the starch molecules to come out and be released from the granules and then enter the solution system. The results of this study are in line with the results of research conducted by Nurjanati et al. (2018), based on the hedonic test, ungerminated red bean milk (0 hours) has a higher viscosity than germinated red bean milk (8, 16 and 24 hours).

Table 3 shows that the viscosity of the green bean germination drink decreased significantly with the duration of germination. The decrease in the viscosity of the material during germination was caused by the hydrolysis of starch during the germination process by the amylase enzymes. The enzyme is formed at the beginning of germination by giberylic acid (Elobuike et al., 2021). Starch will be broken down into simple sugars in the form of glucose which is used as energy and needs for seed growth. Another factor that causes a decrease in the viscosity of mung bean germination drink is the increase in vitamin C during the germination process which results in a decrease in pH as shown in Table 3. A decrease in pH results in hydrolysis of glycosidic bonds which causes a decrease in viscosity. The results of this study are in line with the statement of Widjaja et al. (2019), that a solution will decrease its viscosity if its pH decreases.

STABILITY

The stability of the products can be seen by the presence or absence of precipitate in the product (Farikha et al., 2013). Germinated mung bean drinks in the control treatment (0 hour germination without the addition of nanocalcium) had a higher stability value (91%) compared to all treatments of germinated mung bean drinks fortified with nanocalcium in this study. This was because germinated mung bean seeds absorb more water than ungerminated mung bean seeds. During germination, bean seeds require optimal environmental conditions that are 50-80% RH (Yuwariah et al., 2015). Water absorption in the germination process is needed to maintain cell turgidity, including cell enlargement, photosynthetic reactions, salt solvents, gases and other substances that are transported between cells in tissues to maintain cell growth (Felania, 2017).

Table 3 shows that the stability of the germinated mung bean drinks decreased significantly as the duration of germination increased. This was due to an increase in the water content of germinated mung beans along with the length of germination due to the watering the mung bean seeds every 6 hours during germination time. The water content of germinated mung bean with a time of 0, 6, 12 and 18 hours were 4.32%, 4.99%, 5.42% and 7.22%, respectively. The increase in water content in germinated mung beans resulting germinated mung bean drinks have more water content in turns it affects the stability value of the drinks. In line with the viscosity value of the germinated mung bean drinks, the average value of stability of each treatment decreased with the duration of germination. The decrease in viscosity at germinated mung bean drinks correlated to that in stability.

PROTEIN CONTENT

The protein of germinated mung bean drinks in treatment A2 (12 hours of germination time) was not significantly different from that of treatment A1 (6 hours of germination time) and treatment A3 (12 hours of germination time). The decreasing of protein content in germinated mung bean drinks was due to the hydrolysis of protein into amino acids that are used for embryo growth. The results of this study were in line with the statement of Masood et al. (2014) which stated a decrease in protein content along with an increase in amino acid content during germination of green beans. This is due to an increase in the activity of the protease enzyme which is an endogenous enzyme that is active during the imbibition process, when green beans are in contact with water. Proteases hydrolyze proteins into peptides and amino acids (Ferdiawan et al., 2019). Free amino acids along with glutamic and aspartic acids (in the form of amides) will be translocated to the embryo in the formation of new structures in line with the ongoing germination stage (Pertiwi et al., 2013).

pH VALUE

pH is a parameter used to determine changes in the acidity level of a food product (Widowati et al., 2020). Table 3 shows that the longer the germination time, the lower the pH of the germinated mung bean drinks significantly. According to Wea et al. (2014), in germinated seed plants there was an increase in vitamin synthesis, especially vitamin C (Wea et al., 2014). This situation is in accordance with the vitamin C content in the germinated mung bean drinks in this study (Table 3) so that the pH of the germinated mung bean drinka is low. Based on Indonesian National Standard 01-3830-1995, the pH standard for bean drinks is 6.5-7.0. The pH of germinated mung bean drinks with nanocalcium fortification in this study has a value of 6.62-6.79; it has met the pH standard of bean drinks.

VITAMIN C

Table 3 shows that the vitamin C of the germinated mung bean drinks increased significantly with increasing of germination time. The increase in vitamin C along with germination time is caused by several enzyme systems being active during germination. This happens because of the accumulation of ascorbic acid as a result of biosynthesis. Germination causes reactivation of the enzyme L-Galactono-γ-lactone dehydrogenase which is involved in the oxidation of L-galactono-1,4-lactone to ascorbic acid. The activity of this enzyme increased in parallel to the biosynthesis of ascorbic acid during the seed germination process. Differences in the level of ascorbic acid biosynthesis in germination of mung bean seeds can also be influenced by legume varieties, maturity, climate, light, harvesting and storage methods (Masood et al., 2014). Increasing the content of vitamin C in germination of mung beans can help the solubility of nanocalcium fortification in the germinated mung bean drinks. This is in line with the statement of Yonata et al. (2017), that pH can affect the solubility of minerals. Acidic conditions and small particle size can increase the speed of nanocalcium dissolution from both duck and chicken eggshells, so that the germinated mung bean drinks with eggshells nanocalcium fortification has good digestibility.

Calcium content

Based on the results of the analysis, calcium content of nanocalcium in chicken and duck eggshells was 35.50% and 45.50%, respectively. Calcium content in eggshells is influenced by the thickness and type of eggshell. Duck eggshells have an average thickness of 0.36 - 0.46 mm (Septiana et al., 2015), while the average thickness of chicken eggshells was 0.33 - 0.35 mm (Azizah et al., 2015). Differences in eggshell thickness can affect the amount of mineral content and organic salts, especially calcium carbonate (CaCO3) which acts as a source of calcium in eggshells.

Fortification is a process of increasing the content of essential micronutrients in the form of vitamins and minerals into food, aiming to increase nutrients that do not yet exist or to enrich existing nutrients (Valentina et al., 2014). According to the Indonesian Food and Drug Supervisory Agency (2019), the minimum amount of micronutrient fortification in foodstuffs is 10% of the RDI per serving. In this study, the amount of nanocalcium fortification of chicken and duck eggshells in germinated mung bean drink was 20% of the RDI for calcium per 250 mL serving.

Calcium content of nongerminated mung bean drinks with nanocalcium fortification of chicken and duck eggshells was 18.97% and 25%, respectively. This showed that the calcium content of germinated mung bean drinks both fortified with nanocalcium chicken and duck eggshells decreased with the increasing of germination time (Table 1). This was in accordance with the statement of Oghbaei and Prakash (2020) that the calcium content decreased significantly in germinated and peeled bean seeds. Peeling can reduce some of the minerals present in the skin of green beans. However, this germination and skin peeling treatment had an impact on increasing the bioavailability of mung beans which contributed to stimulating the reduction of anti-nutritional substances. The percentage of bioavailability in the seeds before germination was 14.91–17.19% and in the germinated beans which were peeled 22.96–25.27%.

In addition, the decrease in calcium content of germinated mung bean drinks in this study was thought to be due to the treatment with germination time less than 24 hours so that the phytase enzyme had not worked optimally, causing the complex phytic acid that binds to minerals such as calcium could not be completely released. Based on the results of research by Ghavidel and Davoodi (2011), enzyme activity (phytase, amylase and protease) increased significantly at 24, 48, and 72 hours of germination in several types of legumes, one of which was green beans. The maximum enzymatic activity occurred at 72 hours of germination. Although the calcium of germinated mung bean drinks decreased in the 6, 12 and 18 hour germination treatment, but with an increase in vitamin C at the same germination time treatment it could increase the calcium absorption efficiency of the germinated mung bean drinks.

