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Nutritional studies on improvement of phosphorus availability in chickens using buckwheat

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ソバを活用したニワトリのリン利用性改善に関する栄養学的研究

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Chapter I

General Introduction

In Bangladesh, poultry industry has been expanded rapidly to meet the growing demand for meat and eggs by the increasing human population. According to the annual report of Department of Livestock Services, Bangladesh, the country has approximately 268 million chicken population, which generates 4.52 million tons fresh poultry litter annually, of which 3.10 million ton is produced under commercial poultry farming, and there are 20,466 tons of annual phosphorus (P) production from poultry manure (MFL, 2015; DLS, 2016). Miah *et al*. (2016) estimated that approximately 1.56 million tons of poultry manure is produced in Bangladesh each year, and mostly these are dumped into the nearby sites. As a result, large quantities of P subsequently accumulate in the soil. Environmental impact of P from poultry manure, is a great problem not only in Bangladesh but also in the world.

Phytic acid is the primary storage form of P in plants, exists as the phytate salt (myo-inositol 1,2,3,4,5,6-hexakisphosphate), and accounts for approximately 50 to 90% of the total P in cereals and legumes (Ravindran *et al*., 1995). Phytate accumulates in the seed during the ripening period where it is localized in the aleurone layer of most cereals, except maize, where 88% of the phytic acid is found in the germ (Hídvégi and Lásztity, 2002). Poultry diets primarily comprise plant ingredients that have a large portion of phytate P, which is poorly digested by them due to the insufficient endogenous phytase enzyme, and induces an environmental pollutant through P excretion.

Phytase is the enzyme that catalyzes the step wise removal of orthophosphate from phytic acid. It has an established role in releasing a significant portion of the phytate P present in plant ingredient based rations and making it available to poultry, and reduce the amount of P excretion into the environment (Dersjant-Li *et al*., 2015). The International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB) recognize two classes of phytase depending on the initiation of dephosphorization: the 3-phytase and the 6-phytase. However, the efficiency of phytase activity is not influenced by this characterization, but some other factors, such as, type, pH profile range, and pepsin stability of phytase, and species and age of animal receiving phytase (Dersjant-Li *et al*., 2015). The first generation of commercialized phytase (fungal phytase) was launched in 1991. After that new generations of bacterial phytase was developed in 1999, and it was observed that bacterial phytase possess higher resistance to proteolytic digestion, which partly explain their higher efficacy than fungal phytase.

Phytase is also known to be present in the seeds of plants: cereals, leguminous seeds, and oil meal. Phytase activity of the major feedstuffs used in poultry feed is extremely variable: wheat and barley have high phytase activity, soybean and maize have low phytase activity, and oats and sorghum have almost no phytase activity (Barrier-Guillot *et al*., 1996a; Eeckhout and De Paepe, 1994). It is found that in cereals, phytase was synthesized during maturation and its activity rose remarkably during germination (Azeke *et al*., 2011; Ma and Shan, 2002;). Published scientific data concerning the effects of germination on chemical composition, anti-nutritional factors, and phytase activity in cereals and legumes seeds shows that an increase in phytase activity was accompanied by a concomitant decrease in phytate content (Azeke *et al*., 2011; Kyriakidis *et al*., 1998; Bau *et al*., 1997).

Buckwheat is grown throughout a large area of Asia and Southeast Asia as a crop that fits the farming system on marginal and unproductive land. The crop is not a cereal, but the seeds are usually rich in carbohydrates and have similar uses like other cereal grains, therefore, it is referred to as a pseudocereal. The grain is generally used as human food and as animal or poultry feed. The main producers are Russia, China, Kazakhstan, Ukraine, France, Poland, USA and Japan. In Bangladesh, buckwheat ("Bazra" in Bengali language) is considered as minor cereal, which produced in limited area. During 2014-2015, it has a production around 50 metric tons in 94 acres cultivation area. However, farmers are now motivated towards its production as emphasis is given by the Bangladesh Agricultural Research Institute (BARI), Bangladesh.

Buckwheat (*Fagopyrum esculentum*) is a rich source of phytase activity, although the activity is lower than the phytase activity found in wheat (*Triticum aestivum*), higher than maize (*Zea mays*), barley (*Hordeum vulgare*) and soybean (*Glycine max*) (Egli *et al.*, 2002): some commonly used ingredients in poultry diet. Energy content of buckwheat is comparable with barley but lower than maize and wheat (NRC, 1994). The presence of antinutritional factors, such as, soluble non-starch polysaccharide (arabinoxylans and *β*-glucans) (NSP) in wheat and barley are matter of consideration, because these resulted low nutrient digestibility and poor growth rate in chickens (Esmaeilipour *et al*., 2011; Ravindran *et al*., 1999). These antinutritive effects of NSP are attributed to an increase in intestinal digesta viscosity (Choct and Annison, 1992). Contrarily, buckwheat contains only 0.1-0.2% NSP and the presence of β-glucans also lower than that in barley and wheat (Havrlentová *et al*., 2011; Campbell, 1997). Based on these factors, buckwheat may be considered as good source of phytase in chickens feed.

Efficacy of the microbial phytase in increasing the digestibility and utilization of phytate P in poultry has well examined (Woyengo *et al*., 2010; Ebrahimnezhad *et al*., 2008; Hughes *et al*., 2008; Wu *et al*., 2006), whereas, very limited in case of the plant phytase. Although, intrinsic phytase activity in wheat, barley and triticale was evaluated in broilers and laying hens (Jondreville *et al.*, 2007; Francesch *et al*., 2005; Juanpere *et al*., 2004; Barrier-Guillot *et al*., 1996b), according to the author knowledge, no such findings in case of buckwheat, which is also a rich source of phytase. Therefore, there is a scope to characterize buckwheat phytase activity, and judge its potentiality to use as phytase source.

The present study deals primarily with the questions as to whether phytase activity from buckwheat is effective to improve P availability in chickens, and whether buckwheat can be used as an alternative phytase source for chicken feed. Accordingly, this study was composed of following experiments: in chapter II, an *in vitro* study was conducted to know the chemical composition and phytase activity in different buckwheats, and effects of germination on these parameters. In chapter III, the efficacy of buckwheat phytase to improve P availability was investigated by measuring growth performance, bone quality, and P retention in broilers given non-phytate P deficient diets with buckwheat. In chapter IV, the efficacy of buckwheat phytase was investigated in laying hens by measuring production performance, egg quality, and P retention. In chapter V, phytase activity in digesta from different parts of the digestive tract, and ileal digestibility of nutrient in broilers given buckwheat diets were measured to identify the part of digestive tract that involved in phytate degradation by buckwheat phytase, and to verify if dietary buckwheat affects nutrient digestibility. Finally, chapter VI discussed from the viewpoint of practical use of buckwheat as an alternative phytase source for chicken feed.

Chapter II

Buckwheat Chemical Composition and Phytase Activity: an *in vitro* Study

Abstract

In this study, chemical composition, phytase activity and *in vitro* digestibility of nutrients in buckwheat were measured to assay the variability in buckwheat. Three common buckwheat varieties namely, Shinano No. 1, Kitawase and Harunoibuki, and one tartary buckwheat namely, Dattan were used for the evaluation and each variety seed was purchased from three different prefectures in Japan. For germination, seeds were soaked in water for 12 h, and then placed in a tray lined with wet paper for 36 h at room temperature (23±2°C) maintaining a dark condition, and then dried at 50°C in a forced air oven for 7 h. After that, both non-germinated buckwheat (BU) and germinated buckwheat (GBU) seeds were ground to pass through 1.0-mm aperture for analysis of proximate components, total P, phytate P and phytase activity. *In vitro* digestibility of dry matter, crude protein and phytate P in BU and GBU were also measured. Results of the study clarified that chemical composition and phytase activity varied among different buckwheats, and germination tended to increase phytase activity with concomitant decrease of phytate P content in buckwheat. Germination increased the *in vitro* digestibility of dry matter (DM), crude protein (CP) and phytate P. In conclusion, among the examined buckwheats, Shinano No. 1 BU and GBU showed highest phytase activity and *in vitro* digestibility of phytate P and seemed to be effective to use as a source of phytase in chicken diet.

Introduction

Phytate is the main storage form of plant P and is unavailable or poorly utilized by poultry because of the nominal phytase activity in their digestive tract. Therefore, poultry diets are often supplemented with the source of inorganic P, which increases the cost of the diets. Moreover, this is not an ecological solution, because phytate P is excreted undigested and can be a cause of environmental pollution. In this context, the benefit of phytase supplementation which can degrade phytate and alleviate its negative effects has long been recognized. But his approach is not entirely satisfactory because of the added cost of the enzyme additive and, there is an increasing trend of raising livestock without relying on feed additives. Hence, an alternative approach would be to use feed ingredients having intrinsic phytase activity.

Studies on the phytase activity in cereals have been clarified that a limited number of cereals, such as, wheat (*Triticum aestivum*) and triticale (*Triticosecale*) have a high phytase activity (Jondreville *et al.*, 2007; Barrier-Guillot *et al*., 1996a), and interestingly, their activity increases by germination with the concomitant decrease in phytic acid concentration (Ma and Shan, 2002; Bartnik and Szafrańska, 1987). Buckwheat is a non-glutinous pseudocereal, produced in many countries, but no enough studies have been carried out on the use of buckwheat as feed and hence information about buckwheat phytase is still quite limited (Egli *et al*., 2002). Moreover, it is obscure whether its phytase activity is influenced by germination. Therefore, the present study was conducted to measure chemical composition, phytase activity and *in vitro* digestibility of nutrients in four different buckwheats in non-germinated and germinated form, and to discuss the variability and effects of germination on these parameters.

Materials and Methods

Germination of Buckwheat

Three common buckwheat varieties namely, Shinano No. 1, Kitawase and Harunoibuki, and one tartary buckwheat namely, Dattan were used for the evaluation. Each variety seeds were collected from three different prefectures in Japan, and part of seeds were germinated following the method of Egli *et al*. (2002) with slight modification. Briefly, seeds were soaked in water for 12 h and then transferred to tray lined with wet paper and allowed to germinate for 36 h at room temperature (23±2°C) in a dark condition. During germination, water was sprinkled on seeds every 10 h. After germination, seeds were dried at 50°C in a forced air oven for 7 h. Both BU and GBU seeds were ground to pass through 1.0-mm aperture and approximately 93% hulls were removed by sieving ground seeds. BU and GBU samples were then kept at room temperature until analysis.

Phytase Activity Measurement

Phytase activity was measured following the method described by Eeckhout and De Paepe (1994). In brief, 100 mg of finely ground sample was mixed with Na-phytate solution buffered with acetate at pH 5.5, and then the phosphate ion liberated from phytate was measured colorimetrically. The phytase unit (PU) was defined as that amount of phytase activity which liberates inorganic P from a 0.0015 M Na-phytate solution, at a rate of 1 μmol per min at pH 5.5 and 37°C.

In vitro Digestibility Measurement

The *in vitro* digestibility of DM, CP and phytate P of BU and GBU were determined according to Saunders *et al*. (1973) with slight modifications: briefly, about 250 mg of sample was suspended in 15 ml of 0.1 N HCl containing 1.5 mg pepsin (10,000

U/mg protein) (Nacalai Tesque Inc., Kyoto, Japan), and gently shaken at 41°C for 3 h. After neutralisation with 0.5 N NaOH, the digesta was mixed with 7.5 ml of phosphate buffer at pH 8.0 containing pancreatin (amylase activity 3,220 U/g, protease activity 38,500 U/g and lipase activity 1,600 U/g) (Nacalai Tesque Inc., Kyoto, Japan) and shaken at 41°C for 24 h. The solution was then centrifuged at 1,200 rpm for 10 min, washed with distilled water, filtered and dried.

The DM, CP and phytate P digestibilities of BU and GBU were determined as follows:

$$\text{DM digestibility (\%)} = \frac{\text{Dried sample weight- Dried residue weight}}{\text{Dried sample weight}} \times 100$$

$$\text{CP digestibility (\%)} = \frac{\text{Total N in sample- N in residue}}{\text{Total N in sample}} \times 100$$

$$\text{Phytate P digestibility (\%)} = \frac{\text{Phytate P in sample - Phytate P in residue}}{\text{Phytate P in sample}} \times 100$$

Chemical and Statistical Analysis

Samples of BU and GBU were analyzed for proximate composition according to standard methods (AOAC, 1990). Total P and phytate P of them were measured according to ISO (1998) and Haug and Lantzsch (1983), respectively.

Statistical significances among the means were determined with Tukey's multiple comparison tests at a significance level of 5%. In addition, two-way ANOVA was conducted to test the main effects of buckwheat and germination treatment, and interaction effects between them (SAS Institute, 2015).

Figure 1. Seeds of common buckwheat (a) Shinano No. 1, (b) Kitawase and (c) Harunoibuki, and tartary buckwheat (d) Dattan

Result

Chemical Composition and Phytase Activity (Table 1)

Among the non-germinated buckwheat, highest CP (14.8%), crude fiber (CF) (6.1%), crude ash (CA) (2.9%) and nitrogen-free extract (NFE) (76.6%) contents were recorded in Shinano No. 1, Kitawase, Harunoibuki and Dattan, respectively. However, germination led to increase CP and CF contents, decrease NFE and CA contents numerically in different buckwheats, and affect EE little in most cases. Highest total P (0.46%) and phytate P (0.38%) contents were observed in Kitawase. Although germination did not show any regular effect on total P content but phytate P content decreased numerically. Interestingly, phytase activity was varied remarkably: highest ($P<0.05$) phytase activity was recorded in Shinano No. 1 buckwheat (2.1 PU/g) compared with others, and this trend was continued after germination (2.5 PU/g). However, phytase activity was increased numerically in different buckwheats after germination. Significant differences in phytase activity was found for the main effects of buckwheat and germination treatment.

