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Reevaluation of Phytic Acid in Nutrition

栄養学におけるフィチン酸の再評価

理工学研究科・総合理工学専攻

食品

22D6015・金梓聞

博士論文要旨

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イノシトールに 6 分子のリン酸がエステル結合したフィチン酸は、穀物や豆類などにおいて複数種のミネラルと結合したフィチンとして存在している。これまで、食事中のフィチン酸は亜鉛などのミネラル類を不溶化し、ミネラルの吸収を阻害する反栄養物質と認識されてきた。しかし近年では、フィチン酸の摂取が血清脂質や尿酸値を改善し、疾患の予防や治療に機能するという報告が提出されている。フィチン酸摂取の影響を調べた研究の多くは、反栄養物質・機能性性成分のどちらか一方に偏った視点で行われており、フィチン酸摂取の影響を公平な立場から再評価することが求められている。本研究では、複数のミネラルが結合した不溶性のフィチンと食品添加物に使用されている可溶性のフィチン酸ナトリウムの双方を用いて、フィチン酸のミネラル栄養に及ぼす影響と血清尿酸低下などの機能性について、ラットを用いた栄養試験を中心に検討した。

第 1 章　調製法がフィチン酸のミネラル組成に及ぼす影響

米糠と小麦ふすまを原料としていくつかの方法でフィチン酸を調製した。0.1M 塩酸による抽出後、中和によって回収されるフィチン酸は、原料に含まれるミネラル組成を反映し、マグネシウム、亜鉛、マンガンを結合したフィチンの状態で回収されるが、中和時にマグネシウム塩またはカルシウム塩を添加した場合にはそれぞれ大半がフィチン酸マグネシウムまたはフィチン酸カルシウムとして回収された。さらにエタノールを添加して沈殿させた場合、フィチン酸はこれらのミネラルを含まない状態で回収された。このことは、米糠などから調製したフィチン酸は、調製法によってミネラル類の組成が異なることを意味している。これまでに行われてきたフィチン酸に関する動物実験は、使用しているフィチン酸に結合しているミネラル類に言及していないことから、再検討する必要があると考えられた。

第 2 章　フィチン酸ナトリウムとフィチンのミネラル栄養に及ぼす影響

ラットに水溶性のフィチン酸ナトリウムまたは米糠中と同様にマグネシウム、亜鉛、マンガンを結合した不溶性のフィチンを与えた。フィチン酸ナトリウムを与えたラットは、マグネシウム摂取量が対照群と等しいにもかかわらず糞のマグネシウム排泄量が多く、フィチン酸がマグネシウムの吸収を阻害することが確認された。また、この群では血清と骨の亜鉛濃度が低下しており、亜鉛吸収も阻害されている可能性が考えられた。一方、フィチンを与

えたラットでは、対照群と鉄の摂取量が等しいにもかかわらず糞への鉄排泄量が多く、かつ血清、肝臓、脾臓鉄濃度が低下しており、鉄の吸収が阻害されていると考えられた。フィチン酸ナトリウムを与えたラットに鉄吸収の阻害は認められなかった。対照群に比べてリンの摂取量が多いフィチン酸ナトリウム、またはフィチンを与えたラットでは、リンの見かけの吸収率は低下していたが、見かけの吸収量自体は増加し、血清リン濃度も上昇していた。また、亜鉛摂取量が多いフィチンを与えたラットは血清と骨の亜鉛濃度がフィチン酸ナトリウム投与ラットよりも高かった。これらのことは、これまでフィチン酸の影響と考えられてきた鉄吸収阻害作用が、フィチン酸に結合しているミネラル類の影響であることと、フィチン酸中のリン酸とフィチン酸に結合した亜鉛の一部が利用できている可能性を示している。

第 3 章　亜鉛を含むフィチンの亜鉛栄養に及ぼす影響

米糠や小麦ふすまに存在し亜鉛を構成成分に含むフィチンの亜鉛栄養に及ぼす影響を検討するため、亜鉛状態が適切、または低いラットに亜鉛含有フィチンを投与した。亜鉛状態が適切なラットでは、フィチン酸による亜鉛吸収阻害が確認されたが、低亜鉛栄養状態には至らなかった。一方、低亜鉛状態のラットでは、亜鉛含有フィチンの投与によって、低亜鉛状態がもたらす成長阻害が解消し、亜鉛の栄養状態が改善していることが確認された。このことは、亜鉛不足の場合にフィチン酸に結合した亜鉛が亜鉛源として積極的に利用されることを示している。これまで米糠や小麦ふすまはフィチン酸を含むことから低亜鉛状態を重症化するとされてきたが、むしろ低亜鉛状態を緩和する可能性が大きいと考えられた。