CONCLUSION

Nanocalcium fortification of chicken and duck eggshells by 20% of the RDI for calcium per 250 mL serving had no significant effect on the physical (viscosity and stability) and chemical (protein, pH, and vitamin C) characteristics of germinated mung bean drinks. Germination of mung beans at different times was significantly increased value of pH, vitamin C, protein, and stability; but significantly decreased value of viscosity. Germinated mung bean drinks are a good carrier for chicken and duck eggshells nanocalcium fortification.

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Author Queries???

AQ1: Kindly check the reference

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3. Bukti Comment untuk Reviewer

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REVISION

No.		Queries	Comment
AQ 1	:	The references have already checked	
AQ 2	:	Azis, M. Y. et al., 2018. Eksplorasi Kadar Kalsium (Ca) dalam Limbah Cangkang Kulit Telur Bebek dan Burung Puyuh Menggunakan Metode Titrasi dan AAS. *Al-Kimiya,* 5(2), Pp. 74-77.	The reference cited in line number 65
AQ 3	:	The references have already provided in English language	
AQ 4	:	Badan Penelitian dan Pengembangan (BaLitbang). 2019. *Balai Penelitian Tanaman Aneka Kacang dan Umbi*. (Online). http://www.litbang.pertanian.go.id/varietas/546/. Diakses pada 18 Maret 2021.	The reference cited in line number 34
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AQ 6	:	Badan Standarisasi Nasional.1995. *Susu Kedelai*. Sni 01-3830-1995. Badan Standarisai Nasional. Jakarta: BSN.	The reference cited in line number 278
AQ 7	:	Daftar Komposisi Bahan Makanan (DKBM). 2017. *Tabel Komposisi Pangan Indonesia*. Jakarta: PT. Elex Media Komputindo.	The reference cited in line number 32
AQ 8	:	Handayani, L. dan Syahputra, F., 2017. Isolasi dan Karakterisasi Nanokalsium dari Cangkang Tiram (*Crassostrea gigas*). *JPHP,* 20(3), pp. 515-523.	The reference cited in line number 84
AQ 9	:	Hanura, A. B., Trilaksani, W. dan Suptijah, P., 2017. Karakterisasi Nanohidroksiapatit Tulang Tuna Thunnus Sp. Sebagai Sediaan Biomaterial. *Jurnal Ilmu Dan Teknologi Kelautan Tropis,* 9(2), Pp. 619-629.	The reference cited in line number 84
AQ 10	:	Author initial: NM, TWW. MIS, RCP	
AQ 11	:	Purwanto, M. G. M., 2014. Perbandingan Analisa Kadar Protein Terlarut dengan Berbagai Metode Spektroskopi UV-Visible. *Jurnal Ilmiah Sains dan Teknologi,* 7(2), pp. 64-71.	The reference cited in line number 149
AQ 12	:	Sunardi, S., Krismawati, E. D. dan Mahayana, A., 2020. Sintesis dan Karakterisasi Nanokalsium Oksida dari Cangkang Telur. *ALCHEMY Jurnal Penelitian Kimia,* 16(2), pp. 250-259.	The reference cited in line number 85
AQ13	:	Received: September 26, 2022 Accepted: January 13, 2023.	
AQ14	:	Research Article	

Effect of germination and eggshells nanocalcium fortification on characteristics of germinated mung bean drink (*Vigna radiata*.)

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ABSTRACT

Germinated mung bean drinks with eggshell nanocalcium fortification are an alternative beverage to replace milk. This study aimed to determine the effect of germination time and eggshells nanocalcium fortification on physicochemical characteristics of the germinated mung bean drink. This study used a Factorial Completely Randomized Design with two treatments factors. The first factor was germination time which consisted of three treatment levels (6, 12 and 18 hours) and the second factor was the type of eggshell nanocalcium which consisted of two type of treatment (chicken and duck eggshell), plus the control treatment as mung bean drink without germination and addition of eggshell nanocalcium. Each experiment was repeated three times. Observed parameters in this study were chemical characteristics (protein, calcium, vitamin C, and pH) and physical characteristics (viscosity and stability) of germinated mung bean drink. The results showed that the germination time treatment significantly affected the value of protein, calcium, vitamin C, pH, viscosity, and stability of the germinated mung bean drink. Germination increased vitamin C in mung beans with an average value of 8,21-14,66 mg/100 g. Eggshell nanocalcium fortification showed a significant effect on increasing calcium of germinated mung bean drink. Germinated mung bean drink produced from 6 hours of germination and fortification of duck nanocalcium had the highest calcium content with value of 24.82%.

Key words: physicochemical, chicken, duck, calcium, vitamin C

Introduction

To meet the calcium needs of the Indonesian people who generally suffer from lactose intolerance and digestive disorders after consuming milk, it is necessary to drink milk substitutes made from plant-based foods that contain high protein and calcium. Mung bean is the third highest source of vegetable protein after soybeans and peanuts with a content of protein around 20-25% (Purwono and Hartono, 2005; Food Composition List, 2017; Hastuti et al., 2018). One of the superior varieties of mung beans is VIMA-1 (Vigna sinensis–Malang). VIMA-1 has a promising market potential due to its high protein content (28.02%), low fat (0.40%), and high starch content (67.62%) (Agency for Research and Development, 2019). However, mung beans have anti-nutritional compounds, compounds found naturally in various types of beans, which can prevent the absorption of nutrients in the body.

One process that can increase the nutrition in beans is germination. Germinated mung beans have improved nutritional, functional and biological properties by changing the content, nutritional composition and bioactive compounds, as well as eliminating anti-nutritional factors in beans (Liu et al., 2020). Germination is a condition in which dormant seeds begin to germinate and grow into seedlings under the right growing conditions. Germination in brown rice refers to the point where the growth of a radicle ranges from 2 mm to 5 mm, and pre-germination brown rice is the stage with an expanded a radicle exposed approximately 0.5–1 mm (Watanabe et al., 2004). Moreover, Palmiano and Juliano (1972) stated that the optimal germination period was 18 to 24 hours.

The germination process in mung bean seeds is able to increase the nutritional value by activating enzymes that can reduce or eliminate anti-nutritional factors (Ebert et al. 2017). Phytic acid, the main anti-nutritional substance found in green beans, can be reduced from 12.0 mg/g to 4.03 mg/g through the germination process (Faradilla et al., 2012). Furthermore, germination is able to reduce anti-nutritional substances in germinated bean flour by 66.70% (Nur et al., 2019), so the bioavailability of vitamins and minerals in the flour increase. This is because the germination process can release the bound form of vitamins and minerals into a freer form so that it is more easily digested and absorbed by the human digestive tract. Previous research explained that during germination there is a breakdown of complex molecules into simpler forms which causes germination of green beans to be more easily digested and absorbed by the human body (Ghavidel and Prakash, 2007; Masood et al., 2014). In addition, the content of vitamin C increased with the increasing duration time of germination. Ebert et al. (2017) stated that the average vitamin C in germinated bean was 2.7 times higher than that without germination.