In vitro digestibility (Table 2)

Highest ($P>0.05$) *in vitro* digestibilities of CP (89.0%) and phytate P (30.6%) were recorded in Shinano No. 1, whereas, Dattan showed the highest DM digestibility (75.0%), among the non-germinated buckwheat. Interestingly, in most cases after germination the digestibility values increased ($P>0.05$) in low magnitude in different buckwheats: comparing with others, germinated Shinano No. 1 buckwheat showed greater digestibility values. Significant differences in DM and CP digestibilities were found for the main effects of buckwheat and germination treatment.

Table 1. **Chemical composition and phytase activity in buckwheat**[1]

Buckwheat	GT	%							PA, PU^2/g dry matter
		CP	EE	CF	CA	NFE	TP	PP	
Shinano No. 1	-	14.8±0.7^{ab}	3.1±0.1^{ab}	4.9±0.1^{c}	2.2±0.1^{bc}	74.9±0.6^{ab}	0.40±0.01	0.33±0.01	2.1±0.2^{a}
	+	16.4±0.7^{a}	3.1±0.1^{ab}	5.3±0.3^{ab}	2.0±0.1^{bc}	71.8±0.6^{b}	0.46±0.01	0.27±0.01	2.5±0.2^{a}
Kitawase	-	13.5±0.7^{ab}	3.7±0.3^{a}	6.1±0.2^{abc}	2.7±0.1^{ab}	74.1±1.1^{ab}	0.46±0.04	0.38±0.03	1.4±0.1^{bc}
	+	14.1±0.9^{ab}	3.0±0.3^{ab}	7.0±0.1^{a}	2.4±0.1^{abc}	73.1±1.1^{ab}	0.44±0.04	0.33±0.02	1.6±0.03^{b}
Harunoibuki	-	14.4±0.3^{ab}	2.9±0.1^{ab}	5.1±0.4^{bc}	2.9±0.1^{a}	74.7±0.1^{ab}	0.45±0.03	0.34±0.03	1.3±0.1^{bc}
	+	14.7±0.3^{ab}	2.8±0.1^{b}	5.7±0.5^{bc}	2.6±0.1^{abc}	74.3±0.3^{ab}	0.44±0.03	0.31±0.04	1.4±0.1^{bc}
Dattan	-	12.7±0.7^{b}	3.1±0.1^{ab}	5.2±0.1^{bc}	2.5±0.3^{abc}	76.6±0.9^{a}	0.37±0.02	0.31±0.02	1.0±0.1^{c}
	+	13.4±0.9^{ab}	3.0±0.2^{ab}	5.6±0.1^{bc}	2.0±0.1^{c}	75.9±0.8^{a}	0.39±0.02	0.28±0.02	1.2±0.1^{bc}
Source of variation	----------*P*-value----------								
Buckwheat		0.0119	0.0053	0.0019	0.0008	0.0064	0.0867	0.0779	<0.0001
GT		0.0636	0.3223	0.0005	0.0062	0.0266	0.4880	0.0344	0.0169
Buckwheat × GT		0.7829	0.8898	0.3076	0.6980	0.2788	0.5847	0.9447	0.7101

[1]Values for each parameter represent mean values with three observations (dry matter basis).

[1]GT = germination treatment, CP = crude protein, EE = ether extract, CF = crude fibre, CA = crude ash, NFE = nitrogen-free extract, TP = total phosphorus, PP = phytate phosphorus, PA = phytase activity.

[2]Phytase unit (PU) equivalent to the enzymatic activity, which liberates 1 μmol inorganic phosphate per min at pH 5.5 and 37°C.

[a-c]Means within a column not followed by common superscripts are different at $P<0.05$.

Table 2. ***In vitro*** **digestibilities of dry matter, crude protein and phytate phosphorus in buckwheat** [1]

Buckwheat	GT	%		
		DMD	CPD	PPD
Shinano No. 1	-	73.8±0.8	89.0±0.6^{ab}	30.6±1.2
	+	76.8±0.3	91.1±0.9^{a}	33.5±2.3
Kitawase	-	71.5±0.1	85.4±0.2^{b}	25.3±1.5
	+	73.1±1.3	87.8±0.6^{ab}	25.4±1.8
Harunoibuki	-	73.9±1.6	88.5±0.9^{ab}	27.2±2.7
	+	74.7±2.2	90.8±1.4^{a}	28.1±2.2
Dattan	-	75.0±0.8	86.3±1.0^{b}	25.2±2.3
	+	76.7±0.2	89.0±0.7^{ab}	26.6±2.7
Source of variation		----------*P*-value----------		
Buckwheat		0.0331	0.0025	0.1880
GT		0.0444	0.0012	0.4946
Buckwheat × GT		0.8084	0.8949	0.9691

[1]Values for each parameter represent mean values with three observations.

[1]GT = germination treatment, DMD = dry matter digestibility, CPD = crude protein digestibility, PPD = phytate phosphorus digestibility.

[a-b]Means within a column not followed by common superscripts are different at $P<0.05$.

Discussion

This study revealed that chemical composition varied among different buckwheat: CP, CF, and CA varied markedly (maximal value/minimal value = 1.2 to 1.3), whereas, NFE varied less markedly (maximal value/minimal value = 1.0). Variation among buckwheat cultivars in terms of CP, CF, and ash contents were reported by Dziadek *et al*. (2016). Interestingly, phytase activity varied considerably among different buckwheats (maximal value/minimal value = 2.1). Such variation also observed in wheat and triticale, and it was explained by the genetic characteristics, that play a role in the determination of phytase activity in different varieties in those cereals (Jondreville *et al.*, 2007; Barrier-Guillot *et al*., 1996a). Therefore, it would be interesting to confirm this effect in buckwheat by testing large number of buckwheat varieties. The common buckwheat Shinano No. 1 showed the highest phytase activity, compared with Kitawase, Harunoibuki, and tartary buckwheat Dattan. The efficacy of phytase enzyme in determining grain P availability makes buckwheat especially Shinano No. 1 interesting to use for poultry nutrition.

It is well known that germination can alter chemical composition in grains (Donkor *et al*., 2012, Bau *et al*., 1997), which was true in the present study: CP content tended to increase, whereas, CA and NFE content decreased in different buckwheats after germination treatment. Moreover, germination led to increase phytase activity in buckwheat: such effect also observed in barley, oat, rye, triticale and wheat (Ma and Shan, 2002; Bartnik and Szafrańska, 1987). In addition, decreased phytate P content after germination may be resulted from the increased phytase activity by germination. Similar observation was reported in rice, maize, millet, sorghum and wheat (Azeke *et al.,* 2011).

In this study, *in vitro* digestibilities of CP and phytate P were greater in Shinano No. 1 buckwheat and germination further increased these values numerically. The availability of P in plant ingredients depends primarily on the amount of phytate P present and the concentration of phytase in the plant sources (Liu *et al*., 1998). In current study, highest phytase activity in Shinano No. 1 buckwheat compared with others resulted in highest phytate P digestibility in this buckwheat. Moreover, the numerically increased CP digestibility in different buckwheats after germination may be due to the little alleviation of the negative impact of phytate on enzymes because of the decreased phytate content. However, these *in vitro* results may be useful for the prediction of bioavailability, but for better clarification it may be necessary to do *in vivo* study.

Conclusion

The obtained results revealed that chemical composition, phytase activity and *in vitro* digestibility varied among different buckwheats. Shinano No. 1 buckwheat possesses highest CP content and phytase activity compared with others and this trend was continued after germination. However, germination tended to increase phytase activity with concomitant decrease in phytate P content in different buckwheat. Highest digestibility of phytate P was recorded in Shinano No. 1 buckwheat, which was further increased after germination. In general, buckwheat possesses high phytase activity and germination can increase this activity, therefore, buckwheat can be a potential source of phytase rich ingredient in chicken feed. However, in current study Shinano No. 1 buckwheat showed the highest phytase activity, therefore, this buckwheat may be considered to use in *in vivo* study to examine the efficacy of buckwheat phytase to improve P availability in chicken.

Chapter III

Effects of Buckwheat Added Diets on Growth Performance, Bone Quality and Phosphorus Availability in Broilers

Abstract

To determine whether buckwheat phytase can be used as an alternative phytase source, growth performance, bone quality, and P balance were measured in broilers given non-phytate P deficient diets. A total of 120 male broiler chicks (8 d of age) were divided into eight groups (15 birds each), and given one of the following diets until 42 d of age: positive control (PC) diet satisfying recommended levels of all nutrients, negative control (NC) diet formulated to contain 0.16% lower non-phytate P than in the PC diet, and six other diets, formulated by replacing maize in NC diet with BU or GBU at 10%, 15% and 20% levels. Compared with the PC group, NC group showed impaired growth performance (BW gain, FI, and FCR) and bone quality (dry weight, breaking strength, and contents of ash and P in tibia). However, in most cases, these impairments were ameliorated dose-dependently by the addition of BU and GBU in diets, and the restoration magnitude was greater in GBU than in BU. Total P excretion decreased in NC group and further decreased dose-dependently with increasing levels of BU and GBU. Except for the values in PC group, total P retention increased as the total P excretion decreased. In conclusion, dietary BU and GBU restored the growth performance and bone quality impaired by the non-phytate P deficiency, and improved P retention in broilers, which suggested that buckwheat, especially when germinated, can be used as an alternative phytase source in broiler diets.

Introduction

Feed supplementation with phytase rich cereals have been investigated as a solution for the reduction of P excretion in poultry excreta. According to Barrier-Guillot *et al*. (1996b), wheat (*Triticum aestivum*) have a high phytase activity and can improve P digestibility in broilers. Moreover, Jondreville *et al.* (2007) reported that it may be possible to decrease dietary P supplementation in broilers diet by adding triticale as a phytase rich ingredient.

In chapter II, it was discussed that chemical composition and phytase activity varied among different buckwheats, and germination treatment led to increase phytase activity with concomitant decrease of phytate P content in different buckwheats. Moreover, *in vitro* digestibilities of CP and Phytate P also varied remarkably. Considering the phytase activity and phytase P digestibility values, Shinano No. 1 BU and GBU was found to be the best among them for using in chicken diets as phytase rich ingredient.

Present study was conducted to know whether buckwheat phytase can be used as an alternative phytase source, therefore, the growth performance, bone quality, and P balance were measured in broilers reared on non-phytate P deficient diets containing Shinano No. 1 BU and GBU at different levels and discussed the efficacy of phytase from BU and GBU in broilers.

Materials and Methods

This research was conducted in accordance with the guidelines for regulation of animal experimentation of Shinshu University, Japan.

Germination of Buckwheat

Seeds of Shinano No. 1 buckwheat were purchased commercially, and part of seeds were germinated, as explained detail in chapter II, and after germination both BU and GBU seeds were ground to pass through 1.0-mm aperture, and approximately 93% of the hulls were removed by sieving whole ground seeds and used to formulate broilers diet. Samples of BU and GBU were analyzed for chemical composition and phytase activity (Table 3).

Birds and Diets

One hundred and twenty (8 d of age), male broiler chicks (Ross 308) were divided into eight dietary groups (15 birds each) having similar BW and housed in floor pens at optimum temperature under 24-h light condition. Each dietary group was assigned to one of the following experimental diets: PC diet formulated according to the NRC (1994) recommendations, NC diet formulated to contain 0.16% lower non-phytate P than in the PC diet, and six other diets, formulated to contain 10%, 15%, and 20% of BU or GBU in the NC diet, at the expense of maize (Table 2 and 3). Starter diets contained 23.5% CP and 3,200 kcal of ME/kg and were used for 8–21 d of age. Then, grower diets with 20.5% CP and 3,250 kcal of ME/kg were provided for 22–42 d of age. Birds were transferred from floor pans to cages on d 36, in an open-air house. Each cage was equipped with feeder, nipple drinker and birds were offered the corresponding diet. Diets and water were provided *ad libitum* for the 35-d experimental period (from 8 to 42 d of age).

Data and Sample Collection

Feed intake (FI) and body weight were recorded daily and weekly, respectively. Feed conversion ratio (FCR) was calculated at the end of trial. The digestibility trial was composed of a 3-d preliminary cage adaptation period, followed by 3-d of excreta collection period (d 39–41). Immediately after collection, excreta samples were stored at -20°C in a freezer. Frozen excreta samples were then thawed, homogenized, dried and ground before analysis.

On 43 d of age, 12 birds per dietary group were killed by cervical dislocation and then dressed: carcass, breast and leg meat, and internal organs (heart, spleen, liver, and gizzard) were weighed. Tibia and femur bones (2 tibias and 2 femurs per bird) were collected from 10 birds in each dietary group, measured in length and width, and then subjected to measurement of breaking strength (kgf/cm^2) using a force gauge (ZTA-5000N, IMADA Co., Ltd., Japan). Subsequently, they were measured for dry weight after drying at 100°C for 24 h, and then ashed at 600°C for 24 h (Chung and Baker, 1990). On the same day, for body composition analysis, three birds per group were killed without bleeding. After defeathering, they are ground to be homogenous and then stored at -20°C until further processing.

Chemical Analysis

Samples of BU, GBU, diets, excreta and whole body of birds were analyzed for proximate composition following the standard methods (AOAC, 1990). Total P and phytate P were measured according to ISO (1998) and Haug and Lantzsch (1983), respectively: non-phytate P was calculated by subtracting the phytate P from total P. Phytase activity was measured following Eeckhout and De Paepe (1994) as explained detail in chapter II.

Calculation and Statistical Analysis

The percentage ash was determined relative to dry weight of the bone. Tibia weight/length index and femur weight/length index were calculated by dividing the weight by its length (Seedor *et al*., 1991).