第 4 章　リン源としてのフィチン酸の意義

フィチン酸中のリンの利用性を検討するため、リン欠乏ラットにフィチン酸ナトリウムを段階的に投与した。リン欠乏によってもたらされる成長阻害と骨形成障害はフィチン酸ナトリウム投与によって大幅に改善された。リン欠乏ラットではホスファターゼ活性が有意に上昇していたことから、リン欠乏時にはホスファターゼ活性の上昇によってイノシトールに結合しているリン酸が遊離し、リン源として有効に利用されると結論した。

第 5 章　フィチン酸による血清尿酸値低下作用

ミネラルの栄養状態が十分なラットにフィチン酸ナトリウムを段階的に与えた。フィチン酸は一部のミネラルの吸収を阻害したが、欠乏症を惹起するほどではなかった。一方、フィチン酸ナトリウム投与は血清尿酸値を量依存的に低下させた。肝臓のモリブデン濃度とモリブデン含有酵素であるキサンチンオキシダーゼ（XOX）活性もフィチン酸投与により量依存的に低下していた。これまで報告されてきたフィチン酸投与に伴う血清尿酸の低下は、フィチン酸によるモリブデン吸収阻害に伴う XOX 活性低下によって生じたことが明らかとなった。

まとめ

フィチン酸とミネラルとの関係はミネラルの栄養状態によって変化することが明らかとなった。すなわち、フィチン酸は、ミネラル栄養状態が十分な場合には、一部のミネラルの吸収を阻害するもののその程度は小さく、ミネラル栄養状態の低下を起こすほどのものではないと判断できた。一方、ミネラルの栄養状態が低い場合には、ミネラルの供給源として利用されることを亜鉛またはリン欠乏ラットを用いて証明した。さらにフィチン酸による血清尿酸低下作用にモリブデンの吸収阻害が関わることを示した。

以上

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Preface

Phytic acid is a hexakisphosphate ester of myo-inositol, the molecular structure of inositol hexakisphosphate was revealed in 1914 (Figure 1), and current analyses have verified this theory [1]. Phytate, a salt of phytic acid, which is abundant in beans and cereals and is commonly found as an insoluble mineral-mixed salt also called phytin [2]. It acts as a storage form for phosphorus and minerals, comprising over 75% of total phosphorous found in nuts [3]. Beyond this, seeds carry other phosphoric acid salts such as myoinositol pentaphosphate and myoinositol tris (hydrogen phosphate), but their presence is notably negligible [4]. The importance of the presence of phytic acid in plants is illustrated by the fact that it is hydrolyzed during seed germination allowing the release of phosphorus and other minerals stored in phytin [5].

In the last decades, phytic acid has been recognized as an anti-nutrient factor. In monogastric animals, the limited ability to digest phytic acid may result in phosphorus deficiency and growth inhibition when phytic acid is used as a phosphorus source [2]. This is due to the lack of phytase in the intestines, and phytic acid is often considered indigestible [6,7]. Moreover, phytic acid with its unique inositol hexakisphosphate structure gives it strong chelating ability and stability [2]. It is believed that phytic acid intake particularly affects zinc absorption, as severe growth suppression due to zinc deficiency was commonly observed in Middle East villagers that consumed whole grain bread with high concentrations of phytic acid and no animal protein at all [8,9]. Furthermore, deficiencies in calcium and iron as well as other minerals also have been reported to be induced by the intake of phytic acid [6,10–13]. In the gastrointestinal tract, phytic acid is thought to bind to various micronutrients to form insoluble precipitates,

such as divalent metal cations [14], free fatty acids [15] and small-molecule proteins [16,17]. Since no suitable carrier has yet been identified, it has long been assumed that phytate cannot cross the lipid bilayer of the plasma membrane, and therefore it was thought that phytate would have little chance of being absorbed in the intestinal tract. However, recent studies have shown that phytic acid is detected in tissues and urine after administration of sodium phytate [18,19]. Owing to the particular way phytic acid doesn't absorb specific light wavelengths, and the diverse range of its derivatives [2], an effective and precise method for analyzing phytic acid hasn't yet established.