The eggshells of chickens and ducks are household waste that has not been utilized optimally due to the lack of public knowledge about the contents in it. Egshells consist of 95.1% salt, 3.3% organic matter, and 1.6% water. The main component of inorganic salts in eggshells is dominated by calcium carbonate (CaCO3) of 98.5% (Nurjayanti et al., 2012; Azis et al., 2018). The bioavailability of eggshells is ± 40% (Szeleszczuk et al., 2015) with calcium content in chicken and duck eggshells of 25.73% and 23.67%, respectively (Aminah and Meikawati, 2016).

Calcium contained in eggshells has the potential as an alternative source of calcium that can be fortified in germinated mung bean drinks as a substitute for milk. Calcium is generally available in micro size which is thought to be only able to absorb 50% of the total calcium consumed in its metabolism. The nano form of eggshell calcium can make it easier for calcium to be more soluble and optimally absorbed. According to Widyastuti and Kusuma (2017), nanocalcium is the manufacture of particles with a size of less than 100 nm by changing the nature or function of a material.

There are two methods in the manufacture of nanoparticles, namely top down (physical method) and bottom up (chemical method). The bottom up method is a method of making nanoparticles by arranging atoms or molecules to form nanometer-sized particles from a solution. In the bottom up method, the formation of nanoparticles has a high degree of cohesion so that it is able to produce a more uniform size. One of the bottom-up methods that is often used is precipitation because this method is very effective in the manufacture of nanoparticles, the process is simple, and requires low costs. The precipitation method is carried out by controlling the solubility of the material in the solution through changes in pH (Suptijah et al., 2012; Handayani and Syahputra, 2017; Hanura et al., 2017; Sunardi et al, 2020). Therefore, the objective of this study was to investigate the effect of mung bean germination time and nanocalcium fortification of eggshells on the characteristics of germinated mung bean drink (*Vigna radiata*).

The hypothesis of this study was it is suspected that germination time and type of eggshell nanocalcium fortification has a significant effect on the physico-chemical characteristics of the resulting germinated mung bean drink.

Material and Methods

The materials used in this study were VIMA-1 mung beans, eggshells (chicken and duck), 1N HCL, 3N NaOH, aquabidest, and other chemicals for analysis. The tools used were 100 mesh sieve, Beaker glass (Phyrex, Japan), blender (Philips HR 2116, China), Erlenmeyer (Iwaki, Japan), hot plate, muffle furnace (Barnstead FB1410M-26, USA), mortar, electric oven (Memmert, USA), measuring pipette (Iwaki, Japan), pH meter (Oakton do-35613, USA), Spectrophotometer (UV-VISAE S80, China), AAS (Thermo Scientific Nicolet^{TM}10, USA), analytical balance (Ohaus AR2140, USA), and a Brookfield viscometer (NDJ-8S, China).

Research Methods

This research was carried out at the Agricultural Product Chemistry Laboratory and Agricultural Product Processing Laboratory, Faculty of Agriculture, Sriwijaya University, South Sumatera, Indonesia (-3.2196118175204167, 104.64789811916464). This research was conducted using a completely randomized factorial design with two treatment factors, germination time of mung bean (A) which consisted of 3 treatment levels (A1 = 6 hours; A2 = 12 hours; and A3 = 18 hours) and the type of eggshell nanocalcium (B) which consisted of 2 treatment (B1 = chicken eggshell, and B2 = duck eggshell). The treatment was repeated 3 times. Mung bean drink without germination and addition of eggshell nanocalcium was a control. Addition of eggshell nanocalcium to all treatments was 20% of the Recommended Dietary Intake for calcium, which was 1200 mg/day per serving size (240 mL) (Food and Drug Supervisory Agency of the Republic of Indonesia, 2019).

Research Implementation

Eggshell micro powder preparation (Rahmawati and Nisa, 2015)

Preparation of eggshell micro powder was carried out by preparing 500 g for each chicken and duck eggshells and washed with running water until clean. The eggshells were boiled at 100^{o}C for 3 minutes to kill pathogenic microbes, and then the eggshells were drained. The eggshells were baked for 3 hours at 60^{o}C, and placed at room temperature. The cold eggshells were mashed using a blender at a speed of ±18.000 rpm for 3 minutes. The eggshell powder was then sieved through a 100 mesh sieve. The finished eggshell powder was put into OPP (Oriented Polystyrene) plastic and stored at 4^{o}C for analysis.

Eggshell nanocalcium powder preparation (Khoerunnisa, 2011)

Eggshell powder was soaked in 1N HCl (1:5) for 48 hours and extracted at 90^{o}C for 1 hour. The extracts were filtered using filter paper to obtain the filtrate and precipitate. The filtrate was precipitated with the addition of 3N NaOH and stirred, and then allowed to stand until a precipitate was formed. The precipitate was then neutralized using aquabidest until the pH was neutral. The solution is separated from the precipitate by pouring it slowly so that the precipitate does not get wasted. The precipitate was dried in oven for 3 hours at a temperature of 105^{o}C, followed by burning in muffle furnace at a temperature of 600°C for 5 hours, and then crushed using a mortar to obtain nanocalcium powder. The nanocalcium powder was vacuum packed and stored at 4°C.

Mung bean germination (Wea et al., 2014)

The mung beans were sorted and washed with running water until clean and then soaked for 6 hours. The mung beans are drained and placed on tray with a damp cloth. The green beans is placed in room temperature and germinated for 6, 12 and 18 hours, where every 6 hours watering is done with water. Germinated mung beans are separated from the skin and taken as much as 100 grams for further processing.

Germinated mung bean drinks preparation (Wea et al., 2014)

The germinated mung beans were blanched by boiling at 80°C for 2 minutes, peeled and then mashed with a blender for 3 minutes, added with water in a ratio of 1: 7.5. The germinated mung bean drinks is filtered and then cooked by adding nanocalcium eggshell and 7% sugar. Cooking is carried out until it boils and stirred for 5 minutes then cooled for 30 minutes.

Analysis

The analysis of the germinated mung bean drinks were chemical characteristics: protein content using Lowry method (Purwanto, 2014; Harjanto, 2017), calcium content was using AAS method (AOAC, 2005), vitamin C content (Sudarmadji et al. 2007), pH value (Sudarmadji et al., 2014), and physical characteristics: viscosity (Yuwono and Susanto, 1998) and stability (Wibowo et al., 2014).

Statistical analysis

All analyses were performed in triplicates. The data were subjected to the analysis of variance followed by Fisher's least significant difference (LSD) test to compare among treatment means and control. Differences were considered at a significant level of 95% (p <0.05) by using SPSS v.19 software.

Results

Analysis of the characteristics of germinated mung bean drink with nanocalcium fortification

The interaction between germination time and the type of eggshell nanocalcium no significant effect on all characteristics of germinated mung bean drink, excepted for Vitamin C and calcium that were higer in all treatments compared to control (Table 1). The non-significance among the treatments was attributed to addition of the nanocalcium (CaO) powder of chicken and duck eggshells to the germinated mung bean drinks in the same amount (20% of nutrition facts label for calcium). Moreover, the characteristics of the eggshell nanocalcium (CaO) powder were white color, very fine texture (particle size of CaO in chicken and duck eggshells is 41 .54 nm and 24.90 nm respectivelly), and neutral pH so that it take to the no significant effect on the value of viscosity, stability, protein, pH and vitamin C of germinated mung bean drink (Table 2).