Statistical significances among the dietary groups were determined using Tukey's multiple comparison tests at a significance level of 5%, after one-way ANOVA (SAS Institute, 2015). In addition, two-way ANOVA was performed by omitting the PC and NC groups, to test for the main and interaction effects between the BU and GBU groups. Linear regression analysis was conducted to obtain the equations relating phytase levels in diets with response (BW gain, FI, tibia ash, and tibia P), using the SAS statistical package. The linear regression model used was:

$$Y = a + b \times X$$

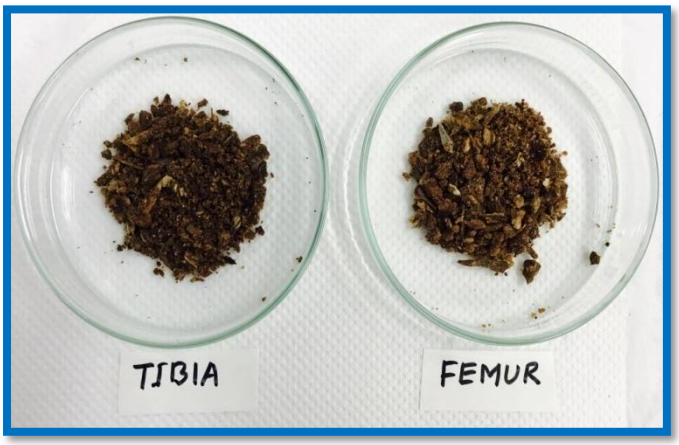
where ‘Y’ is the response criterion, ‘a’ is the ‘Y’ intercept, ‘b’ is the slope of the response criterion and ‘X’ is the level of phytase (PU/kg diet) from BU or GBU (calculated values were used).

Figure 2. Non-germinated and germinated Shinano No. 1 buckwheat (*Fagopyrum esculentum*) seeds and ground buckwheat

Minced broiler

Collected tibia and femur bone

Dried tibia and femur bone



Ground tibia and femur bone

Figure 3. Collection and preparation of samples for analysis of broilers body composition and bone quality

Table 3. **Chemical composition and phytase activity in Shinano No. 1 buckwheat**[1]

Components, %	Shinano No. 1 buckwheat	
	BU	GBU
Crude protein	15.26	17.08
Ether extract	3.17	3.08
Crude fiber	5.06	6.23
Crude ash	2.04	2.07
Total P	0.38	0.42
Phytate P	0.32	0.27
Phytase activity, PU^2/g	2.10	2.60

[1]Values based on analysis of triplicate samples (dry matter basis).

[1]BU = non-germinated buckwheat; GBU= germinated buckwheat.

[2]Phytase unit (PU) equivalent to the enzymatic activity, which liberates 1 μmol inorganic phosphate per min at pH 5.5 and 37°C.

Table 4. **Ingredients and chemical composition of the experimental diets (d 8-21)[1]**

Ingredients, %	PC	NC	NC+BU			NC+GBU		
			10%	15%	20%	10%	15%	20%
Commercial diet[2]	55.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0
Soybean meal	20.0	20.7	20.0	18.8	17.5	20.0	18.8	17.5
Maize	20.1	24.1	13.0	8.4	3.95	13.0	8.4	3.95
BU	-	-	10.0	15.0	20.0	-	-	-
GBU	-	-	-	-	-	10.0	15.0	20.0
Maize oil	2.50	2.00	3.80	4.60	5.35	3.80	4.60	5.35
$Ca_3(PO_4)_2$	0.8	-	-	-	-	-	-	-
$CaCO_3$	0.7	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Vit-min Premix[3]	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Analyzed composition, % (as fed basis)								
Crude protein	23.57	23.49	23.53	23.56	23.68	23.52	23.55	23.63
Crude fiber	3.98	3.91	4.16	4.28	4.41	4.31	4.53	4.72
Total P	0.74	0.59	0.59	0.58	0.58	0.58	0.59	0.59
Phytate P	0.28	0.29	0.28	0.28	0.28	0.29	0.29	0.29
Non-phytate P	0.46	0.30	0.31	0.30	0.30	0.29	0.30	0.30
Calcium[4]	0.99	0.99	1.00	1.00	1.00	0.99	1.00	1.00
ME, kcal/kg[4]	3,204	3,207	3,206	3,209	3,211	3,206	3,209	3,211
Phytase, PU/kg of diet on DM basis[5]	ND	ND	210	315	420	260	390	520

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat, DM = dry matter, ND = not-determined.

[2]Commercial starter diet (CP≥23.5%, ME≥3050 kcal/kg, Feedone Co., Ltd. Kanagawa, Japan).

[3]Vitamin-mineral premix provided with the following concentrations per kg of diet: vitamin A, 5,000 IU; vitamin D_3, 1,000 IU; vitamin E, 11 IU; vitamin K_3, 1.1 mg; vitamin B_1, 6.0 mg; vitamin B_2, 23.0 mg; vitamin B_6, 8 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 18 mg; niacin, 35 mg; choline chloride, 1,700 mg; folacin, 2.20 mg; iron, 95 mg; copper, 11 mg; zinc, 180 mg; manganese, 280 mg; iodine, 3.4 mg.

[4]Calculated nutrient content was based on ingredient composition data from NRC (1994).

[5]Phytase activity value was calculated on the dry matter basis of diets based on the analyzed phytase activity in Shinano No. 1 BU and GBU.

Table 5. **Ingredients and chemical composition of the experimental diets (d 22-42)[1]**

Ingredients, %	PC	NC	NC+BU			NC+GBU		
			10%	15%	20%	10%	15%	20%
Commercial diet[2]	46.3	33.0	33.0	33.0	33.0	33.0	33.0	33.0
Soybean meal	23.0	24.5	23.3	22.9	21.8	23.3	22.9	21.8
Maize	27.5	39.9	30.0	24.7	20.2	30.0	24.7	20.2
BU	-	-	10.0	15.0	20.0	-	-	-
GBU	-	-	-	-	-	10.0	15.0	20.0
Maize oil	0.9	0.4	1.5	2.2	2.8	1.5	2.2	2.8
$Ca_3(PO_4)_2$	0.6	-	-	-	-	-	-	-
$CaCO_3$	0.8	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Vit-min Premix[3]	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Analyzed composition, % (as fed basis)								
Crude protein	20.68	20.59	20.61	20.53	20.63	20.45	20.59	20.56
Crude fiber	3.88	3.68	3.92	4.20	4.56	4.18	4.37	4.80
Total P	0.67	0.50	0.50	0.51	0.51	0.51	0.50	0.50
Phytate P	0.30	0.30	0.29	0.30	0.31	0.30	0.30	0.29
Non-phytate P	0.37	0.20	0.21	0.21	0.20	0.21	0.20	0.21
Calcium[4]	0.81	0.83	0.83	0.84	0.84	0.83	0.84	0.83
ME, kcal/kg[4]	3,257	3,268	3,255	3,244	3,236	3,255	3,244	3,236
Phytase, PU/kg of diet on DM basis[5]	ND	ND	210	315	420	260	390	520

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat, DM = dry matter, ND = not-determined.

[2]Commercial grower diet (CP≥18.0%, ME≥3270 kcal/kg, Feedone Co., Ltd. Kanagawa, Japan).

[3]Vitamin-mineral premix provided with the following concentrations per kg of diet: vitamin A, 5,000 IU; vitamin D_3, 1,000 IU; vitamin E, 11 IU; vitamin K_3, 1.1 mg; vitamin B_1, 6.0 mg; vitamin B_2, 23.0 mg; vitamin B_6, 8 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 18 mg; niacin, 35 mg; choline chloride, 1,700 mg; folacin, 2.20 mg; iron, 95 mg; copper, 11 mg; zinc, 180 mg; manganese, 280 mg; iodine, 3.4 mg.

[4]Calculated nutrient content was based on ingredient composition data from NRC (1994).

[5]Phytase activity value was calculated on the dry matter basis of the diets based on the analyzed phytase activity in Shinano No. 1 BU and GBU.

Results

Growth Performance (Table 6)

Final BW in the birds given PC diet (2,925 g) was slightly lower than the corresponding value of male ROSS 308 broiler (3,023 g) (Aviagen, 2014). This value was further decreased by about 10% in the NC group, but restored dose-dependently with the increasing levels of BU and GBU in the diets. Comparing the BU and GBU groups, the degree of restoration was greater in the GBU groups than in the BU groups. BW gain showed a similar trend as the final BW. FI was decreased slightly but significantly in the NC and BU groups, however, FI did not decrease in all GBU groups. FCR in the PC group was similar with the corresponding value of male ROSS 308 broiler (Aviagen, 2014), which was deteriorated in the NC group. Although restoration of the FCR was dose-dependent in the BU groups, such a trend was not observed in the GBU groups. However, among the BU and GBU groups, the overall growth performance of 20% GBU group was comparable with that of the PC group.

Body Composition (Table 7)

Moisture content ranged from 68.8% to 70.9%, and ash content from 2.3% to 2.6%, and both were not affected by dietary treatments. CP content was numerically higher in BU and GBU groups compared with PC and NC, but it did not follow any regular trend and EE content was almost similar in all groups.

Carcass Quality (Table 8)

The difference in live weight among the groups was reflected also in carcass weight. Compared with PC group carcass weight was lower ($P<0.05$) in all groups, except 15 and 20% GBU added groups. Although decreased carcass weight was restored dose-dependently in case of both BU and GBU added groups, no difference was found among the groups in carcass yield in % of live weight. Moreover, head, shank, giblets and

abdominal fat contents, and % of wing, breast, and leg muscle were not affected by dietary groups.

Bone Quality (Table 9 and Table 10)

Length and width of the tibia and femur bones were not affected by the experimental diets. All values in tibia decreased significantly in the NC group compared with the PC group, and the values were restored in most cases by the addition of BU and GBU in the diets. Non-significant differences were observed among the levels of BU and GBU in terms of dry tibia weight, tibia weight/length index, tibia ash, and P content. Restoration of tibia breaking strength was dose-dependent in the BU groups, but not in the GBU groups. Similar tendency was observed in femur breaking strength. Comparing the values, the degree of restoration was grater in the GBU groups than the BU groups. Decreased dry femur weight in the NC group was restored in the 20% GBU group. Significant difference in femur weight/length index was found for the main effect of germination, but the main effect of level was not significant. Moreover, no significant differences in femur ash and P contents were observed for both main effects.

Balance of Total P and Nitrogen (Table 11)

Total P intake varied ($P<0.05$) between the PC and other groups because of the varying dietary level of P. Interestingly, the total P excretion decreased, and retention increased dose-dependently, with the addition of BU and GBU in the diets. Lowest ($P<0.05$) excretion was observed in the 20% BU and GBU groups compared with others, except 15% BU and GBU. Consequently, the retention of total P was increased in those groups significantly. Although intake was lower, retention of total P in 15% and 20% BU and GBU groups were comparable with the PC group. On the other hand, nitrogen intake was similar in all groups, but excretion varied considerably. Lowest ($P>0.05$) excretion of nitrogen resulted in highest ($P>0.05$) retention in the 20% GBU group, compared with the PC group.

Table 6. **The effects of non-germinated and germinated buckwheat added diets on the performance parameters in broilers**[1]

Dietary groups	FBW, g	BWG, g	FI, g/bird/d	FCR, g feed/g BW
PC	2,925±31.1^{a}	2,729±31.3^{a}	130.2±0.4^{ab}	1.67±0.02^{d}
NC	2,614±18.6^{e}	2,419±17.7^{e}	127.5±0.2^{c}	1.85±0.01^{a}
NC + 10% BU	2,651±22.4^{de}	2,457±21.6^{de}	128.4±0.5^{c}	1.83±0.02^{ab}
NC + 15% BU	2,715±14.8^{cd}	2,520±14.8^{cd}	128.0±0.4^{c}	1.78±0.01^{bc}
NC + 20% BU	2,761±16.9^{bc}	2,567±15.6^{bc}	128.6±0.3^{c}	1.76±0.01^{c}
NC + 10% GBU	2,751±15.9^{bcd}	2,556±14.9^{bc}	128.7±0.4^{bc}	1.76±0.01^{c}
NC + 15% GBU	2,801±24.9^{bc}	2,605±23.5^{bc}	130.2±0.2^{ab}	1.75±0.02^{c}
NC + 20% GBU	2,830±26.7^{ab}	2,635±25.8^{ab}	130.3±0.3^{a}	1.73±0.02^{cd}
Source of variation	----------*P*-value----------			
Germination	0.0204	<0.0001	<0.0001	0.0006
Level	0.0008	<0.0001	0.0363	0.0013
Germination × Level	0.2680	0.7492	0.0587	0.2732

[1]Values for each parameter represent mean±standard error values with fifteen observations (d 8 to d 42).

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat, FBW = final body weight, BWG = body weight gain, FI = feed intake, FCR = feed conversion ratio.

[a-e]Means within a column not followed by common superscripts are different at $P<0.05$.

Table 7. **The effects of non-germinated and germinated buckwheat added diets on the body composition in broilers**[1]

Dietary groups	%			
	Moisture	Crude protein	Ether extract	Ash
PC	68.8±1.3	12.1±0.3	12.3±1.0	2.3±0.03
NC	70.9±1.3	11.9±0.7	10.2±0.2	2.3±0.08
NC + 10% BU	69.9±0.4	12.3±0.6	10.5±0.4	2.3±0.20
NC + 15% BU	70.1±0.9	13.1±0.3	10.2±1.0	2.5±0.07
NC + 20% BU	69.8±1.1	13.4±0.8	10.7±0.6	2.5±0.17
NC + 10% GBU	69.6±0.9	13.7±0.5	11.1±0.5	2.5±0.06
NC + 15% GBU	70.5±0.4	13.2±0.1	10.2±1.2	2.6±0.04
NC + 20% GBU	69.7±0.3	13.7±0.2	10.6±0.3	2.6±0.03
Source of variation	----------*P*-value----------			
Germination	0.9794	0.1641	0.7587	0.2116
Level	0.6713	0.5053	0.7044	0.1727
Germination × Level	0.8466	0.3651	0.9096	0.6115

[1]Values for each parameter represent mean±standard error values with three observations (43-d of age).