On the other hand, there has been growing interest in exploring the health advantages of phytic acid administration in recent years. Several studies have reported that phytic acid intake has powerful prevention and treatment on different symptoms, including enterocolitis [20], cancer [21] and neurodegenerative diseases [22] in human and animal models. Furthermore, epidemiological studies and animal experiments have shown that phytic acid is effective in preventing hyperlipidemia [23,24], and hyperuricemia [25]. As a result, phytic acid is expected to have great potential for improving human health and preventing diseases. Furthermore, it has been noted that phytic acid-induced zinc deficiency only occurs when zinc intake is insufficient, and it is not necessary to worry about phytic acid-induced zinc deficiency when the zinc intake is sufficient [26].

Most studies focus solely on the potential health benefits or detriments of phytic acid. This approach has sparked considerable debate regarding whether phytic acid should be commercialized as a health supplement, with an added emphasis on minimizing its daily intake to enhance mineral utilization.

Since phytic acid is abundant in unrefined grains and legumes, its intake level varies depending on the content of the dish. In a recent review, phytic acid intake is roughly

divided into three types according to eating habits [2]: (i) general Western-style diets low in phytate rich plant foods results in a low intake level of 200 to 350 mg/d, (ii) Western-style diets with enhanced portions of whole grain products or other phytate rich foods results in a higher intake level of 500 to 800 mg/d, and (iii) diets dominated by legumes and unrefined grains such as vegetarian diets or some diets in developing countries in Asia, Africa, and Latin America result in a high intake level of >1000 mg/d. The diets eaten by Egyptian boys with zinc deficiency [9] would fall into this category (iii). In some developing countries corresponding to category (iii), phytic acid intakes in excess of 2000 mg/d have been reported [27–30]. Regarding East Asia, where the Westernization of eating habits is progressing, the daily phytate intake for adult males (21–70 years) was 839 ± 400 mg and for females 752 ± 407 mg in the Republic of Korea [31]. The consumption of phytate in China is of the type (ii) for people 648 mg/d living in cities, while the consumption of phytate is much higher in the rural (1342 mg/d) [32].

However, phytic-acid-enriched foods and supplements are designed for phytic acid intakes of more than 500 mg/d [33]. Therefore, even in East Asia including Japan, where Westernization is progressing, it can be estimated that there are cases where the phytic acid intake falls into category (iii) due to the intake of phytic-acid-enriched foods. Since the amount of human diet is 400 to 500 g/d by dry weight, the phytic acid dose in this study corresponds to 2000 to 5000 mg/d; that is, it corresponds to the phytic acid intake of category (iii).

For these reasons, an enhanced nutritional understanding of phytic acid is imminent at a time when the occurrence of lifestyle diseases is progressively higher [34] and a vegan lifestyle is being promoted [35]. In this study, I investigated the mineral composition of various phytin in Chapter 1 and compared the effects of different forms of phytic acid on

mineral metabolism in Chapter 2. The bioavailability of zinc and phosphorous contained in phytates is described in Chapters 3 and 4, respectively. In Chapter 5 the health functions of phytic acid are mainly discussed.

Chapter 1

Effects of differences in manufacturing methods and raw materials on the mineral composition of bran-derived phytin

Summary

Phytic acid is usually deposited in dormant seeds bound to minerals in the form of phytin. Bran is a byproduct of grain processing and used as a raw material for phytin production. In this study, commercially available rice bran and wheat bran were used as raw materials, and phytin was extracted and prepared by four typical production methods: alkali addition, calcium salt addition, magnesium salt addition, and ethanol precipitation. In addition to phosphorus, the raw bran contained magnesium, calcium, manganese, zinc, and iron, and these minerals were also recovered in all phytin products. The phytin products produced by alkali addition showed a mineral composition that was almost identical to the proportions found in the bran. The addition of alkali metal salts increased the corresponding metal content in the phytin products, suggesting that magnesium and calcium may be antagonistic in their binding to phytic acid. Ethanol precipitation resulted in lower calcium and magnesium recoveries and a relatively higher iron content. Among the phytate products obtained, those with a high iron ratio were amber in color, while those with a high manganese ratio were pink. The mineral composition in phytin is affected and complicated by the type of raw material and method of production, and the mineral composition in phytin and phytic acid must be noted when interpreting the results of animal studies on phytin and phytic acid.