Table 1. Physical and chemical characteristics of germinated mung bean drinks with nanocalcium fortification

Treatments	Parameter					
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)	Calcium (%)
A_1B_1	27.60±0.20[a]	87.00±1.00[a]	1.90±0.05[a]	6.81±0.02[a]	8.21±1.02[a]	17.82
A_1B_2	27.13±0.15[a]	85.67±1.53[a]	1.81±0.09[a]	6.77±0.04[a]	7.62±1.02[a]	24.82
A_2B_1	26.17±0.29[a]	73.00±1.00[a]	1.77±0.11[a]	6.74±0.04[a]	11.73±1.01[a]	16.45
A_2B_2	26.03±0.45[a]	71.67±1.53[a]	1.66±0.14[a]	6.69±0.04[a]	11.14±1.01[a]	23.66
A_3B_1	25.53±0.21[a]	65.67±0.58[a]	1.56±0.14[a]	6.63±0.04[a]	14.66±1.01[a]	15.66
A_3B_2	24.97±0.76[a]	64.67±1.53[a]	1.50±0.18[a]	6.62±0.01[a]	13.49±1.02[a]	22.49
Control	28.80±0.01[a]	91.33±0.58[a]	2.01±0.16[a]	6.81±0.02[a]	5.28±1.76[b]	nd

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation

Control = germination time of green beans 0 hours without the addition of nanocalcium
A_1 = mung bean germination time for 6 hours B_1 = nanocalsium of chicken eggshell
A_2 = mung bean germination time for 12 hours B_2 = nanocalcium of duck eggshell
A_3 = mung bean germination time for 18 hours

Table 2. Effect of eggshell nanocalcium fortification on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH*	Vitamin C (mg/100g)
B_1	26.43±0.23^a	75.22±0.86^a	1.74±0.10^a	6.73±0.03^a	11.54±1.02^a
B_2	26.04±0.46^a	74.00±1.53^a	1.66±0.13^a	6.69±0.03^a	10.75±1.02^a

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
B_1 = nanocalcium of chicken eggshell
B_2 = nanocalcium of duck eggshell

Germination time was significantly effect the characteristics of germinated mung been drinks (Table 3).

Table 3.The effect of germination time on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)
A_1	27.37±0.49^a	86.33±1.05^c	1.85±0.16^b	6.79±0.02^c	7.92±1.02^a
A_2	26.10±0.37^b	72.33±1.26^b	1.72±0.13^{ab}	6.72±0.04^b	11.44±1.01^b
A_3	25.25±0.18^c	65.17±1.25^a	1.53±0.07^a	6.62±0.03^a	14.08±1.02^c

Note: Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
A_1 = mung bean germination time for 6 hours
A_2 = mung bean germination time for 12 hours
A_3 = mung bean germination time for 18 hours

Discussion

Table 1 shows a significant increase in the vitamin C and calcium content of germinated mung bean drinks to the control. The increase in vitamin C in germinated mung bean drinks in all treatments in this study was due to the food reserves contained in the cotyledons in the form of starch hydrolyzed by the amylase enzyme into simple sugars which were then synthesized into vitamin C during the germination process. The results of this study were in line with the opinion of Ebert et al. (2017) which stated that the content of vitamin C in the germinated peanut seeds increased by 2.7 times compared to the ungerminated seed.

The calcium content of germinated mung bean drinks fortified with nanocalcium of duck eggshell was higher than the calcium content of germinated mung bean drinks fortified with nanocalcium of chicken eggshell. This was because the calcium content in duck eggshell nanocalcium (45.50%) was higher than the calcium level in chicken eggshell nanocalcium (35.50%).

Viscosity

Viscosity is a measure of the size of the friction that occurs in the fluid. The greater the viscosity of a food, the thicker the material, and vice versa (Srihidayati, 2017). The viscosity of germinated mung bean drinks in the control treatment (0 hour germination without nanocalcium fortification) was higher (28.80±0.01 m.Pa.s) compared to that of nanocalcium fortified germinated mung bean drinks for all treatments in this study. This is due to the fact that ungerminated mung bean starch has not been hydrolyzed so that it still has higher starch content than germinated mung bean starch. In the preparation of mung bean drinks, starch will undergo gelatinization in the presence of heat treatment which will cause the starch granules to break and the starch molecules to come out and be released from the granules and then enter the solution system. The results of this study are in line with the results of research conducted by Nurjanati et al. (2018), based on the hedonic test, ungerminated red bean milk (0 hours) has a higher viscosity than germinated red bean milk (8, 16 and 24 hours).

Table 3 shows that the viscosity of the green bean germination drink decreased significantly with the duration of germination. The decrease in the viscosity of the material during germination was caused by the hydrolysis of starch during the germination process by the amylase enzymes. The enzyme is formed at the beginning of germination by giberylic acid (Elobuike et al., 2021). Starch will be broken down into simple sugars in the form of glucose which is used as energy and needs for seed growth. Another factor that causes a decrease in the viscosity of mung bean germination drink is the increase in vitamin C during the germination process which results in a decrease in pH as shown in Table 3. A decrease in pH results in hydrolysis of glycosidic bonds which causes a decrease in viscosity. The results of this study are in line with the statement of Widjaja et al. (2019), that a solution will decrease its viscosity if its pH decreases.

Stability

The stability of the products can be seen by the presence or absence of precipitate in the product (Farikha et al., 2013). Germinated mung bean drinks in the control treatment (0 hour germination without the addition of nanocalcium) had a higher stability value (91%) compared to all treatments of germinated mung bean drinks fortified with nanocalcium in this study. This was because germinated mung bean seeds absorb more water than ungerminated mung bean seeds. During germination, bean seeds require optimal environmental conditions that are 50-80% RH (Yuwariah et al., 2015). Water absorption in the germination process is needed to maintain cell turgidity, including cell enlargement, photosynthetic reactions, salt solvents, gases and other substances that are transported between cells in tissues to maintain cell growth (Felania, 2017).

Table 3 shows that the stability of the germinated mung bean drinks decreased significantly as the duration of germination increased. This was due to an increase in the water content of germinated mung beans along with the length of germination due to the watering the mung bean seeds every 6 hours during germination time. The water content of germinated mung bean with a time of 0, 6, 12 and 18 hours were 4.32%, 4.99%, 5.42% and 7.22%, respectively. The increase in water content in germinated mung beans resulting germinated mung bean drinks have more water content in turns it affects the stability value of the drinks. In line with the viscosity value of the germinated mung bean drinks, the average value of stability of each treatment decreased with the duration of germination. The decrease in viscosity at germinated mung bean drinks correlated to that in stability.

Protein Content

The protein of germinated mung bean drinks in treatment A2 (12 hours of germination time) was not significantly different from that of treatment A1 (6 hours of germination time) and treatment A3 (12 hours of germination time). The decreasing of protein content in germinated mung bean drinks was due to the hydrolysis of protein into amino acids that are used for embryo growth. The results of this study were in line with the statement of Masood et al. (2014) which stated a decrease in protein content along with an increase in amino acid content during germination of green beans. This is due to an increase in the activity of the protease enzyme which is an endogenous enzyme that is active during the imbibition process, when green beans are in contact with water. Proteases hydrolyze proteins into peptides and amino acids (Ferdiawan et al., 2019). Free amino acids along with glutamic and aspartic acids (in the form of amides) will be translocated to the embryo in the formation of new structures in line with the ongoing germination stage (Pertiwi et al., 2013).

pH value

pH is a parameter used to determine changes in the acidity level of a food product (Widowati et al., 2020). Table 3 shows that the longer the germination time, the lower the pH of the germinated mung bean drinks significantly. According to Wea et al. (2014), in germinated seed plants there was an increase in vitamin synthesis, especially vitamin C (Wea et al., 2014). This situation is in accordance with the vitamin C content in the germinated mung bean drinks in this study (Table 3) so that the pH of the germinated mung bean drinka is low. Based on National Standardization Agency (1995) SNI 01-3830-1995, the pH standard for bean drinks is 6.5-7.0. The pH of germinated mung bean drinks with nanocalcium fortification in this study has a value of 6.62-6.79; it has met the pH standard of bean drinks.