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

Table 8. **The effects of non-germinated and germinated buckwheat added diets on different organs weight in broilers[1]**

Dietary groups	Live weight, g	Carcass weight, g	% live weight					% carcass weight		
			Head	Shank	Giblet	Abdominal fat	Carcass yield	Wing	Breast	Leg
PC	2,909±45.3[a]	2,039±32.8[a]	2.4±0.02	4.5±0.06	3.7±0.04	0.91±0.10	70.1±0.5	11.0±0.2	37.5±1.2	34.3±0.7
NC	2,641±13.9[b]	1,829±15.3[b]	2.3±0.04	4.6±0.11	3.9±0.07	0.81±0.14	69.3±0.6	11.0±0.2	38.1±0.6	30.4±0.7
NC + 10% BU	2,687±25.4[b]	1,895±22.2[b]	2.4±0.03	4.7±0.09	3.9±0.09	0.72±0.09	70.7±1.4	10.8±0.4	35.8±0.8	32.6±0.9
NC + 15% BU	2,725±19.2[b]	1,900±37.0[b]	2.3±0.03	4.8±0.10	3.8±0.10	0.92±0.11	69.5±0.5	10.9±0.3	39.3±1.5	33.2±1.5
NC + 20% BU	2,754±19.8[b]	1,919±24.9[b]	2.4±0.05	4.7±0.08	3.8±0.04	0.92±0.08	69.7±0.6	10.6±0.3	36.5±0.8	32.3±1.2
NC + 10% GBU	2,755±19.2[b]	1,895±17.7[b]	2.5±0.03	4.7±0.11	3.8±0.07	0.85±0.10	68.8±0.7	11.3±0.2	39.0±0.9	34.8±0.9
NC + 15% GBU	2,814±38.4[a]	1,968±28.6[a]	2.4±0.03	4.7±0.08	3.7±0.14	0.88±0.09	69.3±0.5	10.5±0.3	37.1±1.1	33.7±1.0
NC + 20% GBU	2,838±32.3[a]	1,972±34.8[a]	2.5±0.03	4.6±0.09	3.6±0.09	0.88±0.12	70.1±0.7	10.7±0.3	37.3±1.1	34.7±1.1

[1]Values for each parameter represent mean±standard error values with ten observations.

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

[a,b]Means within a column not followed by common superscripts are different at $P<0.05$.

Table 9. **The effects of non-germinated and germinated buckwheat added diets on tibia characteristics in broilers[1]**

Dietary groups	Length, cm	Width, mm	Dry wt., g	Wt./length index, mg/mm	Breaking strength, kgf/cm^2	Ash, %	P, %
PC	9.9±0.6	10.4±0.2	7.84±0.22^{a}	78.8±2.0^{a}	41.97±1.53^{a}	39.45±0.30^{a}	9.01±0.18^{a}
NC	9.9±0.5	10.1±0.2	6.68±0.14^{c}	67.3±1.5^{c}	30.08±1.26^{d}	35.55±0.21^{c}	7.38±0.08^{c}
NC + 10% BU	9.9±0.6	10.0±0.1	6.73±0.15^{c}	68.0±1.7^{c}	31.77±1.21^{cd}	36.80±0.68^{bc}	7.93±0.23^{bc}
NC + 15% BU	9.9±0.4	10.1±0.2	6.89±0.18^{bc}	69.2±1.9^{bc}	35.06±1.33^{bcd}	37.61±0.74^{abc}	8.25±0.19^{b}
NC + 20% BU	9.9±1.0	10.3±0.2	7.25±0.17^{abc}	73.2±2.1^{abc}	38.71±1.47^{ab}	38.41±0.60^{ab}	8.39±0.15^{ab}
NC + 10% GBU	9.9±0.8	10.1±0.1	7.26±0.17^{abc}	73.2±1.4^{abc}	37.31±1.11^{abc}	37.85±0.49^{abc}	8.16±0.15^{b}
NC + 15% GBU	9.9±0.5	10.2±0.2	7.63±0.22^{ab}	77.0±2.2^{ab}	40.00±1.79^{ab}	38.08±0.43^{ab}	8.21±0.15^{b}
NC + 20% GBU	9.9±0.6	10.6±0.3	7.94±0.24^{a}	80.2±2.5^{a}	40.72±1.37^{ab}	39.09±0.43^{ab}	8.42±0.12^{ab}
Source of variation	----------*P*-value----------						
Germination	0.7223	0.2300	0.0001	0.0001	0.0006	0.1486	0.6060
Level	0.8961	0.1124	0.0108	0.0126	0.0021	0.0674	0.1332
Germination × Level	0.9088	0.7587	0.8491	0.7973	0.4057	0.8878	0.7270

[1]Values for each parameter represent mean±standard error values with ten observations.

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

[a-d]Means within a column not followed by common superscripts are different at $P<0.05$.

Table 10. **The effects of non-germinated and germinated buckwheat added diets on femur characteristics in broilers[1]**

Dietary groups	Length, cm	Width, mm	Dry wt., g	Wt./length index, mg/mm	Breaking strength, kgf/cm^2	Ash, %	P, %
PC	7.4±0.6	10.7±0.2	6.20±0.16^{a}	84.2±2.3	37.11±1.19^{a}	37.24±0.50	7.46±0.14
NC	7.3±0.6	10.4±0.1	5.54±0.13^{b}	75.5±2.0	27.45±1.13^{b}	34.46±0.62	6.88±0.15
NC + 10% BU	7.3±0.6	10.4±0.2	5.66±0.09^{ab}	77.2±1.3	28.20±0.79^{b}	35.34±0.62	7.12±0.21
NC + 15% BU	7.3±0.5	10.2±0.1	5.82±0.10^{ab}	79.3±1.6	33.78±0.80^{a}	35.27±0.80	7.23±0.28
NC + 20% BU	7.4±0.6	10.3±0.2	6.07±0.17^{ab}	82.5±2.3	34.40±1.09^{a}	36.32±0.36	7.36±0.27
NC + 10% GBU	7.3±0.7	10.4±0.1	5.95±0.17^{ab}	81.2±2.6	34.19±0.70^{a}	35.07±0.38	7.04±0.20
NC + 15% GBU	7.4±0.7	10.3±0.2	6.11±0.14^{ab}	83.2±1.9	36.61±1.45^{a}	35.86±0.85	7.34±0.15
NC + 20% GBU	7.3±0.7	10.6±0.1	6.16±0.16^{a}	84.2±1.6	36.72±1.46^{a}	36.07±0.73	7.30±0.13
Source of variation	----------*P*-value----------						
Germination	0.7960	0.3046	0.0449	0.0495	0.0001	0.9631	0.9586
Level	0.9874	0.4512	0.0711	0.1123	0.0002	0.3497	0.4964
Germination × Level	0.9231	0.3870	0.6906	0.0731	0.2074	0.7747	0.8968

[1]Values for each parameter represent mean±standard error values with ten observations.

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

[a-c]Means within a column not followed by common superscripts are different at $P<0.05$.

Table 11. **The effects of non-germinated and germinated buckwheat added diets on the balance of total phosphorus and nitrogen in broilers**[1]

Dietary groups	Total phosphorus			Nitrogen		
	Intake, g/bird/d	Excretion, g/ bird/d	Retention, g/ bird/d	Intake, g/ bird/d	Excretion, g/ bird/d	Retention, g/ bird/d
PC	1.35±0.01^{a}	0.74±0.04^{a}	0.61±0.03^{a}	6.59±0.07	2.88±0.09^{ab}	3.72±0.04^{abc}
NC	0.98±0.01^{b}	0.58±0.02^{b}	0.39±0.03^{b}	6.43±0.04	3.08±0.12^{a}	3.35±0.13^{c}
NC + 10% BU	0.97±0.01^{b}	0.57±0.02^{bc}	0.41±0.02^{b}	6.45±0.08	2.95±0.06^{a}	3.50±0.13^{bc}
NC + 15% BU	1.00±0.01^{b}	0.51±0.02^{bcd}	0.50±0.01^{ab}	6.54±0.07	2.89±0.08^{ab}	3.65±0.08^{abc}
NC + 20% BU	1.00±0.01^{b}	0.43±0.02^{d}	0.57±0.02^{a}	6.48±0.07	2.62±0.10^{ab}	3.85±0.09^{ab}
NC + 10% GBU	1.00±0.01^{b}	0.57±0.03^{bc}	0.43±0.02^{b}	6.43±0.08	2.99±0.10^{a}	3.44±0.13^{bc}
NC + 15% GBU	1.00±0.01^{b}	0.44±0.04^{cd}	0.56±0.04^{a}	6.58±0.06	2.74±0.13^{ab}	3.85±0.13^{ab}
NC + 20% GBU	0.98±0.01^{b}	0.40±0.01^{d}	0.58±0.02^{a}	6.53±0.06	2.46±0.07^{b}	4.07±0.07^{a}
Source of variation	----------*P*-value----------					
Germination	0.9659	0.7844	0.1332	0.6612	0.2499	0.1757
Level	0.4267	0.0077	<0.0001	0.3001	0.0004	0.0002
Germination × Level	0.0771	0.6495	0.5139	0.8820	0.5118	0.3736

[1]Values for each parameter represent mean±standard error values with eight observations.

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

[a-d]Means within a column not followed by common superscripts are different at $P<0.05$.

Discussion

In the present study, the diets were formulated based on the NRC (1994), in which the nutrient specifications were slightly lower than those in "ROSS 308 nutrition specification". This may be one of the reasons for the slightly decreased final BW in PC group than corresponding value of ROSS 308 broilers (Aviagen, 2014). The deteriorated growth performance and bone quality (tibia and femur breaking strength, tibia ash, and P contents) in the NC group were restored by the addition of BU and GBU in the diets, and a near-complete restoration was observed in the 20% GBU group, suggesting that birds complemented the shortage of P in the NC diet with non-phytate P released from phytate P by the action of BU and GBU phytase. The degree of restoration was greater in the GBU groups than in BU groups, which could be due to the increased phytase activity by germination. It is noteworthy that 20% GBU group showed a growth performance and bone quality comparable with the PC group. This suggests that phytase activity in the 20% GBU diet may be close enough to minimize the deficiency of non-phytate P in this group.

The above findings lead to expectations of decreased P excretion in the BU and GBU groups, which were found to be true: NC group showed decreased P excretion because of the P deficient diet, and BU and GBU groups showed further decrease in the values (compared with the PC group, 23% to 42% decrease in the BU groups, and 23% to 46% decrease in the GBU groups). The highest decrease rate (46%) was recorded in the 20% GBU group, which was greater than the values reported by Paik (2003), who found that addition of microbial (derived from *Aspergillus oryzae*) or plant (wheat bran) phytase (600 PU/kg diet) in a P deficient diet could decrease the P excretion approximately 28%-30% in broilers. In addition, nitrogen retention tended to increase in

the 20% GBU group, although all diets were isonitrogenous. Similar observations have been reported by Selle *et al*. (2003), who explained that phytate-protein complexes resistant to digestion were decreased by the action of phytase (Selle *et al*., 2000; Ravindran *et al*., 1995).

According to Takemasa *et al*. (1996), the phytase unit (PU) equivalent to 0.10% of non-phytate P was 648–1,055 PU/kg diet (yeast phytase, at pH 5.5). An equivalency of 750 PU/kg diet of triticale phytase to 0.10% P was observed in terms of final weight, feed intake, and tibiotarsi dry matter, whereas it was 0.79% P in terms of tibiotarsi ash (Jondreville *et al*., 2007). It is mentionable that in most cases, BW gain, FI, and bone ash were used to calculate the equivalency value (Jendza *et al.*, 2006; Denbow *et al*., 1995), and linear regression was considered more accurate over quadratic or other polynomial fits (Ribeiro Jr *et al*., 2016; Han *et al*., 2009). Therefore, to express the effect of buckwheat phytase as the amount of non-phytate P in this study, the relationships between the levels of phytase unit and BW gain, FI, tibia ash, and tibia P content were determined, resulting in the following regression equations:

$Y_1 = 2408 + 0.428X$, $r^2 = 0.86$

$Y_2 = 127.3 + 0.005X$, $r^2 = 0.63$

$Y_3 = 35.63 + 0.006X$, $r^2 = 0.96$

$Y_4 = 7.52 + 0.002X$, $r^2 = 0.87$

where Y_1 = BW gain (g), Y_2 = FI (g/bird/day), Y_3 = Tibia ash (%), Y_4 = Tibia P (%), and X = buckwheat phytase (PU/kg diet).

All the regression equations were significant (P<0.05). Consequently, the levels of phytase unit required to obtain the same BW gain, FI, tibia ash, and tibia P in the PC group were calculated to be 750, 580, 636, and 745 PU/kg diet, respectively. Taking the BW gain as the most important parameter, 750 PU/kg diet should be included in the NC

diet formulated on the basis of NRC requirement, and therefore, 470 PU/kg diet of buckwheat phytase may be equivalent to 0.10% non-phytate P; the NC diet was deficient in 0.16% non-phytate P compared with the PC diet.