Introduction

Grains and legumes are major sources of energy, protein, and minerals. Minerals in the seeds exist bound to phytic acid, an inositol hexakisphosphate, and such a mineral-phytic acid complex in seeds is referred to as phytin [2]. Phytin is the primary raw material used to produce inositol and is applied in various fields such as pharmaceuticals, nutraceuticals, and food additives [36]. Under physiological conditions, the negatively charged phosphate groups of phytic acid bind to metal cations such as calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn), making them insoluble and inhibiting their absorption in the gut [14]. The inhibitory effects of phytate on mineral absorption have been extensively studied. These studies have used various grades of phytate, ranging from purified phytate to naturally occurring phytin [7,12,13,37,38]. Although the physicochemical properties of phytates have been studied under laboratory conditions[39–41], few studies on the mineral composition of natural and commercial phytin can be found. In addition, the mineral concentrations in the diets used in animal experiments did not been validated in these studies.

Phytin in seeds is mainly concentrated in the external covering layers of the seed pericarp and aleurone layer, with lower levels in the germ [42]. These parts are removed during the refining process of grains, referred to as bran. In Japan, rice bran, an abundant byproduct of rice polishing, is used as a raw material for producing phytate and inositol[36,43]. Taking advantage of the fact that phytic acid is soluble in acids but insoluble in organic solvents and the insolubility of divalent metal salts of phytic acid, several methods using organic solvents and alkaline metal salts have been used in the industrial production of phytin and purified phytate from bran [36,43]. However, the diversity of phytin production methods leads to variation in the mineral composition of

the produced phytin. Since phytin is often used in nutritional studies on phytic acid, clarifying the relationship between the mineral composition of phytin and method of production is important for the accurate interpretation of nutritional studies on phytic acid.

In this study, in order to clarify the effect of manufacturing methods on the mineral composition of phytin, commercially available fresh rice bran and wheat bran were used as raw materials, and the contained phytin was extracted, precipitated, and recovered according to several typical industrial manufacturing methods, and the mineral composition of the obtained phytin was investigated.

Materials and methods

Materials

Domestic wheat bran (Yutec Co., Ltd. Hokkaido, Japan) and rice bran (Uchino Beikoku Co., Ltd. Saitama, Japan) were purchased by mail order. Three samples of each type of bran produced in the same batch were randomly selected for mineral analysis, while the remaining bran samples were used as raw materials for phytin preparation. Sodium phytate was purchased from FUJIFILM Wako Pure Chemical Co. (Osaka). Ferric phytate and manganese phytate were prepared as follows: 1 M $FeCl_3$ or 1 M $MnCl_2$ solution was mixed with an equal volume of 0.2 M sodium phytate, and the resulting precipitates were collected as ferric phytate or manganese phytate, respectively.

Preparation of phytin

Four different preparation methods were applied in this experiment, including the alkali addition method, alkaline earth metal (Ca or Mg) addition method, and ethanol precipitation method [36,43]. In the alkali addition method or alkaline earth metal addition method, 5 g (precisely weighed) of wheat bran or rice bran was placed in a 50-mL triangular flask with 50 mL of 1% HCl and extracted overnight at 30°C with constant shaking in a water bath, and the supernatant was collected as an acid extract. The acid extract was neutralized to pH 4.0 using 3 M NaOH (alkali addition method) or a solution containing 3.6 M $Ca(OH)_2$ and 0.9 M $CaCl_2$ (calcium salt addition method) or a solution containing 2 M $Mg(OH)_2$ and 1 M $MgCl_2$ (magnesium salt addition method), followed by pH adjustment to 7.0 using 1 M NaOH. Each mixture was filtered, and the resulting precipitate was collected, washed with pure water, and then dried to obtain the three phytin samples. For the ethanol precipitation method, 50 mL of the acid extract was

prepared from the defatted wheat or rice bran. Anhydrous ethanol (50 mL) was added to the acid extract for the first sedimentation. After separation by centrifugation, the clear supernatant was subjected to second sedimentation with 100 mL of anhydrous ethanol, and the resulting precipitate was obtained by centrifugation. The two precipitates were combined, washed with ethanol, and then dried to obtain the fourth phytin sample.