Vitamin C

Table 3 shows that the vitamin C of the germinated mung bean drinks increased significantly with increasing of germination time. The increase in vitamin C along with germination time is caused by several enzyme systems being active during germination. This happens because of the accumulation of ascorbic acid as a result of biosynthesis. Germination causes reactivation of the enzyme L-Galactono-γ-lactone dehydrogenase which is involved in the oxidation of L-galactono-1,4-lactone to ascorbic acid. The activity of this enzyme increased in parallel to the biosynthesis of ascorbic acid during the seed germination process. Differences in the level of ascorbic acid biosynthesis in germination of mung bean seeds can also be influenced by legume varieties, maturity, climate, light, harvesting and storage methods (Masood et al., 2014). Increasing the content of vitamin C in germination of mung beans can help the solubility of nanocalcium fortification in the germinated mung bean drinks. This is in line with the statement of Yonata et al. (2017), that pH can affect the solubility of minerals. Acidic conditions and small particle size can increase the speed of nanocalcium dissolution from both duck and chicken eggshells, so that the germinated mung bean drinks with eggshells nanocalcium fortification has good digestibility.

Calcium Content

Based on the results of the analysis, calcium content of nanocalcium in chicken and duck eggshells was 35.50% and 45.50%, respectively. Calcium content in eggshells is influenced by the thickness and type of eggshell. Duck eggshells have an average thickness of 0.36 - 0.46 mm (Septiana et al., 2015), while the average thickness of chicken eggshells was 0.33 - 0.35 mm (Azizah et al., 2015). Differences in eggshell thickness can affect the amount of mineral content and organic salts, especially calcium carbonate (CaCO3) which acts as a source of calcium in eggshells.

Fortification is a process of increasing the content of essential micronutrients in the form of vitamins and minerals into food, aiming to increase nutrients that do not yet exist or to enrich existing nutrients (Valentina et al., 2014). According to the Indonesian Food and Drug Supervisory Agency (2019), the minimum amount of micronutrient fortification in foodstuffs is 10% of the RDI per serving. In this study, the amount of nanocalcium fortification of chicken and duck eggshells in germinated mung bean drink was 20% of the RDI for calcium per 250 mL serving.

Calcium content of nongerminated mung bean drinks with nanocalcium fortification of chicken and duck eggshells was 18.97% and 25%, respectively. This showed that the calcium content of germinated mung bean drinks both fortified with nanocalcium chicken and duck eggshells decreased with the increasing of germination time (Table 1). This was in accordance with the statement of Oghbaei and Prakash (2020) that the calcium content decreased significantly in germinated and peeled bean seeds. Peeling can reduce some of the minerals present in the skin of green beans. However, this germination and skin peeling treatment had an impact on increasing the bioavailability of mung beans which contributed to stimulating the reduction of anti-nutritional substances. The percentage of bioavailability in the seeds before germination was 14.91–17.19% and in the germinated beans which were peeled 22.96–25.27%.

In addition, the decrease in calcium content of germinated mung bean drinks in this study was thought to be due to the treatment with germination time less than 24 hours so that the phytase enzyme had not worked optimally, causing the complex phytic acid that binds to minerals such as calcium could not be completely released. Based on the results of research by Ghavidel and Davoodi (2011), enzyme activity (phytase, amylase and protease) increased significantly at 24, 48, and 72 hours of germination in several types of legumes, one of which was green beans. The maximum enzymatic activity occurred at 72 hours of germination. Although the calcium of germinated mung bean drinks decreased in the 6, 12 and 18 hour germination treatment, but with an increase in vitamin C at the same germination time treatment it could increase the calcium absorption efficiency of the germinated mung bean drinks.

Conclusion

Nanocalcium fortification of chicken and duck eggshells by 20% of the RDI for calcium per 250 mL serving had no significant effect on the physical (viscosity and stability) and chemical (protein, pH, and vitamin C) characteristics of germinated mung bean drinks. Germination of mung beans at different times was significantly increased value of pH, vitamin C, protein, and stability; but significantly decreased value of viscosity. Germinated mung bean drinks are a good carrier for chicken and duck eggshells nanocalcium fortification.

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4. Bukti Decision Letter untuk Article Accepted

(13 Januari 2023)

January 13, 2023

Dear Nura Malahayati

I am pleased to inform you that your manuscript titled as "Effect of germination and eggshells nanocalcium fortification on characteristics of germinated mung bean drink (Vigna radiata.)" (Manuscript Number: EJFA-2022-09-277 was accepted for publication in the Emirates Journal of Food and Agriculture.
As we declared in "Instructions for Authors", you need to contribute to Emirates Journal of Food and Agriculture for Publication Fee (APC).

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RESEARCH ARTICLE

Effect of germination and eggshells nanocalcium fortification on characteristics of germinated mung bean drink (*Vigna radiata*.)

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ABSTRACT

Germinated mung bean drinks with eggshell nanocalcium fortification are an alternative beverage to replace milk. This study aimed to determine the effect of germination time and eggshells nanocalcium fortification on physicochemical characteristics of the germinated mung bean drink. This study used a Factorial Completely Randomized Design with two treatments factors. The first factor was germination time which consisted of three treatment levels (6, 12 and 18 hours) and the second factor was the type of eggshell nanocalcium which consisted of two type of treatment (chicken and duck eggshell), plus the control treatment as mung bean drink without germination and addition of eggshell nanocalcium. Each experiment was repeated three times. Observed parameters in this study were chemical characteristics (protein, calcium, vitamin C, and pH) and physical characteristics (viscosity and stability) of germinated mung bean drink. The results showed that the germination time treatment significantly affected the value of protein, calcium, vitamin C, pH, viscosity, and stability of the germinated mung bean drink. Germination increased vitamin C in mung beans with an average value of 8,21-14,66 mg/100 g. Eggshell nanocalcium fortification showed a significant effect on increasing calcium of germinated mung bean drink. Germinated mung bean drink produced from 6 hours of germination and fortification of duck nanocalcium had the highest calcium content with value of 24.82%.

Key words: Physicochemical, Chicken, Duck, Calcium, Vitamin C

INTRODUCTION

To meet the calcium needs of the Indonesian people who generally suffer from lactose intolerance and digestive disorders after consuming milk, it is necessary to drink milk substitutes made from plant-based foods that contain high protein and calcium. Mung bean is the third highest source of vegetable protein after soybeans and peanuts with a content of protein around 20-25% (Purwono and Hartono, 2005; Hastuti et al., 2018). One of the superior varieties of mung beans is VIMA-1 (Vigna sinensis–Malang). VIMA-1 has a promising market potential due to its high protein content (28.02%), low fat (0.40%), and high starch content (67.62%) (Agency for Research and Development, 2019). However, mung beans have anti-nutritional compounds, compounds found naturally in various types of beans, which can prevent the absorption of nutrients in the body.