Compared with barley, triticale, and wheat, which are natural phytase sources (Jondreville *et al*., 2007; Juanpere *et al*., 2004; Barrier-Guillot *et al*., 1996b), buckwheat may be a more suitable ingredient for poultry feed, as barley, triticale, and wheat contain high levels of ß-glucans (Havrlentová and Kraic, 2006), which are indigestible polysaccharides that decrease nutrient digestibility (Moharrery, 2006; Wang *et al*., 2005; He *et al*., 2003), but are found in relatively low concentrations in buckwheat (Havrlentová *et al*., 2011). Although buckwheat has not yet been produced for animal feed to the knowledge, low quality seeds unsuitable for human consumption may probably be used for this purpose.

Conclusion

Results obtained here revealed that broilers received NC diet showed deteriorated growth performances and bone quality compared with those received PC diet. Interestingly, all deteriorated parameters restored dose-dependently by the addition of BU and GBU. The degree of restoration was greater in the GBU groups than in BU groups. Decreased excretion of total P and nitrogen resulted in their higher retention in BU and GBU added groups, however, effect of GBU was dominant on BU. In conclusion, addition of phytase rich Shinano No. 1 GBU at 20% level can maintain the growth performance and bone quality, and improve P availability in broilers given diets deficient in 0.16% non-phytate P. Therefore, it was clarified that phytase in BU and GBU can function in the digestive tract of broilers and can contribute to reduce the dietary supplementation and excretion of P, suggesting that buckwheat, especially when germinated, can be used as an alternative phytase source to improve P availability in broilers.

Chapter IV

Effects of Buckwheat Added Diets on Production Performance, Egg Quality and Phosphorus Availability in Laying Hens

Abstract

To evaluate the efficacy of buckwheat phytase to improve P availability in laying hens, production performance, egg quality, and P balance were measured in laying hens given non-phytate P deficient diets. Fifty-six Lohmann LSL-Lite laying hens (46 weeks of age) were divided into eight dietary groups (7 birds each) having similar body weight. Diets were namely, PC diet formulated according to the NRC recommendations, NC diet formulated to contain 0.16% lower non-phytate P than in the PC diet, and six other diets formulated by replacing maize in the NC diet with BU or GBU buckwheat at 10%, 15%, and 20% levels. Deteriorated production performance (hen-day egg production, feed intake, egg weight and egg mass) and eggshell quality (shell breaking strength, shell weight and shell thickness) in laying hens given non-phytate P deficient NC diet was restored by the addition of at least 15 % BU and 10% GBU to the NC diet. Total P retention significantly increased in 20% BU, 15% GBU and 20% GBU groups as the excretion decreased considerably in those groups compared with the NC group. This study results indicated that dietary buckwheat can restore impaired production performance, eggshell quality, and leads to improve P balance in laying hens given non-phytate P deficient diets, and therefore, buckwheat phytase seems to be efficacious in improving the availability of P in laying hens.

Introduction

Earlier studies reported that efficiency of phytate utilization varied between laying hens and broilers: Edwards Jr. (1982) reported that laying breed can utilize phytate P more efficiently than the meat type breed, whereas, Nelson (1976) observed very little difference between them. Contribution of high phytase activity in the small intestine in laying hens in this regard is still in controversy, as because, several reports mentioned that crop is the main site of phytate degradation in chicken (Takemasa *et al*., 1996; Takemasa and Murakami, 1995). Contrarily, Saitoh (2001) reported that conversion rate of phytate to non-phytate P was better in broilers than in laying hens.

In chapter III, it was observed that impaired growth performances and bone quality in broilers given non-phytate P deficient diet was restored dose-dependently with the addition of Shinano No. 1 BU and GBU. Moreover, excretion of P decreased about 23% to 43% in BU and GBU added groups. It is noteworthy that, 470 units of buckwheat phytase per kg diet was equivalent to 0.10% of non-phytate P in broilers. All those results indicated that, buckwheat can be used as phytase source in broiler diets. However, in order to use buckwheat in chicken feed practically, experiment with laying hens should be essential.

Therefore, the study was conducted to measure the production performance, egg quality and P balance in laying hens given non-phytate P deficient diets containing Shinano No. 1 BU and GBU at different levels, and to discuss the efficacy of buckwheat phytase in laying hens, especially in comparison with broilers.

Materials and Methods

This research was conducted in accordance with the guidelines for regulation of animal experimentation of Shinshu University, Japan.

Germination of Buckwheat

Seeds of Shinano No. 1 buckwheat was purchased commercially, and a proportion of the seeds were germinated, as explained detail in chapter II, and after germination both BU and GBU seeds were ground to pass through 1.0-mm aperture, and approximately 93% of the hulls were removed by sieving whole ground seeds and used to formulate broilers diet. Samples of BU and GBU were analyzed for chemical composition and phytase activity (Table 12).

Hens, Diets and Sampling

Fifty-six Lohmann LSL-Lite laying hens (46 weeks of age) were divided into eight groups (seven hens each) with similar body weight (BW) and reared in individual cages under 16L:8D light condition. Hens were allocated to one of the eight experimental diets: PC diet formulated to meet or exceed the nutrients recommended by NRC (1994), NC diet formulated to contain 0.16% lower non-phytate P than in the PC diet, and six other diets formulated to contain 10%, 15%, and 20% of Shinano No. 1 BU or GBU in the NC diet, at the expense of maize (Table 13). Diets with 2,988 kcal/kg ME, 17.8% CP, and 3.62% calcium were given for six weeks: first one week was employed for adaptation, and the subsequent five weeks for data collection. Excreta were collected, and immediately stored at -20°C in a freezer. Excreta of each hen were pooled for the three day of collection period. Frozen excreta samples were then thawed, homogenized, dried, and ground before analysis.

Production Performance and Egg Quality

Egg production and feed intake (FI) were recorded daily. Egg production was calculated on a hen per day basis, and egg mass was calculated from collected data of egg production and egg weight, using: egg mass (g/hen) = (egg production × egg weight)/ period (day). Feed conversion ratio was calculated as the ratio of grams of feed consumed as grams of egg mass. Eggs were analysed every day to determine the egg quality (egg weight, specific gravity, Haugh unit, and albumen height), and eggshell quality (shell breaking strength, shell weight, and shell thickness). Specific gravity was determined by using a graded series of saline solutions varying in specific gravity from 1.060 to 1.146. Egg weight, eggshell breaking strength, Haugh unit, albumen height, and yolk color were measured using Digital Egg Tester (Nabel Co., Ltd., Japan). Eggshell was weighed after drying at 100°C for 2 h, and thickness was measured using a micrometer (PK-1012CPX, Mitutoyo Corporation, Japan).

Chemical and Statistical Analysis

Samples of BU, GBU, diets and excreta were analyzed for proximate composition following the standard methods (AOAC, 1990). Total P and phytate P of them were measured according to ISO (1998) and Haug and Lantzsch (1983), respectively: non-phytate P was calculated by subtracting the phytate P from total P. Phytase activity was measured following the method described by Eeckhout and De Paepe (1994), as explained detail in chapter II.

Statistical significances among the dietary groups were determined with Tukey's multiple comparison tests at a significance level of 5% after one-way ANOVA (SAS Institute, 2015). Two-way ANOVA was performed by omitting the PC and NC groups to test for the main and interaction effects between BU and GBU groups. Moreover, linear regression analysis was conducted to obtain the equations relating phytase levels in diets with response (hen-day egg production, FI, and eggshell breaking strength), using the SAS statistical package and the linear regression model was followed as described in chapter III.

Table 12. **Chemical composition and phytase activity in Shinano No. 1 buckwheat**[1]

Components, %	Shinano No. 1 buckwheat	
	BU	GBU
Crude protein	15.71	17.02
Ether extract	2.92	2.87
Crude fiber	5.16	5.74
Crude ash	2.13	1.91
Total P	0.39	0.45
Phytate P	0.33	0.27
Phytase activity, PU^2/g	2.20	2.77

[1]Values based on analysis of triplicate samples (dry matter basis).

[1]BU = non-germinated buckwheat; GBU = germinated buckwheat.

[2]Phytase unit (PU) equivalent to the enzymatic activity, which liberates 1 μmol inorganic phosphate per min at pH 5.5 and 37°C.

Table 13. **Ingredients and chemical composition of experimental diets (week 46-51)[1]**

Ingredients, %	PC	NC	NC+BU			NC+GBU		
			10%	15%	20%	10%	15%	20%
Commercial diet[2]	33.5	33.7	34.0	34.2	34.7	34.0	34.2	34.7
Soybean meal	19.3	19.3	17.7	17.2	16.4	17.7	17.2	16.4
Maize	38.8	38.8	28.5	22.8	17.2	28.5	22.8	17.2
BU	-	-	10.0	15.0	20.0	-	-	-
GBU	-	-	-	-	-	10.0	15.0	20.0
Maize oil	0.45	0.45	2.05	3.05	3.95	2.05	3.05	3.95
$CaCO_3$	5.7	6.4	6.4	6.4	6.4	6.4	6.4	6.4
$Ca_3(PO_4)_2$	0.9	-	-	-	-	-	-	-
Vit-min premix[3]	1.35	1.35	1.35	1.35	1.35	1.35	1.35	1.35
Analyzed composition, % (as fed basis)								
Crude protein	17.9	17.8	17.9	17.7	17.8	17.9	17.8	17.7
Crude fiber	3.9	3.8	4.0	4.1	4.2	4.1	4.3	4.3
Total P	0.62	0.47	0.46	0.46	0.47	0.46	0.47	0.47
Phytate P	0.26	0.27	0.25	0.26	0.26	0.26	0.26	0.27
Non-phytate P	0.36	0.20	0.21	0.20	0.21	0.20	0.21	0.20
Calcium[4]	3.60	3.60	3.61	3.62	3.64	3.62	3.62	3.64
ME, kcal/kg[4]	2,984	2,990	2,985	2,990	2,990	2,985	2,990	2,990
Phytase, PU/kg of diet on DM basis[5]	ND	ND	220	328	440	280	420	564

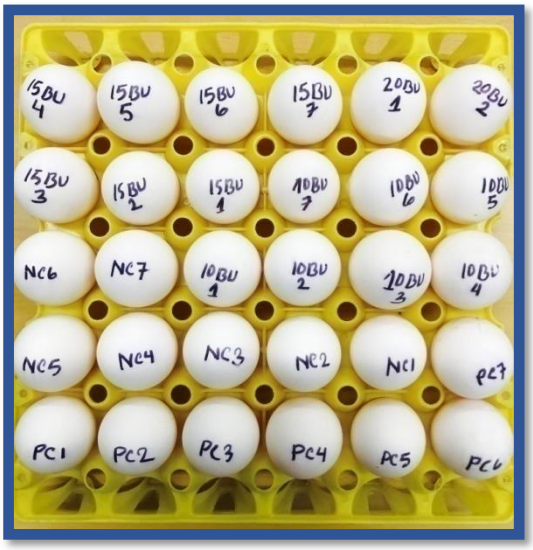
[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat, DM = dry matter, ND = not-determined.

[2]Layer diet (CP≥17.0 %, ME≥2,850 kcal/kg, Feedone Co., Ltd. Kanagawa, Japan).

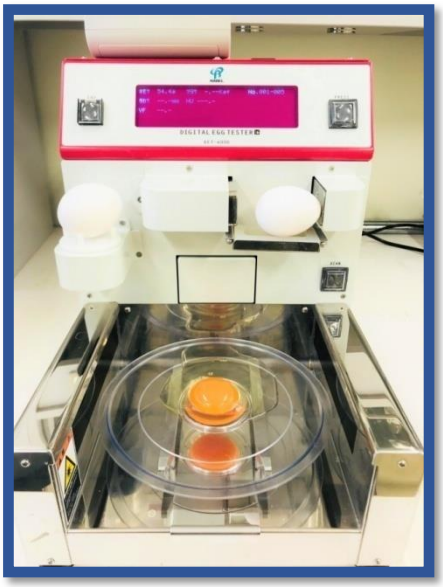
[3]Vitamin-mineral premix provided with the following concentrations per kg of diet: vitamin A, 1.5 mg; vitamin D_3, 0.03 mg; vitamin E, 7.37 mg; vitamin K_3, 1.1 mg; vitamin B_1, 6.0 mg; vitamin B_2, 23.0 mg; vitamin B_6, 8 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 18 mg; niacin, 35 mg; choline chloride, 1,700 mg; folacin, 2.20 mg; iron, 95 mg; copper, 11 mg; zinc, 180 mg; manganese, 280 mg; iodine, 3.4 mg.

[4]Calculated nutrient content was based on ingredient composition data from NRC (1994).

[5]Phytase activity value was calculated on the dry matter basis of the diets based on the analyzed phytase activity in Shinano No. 1 BU and GBU.

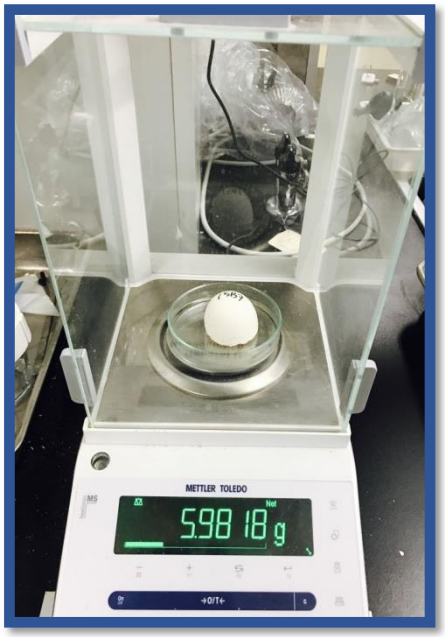


Collected eggs



Eggs quality measurement

Dried eggshell



Shell weight measurement

Figure 4. Collection of eggs and analysis of egg quality

Results

Production Performances (Table 14)

Hen-day egg production in the PC group was about 99%, which decreased (P<0.05) to 93% in the NC group but restored by the addition of at least 15% BU and 10% GBU. Although FI decreased (P<0.05) in the NC and 10% BU groups, other groups did not show any difference (P>0.05) with the PC group. Egg weight and egg mass showed a similar trend as the FI, excepting that the 10% BU group showed restored value, whereas FCR was almost similar in all groups. Comparing the values in BU and GBU groups, the degree of restoration was greater in GBU groups than in BU groups, in all parameters. Significant differences in FI and egg mass were found for the main effects of germination and level, whereas, that in hen-day egg production was found only for the main effect of level. No significant differences in egg weight and FCR were observed for both main effects.

