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Research Article
Reducing Osteoporosis by Phytase Supplemented Diet in Albino Rats

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Abstract

Background and Objective: Phytases are considered to be prospect nominee for use as an enzyme that have great value in enhancing the nutritional quality of phytate-rich foods and feed. The present study aimed at production of high value of phytase enzyme from new microbial isolates and investigates the effect of this enzyme as supplemented diet on bone performance in albino rats. **Materials and Methods:** Screening of seventy lactobacillus sp. for greatest phytase productivity. Basal diet would be supplemented with 1.6 mg kg^{-1} zinc carbonate or 520 U kg^{-1} of prepared phytase for 6 weeks. Thirty Wister rats 150±7 g (15 males and 15 females) were divided into 3 groups (5 for each sex). G1: Control group fed with basal diet, G2: Fed with basal diet supplemented with 1.6 mg kg^{-1} zinc carbonate and G3: Fed with basal diet supplemented with 520 U kg^{-1} phytase enzyme. Levels of bone porosity and minerals in serum and bone (iron, zinc, phosphorus and calcium) were measured in each animal. Data were statistically analyzed using students t-test according to Snedecor and Cochran and values are expressed as Mean±SE ($p \leq 0.05$). **Results:** A significant elevation of phosphorus, calcium and zinc were recorded in serum and bone for both sex in phytase treated rats, as well as pronounced reduction in the percentage of bone porosity was observed 3.42 and 0.41 for female and male, respectively in the same groups. **Conclusion:** Diet rich with phytase enzyme induced promise improvement in mineral metabolism and bone structure throughout the experiment. That might reduce osteoporosis in long time usage.

Key words: Phytase, growth factor, zinc supplemented, minerals, osteoporosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

This study discovers the possible synergistic effect of microbial phytase that can be beneficial for osteoporosis. Phytic acid is known as (myo-inositol 1, 2, 3, 4, 5, 6-Hexakis dihydrogenphosphate) and mixed cation salts of phytic acid, designated as phytate, are a group of organic phosphorus (P) compounds found widely in nature especially in legumes, cereals and oil seed crops[1]. Phytic acid, which is the main constituent of animal diet, is not digested by monogastric animals and hence, create problem in the availability of phosphorus in their diet. Various reported phytase-producing isolates and showed that the phytases with broader substrate specificity generally had low specific activities. Despite the considerable economic interest, low yield and high cost of enzyme production are the limiting factors in using this enzyme in animal diet. It also causes environmental pollution by extra supplemented phosphorus in animal's diet[2]. Phytases are the primary enzymes responsible for the hydrolysis of phytic acid[3]. Hence, phytases are considered to be potential candidate for use as an enzyme that have great value in enhancing the nutritional quality of phytate-rich foods and feed[4,5].

Phytate is of great interest in human nutrition, feed technology, food and medical science[6]. Researchers, as this interest has generated a wealth of relevant information. The negative effect of phytate on the availability of Ca and zinc, in food stuffs has been extensively investigated. Phytate in human diets is also claimed to have benefits, such as anti-carcinogenic effect[7]. Animal nutritionists have long regarded phytate as both indigestible and an anti-nutritional factor for non-ruminant animals[8]. Phytate is a polyanionic molecule with the potential to chelate positively charged nutrients, which is almost certainly fundamental to the anti-nutritive properties of phytate. These anti-nutritive properties require further investigation but phytate probably compromises the utilization of protein/amino acids, energy, calcium and trace minerals. Phytase, which occurs widely throughout nature, is the requisite enzyme to degrade and release inorganic P[9]. If the practical acceptance of microbial phytase in poultry diets continues, it is likely that phytase feed enzymes will re-define nutrient requirements for sustainable poultry production in the future. Nevertheless, three decades elapsed before an *Aspergillus niger*-derived phytase feed enzyme, with the capacity to liberate phytate-bound P and reduce P excretion, was commercially introduced in 1991[10].

In addition, phytase would be an eco-friendly product, reducing the amount of phosphorus entering the environment or problems resulted by eutrophication and constant chelating of nutrient factors from soil, as supplementation of phytase in the diets for monogastric animals reduces the fecal phosphate excretion up to 50%[11]. Thus, in the past decade, there has been a great deal of interest on the study of microbial phytase producers and the optimization of media and conditions for maximum production of the enzyme with the aim to increase yields to make it economical as a commercial product[12,13].