Analysis of metals

A portion of the bran or phytin samples was accurately weighed, placed in a Kjeldahl flask, a mixture of HNO_3 and $HClO_4$ (5/1, v/v) was added, and the mixture was heated until the solids disappeared and white fumes appeared. After cooling to room temperature, ultrapure water was added and heated again to remove any remaining acid until the white fumes disappeared, indicating that digestion was complete.

The digests were diluted with 0.1 M HCl and filtrated through a 0.45-μm filter. The standard metal element solutions and sample solutions were analyzed using a flame atomic absorption spectrophotometer (AA-7000, Shimadzu, Kyoto, Japan). The inorganic phosphorus determination was performed using the vanadomolybdate method [44].

Results and discussion

Table 1 shows the mineral composition of the bran used in this study compared with the values listed in the Food Standard Composition Tables [45] or US food database [46]. The quantitative results for each mineral in rice bran were very close to the values in the Standard Tables of Food Composition in Japan [45]. In contrast, the mineral content of the wheat bran used was slightly lower in phosphorus and Mg than the values listed in the U.S. food database [46]. This is probably due to the fact that the wheat bran used in this study and the wheat bran from which the U.S. values were derived differ in the type of wheat (e.g., hard, ordinary, and soft wheat) as well as the region of origin. Still, in both brans, the Mg content was much higher than the Ca content and small amounts of Zn, Mn, and Fe were contained. Rice bran was high in phosphorus and Mg contents, and its total mineral content was markedly higher than that of wheat bran. Thus, rice bran, a byproduct of rice polishing, has been widely used for the production of phytic acid and inositol because of its high phosphorus content [36,43]. On the other hand, wheat bran, a byproduct of wheat flour production, does not contain much phosphorus and has not been widely applied for phytic acid production.

Table 2 summarizes the mineral composition of phytin prepared from rice or wheat bran by different production methods. The composition and percentage of minerals in phytin prepared by the alkaline addition method using only NaOH is almost the same as that of the raw bran. This indicates that when only NaOH, the most common neutralizing agent, is used, the cations precipitate in approximately the same proportion as in the acid extract. In other words, the phytin prepared by this method is considered to be the pre-extracted form of phytin, with magnesium phytate as the main component and various metals in the form of phytates.

In addition to the use of NaOH, the addition of alkaline earth metals such as Ca and Mg to produce phytin has been effective in improving the yield of phytin [36,43]. However, when these alkaline earth metal salts were added, the total mineral concentration of the obtained phytin was clearly higher than that of other methods, as shown in Table 2. This indicates that the phytin precipitate obtained by alkaline earth metal addition contains not only calcium phytate or magnesium phytate, but also some of the added alkaline earth metal salts that have become insoluble and precipitated. Thus, the introduction of Ca or Mg resulted in a significant difference in the metal ratio of the resulting phytate precipitate from that of the raw bran. Furthermore, when calcium salts were used as precipitants, the ratio of Mg in the precipitates was much lower, and when magnesium salts were used, the ratio of Ca was even lower than in the original bran. This means that Ca and Mg inhibit each other's binding to phytic acid, i.e., there is an antagonistic relationship between the binding of calcium and magnesium ions to phytic acid in solution. On the other hand, adding large amounts of Ca and Mg did not significantly affect the recovery of Mn, Zn, and Fe, irrespective of whether the source material was rice or wheat bran.

Phytin is known to be insoluble in organic solvents such as alcohols, but soluble in acids [36,43]. In our experiment, the addition of ethanol could also precipitate phytin. The addition of ethanol to the acid extract did indeed decrease the solubility of phytate, but the hydrogen ion concentration of the solution was not markedly changed, suggesting that a large amount of phytin was not precipitated. Compared with other methods, the phytin obtained by ethanol precipitation had lower percentages of Ca and Mg, and a higher percentage of Fe. This difference is attributed to the fact that calcium or magnesium phytates remain soluble at pH 4 to 5, whereas ferric phytate is insoluble under even lower

pH conditions [47]. Therefore, the phytate product obtained by the ethanol precipitation method is considered to have a high percentage of free phytic acid or alkali metal salts of phytic acid.