Egg quality (Table 15 and Table 16)

Egg specific gravity ranged from 1.126 to 1.129, and yolk color from 10.0 to 10.4, and both were not significantly varied among the dietary groups. Moreover, Haugh unit and albumen height were almost similar in all dietary groups. Eggshell quality, such as shell breaking strength and shell weight were deteriorated in the NC group (P<0.05), and the deteriorated values were restored dose-dependently by the addition of BU and GBU in diets. Although eggshell thickness decreased (P<0.05) in the NC and 10% BU groups, other groups did not show any difference (P>0.05) with the PC group. Significant differences in shell breaking strength, shell weight and shell thickness were found for the main effects of germination and level, whereas, no significant differences in Haugh unit and albumen height were observed for both main effects.

Balance of Total P and Nitrogen (Table 17)

Total P intake was varied (P<0.05) between the PC and other groups, because of the varied dietary level of P, whereas, the variation among the groups except PC, was due to the varied FI. Excretion of total P decreased considerably in 20% BU, 15% GBU and 20% GBU groups and concomitantly retention of total P increased (P<0.05) in those groups compared with the NC group. In this study all the diets were isonitrogenous, therefore, the decreased (P<0.05) nitrogen intake in the NC and 10% BU groups was because of the lower FI, that resulted in lower (P<0.05) retention of nitrogen in those groups, compared with others. Significant differences in the excretion of total P and nitrogen were found for the main effect of level, whereas, that in the retention of total P and nitrogen were observed for the main effects of germination and level.

Table 14. **The effects of non-germinated and germinated buckwheat added diets on production performance in laying hens[1]**

Dietary groups	Hen-day egg production, %	FI, g/hen/d	Egg weight, g	Egg mass, g/hen/day	FCR, g feed/g eggs	BWC, g/hen/6 weeks
PC	99±0.6[a]	117.3±0.2[a]	61.2±0.3[a]	60.4±0.3[ab]	1.94±0.01	6.4±13.0
NC	93±0.8[c]	111.7±0.5[c]	58.3±0.3[b]	54.3±0.8[c]	2.06±0.02	-51.4±16.3
NC + 10% BU	95±1.3[bc]	115.9±0.2[b]	60.2±0.5[ab]	57.0±1.1[bc]	2.04±0.04	-29.3±20.1
NC + 15% BU	97±0.9[ab]	117.0±0.2[ab]	60.4±0.6[ab]	58.7±1.0[ab]	2.00±0.03	-15.0±19.1
NC + 20% BU	98±0.8[ab]	117.2±0.3[a]	60.6±0.7[a]	59.6±1.0[ab]	1.97±0.03	-14.7±22.2
NC + 10% GBU	97±1.1[ab]	117.7±0.2[a]	60.6±0.5[a]	58.9±0.9[ab]	2.00±0.03	-26.4±18.0
NC + 15% GBU	99±0.6[a]	117.7±0.2[a]	61.2±0.5[a]	60.5±0.6[ab]	1.95±0.02	-20.7±23.5
NC + 20% GBU	99±0.6[a]	118.1±0.1[a]	61.6±0.4[a]	60.8±0.3[a]	1.94±0.01	-12.9±19.0
Source of variation	----------*P*-value----------					
Germination	0.0544	<0.0001	0.0969	0.0242	0.1868	0.9842
Level	0.0171	0.0007	0.4543	0.0322	0.7499	0.7791
Germination × Level	0.5634	0.0681	0.8240	0.9202	0.9763	0.9740

[1]Values for each parameter represent mean±standard error values with seven observations (from 47 to 51 weeks of age).

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat, FI = feed intake, FCR = feed conversion ratio, BWC = body weight change.

[a-c]Means within a column not followed by common superscripts are different at $P<0.05$.

Table 15. **The effects of non-germinated and germinated buckwheat added diets on egg quality parameters in laying hens**[1]

Dietary groups	Specific gravity	Haugh unit	Albumen height, mm	Yolk color
PC	1.126±0.001	88.7±1.4	8.1±0.2	10.4±0.1
NC	1.126±0.001	88.6±1.0	7.9±0.1	10.2±0.1
NC + 10% BU	1.126±0.000	89.1±0.4	8.0±0.1	10.2±0.1
NC + 15% BU	1.129±0.002	89.7±0.9	8.2±0.2	10.1±0.1
NC + 20% BU	1.129±0.001	90.1±0.6	8.2±0.1	10.4±0.0
NC + 10% GBU	1.129±0.000	90.9±0.7	8.3±0.1	10.3±0.1
NC + 15% GBU	1.129±0.001	90.7±1.2	8.4±0.2	10.0±0.1
NC + 20% GBU	1.126±0.001	91.0±0.6	8.1±0.2	10.4±0.1
Source of variation	----------*P*-value----------			
Germination	0.1710	0.0677	0.0651	0.8070
Level	0.0663	0.7688	0.5925	0.0728
Germination × Level	0.1551	0.8271	0.7854	0.4906

[1]Values for each parameter represent mean±standard error values with seven observations (from 47 to 51 weeks of age).

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

Table 16. **The effects of non-germinated and germinated buckwheat added diets on eggshell quality parameters in laying hens**[1]

Dietary groups	Shell breaking strength, kgf/cm^2	Shell weight, g	Shell thickness, mm
PC	5.65±0.05^{b}	5.93±0.04^{ab}	0.60±0.003^{a}
NC	4.31±0.04^{f}	5.54±0.04^{d}	0.58±0.004^{b}
NC + 10% BU	4.76±0.04^{e}	5.72±0.03^{c}	0.58±0.004^{b}
NC + 15% BU	5.24±0.04^{c}	5.85±0.02^{bc}	0.59±0.002^{ab}
NC + 20% BU	5.74±0.03^{ab}	6.00±0.02^{a}	0.60±0.001^{a}
NC + 10% GBU	5.02±0.04^{d}	5.90±0.03^{ab}	0.59±0.001^{ab}
NC + 15% GBU	5.65±0.04^{b}	5.95±0.03^{ab}	0.60±0.003^{a}
NC + 20% GBU	5.90±0.05^{a}	6.04±0.05^{a}	0.60±0.003^{a}
Source of variation	----------*P*-value----------		
Germination	<0.0001	0.0002	0.0032
Level	<0.0001	<0.0001	<0.0001
Germination × Level	0.0583	0.0602	0.2015

[1]Values for each parameter represent mean±standard error values with seven observations (from 47 to 51 weeks of age).

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

[a-f]Means within a column not followed by common superscripts are different at $P<0.05$.

Table 17. **The effects of non-germinated and germinated buckwheat added diets on the balance of total phosphorus and nitrogen in laying hens**[1]

Dietary groups	Total phosphorus			Nitrogen		
	Intake, g/hen/d	Excretion, g/hen/d	Retention, g/hen/d	Intake, g/hen/d	Excretion, g/hen/d	Retention, g/hen/d
PC	0.74±0.01[a]	0.51±0.02[a]	0.23±0.01[abc]	3.43±0.02[a]	1.43±0.03	2.00±0.03[a]
NC	0.52±0.01[cd]	0.34±0.01[b]	0.18±0.01[d]	3.17±0.06[b]	1.37±0.03	1.79±0.05[b]
NC + 10% BU	0.51±0.01[d]	0.33±0.02[bc]	0.19±0.02[cd]	3.18±0.06[b]	1.39±0.06	1.80±0.06[b]
NC + 15% BU	0.54±0.003[bc]	0.31±0.01[bc]	0.23±0.01[abcd]	3.41±0.02[a]	1.38±0.04	2.03±0.04[a]
NC + 20% BU	0.55±0.004[b]	0.29±0.01[bc]	0.26±0.02[ab]	3.40±0.02[a]	1.35±0.05	2.05±0.06[a]
NC + 10% GBU	0.55±0.003[b]	0.34±0.01[bc]	0.21±0.01[bcd]	3.46±0.02[a]	1.49±0.01	1.96±0.03[ab]
NC + 15% GBU	0.57±0.002[b]	0.30±0.01[bc]	0.27±0.01[a]	3.43±0.01[a]	1.37±0.03	2.06±0.03[a]
NC + 20% GBU	0.56±0.003[b]	0.29±0.01[c]	0.28±0.01[a]	3.41±0.02[a]	1.33±0.03	2.09±0.04[a]
Source of variation	----------*P*-value----------					
Germination	<0.0001	0.5037	0.0046	0.0001	0.4759	0.0310
Level	<0.0001	0.0040	<0.0001	0.0030	0.0473	0.0002
Germination × Level	0.0562	0.5995	0.7225	0.0630	0.2109	0.2199

[1]Values for each parameter represent mean±standard error values with seven observations.

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

[a-d]Means within a column not followed by common superscripts are different at $P<0.05$.

Discussion

So far, several researches on dietary microbial phytase has been performed to evaluate its efficacy in laying hens and reported that this enzyme restored impaired production performance in laying hens received P deficient diets (Hughes *et al*., 2008; Panda *et al*., 2005); moreover, it was reported that 250 PU/kg diet was equivalent to 0.10% non-phytate P in laying hens (Van Der Klis *et al*., 1997). On the other hand, researches on dietary plant phytase are still limited: Scott *et al*. (2000) suggested wheat phytase degraded phytate P in diets, and improved phytate P utilization in laying hen, although phytase units equivalent to 0.10% non-phytate P was not shown in this report.

In the present study, impaired production performance, such as hen-day egg production, FI, egg weight, and egg mass, and eggshell quality, such as shell breaking strength, shell weight, and shell thickness, in the NC group were restored by the addition of at least 15% of BU and 10% GBU to NC diet. Furthermore, excretion of P decreased in laying hens by 35% to 43% in BU and GBU added groups, compared with the PC group, and led to higher retention of total P in 20% BU, 15% GBU and 20% GBU groups, which may be explained by the increased availability of P from phytate P by the activity of buckwheat phytase. When expressing the effect of buckwheat phytase as the amount of non-phytate P, linear relationships between the levels of phytase unit and economically important parameters, such as hen-day egg production, FI, and eggshell strength, were determined, resulting in the following regression equations:

$Y_1 = 93 + 0.011X$, $r^2 = 0.75$

$Y_2 = 115.6 + 0.005X$, $r^2 = 0.53$

$Y_3 = 4.07 + 0.003X$, $r^2 = 0.94$

where Y_1 = hen-day egg production (%), Y_2 = FI (g/hen/day), Y_3 = shell breaking strength (kgf/cm^2), and X = buckwheat phytase (PU/kg diet).

All the regression equations were significant (P<0.05), except for FI (P=0.0942). Consequently, the levels of phytase unit required to obtain the same hen-day egg production, FI, and eggshell strength in the PC group were calculated to be 545, 340 and 395 PU/kg diet, respectively. Taking the hen-day egg production as the most important parameter, 545 PU/kg diet should be included in the NC diet formulated based on the NRC requirement, and therefore, 340 PU/kg diet of buckwheat phytase may be equivalent to 0.10% non-phytate P. This value is 1.4 times greater than that in microbial phytase (derived from *Aspergillus niger*), which was 250 PU/kg diet (Van Der Klis *et al*., 1997), suggesting that microbial phytase is superior to some extent to plant phytase.

It is noteworthy that there are some reports showing a difference in the efficiency of phytate utilization between laying hens and broilers: Edwards Jr. (1982) reported that Leghorn type chickens utilized phytate P more efficiently than broiler chickens, whereas Nelson (1976) observed that broiler chicks and laying hens showed almost similar efficiency. Recently, Saitoh (2001) reported that the efficiency of phytate utilization was better in broilers than in laying hens. In broiler study (chapter III) it was observed that 470 PU/kg diet of buckwheat phytase was equivalent to 0.10% non-phytate P in broilers, which is greater than the corresponding value in the present study, and phytase activity in buckwheat used in the present study was much the same as used in broiler study (chapter III). Consequently, it indicated that laying hens has better efficiency of phytate utilization than broilers at least in case of buckwheat phytase.

The reason why the phytase efficacy was different between chicken strains is still obscure. To explain this reason, the main site of phytate P degradation should be identified. The intestine may not be the site, because there are almost no reports showing

that the chicken intestine contributes to the phytate degradation, and intestinal phytase activity is often diminished as having little importance on phytate degradation (Humer *et al*., 2015), although phytase activity in the small intestine of laying hens was 35% higher than in broilers (Maenz and Classen, 1998). On the other hand, the crop is promising, because high phytase activity was measured in the crop in several reports (Takemasa *et al*., 1996; Takemasa and Murakami, 1995). Interestingly, Shires *et al*. (1987) observed the slow rate of feed passages in the crop and esophagus in laying hens compared with broilers, which can be a clue to explain the reason.

Nitrogen retention decreased in NC group compared with PC group, and recovered with the increasing level of BU and GBU dose-dependently: the magnitude of recovery was greater in GBU then in BU. This trend was in accordance with the results of egg weight and egg mass, both of which may need nitrogen to increase their values (Novak *et al*., 2006; Bunchasak *et al*., 2005; Keshavarz and Nakajima, 1995). The changes in nitrogen retention may be explained mainly by changes in nitrogen intake, because there was no statistical difference in nitrogen excretion among groups. Interestingly, broilers (chapter III) received non-phytate P deficient diets containing BU and GBU, nitrogen retention showed similar trend to that in laying hens, but the mechanism was somewhat different: nitrogen excretion differed significantly among groups, but nitrogen intake (chapter III). This suggests that there is a difference in the response of nitrogen balance to non-phytate P deficiency between laying hens and broilers.