One process that can increase the nutrition in beans is germination. Germinated mung beans have improved nutritional, functional and biological properties by changing the content, nutritional composition and bioactive compounds, as well as eliminating anti-nutritional factors in beans (Liu et al., 2020). Germination is a condition in which dormant seeds begin to germinate and grow into seedlings under the right growing conditions. Germination in brown rice refers to the point where the growth of a radicle ranges from 2 mm to 5 mm, and pre-germination brown rice is the stage with an expanded a radicle exposed approximately 0.5–1 mm (Watanabe et al., 2004). Moreover, Palmiano and Juliano (1972) stated that the optimal germination period was 18 to 24 hours.

The germination process in mung bean seeds is able to increase the nutritional value by activating enzymes that can reduce or eliminate anti-nutritional factors (Ebert et al. 2017). Phytic acid, the main anti-nutritional substance

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found in green beans, can be reduced from 12.0 mg/g to 4.03 mg/g through the germination process (Faradilla et al., 2012). Furthermore, germination is able to reduce anti-nutritional substances in germinated bean flour by 66.70% (Nur et al., 2019), so the bioavailability of vitamins and minerals in the flour increase. This is because the germination process can release the bound form of vitamins and minerals into a freer form so that it is more easily digested and absorbed by the human digestive tract. Previous research explained that during germination there is a breakdown of complex molecules into simpler forms which causes germination of green beans to be more easily digested and absorbed by the human body (Ghavidel and Prakash, 2007; Masood et al., 2014). In addition, the content of vitamin C increased with the increasing duration time of germination. Ebert et al. (2017) stated that the average vitamin C in germinated bean was 2.7 times higher than that without germination.

The eggshells of chickens and ducks are household waste that has not been utilized optimally due to the lack of public knowledge about the contents in it. Egshells consist of 95.1% salt, 3.3% organic matter, and 1.6% water. The main component of inorganic salts in eggshells is dominated by calcium carbonate (CaCO3) of 98.5% (Nurjayanti et al., 2012). The bioavailability of eggshells is ± 40% (Szeleszczuk et al., 2015) with calcium content in chicken and duck eggshells of 25.73% and 23.67%, respectively (Aminah and Meikawati, 2016).

Calcium contained in eggshells has the potential as an alternative source of calcium that can be fortified in germinated mung bean drinks as a substitute for milk. Calcium is generally available in micro size which is thought to be only able to absorb 50% of the total calcium consumed in its metabolism. The nano form of eggshell calcium can make it easier for calcium to be more soluble and optimally absorbed. According to Widyastuti and Kusuma (2017), nanocalcium is the manufacture of particles with a size of less than 100 nm by changing the nature or function of a material.

There are two methods in the manufacture of nanoparticles, namely top down (physical method) and bottom up (chemical method). The bottom up method is a method of making nanoparticles by arranging atoms or molecules to form nanometer-sized particles from a solution. In the bottom up method, the formation of nanoparticles has a high degree of cohesion so that it is able to produce a more uniform size. One of the bottom-up methods that is often used is precipitation because this method is very effective in the manufacture of nanoparticles, the process is simple, and requires low costs. The precipitation method is carried out by controlling the solubility of the material in the solution through changes in pH (Suptijah et al., 2012). Therefore, the objective of this study was to investigate the effect of mung bean germination time and nanocalcium fortification of eggshells on the characteristics of germinated mung bean drink (*Vigna radiata*).

The hypothesis of this study was it is suspected that germination time and type of eggshell nanocalcium fortification has a significant effect on the physico-chemical characteristics of the resulting germinated mung bean drink.

MATERIAL AND METHODS

The materials used in this study were VIMA-1 mung beans, eggshells (chicken and duck), 1N HCL, 3N NaOH, aquabidest, and other chemicals for analysis. The tools used were 100 mesh sieve, Beaker glass (Phyrex, Japan), blender (Philips HR 2116, China), Erlenmeyer (Iwaki, Japan), hot plate, muffle furnace (Barnstead FB1410M-26, USA), mortar, electric oven (Memmert, USA), measuring pipette (Iwaki, Japan), pH meter (Oakton do-35613, USA), Spectrophotometer (UV-VISAE S80, China), AAS (Thermo Scientific NicoletTM10, USA), analytical balance (Ohaus AR2140, USA), and a Brookfield viscometer (NDJ-8S, China).

RESEARCH METHODS

This research was carried out at the Agricultural Product Chemistry Laboratory and Agricultural Product Processing Laboratory, Faculty of Agriculture, Sriwijaya University, South Sumatera, Indonesia (-3.2196118175204167, 104.64789811916464). This research was conducted using a completely randomized factorial design with two treatment factors, germination time of mung bean (A) which consisted of 3 treatment levels (A1 = 6 hours; A2 = 12 hours; and A3 = 18 hours) and the type of eggshell nanocalcium (B) which consisted of 2 treatment (B1 = chicken eggshell, and B2 = duck eggshell). The treatment was repeated 3 times. Mung bean drink without germination and addition of eggshell nanocalcium was a control. Addition of eggshell nanocalcium to all treatments was 20% of the Recommended Dietary Intake for calcium, which was 1200 mg/day per serving size (240 mL).

RESEARCH IMPLEMENTATION

Eggshell micro powder preparation (Rahmawati and Nisa, 2015)

Preparation of eggshell micro powder was carried out by preparing 500 g for each chicken and duck eggshells and washed with running water until clean. The eggshells were boiled at 100°C for 3 minutes to kill pathogenic microbes, and then the eggshells were drained. The eggshells were baked for 3 hours at 60°C, and placed at room temperature. The cold eggshells were mashed using a blender at a speed of ±18.000 rpm for 3 minutes. The eggshell powder was then sieved through a 100 mesh sieve. The finished eggshell powder was put into OPP (Oriented Polystyrene) plastic and stored at 4°C for analysis.

Eggshell nanocalcium powder preparation (Khoerunnisa, 2011)

Eggshell powder was soaked in 1N HCl (1:5) for 48 hours and extracted at 90°C for 1 hour. The extracts were filtered using filter paper to obtain the filtrate and precipitate. The filtrate was precipitated with the addition of 3N NaOH and stirred, and then allowed to stand until a precipitate was formed. The precipitate was then neutralized using aquabidest until the pH was neutral. The solution is separated from the precipitate by pouring it slowly so that the precipitate does not get wasted. The precipitate was dried in oven for 3 hours at a temperature of 105°C, followed by burning in muffle furnace at a temperature of 600°C for 5 hours, and then crushed using a mortar to obtain nanocalcium powder. The nanocalcium powder was vacuum packed and stored at 4o°C.

Mung bean germination (Wea et al., 2014)

The mung beans were sorted and washed with running water until clean and then soaked for 6 hours. The mung beans are drained and placed on tray with a damp cloth. The green beans is placed in room temperature and germinated for 6, 12 and 18 hours, where every 6 hours watering is done with water. Germinated mung beans are separated from the skin and taken as much as 100 grams for further processing.