Figure 2 shows images of phytin from different sources and methods, and ferric phytate and manganese phytate, along with Fe and Mn content in each phytin product. Ferric phytate is amber and manganese phytate is pink.

Phytin obtained by ethanol precipitation showed an amber color derived from ferric phytate, regardless of the raw material. Since the ethanol precipitation method also produces less calcium phytate and magnesium phytate (Table 2), which are white in color, the amber color of ferric phytate, which has a relatively high content ratio, may have determined the color tone of the obtained phytin. On the other hand, phytin obtained from wheat bran by the alkali addition or calcium salt addition method showed a pink color derived from manganese phytate. This may reflect the high ratio of Mn in these phytin products. The remaining phytate products were white in color, reflecting low ratios of Fe or Mn and high ratios of calcium or magnesium phytate.

The above findings indicate that coloration of the phytin products is due to Fe and Mn. However, wheat bran contains many brown pigments such as flavonoids and lignin [48,49], and it cannot be ruled out that these may be incorporated into the final phytate precipitation and contribute to coloration of the product. Commercially available phytin and phytic acid vary in color from colorless or white to light brown [1,36]. This variation is most likely due to differences in raw materials, methods, and even the degree of purification among different manufacturers.

Although the methods for extracting phytin from bran are relatively similar, different precipitation methods can have a significant impact on the composition of the resulting

phytin product. Phytic acid possesses six strong acidic groups and six moderately weak acidic groups, with pKa values ranging from 1.9 to 9.5 [39]. In the present experiment, to fully extract phytin from the raw material, an extended period is required under strongly acidic solutions (pH < 1). At this stage, phytin in the bran can be completely dissociated into phytic acid. Subsequently, an alkaline neutralizing agent or a precipitating agent is added to adjust the pH to the desired level. As the pH gradually increases, the metal ions present in the acidic extraction solution interact with the added metal ions and progressively bind to the acidic groups of phytic acid. In other words, the positions of the weak acidic groups of phosphate connected to the inositol hexaphosphate ring are occupied by the cations with the highest affinity, followed by the positions of the strong acidic groups. The affinity sequence between phytic acid and multivalent cations is Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+}> Ca^{2+} [40]. According to the previous discussion, we can infer that the affinity of Mg for phytic acid should be like that of Ca. Finally, after all 12 acidic group positions are fully occupied, precipitation occurs, and the product can be recovered as phytin products. The binding mechanism between phytic acid and cations in solution is complex and influenced by factors such as ion strength, pH, and temperature. However, it is clear that without thorough ion exchange, phytin products derived from natural sources will invariably contain certain amounts of elements such as Mn, Fe, Zn, as indicated by our experimental results. Accordingly, the type and origin of the grains used might significantly influence the composition of phytate products, as the types and concentrations of minerals in bran can vary markedly [14].

Phytic acid, as an antinutrient factor inhibiting the absorption of trace elements and leading to trace element deficiencies, has been investigated in various studies using natural phytin-containing substances or phytin [7,12,13,37,38]. In these studies, some

experiments have analyzed the elemental composition of the experimental materials, while others have not. Nevertheless, many experiments lack a detailed understanding of the composition of phytin in the materials, which is critical for discussing minerals. This is because natural phytin-containing substances have abundant and diverse minerals, which can also significantly impact the minerals status within the body. In nutritional studies on phytic acid, the presence of trace elements such as Mn, Fe and Zn in phytates may significantly affect the results of research on the inhibitory effect of phytic acid on the absorption of these elements, as organisms may be able to absorb even very small amounts of these trace elements depending on their nutritional status. In daily life, phytic acid is typically consumed in its natural form from grains and legumes, and even commercial phytin can exhibit variations in composition due to various factors. Therefore, the composition of minerals in phytin and effects of cations in phytin should also be further discussed in future nutritional studies on phytates and minerals.