Two big goals for this study, first is to produce the microbial phytase from new isolate microorganisms. Second, study what is the effect of addition the microbial phytase enzyme on rate diet compared by diet supplemented with zinc. This study will help the researcher to uncover the critical areas of minerals bone loss that many researchers were not able to explore. A new theory on feeding by microbial phytase may be arrived at to prevent osteoporosis.

MATERIALS AND METHODS

Preparation of phytase enzymes

Microorganism: *Seventy lactobacillus* spp. were tested for their ability to produce phytase. The screening sub-cultured in MRS broth and preserved in glycerol solution (20%) at working cell bank at -80°C for further use. Among the tested *Lactobacillus acidophilus* showed the highest production of phytase that give 520 unit mL^{-1} of phytase, it was selected for further investigation.

Phytase production: Production of phytase derived from *Lactobacillus acidophilus* was carried out in a fermentation medium containing the following (g L^{-1}): $(NH_4)_2SO_4$-0.4 g, $MgSO_4.7H_2O$-0.2 g, casein-1 g, KH_2PO_4-0.5 g and K_2HPO_4-0.4 g and 1 g of sodium phytate dissolved in 1000 mL of wheat bran extract. The pH was adjusted to 6.5. The inoculated flasks were incubated in 37°C for 48 h under anaerobic condition[14].

Phytase assay: Phytase activity was determined by measuring the amount of liberated inorganic phosphate. The reaction mixture consisted of 0.9 mL of acetate buffer (0.2 M, pH 5.5) containing 1 mM phytate and 0.1 mL of the enzyme solution. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 1 mL of 10% trichloroacetic acid. The aliquot was subsequently analyzed for inorganic phosphate as described earlier by Pirgozliev *et al.*[10]. Color reagent (1.5 mL) was added which consist of 1:4 v/v of 2.75% ferrous sulphate: 2.5% ammonium molybdate dissolved in 5.5% sulphuric acid). One unit of the phytase activity was expressed as the amount of enzyme required to liberate 1 μ mol of phosphate min^{-1} from sodium phytate.

Biological assay

Animals: Thirty Wistar rats (15 male, 15 female) weighing (150±7 g) were obtained from the National Research Centre. The animals were maintained at a controlled temperature of 23±2°C with relative humidity between 50 and 60%, with alternating 12 h periods of light and dark for 2 weeks. After acclimatization period, animals randomly divided into three groups (5 males and 5 females) of ten rats each for carrying the experiment according to the protocol of National Research Centre[15].

Experimental design: The animal's diets were prepared according to the recommendations of the National Research Centre in March, 2016 (AIN-93G-Mx). Fed on basal control diet with mixture mainly 30% yellow maize, 34% soybeans, cellulose 10%, casein 10% corn oil 10%, Vitamin mixture 1%, L-cysteine 0.018%, Salt mixture 4% and Choline chloride 0.025%. The major nutritional contents of the laboratory diet were 22% protein, 3.48% fat and 3.71% fiber. That supplies the recommended concentration of elements for AIN-93 G and AIN 93 diet[16]. The rats were divided into three groups:

- Group 1 (G1): Was given a diet prepared
- Group 2 (G2): Basal diet which was supplemented with zinc carbonate at a concentration of 1.60 mg kg^{-1} diet
- Group 3 (G3): Basal control diet which was supplemented with phytase enzyme prepared at a concentration 520 U kg^{-1} diet

The rats were fed with diets for six weeks. Body weight change and total food intake were recorded. The daily consumed feed were calculated and the Feed Efficiency Ratio (FER) were estimated (Body weight gain/consumed feed). The feed and water were supplied randomly. The protocol applied throughout this study complies with the NRC Ethical Committee's guidelines and all animals received human care.

Sample collection: At the end of experimental period (6 weeks) rats were fasted over night and classified into females and males. Samples of blood were taken from the orbital plexus of vain according to[17], serum was separated by centrifugation at 3600 rpm. Serum was kept at -40°C for further studies. Animals were dissected for getting femur bone.

Biochemical studies: Measurement of Iron, phosphorus, zinc and calcium in serum was conducted according to the methods of AOAC by flame atomic absorption spectrometry[18].

Bone porosity: Transverse section of the bone of femur of both males and females were taken from the different groups, dried and sent for evaluation of porosity. The Dry Weight (DW) of the studied bones were measured using an electronic balance (0.1 mg precision), whereas the skeleton and grain volume (v_b and v_{sk}) of the studied samples were measured using Automated Helium Pycnometer (Ultra Pyc 1200e by Quantachrome) at 19 psi and the ambient temperature. The bulk volume (v_b) was measured after isolating the bone sample in a Para-film to avoid invading the pores by helium. Porosity 'Ø' of the dry core-shaped samples was calculated by substituting the skeleton volume 'v_{sk}' by the following equation[19]:

$$\varnothing = \frac{v_b - v_{sk}}{v_b} \times 100$$

The same bone samples were fired and ground to be analyzed for the minerals Ca, P, Zn and Fe using atomic absorption.