Conclusion

Results obtained here revealed that laying hens received P deficient NC diet showed deteriorated egg production, FI, egg weight and eggshell quality compared with those received PC diet. However, all those parameters restored by the addition of at least 15% BU and 10% GBU. However, the degree of restoration of production performance and eggshell quality was greater in GBU groups than in BU groups. Moreover, total P excretion decreased considerably in 20% BU, 15% GBU and 20% GBU groups, led to increase total P retention in those groups. In conclusion, addition of buckwheat restored impaired production performance and eggshell quality caused by the non-phytate P deficiency in laying hens, and improved P availability. However, laying hens require smaller amount of phytase than broilers to cope with non-phytate P deficiency.

Chapter V

Phytase Activity in the Digesta From Different Parts of the Digestive Tract and Ileal Digestibility of Nutrients in Broilers Fed with Buckwheat Diets

Abstract

In this study, effects of dietary buckwheat on phytase activity in digesta of different parts of the digestive tract, and ileal digestibility of nutrients were measured in broilers given buckwheat diets. A total of 80 male broilers (29 d of age) were divided into four groups (20 birds each) and given one of the following diets until 36 d of age: PC diet formulated based on the NRC recommendations, NC diet contained 0.15% lower non-phytate P than in the PC diet, and two other diets, formulated by replacing maize in NC diet with either Shinano No. 1 BU or GBU at 20% level. On 36 d of age, birds were sacrificed to collect digesta from crop, gizzard, duodenum, jejunum, ileum, and cecum. Phytase activity was low in PC and NC diets, which increased in BU diet and much increased in GBU diet. Similar trend was found in the crop digesta, but phytase activities in crop digesta of BU and GBU diets were slightly lower, compared to the values for phytase activity in each diet. These values decreased steeply when digesta moved to the gizzard, and then decreased gradually: the values in ileal digesta showed markedly low activity with little effect of dietary treatment. Dietary BU and GBU did not affect ileal digestibilities of DM and CP, but increased ileal phytate P digestibility. These results suggest that in broilers, the crop might be the primary site of phytate degradation by buckwheat phytase, and buckwheat may have little adverse effect on ileal digestibility of nutrients.

Introduction

In chapter II, it was found that Shinano No. 1 BU and GBU possessed high phytase activity and showed greater value of *in vitro* phytate P digestibility compared with others. Based on this, *in vivo* study (chapter III) was conducted in broilers using Shinano No. 1 BU and GBU at different levels. Results showed that it is possible to reduce 0.16% dietary non-phytate P in broilers by adding GBU at 20% level without compromising performance and bone quality, indicating phytate P utilization increased by GBU phytase activity. However, the efficiency of phytate utilization varied between laying hens and broilers (Saitoh, 2001; Edwards Jr.,1982; Nelson, 1976). Therefore, another *in vivo* study conducted in laying hens (chapter IV) and results showed that impaired production performance and eggshell quality in laying hens caused by the deficiency of 0.16% non-phytate P was restored by addition BU or GBU at and above 15% level.

However, to confirm the practical use of buckwheat as phytase source in chicken feed, its activity in the digestive tract should be understood. There is a report showing that phytase from wheat and barley functioned only in crop of chicks (Takemasa and Murakami, 1995). Buckwheat is a plant of the family Polygonaceae and taxonomically different from wheat and barley, and there is a possibility that characteristics of buckwheat phytase are not the same as such cereals. Therefore, the present study was conducted to measure the phytase activity in digesta from different parts of the digestive tract and ileal digestibility of nutrients in broilers given BU and GBU diets, to identify the part of digestive tract that is involved in phytate degradation by buckwheat phytase, and to verify if dietary buckwheat affects nutrient digestibility.

Materials and Methods

This research was conducted in accordance with guidelines for regulation of animal experimentation of Shinshu University, Japan.

Germination of Buckwheat

Seeds of Shinano No. 1 buckwheat was purchased commercially, and part of seeds were germinated, as explained detail in chapter II, and after germination both BU and GBU seeds were ground to pass through 1.0-mm aperture and approximately 93% hulls were removed by sieving the ground seeds and used to formulate experimental diets. Samples of BU and GBU were analyzed for chemical composition and phytase activity (Table 18).

Birds, Diets and Sampling

A total of eighty (29 d of age) male broilers (Ross 308) were divided into 4 groups (20 birds each) having similar body weight, and given one of the following four diets until 36 d of age: PC diet was formulated according to the NRC (1994) recommendations, NC diet was based on the PC diet with reduced level of non-phytate P (0.15% lower than PC diet), and other two diets were formulated by replacing maize in the NC diet with BU and GBU at 20% (Table 19). All diets contained titanium oxide (0.5%) as an indigestible marker. On 36 d of age, after 12 of h fasting birds were allowed to take feed for 1 h and, 30 min after completion of feeding 10 birds from each group were sacrificed to collect crop and gizzard contents, and rests 10 birds were sacrificed after 60 min to collect duodenum, jejunum, ileum and cecum contents and stored at -20ºC until further processing. Frozen samples were thawed, dried and ground to pass through a 1.0-mm screen for measuring of phytase activity and ileal digestibility.

Chemical Analysis

Samples of BU, GBU, diets, and ileal digesta were analyzed for proximate composition according to standard methods (AOAC, 1990). Total P and phytate P of samples were measured according to ISO (1998) and Haug and Lantzsch (1983), respectively: non-phytate P was calculated as subtracting phytate P from total P. pH of the digesta from different parts in the digestive tract was determined following the method described by Esmaeilipour *et al.* (2011). Briefly, one gram of digesta from crop, gizzard, duodenum, jejunum, and ileum were placed individually into clean falcon tubes and mixed with deionized water (1:10 weight/volume), and the pH of the solution was measured with a digital pH meter at room temperature. Phytase activity was measured following Eeckhout and De Paepe (1994) as explained detail in chapter II. Titanium was determined according to the procedure described by Short *et al*. (1996).

Calculation and Statistical Analysis

The following equation was used for calculation of ileal nutrient digestibility (Meng *et al*., 2005):

Ileal nutrients digestibility (%) = $\{1 - [(TiO_{2\ \%\ diet}/TiO_{2\ \%}\ digesta) \times (nutrient_{\%\ digesta}/nutrient_{\%\ diet})]\} \times 100$.

Statistical significances among the dietary groups were determined with Tukey's multiple comparison tests at a significance level of 5% after one-way ANOVA (SAS Institute, 2015). Considering germination and digestive tract parts as factors, two-way ANOVA was conducted omitting the PC and NC groups, to know the effects of germination and digestive tract parts, and their interaction on the phytase activity in digesta.

Table 18. **Chemical composition and phytase activity in Shinano No. 1 buckwheat**[1]

Components, %	Shinano No. 1 buckwheat	
	BU	GBU
Crude protein	13.33	14.98
Ether extract	3.19	3.25
Crude fiber	5.03	6.55
Crude ash	2.34	1.96
Total P	0.40	0.46
Phytate P	0.33	0.28
Phytase activity, PU^2/g	2.46	2.69

[1]Values for each parameter represent mean of triplicate analysis (dry matter basis).

[1]BU= non-germinated buckwheat; GBU= germinated buckwheat.

[2]Phytase unit (PU) equivalent to the enzymatic activity, which liberates 1 μmol inorganic phosphate per min at pH 5.5 and 37°C.

Table 19. **Ingredients and chemical composition of the experimental diets (d 29-36)[1]**

Ingredients, %	PC	NC	NC + 20% BU	NC + 20% GBU
Maize	55.3	55.3	35.3	35.3
Soybean meal	34.5	34.7	33.3	33.3
BU	-	-	20.0	-
GBU	-	-	-	20.0
Maize oil	6.2	6.2	7.6	7.6
$Ca_3(PO4)_2$	1.2	0.35	0.35	0.35
$CaCO_3$	1.2	1.85	1.85	1.85
Vit-min premix[2]	1.1	1.1	1.1	1.1
Titanium oxide	0.5	0.5	0.5	0.5
Analyzed composition, % (as fed basis)				
Crude protein	17.5	17.2	17.7	17.8
Crude fiber	3.6	3.3	4.1	4.3
Total P	0.66	0.49	0.48	0.50
Phytate P	0.30	0.28	0.27	0.28
Non-phytate P	0.36	0.21	0.21	0.22
Phytase, PU/kg	22.5	23.4	446.6	525.7
Calcium[3]	0.95	0.96	0.97	0.97
ME, kcal/kg[3]	3,190	3,195	3,185	3,185

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

[2]Vitamin-mineral premix provided the following per kg of diet: vitamin A, 5,000 IU; vitamin D_3, 1,000 IU; vitamin E, 11 IU; vitamin K_3, 1.1 mg; vitamin B_1, 6.0 mg; vitamin B_2, 23.0 mg; vitamin B_6, 8 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 18 mg; niacin, 35 mg; choline chloride, 1,700 mg; folacin, 2.20 mg; iron, 95 mg; copper, 11 mg; zinc, 180 mg; manganese, 280 mg; iodine, 3.4 mg.

[3]Calculated nutrient content was based on ingredient composition data from NRC (1994).

Results

Digesta pH (Table 20)

In the PC group, the pH value of the digesta in the crop was 5.56, which decreased steeply to 3.55 in the gizzard, and increased to 6.05 in the duodenum, 6.44 in the jejunum and 7.67 in the ileum; this value slightly decreased in the cecum. Other groups showed a similar trend to the PC group.

Digesta Phytase Activity (Table 21)

Phytase activity in PC and NC diets, and in all the digesta of broilers from PC and NC groups were less than 30 PU/kg. The effect of these diets on the phytase activity was negligible. On the other hand, phytase activity in NC diet increased with the addition of BU, and more significantly with the addition of GBU. Such increased activity was observed in the crop digesta. However, phytase activity in the crop digesta of broilers that were fed BU and GBU diets was marginally lower when compared with that of each diet. These values decreased (P<0.0001) sharply as the digesta moved into the lower parts of the digestive tract. The highest decrease in the phytase activity was observed when the digesta moved to the gizzard; the phytase activity in the gizzard digesta was 33% to 37% of that in the diets. Similarly, all the dietary groups exhibited a low phytase activity in the ileal digesta (P=0.1692). Furthermore, two-way ANOVA results showed that the effect of germination was non-significant (P=0.0994), whereas the effect of digestive tract parts (P<0.0001), and their interaction (P=0.0094) were significant on phytase activity in digesta.

Ileal Nutrients Digestibility (Table 22)

Ileal DM and CP digestibilities were affected marginally by the dietary BU and GBU. However, the digestibility of total P tended (P=0.0556) to increase in the BU and GBU diets when compared with that in the PC and NC diets. Furthermore, the digestibility of phytate P increased significantly (P<0.05).

Table 20. **The pH of digesta from different parts of the digestive tract in broilers**[1]

Dietary groups	Crop	Gizzard	Duodenum	Jejunum	Ileum	Cecum
Time after feeding (min)	30	30	60	60	60	60
PC	5.56±0.04	3.55±0.10	6.05±0.11	6.44±0.04	7.67±0.17	7.30±0.13
NC	5.49±0.04	3.71±0.13	5.98±0.08	6.33±0.04	7.75±0.18	7.00±0.14
NC + 20% BU	5.59±0.13	3.54±0.10	6.03±0.14	6.39±0.04	7.81±0.11	7.17±0.12
NC + 20% GBU	5.59±0.04	3.85±0.08	6.06±0.08	6.38±0.05	7.73±0.11	7.18±0.14
P-value	0.2205	0.1152	0.9752	0.3599	0.9269	0.4669

[1]Values for each parameter represent mean±standard error values with ten observations.

[1]PC = positive control; NC = negative control; BU = non-germinated buckwheat; GBU = germinated buckwheat.

Table 21. **Phytase activities (PU/kg) in diets and in digesta from different parts of the digestive tract in broilers**[1]

Dietary groups	Diets	Digestive tract parts			
		Crop	Gizzard	Jejunum	Ileum
PC	22.5	28.5±8.1^{C}	10.0±2.6^{B}	16.3±3.8^{B}	18.5±3.6
NC	23.4	22.7±5.6^{C}	13.5±5.0^{B}	19.2±4.1^{B}	17.3±1.8
NC + 20% BU	446.6	392.5±20.6^{Ba}	163.6±20.0^{Ab}	77.3±9.0^{Ac}	14.6±2.8^{d}
	(100%)	(87.9%)	(36.6%)	(17.3%)	(3.3%)
NC + 20% GBU	525.7	465.3±11.5^{Aa}	175.2±14.9^{Ab}	60.6±9.8^{Ac}	10.5±1.6^{d}
	(100%)	(88.3%)	(33.3%)	(11.5%)	(2.0%)
Main effects					
Germination					
BU			161.9±33.6		
GBU			177.6±40.7		
Digestive tract parts					
Crop			428.4±16.3^{A}		
Gizzard			169.4±11.9^{B}		
Jejunum			68.9±6.9^{C}		
Ileum			12.5±1.7^{D}		
Source of variation			*P*-value		
Germination			0.0994		
Digestive tract parts			<0.0001		
Germination × Digestive tract parts			0.0094		

[1]Values (except for diet) represent mean±standard error values with five observations.

[1]PC = positive control, NC = negative control, BU = non-germinated buckwheat, GBU = germinated buckwheat.

[A-D]Means within column not followed by common superscripts are different at $P<0.05$.

[a-d]Means within row not followed by common superscripts are different at $P<0.05$.