Chapter 2

Effect of sodium phytate and phytin on the absorption and organ concentration of several minerals in rats

Summary

The effects of sodium phytate or phytin administration on several mineral utilizations in rats were examined. Eighteen male 4-week-old Wistar/ST rats were divided into 3 groups. One group (control group) was fed a basal AIN93G diet, the other two groups (SP group and phytin group) were fed the basal diet containing sodium phytate (1.0wt%) or rice bran-derived phytin (1.0wt%), respectively, for 4 weeks. Because the phytin used contained magnesium, zinc, and manganese, the phytin group consumed more of these minerals than the other two groups. The SP group excreted more magnesium in their feces but had no change in retention, which had a similar magnesium intake. In addition, the SP group had lower zinc concentrations in the serum and several organs than the control group. Comparing to the control group, only significant difference was observed in the femoral zinc concentration in the phytin group. The phytin group had lower apparent iron absorption and lower serum and organ iron concentrations than the other two groups. The serum phosphorus concentrations and urinary phosphorus excretion were increased in the SP and phytin groups. These results suggest that 1) phytic acid may inhibit the absorption of magnesium and zinc, but has little effect on their nutritional status; 2) minerals bound to phytic acid, probably manganese, interfere with iron absorption; and 3) phytic acid is partially hydrolyzed in the digestive tract, with a tendency for phosphoric acid and phytic acid-bound minerals to be released and utilized.

Introduction

The Southeast Asian diet, based on plant-based products, tends to have a high intake of phytic acid [2]. For decades, phytic acid has been considered an anti-nutritional factor because it forms insoluble salts with divalent metal cations and affects the absorption of minerals in the small intestine [50]. On the other hand, there has been growing interest in exploring the health advantages of phytic acid administration in recent years [34]. As elucidated in Chapter 1, the mineral composition in phytin is affected and complicated by the type of raw material and method of production [51]. Moreover, the experimental conditions applied in these studies varied, resulting in a questionable reproducibility of phytic acid nutritional assessments. This may have instigated the debate in recent years concerning the impact of phytic acid on mineral assimilation.

There are two types of studies on phytic acid, one using water-soluble sodium phytate and the other using insoluble phytin. Accordingly, the adverse or beneficial health effects of phytic acid is difficult to be interpreted uniformly at present. Therefore, in order to evaluate phytic acid nutritionally, it is necessary to conduct experiments using different forms of phytic acid simultaneously.

In this study, we performed an animal experiment in which rats were administered sodium phytate as a soluble phytate or phytin as an insoluble phytate and the organ accumulation and balance of several minerals were measured.

Materials and methods

Animals and diets

The experimental protocol followed the Guide for the Care and Use of Experimental Animals issued by the Prime Minister's Office of Japan and approved by the Animal Ethics Committee of Kansai University (Approval No. 2113).

Eighteen 4-week-old male Wistar/ST rats (SHIMIZU Laboratory Supplies Co., Kyoto) were divided into 3 groups. One group (control group) was fed a basal diet prepared according to the AIN93G formulation [52], and the other two groups (SP group and phytin group) were fed the basal diet containing 1.0 wt% sodium phytate (Sigma-Aldrich, St. Louis) or 1.0 wt% phytin (Tokyo Chemical Industry, Tokyo), respectively. The animals were allowed to access tap water and the experimental diet *ad libitum*.

Table 3 summarizes the actual mineral content of the diets administered to each group. The phytin used in this study was sold as calcium phytate derived from rice bran, but the analysis showed that it contained no calcium, but 20.0% phosphorus, 11.7% magnesium, 0.1% zinc, and 0.2% manganese. Thus, the diet fed to the phytin group contained higher concentrations of magnesium, zinc, and manganese than the other diets. In addition, the SP and phytin groups also received higher doses of phosphorus than the control group due to the phytate-derived phosphorus.

After 20 days of feeding, each rat was housed separately in a metabolic cage (Natsume Seisakusho Co., Ltd., Osaka), and all feces and urine were collected every 48 hours. One mL of 1M HCl was added to the flasks which were used to collect the urine. The food and water consumption as well as body weight were recorded every 2 days.

On day 28, the rats were weighed and sacrificed under isoflurane (Fujifilm Wako Pure Chemical Co., Tokyo) anesthesia. The liver, kidney, spleen, and femur were removed,

then weighed, rinsed with cold saline, and frozen in liquid nitrogen. Blood was collected from the abdominal aorta, and serum was obtained by centrifugation at 1,500 × *g* for 15 min. All the specimens were stored at -30 °C until analysis.