Bone mineral assay: Femurs from all animals were cleaned of soft tissue using stainless steel scissors and stainless steel forceps. They were dried in an oven at 100°C for 3 h. Dry weights were taken and femurs were ashed in silica glass crucibles[20]. The samples were placed in a cold furnace and temperature was gradually raised to 450°C and kept so for 24 h. One milliliter of concentrated high purity nitric acid was then added. After the bones had dissolved, the solutions were taken to dryness on a hotplate and then fired for 30-60 sec over a Bunsen burner. The resulting white ashes were dissolved in 5 mL of 20% HCl, the crucible was covered and digested at low heat for about 15 min. This was allowed to cool and made up to 25 mL in a volumetric flask. Solutions were further diluted with 1 M HCl as required, after which iron, phosphorus, zinc and calcium determinations were made using atomic absorption spectrophotometry[10].

Statistical analysis: Data were statistically analyzed using students t-test according to Snedecor and Cochran[21]. The t-test was performed to evaluate the difference between mean values of the treated group and those of control group. Values are exposed as Mean±SE $p \leq 0.05$.

RESULTS

The nutritional status of the animals through the course of treatment is showed in Table 1. It was clear that adding

Table 1: Nutritional status of rats challenged with basal diet supplemented with phytase or zinc (Mean±SE)

	Females			Males		
Parameters (g)	G1	G2	G3	G1	G2	G3
Initial body weight	182.00±3.26	184.60±3.80	186.40±13.95	182.40±1.93	182.20±1.59	183.00±2.00
Final body weight	249.00±5.05	251.00±5.20	240.20±12.47	236.60±2.90	226.40±3.05	223.40±4.43
Body weight gain	66.60±2.03^{a}	78.40±1.80^{b}	67.20±2.15^{a}	54.20±1.49^{c}	44.20±1.49^{d}	41.00±1.80^{d}
Total food intake	445.20±16.8^{a}	535.00±39.5^{b}	442.60±11.3^{a}	393.60±5.78^{c}	350.00±11.0^{d}	335.00±11.0^{e}

Different superscripts means significance difference ($p \leq 0.05$), G1: Diet, G2: Diet+zinc carbonate, G3: Diet+phytase enzyme

Table 2: Mineral concentration in serum of male and female rats basal diet supplemented with phytase or zinc

	Females			Males		
Parameters	G1	G2	G3	G1	G2	G3
Phosphorus (mg g^{-1})	17.9±3.26^{a}	18.5±3.6^{ab}	19.0±1.28^{a}	18.7±0.4^{a}	19.0±0.86^{a}	19.3±1.73^{a}
Calcium (mg g^{-1})	8.3±2.24^{ab}	8.6±1.22^{b}	8.8±0.06^{a}	8.5±1.1^{ab}	8.5±0.08^{ab}	9.2±0.06^{ab}
Iron (ppm)	60.0±7.46^{a}	70.0±4.22^{a}	104.0±4.36^{c}	74.0±4.54^{b}	107.0±7.30^{a}	129.0±3.15^{b}
Zinc (ppm)	41.0±2.70^{a}	46.0±5.86^{ab}	87.0±13.8^{c}	27.0±5.78^{ab}	28.0±3.78^{a}	90.0±8.71^{c}

Different superscripts means significance difference ($p \leq 0.05$) using SEM method, G1: Diet, G2: Diet+zinc carbonate, G3: diet+phytase enzyme

Table 3: Mineral concentration in femur of male and female rats basal diet supplemented with phytase or zinc

	Females			Males		
Parameters	G1	G2	G3	G1	G2	G3
Phosphorus (mg g^{-1})	83.90±0.05^{a}	126.53±0.03^{a}	210.16±8.00^{a}	14.71±0.26^{a}	36.34±2.9^{a}	38.37±1.01^{a}
Calcium (mg g^{-1})	32.35±0.09^{a}	49.62±0.16^{b}	69.01±4.88^{b}	82.72±0.31^{a}	94.54±3.6^{a}	103.81±0.70^{ab}
Iron (ppm)	589.00±0.27^{a}	623.00±0.03^{ab}	892.80±5.45^{bc}	216.00±0.03^{a}	295.25±2.8^{a}	346.60±0.37^{a}
Zinc (ppm)	700.00±0.04^{a}	827.00±0.03^{ab}	827.00±4.20^{a}	223.00±0.27^{a}	183.00±6.02^{a}	240.00±0.20^{a}
Bone porosity (%)	20.20±0.21^{a}	14.00±0.16^{a}	10.40±3.42^{ab}	18.61±2.40^{b}	13.52±6.5^{b}	11.20±0. 41^{a}