Table 22. **Ileal digestibilities (%) of DM, CP, total P and phytate P in broilers** [1]

Dietary groups	DM	CP	Total P	Phytate P
PC	90.56±0.40	69.90±2.06	28.82±1.91	20.24±1.20[b]
NC	89.38±0.66	67.33±2.47	26.68±1.57	19.19±1.06[b]
NC + 20% BU	90.49±0.41	72.02±1.79	35.16±3.85	28.11±1.24[a]
NC + 20% GBU	90.56±0.31	73.02±2.24	36.84±2.32	30.31±1.76[a]
P-value	0.2327	0.2143	0.0556	<0.0001

[1]Values for each parameter represent mean±standard error values with five observations.

[1]PC = positive control; NC = negative control; BU = non-germinated buckwheat; GBU = germinated buckwheat.

[a-b]Means within a column not followed by common superscripts are different at $P<0.05$.

Discussion

It has been reported that the activity of dietary phytase is affected by proteolytic stability (Bedford and Partridge, 2010). For instance, Onyango *et al*. (2005) investigated microbial phytases for broilers and reported that the phytase activity in the digesta was kept high from the crop to the ileum when *Escherichia coli*-derived phytase was provided. However, that activity was high only in the crop digesta when the broilers were fed *Peniophora lycii*-derived phytase, which suggested that the former might have higher proteolytic stability than the latter. Takemasa and Murakami (1995) observed the activity of phytases of plants, such as wheat and barley, only in the crop digesta, but not in the gizzard digesta of chicks. In the present study, about 90% of the phytase activity of the diet was found in the crop digesta and 33% to 37% in the gizzard digesta. This suggests that unlike phytases of wheat and barley, the buckwheat phytase might be partially stable to pepsin hydrolysis in the proventriculus.

In this study, the main effect of germination on the phytase activity was not significant; however, the crop digesta exhibited increased activity with germination. On the other hand, dietary GBU has been reported to induce higher P utilization in broilers when compared with that of dietary BU (chapter III). Considering these results, it is suggested that the overall phytate degradation is due to the phytase activity in the crop digesta. The lowers parts, such as the jejunum and ileum, might be little involved in phytate degradation because of significantly decreased phytase activity in these parts. Furthermore, the involvement of gizzard might be limited, because of low phytase activity and digesta storage capacity, when compared with the crop.

On the other, the main effect of digestive tract parts, and the interaction were significant. This suggests that the phytase activity of the digesta decreased as it moved down the digestive tract, and the rate of decrease varied with germination. The decreased might be attributed to the proteolytic enzymes, such as pepsin and trypsin. It is difficult to confirm if the rate of decrease, i.e. proteolytic resistance, in the phytase activity was varied with germination, because sufficient information on the properties of buckwheat phytase was not obtained.

Ileal digestibilities of DM and CP were affected marginally by dietary BU and GBU. In this connection, broilers that were fed wheat- or barley- based diet without supplementation of β-glucanase exhibited low digestibilities of DM and CP (Moharrery, 2006; Wang *et al*., 2005). Therefore, the use of buckwheat instead of such cereals might reduce the extra cost of supplementing enzyme for commercial broiler production.

Dietary BU and GBU tended (P=0.0556) to increase total P digestibility and significantly (P<0.05) increased phytate P digestibility. However, the ileal digestibility of total P was as low as 35% in both BU and GBU groups, although about 60% of the ingested P was retained in broilers in previous study (chapter III), and such low ileal digestibility of P was reported also by Woyengo *et al*. (2010) and Mutucumarana *et al*. (2014). This inconsistency might probably be due to the retention time of digesta in the crop, the possible primary site of phytate degradation. The phytate P in the feed passing through the crop quickly might be partially hydrolyzed by phytase. The time-course change in P digestibility should be determined.

Conclusion

Results obtained here revealed that pH in the digesta in different parts of the digestive tract was not affected by the addition of BU and GBU in the diets. Phytase activities in the crop digesta in broilers given BU and GBU diets were marginally lower compared with that of each diet. These values decreased sharply when digesta moved to the gizzard, and then decreased gradually. The result of two-way ANOVA with germination and digestive tract parts as main factors showed that the effect of germination was non-significant, whereas, that of digestive tract parts, and their interaction were significant on phytase activity in the digesta. Ileal phytate P digestibility increased in BU and GBU added groups, whereas DM and CP digestibilities were almost similar in all groups. In conclusion, the crop digesta exhibited highest phytase activity in broilers given BU and GBU diets, and suggested the primary site of phytate degradation by buckwheat phytase might be the crop. In addition, the buckwheat had little adverse effect on ileal digestibility of nutrients.

Chapter VI

General Discussion

Phytate represents the major binding form of P in plant originated feedstuffs, and the availability of P from them largely depends on the enzymatic hydrolysis of phytate in the digestive tract of poultry. High prices of inorganic P supplements and environmental burden linked with excessive P excretion as well as exhaustion of the global rock phosphate stores demand for maximization of phytate P utilization in poultry feeding. Supplementing phytase enzyme, which can increase the availability of P by hydrolyzing phytate P may be a suitable solution, but using cereals having phytase activity is an easier way for the developing countries, such as Bangladesh, where feed cost is a major concern, and for the developed countries, where relying on feed additives is decreasing day by day.

In this context, earlier studies have been identified that phytase rich cereals such as, wheat, barley, and triticale can be used as phytase source in poultry feed, and these cereals can improve P availability in poultry. Varietal variability and influence of germination on phytase activity in some cereals also have been studied. Buckwheat is known to be a non-glutinous pseudocereal having high phytase activity, however it is obscure, whether its phytase activity is varied among different buckwheats, and whether it can improve P availability in chickens. Taking these points in consideration, the primary objective of this study was to reveal the potentiality of buckwheat as an alternative phytase source for chickens feed, and hence the following studies were conducted to make a concrete conclusion.

In the first study, chemical composition, phytase activity, and *in vitro* digestibility of nutrient in buckwheat have been investigated and described in chapter II. The results revealed that chemical composition varied among different buckwheats: CP, CF, and CA varied markedly, whereas, NFE varied less markedly. Interestingly, phytase activity was varied considerably, and such variation also reported in wheat and triticale (Jondreville *et al.*, 2007; Barrier-Guillot *et al*., 1996a). The common buckwheat Shinano No. 1 showed the highest phytase activity compared with Kitawase, Harunoibuki, and tartary buckwheat Dattan. Moreover, germination led to increase phytase activity with concomitant decrease of phytate P content in buckwheat: such effect of germination reported in barley, oat, rye, triticale, and wheat (Ma and Shan, 2002; Bartnik and Szafrańska, 1987). The *in vitro* digestibilities of CP and phytate P were greater in Shinano No. 1, and germination further increased these values numerically. Highest phytase activity in Shinano No. 1 buckwheat compared with others resulted in highest phytate P digestibility in this buckwheat.

Based on the results obtained in chapter II, second study was conducted to investigate the efficacy of buckwheat phytase to improve P availability in broilers. Therefore, growth performance, bone quality, and P balance were measured in broilers given non-phytate P deficient diets with Shinano No. 1 BU and GBU (chapter III). A total of one hundred and twenty (8 d of age) male broilers were allocated to eight groups and given the following diets until 42 d of age: PC diet formulated according to NRC recommendations, NC diet formulated to contain 0.16% lower non-phytate P than in the PC diet, and six other diets formulated by replacing maize in the NC diet with Shinano No. 1 BU or GBU at 10%, 15%, and 20% level. Comparing with the PC group, NC group showed impaired growth performance (BW gain, FI, and FCR), and bone quality (dry weight, breaking strength, and contents of ash and P in tibia). However, in most cases,

these impairments were ameliorated dose-dependently with the addition of BU and GBU in the diets, and the restoration magnitude was greater in the GBU than in BU. Especially, the growth performance in 20% GBU group was comparable with the PC group.

In addition, total P excretion decreased in the NC group, and further decreased dose-dependently with the increasing levels of BU and GBU. Total P retention increased as the excretion decreased. This may result from the absorption of non-phytate P released from phytate P by the action of phytase in BU and GBU added diets. The highest decreased rate (46%) was recorded in 20% GBU group, this value was higher than the values reported by Paik (2003), who found that addition of microbial (derived from *Aspergillus oryzae*) or plant (wheat bran) phytase (600 PU/kg diet) in a P deficient diet could decrease the P excretion approximately 28%-30% in broilers. Lowest excretion of nitrogen ($P>0.05$) resulted in its highest retention ($P>0.05$) in 20% GBU group compared with the PC group. Such improvement of nitrogen utilization caused by dietary phytase has been reported by Selle *et al*. (2003). Moreover, to express the effect of buckwheat phytase as the amount of non-phytate P in this study, the linear regression analysis was conducted, and it was calculated that 470 PU/kg diet of buckwheat phytase might be equivalent to 0.10% non-phytate P.

However, to confirm the practical use of buckwheat as phytase source, its efficacy in laying hens was needed to be judged. Therefore, third study was conducted using laying hens (chapter IV). Fifty-six Lohmann LSL-Lite laying hens were allocated to eight different diets for six weeks (46 to 51 weeks of age): PC diet formulated to meet or exceed the NRC recommended level of nutrients, NC diet formulated to contain 0.16% lower non-phytate P than in the PC diet, and six other diets were formulated by replacing of maize in the NC diet with Shinano No. 1 BU or GBU at 10%, 15%, and 20% level. Results

of the study showed that production performance and eggshell quality was deteriorated (P<0.05) in the NC group, but in most cases, the deteriorated values were restored dose-dependently with the addition of BU and GBU in diets. Excretion of total P was decreased, and concomitantly retention was increased in the laying hens given BU or GBU added diets, can be explained by the increased availability of P from phytate P due to the phytase activity in the BU and GBU.

It was calculated that 340 PU/kg diet of buckwheat phytase might be equivalent to 0.10% non-phytate P in laying hens, which was lower than the calculated buckwheat phytase equivalency value in broilers. It indicated that efficacy of buckwheat phytase might be exhibited at lower dose in laying hens than in broilers. Around 35% higher intestinal phytase activity in laying hens could contribute to better phytate utilization than broilers (Maenz and Classen, 1998). However, there is controversy as to whether intestinal phytase can contribute to the phytate degradation and utilization, because crop is considered as the main site of phytate degradation in chicken (Takemasa *et al*., 1996; Takemasa and Murakami, 1995). Slow rate of feed passages in the crop and esophagus in laying hens compared with broilers (Shires *et al*., 1987) might have some contribution in this regard.

Efficacy of buckwheat phytase activity to improve P availability in broilers and laying hens were cleared from the previous studies, and it can be assumed that buckwheat can be used as an alternative phytase source for chicken feed. However, because of limited number of studies on the use of buckwheat as a feed resource, much basic information is lacking. Therefore, in another study phytase activity in digesta of different parts of the digestive tract, and ileal digestibility of nutrients in broilers given BU and GBU diets

were measured to identify the part of digestive tract that is involved in phytate degradation by buckwheat phytase, and to verify if dietary buckwheat affects nutrient digestibility.

Eighty, male broilers (from 29 to 36 d of age) were allocated to four different diets: PC diet adequate in non-phytate P, NC diet contained 0.15% lower non-phytate than in the PC diet, and NC diet with 20% BU or GBU at the expense of maize. Digesta from different parts of the digestive tract were collected on 36 d of age, and phytase activity and ileal digestibility were measured. It was found that phytase activity either from BU or GBU was remained active mainly in crop digesta, and the activity decreased as the digesta moved to the gizzard, and then decreased gradually. According to Takemasa and Murakami (1995), phytase activities were found in the crop digesta in chickens given wheat and barley diets, but not in other parts of digestive tract, whereas, some degree (33% to 37%) of phytase activity was found in the gizzard digesta in chickens given BU and GBU diets. This suggests that unlike the phytases of wheat and barley, the buckwheat phytase might be partially stable to pepsin hydrolysis in the proventriculus. Dietary BU and GBU tended (P=0.0556) to increase total P digestibility and significantly increased phytate P digestibility, whereas, DM and CP digestibilities were affected marginally (P>0.05). In practical viewpoint, compared with barley, triticale, and wheat, buckwheat might be more suitable ingredient for poultry feed, because barley, triticale, and wheat contain high levels of β-glucans, which is an indigestible polysaccharide that decrease nutrient digestibility (Havrlentová and Kraic, 2006; Moharrery, 2006; Wang *et al*., 2005). Contrarily, buckwheat contains low levels of ß-glucans (Havrlentová *et al*., 2011). Although buckwheat has not yet been produced for animal feed, low quality seeds for human consumption might probably be used for this purpose.

Concluding Remarks

Poultry industry has increased rapidly in response to increasing demand for poultry products, and therefore, this industry must be environmentally sound to ensure its long-term sustainable growth. Environmental concern is relating to poultry waste, which is rich in phytate P that can contaminate water and create ecological problem. Supplementation of phytase in the poultry diet to increase the utilization of phytate P is a solution of this problem. In this regard, the present study was designed to use buckwheat as an alternative phytase source for chicken feed. The results obtained in this study suggested that buckwheat has high phytase activity, which can further increase by germination, and both non-germinated and germinated buckwheat can be used as a potential phytase rich ingredient in chicken diet. The addition of buckwheat at 20% level in 0.16% non-phytate P deficient diet can maintain growth performance and bone quality in broilers, and production performance and eggshell quality in laying hens. The utilization of phytate P can be increased and reduction of P in excreta can be achieved concomitantly.

For Future Research

From the practical viewpoint, further studies are needed to know the efficacy of other buckwheat as well as suitable maximum inclusion level of buckwheat, increasing the reduction rate of non-phytate P in chicken diets. In addition, buckwheat contains flavonoids which work to reduce blood cholesterol, so there is an opportunity to investigate the effects of buckwheat as medicinal feed source for chicken feed.

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