Germinated mung bean drinks preparation (Wea et al., 2014)

The germinated mung beans were blanched by boiling at 80°C for 2 minutes, peeled and then mashed with a blender for 3 minutes, added with water in a ratio of 1: 7.5. The germinated mung bean drinks is filtered and then cooked by adding nanocalcium eggshell and 7% sugar. Cooking is carried out until it boils and stirred for 5 minutes then cooled for 30 minutes.

Analysis

The analysis of the germinated mung bean drinks were chemical characteristics: protein content using Lowry method (Harjanto, 2017), calcium content was using AAS method (AOAC, 2005), vitamin C content (Sudarmadji et al. 2007), pH value (Sudarmadji et al., 2014), and physical characteristics: viscosity (Yuwono and Susanto, 1998) and stability (Wibowo et al., 2014).

Statistical analysis

All analyses were performed in triplicates. The data were subjected to the analysis of variance followed by Fisher's least significant difference (LSD) test to compare among treatment means and control. Differences were considered at a significant level of 95% (p <0.05) by using SPSS v.19 software.

RESULTS

Analysis of the characteristics of germinated mung bean drink with nanocalcium fortification

The interaction between germination time and the type of eggshell nanocalcium no significant effect on all characteristics of germinated mung bean drink, excepted for Vitamin C and calcium that were higer in all treatments compared to control (Table 1). The non-significance among the treatments was attributed to addition of the nanocalcium (CaO) powder of chicken and duck eggshells to the germinated mung bean drinks in the same amount (20% of nutrition facts label for calcium). Moreover, the characteristics of the eggshell nanocalcium (CaO) powder were white color, very fine texture (particle size of CaO in chicken and duck eggshells is 41.54 nm and 24.90 nm respectivelly), and neutral pH so that it take to the no significant effect on the value of viscosity, stability, protein, pH and vitamin C of germinated mung bean drink (Table 2).

Germination time was significantly effect the characteristics of germinated mung been drinks (Table 3).

DISCUSSION

Table 1 shows a significant increase in the vitamin C and calcium content of germinated mung bean drinks to the control. The increase in vitamin C in germinated mung bean drinks in all treatments in this study was due to the food reserves contained in the cotyledons in the form of starch hydrolyzed by the amylase enzyme into simple sugars which were then synthesized into vitamin C during the germination process. The results of this study were in line with the opinion of Ebert et al. (2017) which stated that the content of vitamin C in the germinated peanut seeds increased by 2.7 times compared to the ungerminated seed.

The calcium content of germinated mung bean drinks fortified with nanocalcium of duck eggshell was higher than the calcium content of germinated mung bean drinks fortified with nanocalcium of chicken eggshell. This was

Table 1: Physical and chemical characteristics of germinated mung bean drinks with nanocalcium fortification

Treatments	Parameter					
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)	Calcium (%)
A_1B_1	27.60 ± 0.20[a]	87.00 ± 1.00[a]	1.90 ± 0.05[a]	6.81 ± 0.02[a]	8.21 ± 1.02[a]	17.82
A_1B_2	27.13 ± 0.15[a]	85.67 ± 1.53[a]	1.81 ± 0.09[a]	6.77 ± 0.04[a]	7.62 ± 1.02[a]	24.82
A_2B_1	26.17 ± 0.29[a]	73.00 ± 1.00[a]	1.77 ± 0.11[a]	6.74 ± 0.04[a]	11.73 ± 1.01[a]	16.45
A_2B_2	26.03 ± 0.45[a]	71.67 ± 1.53[a]	1.66 ± 0.14[a]	6.69 ± 0.04[a]	11.14 ± 1.01[a]	23.66
A_3B_1	25.53 ± 0.21[a]	65.67 ± 0.58[a]	1.56 ± 0.14[a]	6.63 ± 0.04[a]	14.66 ± 1.01[a]	15.66
A_3B_2	24.97 ± 0.76[a]	64.67 ± 1.53[a]	1.50 ± 0.18[a]	6.62 ± 0.01[a]	13.49 ± 1.02[a]	22.49
Control	28.80 ± 0.01[a]	91.33 ± 0.58[a]	2.01 ± 0.16[a]	6.81 ± 0.02[a]	5.28 ± 1.76[b]	nd

Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
Control = germination time of green beans 0 hours without the addition of nanocalcium
A_1 = mung bean germination time for 6 hours B_1 = nanocalsium of chicken eggshell
A_2 = mung bean germination time for 12 hours B_2 = nanocalcium of duck eggshell
A_3 = mung bean germination time for 18 hours

Table 2: Effect of eggshell nanocalcium fortification on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH*	Vitamin C (mg/100g)
B_1	26.43 ± 0.23[a]	75.22 ± 0.86[a]	1.74 ± 0.10[a]	6.73 ± 0.03[a]	11.54 ± 1.02[a]
B_2	26.04 ± 0.46[a]	74.00 ± 1.53[a]	1.66 ± 0.13[a]	6.69 ± 0.03[a]	10.75 ± 1.02[a]

Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
B_1 = nanocalcium of chicken eggshell
B_2 = nanocalcium of duck eggshell

Table 3: The effect of germination time on the characteristics of germinated mung bean drinks

Treatments	Parameter				
	Viscosity (mPa.s)	Stability (%)	Protein (%)	pH	Vitamin C (mg/100g)
A_1	27.37 ± 0.49[a]	86.33 ± 1.05[c]	1.85 ± 0.16[b]	6.79 ± 0.02[c]	7.92 ± 1.02[a]
A_2	26.10 ± 0.37[b]	72.33 ± 1.26[b]	1.72 ± 0.13[ab]	6.72 ± 0.04[b]	11.44 ± 1.01[b]
A_3	25.25 ± 0.18[c]	65.17 ± 1.25[a]	1.53 ± 0.07[a]	6.62 ± 0.03[a]	14.08 ± 1.02[c]

Numbers marked with the same letter notation in the same column indicate not significantly different (5%)
Data = mean ± standard deviation
A_1 = mung bean germination time for 6 hours
A_2 = mung bean germination time for 12 hours
A_3 = mung bean germination time for 18 hours

because the calcium content in duck eggshell nanocalcium (45.50%) was higher than the calcium level in chicken eggshell nanocalcium (35.50%).

VISCOSITY

Viscosity is a measure of the size of the friction that occurs in the fluid. The greater the viscosity of a food, the thicker the material, and vice versa (Srihidayati, 2017). The viscosity of germinated mung bean drinks in the control treatment (0 hour germination without nanocalcium fortification) was higher (28.80±0.01 m.Pa.s) compared to that of nanocalcium fortified germinated mung bean drinks for all treatments in this study. This is due to the fact that ungerminated mung bean starch has not been hydrolyzed so that it still has higher starch content than germinated mung bean starch. In the preparation of mung bean drinks, starch will undergo gelatinization in the presence of heat treatment which will cause the starch granules to break and the starch molecules to come out and be released from the granules and then enter the solution system. The results of this study are in line with the results of research conducted by Nurjanati et al. (2018), based on the hedonic test, ungerminated red bean milk (0 hours) has a higher viscosity than germinated red bean milk (8, 16 and 24 hours).

Table 3 shows that the viscosity of the green bean germination drink decreased significantly with the duration of germination. The decrease in the viscosity of the material during germination was caused by the hydrolysis of starch during the germination process by the amylase enzymes. The enzyme is formed at the beginning of germination by giberylic acid (Elobuike et al., 2021). Starch will be broken down into simple sugars in the form of glucose which is used as energy and needs for seed growth. Another factor that causes a decrease in the viscosity of mung bean germination drink is the increase in vitamin C during the germination process which results in a decrease in pH as shown in Table 3. A decrease in pH results in hydrolysis of glycosidic bonds which causes a decrease in viscosity. The results of this study are in line with the statement of Widjaja et al. (2019), that a solution will decrease its viscosity if its pH decreases.