Different superscripts means significance difference ($p \leq 0.01$) using SEM method, G1: Diet, G2: Diet+zinc carbonate, G3: Diet+phytase enzyme

phytase or zinc carbonate as growth factor to basal diet affect of food consumption on body weight gaining. The description data revealed that female rats consumed basal diet supplemented with zinc more than male rats.

Mineral concentration in serum of male and female rats challenged with based diet supplemented with zinc or phytase as data presented in Table 2. The depicted data revealed significant increase in each of phosphorus, calcium, iron and zinc in serum of both female and male groups supplemented with phytase enzyme as compared to other groups at $p \leq 0.05$, respectively.

The results of the Table 2 measured parameters were confirmed with that recorded in bone investigation, where the parameters were elevated in the same treated groups (Table 3). Zinc supplemented groups recorded significant elevation ($p<0.05$) in all bone investigated parameters also. Screening of bone porosity in groups of rats supplemented with phytase showed better condition especially those in G3 and G2. These results were confirmed by the higher level of bone mineralization in the same groups. Regarding to bone porosity measured in bone of male and female treated rats with zinc and phytase enzyme, significant reduction in bone porosity was recorded in both phytase and zinc treated groups but reduction in porosity was more pronounced in phytase groups than zinc at $p \leq 0.05$.

DISCUSSION

Diet rich with phytase enzyme induced promise improvement in mineral metabolism and bone structure throughout the experiment. The recent developments in the production and/or expression of enzymes in other forms of microorganisms, such as bacteria and yeast, have resulted in new exogenous phytases. There is suggestive evidence that bacterial phytase may be more efficacious in breeding of broilers where bacterial phytase derived from *E. coli* liberated more P in broilers than two recombinant fungal phytases[22]. The bacterial phytase was more resistant to pepsin activity than fungal phytases[23]. The enzyme production by some bacterial isolates from different parts of the gastrointestinal tract of chickens, they found that the species *Bifidobacterium dentium*, *Lactobacillus reuteri* (L-M15) and *Lactobacillus salivarius* (L-ID15) had the highest phytase production and phytate degrading activity when used as starter in whole wheat bread making process[24].

The study of the nutritional status of the animals during the treatment course showed that phytase not acts as growth factor and that coincides with the previous study where it is used as food additive for growing and improve the body weight gain and food conversion rate[25]. These results were in agreement with Nakmmura who mentioned that there were no significant differences between groups of animals supplemented with phytase or Zn and control rats in food intake or body weight gain[26].

Rats supplemented with phytase enzyme especially G3 when compared to those not supplied by the zinc have minerals level in both serum and bone. The enzyme activity showed the least bone porosity in groups of rats supplemented with phytase enzyme. Use of phytase enzyme increased the bioavailability of iron which means protection from Iron deficiency complications such as anemia, significant decreases ($p \leq 0.05$) in psychomotor and mental development and reduced immune status[27]. The use of phytase with diets rich in cereals will also increase the absorption of calcium, affecting the attainment of peak bone mass, which may be important in reducing the risk of fractures and osteoporosis in later life[28]. In the same time the use of enzyme will increase bioavailability of Zinc which is necessary for bone formation and stimulation of growth hormone[29].

In such studies it has to be considered that, this is not the naturally occurring form of phytate in foods. In fact, phytate is mainly found in vegetable seeds as a calcium/magnesium salt called "phytin" [30]. The literature suggested that if the essential elements are present in balanced ratios with respect to phytate, there was no reason for a modification of the calcium magnesium balance[31,32].

CONCLUSION

The study concluded that the usage of microbial phytase enzyme improved mineral bioavailability in cereals and consequently the state of bone mineralization and controls bone fragility. Therefore, the current finding may have important implications in the development of new therapeutic strategy for treatment of osteoporosis.

SIGNIFICANCE STATEMENT

This study identifies the critical of microbial phytase production for supplemented food, that camwood opportunity to be worthwhile for many requisitions bone loss that many researchers were not able to explore. Thus, a new theory on these micronutrients combination and possibly other combinations, may be decreased osteoporosis.

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