STABILITY

The stability of the products can be seen by the presence or absence of precipitate in the product (Farikha et al., 2013). Germinated mung bean drinks in the control treatment (0 hour germination without the addition of nanocalcium) had a higher stability value (91%) compared to all treatments of germinated mung bean drinks fortified with nanocalcium in this study. This was because germinated mung bean seeds absorb more water than ungerminated mung bean seeds. During germination, bean seeds require optimal environmental conditions that are 50-80% RH (Yuwariah et al., 2015). Water absorption in the germination process is needed to maintain cell turgidity, including cell enlargement, photosynthetic reactions, salt solvents, gases and other substances that are transported between cells in tissues to maintain cell growth (Felania, 2017).

Table 3 shows that the stability of the germinated mung bean drinks decreased significantly as the duration of germination increased. This was due to an increase in the water content of germinated mung beans along with the length of germination due to the watering the mung bean seeds every 6 hours during germination time. The water content of germinated mung bean with a time of 0, 6, 12 and 18 hours were 4.32%, 4.99%, 5.42% and 7.22%, respectively. The increase in water content in germinated mung beans resulting germinated mung bean drinks have more water content in turns it affects the stability value of the drinks. In line with the viscosity value of the germinated mung bean drinks, the average value of stability of each treatment decreased with the duration of germination. The decrease in viscosity at germinated mung bean drinks correlated to that in stability.

PROTEIN CONTENT

The protein of germinated mung bean drinks in treatment A2 (12 hours of germination time) was not significantly different from that of treatment A1 (6 hours of germination time) and treatment A3 (12 hours of germination time). The decreasing of protein content in germinated mung bean drinks was due to the hydrolysis of protein into amino acids that are used for embryo growth. The results of this study were in line with the statement of Masood et al. (2014) which stated a decrease in protein content along with an increase in amino acid content during germination of green beans. This is due to an increase in the activity of the protease enzyme which is an endogenous enzyme that is active during the imbibition process, when green beans are in contact with water. Proteases hydrolyze proteins into peptides and amino acids (Ferdiawan et al., 2019). Free amino acids along with glutamic and aspartic acids (in the form of amides) will be translocated to the embryo in the formation of new structures in line with the ongoing germination stage (Pertiwi et al., 2013).

pH VALUE

pH is a parameter used to determine changes in the acidity level of a food product (Widowati et al., 2020). Table 3 shows that the longer the germination time, the lower the pH of the germinated mung bean drinks significantly. According to Wea et al. (2014), in germinated seed plants there was an increase in vitamin synthesis, especially vitamin C (Wea et al., 2014). This situation is in accordance with the vitamin C content in the germinated mung bean drinks in this study (Table 3) so that the pH of the germinated mung bean drinka is low. Based on Indonesian National Standard 01-3830-1995, the pH standard for bean drinks is 6.5-7.0. The pH of germinated mung bean drinks with nanocalcium fortification in this study has a value of 6.62-6.79; it has met the pH standard of bean drinks.

VITAMIN C

Table 3 shows that the vitamin C of the germinated mung bean drinks increased significantly with increasing of germination time. The increase in vitamin C along with germination time is caused by several enzyme systems being active during germination. This happens because of the accumulation of ascorbic acid as a result of biosynthesis. Germination causes reactivation of the enzyme L-Galactono-γ-lactone dehydrogenase which is involved in the oxidation of L-galactono-1,4-lactone to ascorbic acid. The activity of this enzyme increased in parallel to the biosynthesis of ascorbic acid during the seed germination process. Differences in the level of ascorbic acid biosynthesis in germination of mung bean seeds can also be influenced by legume varieties, maturity, climate, light, harvesting and storage methods (Masood et al., 2014). Increasing the content of vitamin C in germination of mung beans can help the solubility of nanocalcium fortification in the germinated mung bean drinks. This is in line with the statement of Yonata et al. (2017), that pH can affect the solubility of minerals. Acidic conditions and small particle size can increase the speed of nanocalcium dissolution from both duck and chicken eggshells, so that the germinated mung bean drinks with eggshells nanocalcium fortification has good digestibility.

Calcium content

Based on the results of the analysis, calcium content of nanocalcium in chicken and duck eggshells was 35.50% and 45.50%, respectively. Calcium content in eggshells is influenced by the thickness and type of eggshell. Duck eggshells have an average thickness of 0.36 - 0.46 mm (Septiana et al., 2015), while the average thickness of chicken eggshells was 0.33 - 0.35 mm (Azizah et al., 2015). Differences in eggshell thickness can affect the amount of mineral content and organic salts, especially calcium carbonate (CaCO3) which acts as a source of calcium in eggshells.

Fortification is a process of increasing the content of essential micronutrients in the form of vitamins and minerals into food, aiming to increase nutrients that do not yet exist or to enrich existing nutrients (Valentina et al., 2014). According to the Indonesian Food and Drug Supervisory Agency (2019), the minimum amount of micronutrient fortification in foodstuffs is 10% of the RDI per serving. In this study, the amount of nanocalcium fortification of chicken and duck eggshells in germinated mung bean drink was 20% of the RDI for calcium per 250 mL serving.

Calcium content of nongerminated mung bean drinks with nanocalcium fortification of chicken and duck eggshells was 18.97% and 25%, respectively. This showed that the calcium content of germinated mung bean drinks both fortified with nanocalcium chicken and duck eggshells decreased with the increasing of germination time (Table 1). This was in accordance with the statement of Oghbaei and Prakash (2020) that the calcium content decreased significantly in germinated and peeled bean seeds. Peeling can reduce some of the minerals present in the skin of green beans. However, this germination and skin peeling treatment had an impact on increasing the bioavailability of mung beans which contributed to stimulating the reduction of anti-nutritional substances. The percentage of bioavailability in the seeds before germination was 14.91–17.19% and in the germinated beans which were peeled 22.96–25.27%.

In addition, the decrease in calcium content of germinated mung bean drinks in this study was thought to be due to the treatment with germination time less than 24 hours so that the phytase enzyme had not worked optimally, causing the complex phytic acid that binds to minerals such as calcium could not be completely released. Based on the results of research by Ghavidel and Davoodi (2011), enzyme activity (phytase, amylase and protease) increased significantly at 24, 48, and 72 hours of germination in several types of legumes, one of which was green beans. The maximum enzymatic activity occurred at 72 hours of germination. Although the calcium of germinated mung bean drinks decreased in the 6, 12 and 18 hour germination treatment, but with an increase in vitamin C at the same germination time treatment it could increase the calcium absorption efficiency of the germinated mung bean drinks.

CONCLUSION

Nanocalcium fortification of chicken and duck eggshells by 20% of the RDI for calcium per 250 mL serving had no significant effect on the physical (viscosity and stability) and chemical (protein, pH, and vitamin C) characteristics of germinated mung bean drinks. Germination of mung beans at different times was significantly increased value of pH, vitamin C, protein, and stability; but significantly decreased value of viscosity. Germinated mung bean drinks are a good carrier for chicken and duck eggshells nanocalcium fortification.

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