Analysis of metals

Approximately 1 g of liver, kidney, spleen, femur, experimental diets, and 1 mL of serum were heated with 5 mL of nitric acid until there were no solids. The obtained solution was diluted with pure water and filtrated through a 0.45 μm filter. The feces samples were freeze-dried overnight and then ground into a mill. The feces powder was partially weighed in crucibles and then heated in an electric furnace at 500 ˚C for 16 hours. The ashes obtained were dissolved in 0.1M nitric acid and filtrated through a 0.45 μm filter. The urine samples were centrifuged at 20,000 x *g* for 5 min and the supernatant obtained was used for analysis.

The contents of calcium, magnesium, iron, zinc, manganese, and copper in the solutions were determined using an atomic absorption spectrophotometer (AA-7000, Shimadzu, Kyoto) or an inductively coupled plasma mass spectrometer (ICPMS-2030, Shimadzu, Kyoto). For the determination of calcium and magnesium, lanthanum chloride solution was added to a final concentration of 3000 ppm to eliminate the interference from coexisting phosphorus. In the analysis by ICPMS, ^{115}indium was used as an internal standard. The inorganic phosphorus determination was performed using the vanadomolybdate method [44].

It is difficult to avoid contamination of a very small amount of the experimental diets when collecting the urine. Therefore, only the concentrations of calcium, phosphorus, and magnesium were measured because urine is the main excretion route and the effect of

dietary contamination is negligible.

Statistical analysis

The statistical differences among the groups were evaluated by Tukey's honestly significant difference test after one-way analysis of variance (ANOVA). SPSS Statistics 27 for windows (IBM Japan, Ltd., Tokyo) was used as the statistical analysis application.

Results

Table 4 shows the body weight, feed intake and water consumption in each group. After 4 weeks of feeding, the weight gain of the rats in each group was almost equal, and the addition of sodium phytate or phytin to the diet did not affect the growth of the rats.

Table 5 summarizes the balance calculated from fecal and urinary excretion for several minerals. For calcium, there were no differences among the three groups for all parameters.

For magnesium, the apparent absorption rate was lower in the phytin group, which had a very high intake, than in the other two groups. On the other hand, when the SP group was compared to the control group with equal magnesium intake, the SP group had higher fecal excretion and lower amounts of apparent absorption. Thus, although there was a difference in apparent absorption among the groups, there was no difference in the final retention amounts among the three groups because urinary excretion was higher in the phytin group, the control group, and the SP group, in that order.

Regarding phosphorus, due to the phytic-acid-derived phosphorus, the SP and phytin groups, which had a higher intake than the control group, had a higher apparent absorption, but these two groups also had higher urinary excretion, and there was no difference among the three groups in the final amount of phosphorus retained.

For iron, fecal excretion was higher in the phytin group, and the amount and rate of apparent absorption was lower than in the other two groups. Although the intake of zinc was higher in the SP and phytin groups than in the control group, the apparent absorption amounts of zinc was not different among the three groups because the fecal excretion was also higher in these groups. For copper, no difference in apparent absorption was observed among the three groups. For manganese, there was no difference in the apparent

absorption among the three groups because the phytin group, which had a higher intake, also excreted more manganese in their feces.

Table 6 shows the mineral concentrations in the serum and organs of each group. For calcium, the SP group showed higher levels in the kidneys. For magnesium, there was no difference in the serum and organ concentrations among the three groups. For phosphorus, the serum concentrations in the SP and phytin groups were significantly higher than in the control group. In addition, the liver and spleen concentrations were higher in the phytin group than in the other two groups.

For iron, the phytin group had significantly lower concentrations in the serum, liver, and spleen than the other two groups. The zinc concentration in the SP group was significantly lower than that in the control group in the serum, kidney, spleen, and femur. The phytin group also tended to show lower values than the control group, and there was a significant difference in the concentration in the femur. For copper, the serum levels in the SP and phytin groups were significantly lower than in the control group. For manganese, there was no difference in the serum and organ concentrations among the three groups.

Discussion

As mentioned in the materials and methods section, the rice-bran-derived phytin used in this study contained magnesium, zinc, and manganese, not calcium, even though it was clearly labeled calcium phytate. As described in Chapter 1, to separate phytin from rice bran, phytic acid has been extracted from rice bran with acid, and ethanol, magnesium oxide, calcium chloride, etc. are added to the resulting acid extract solution to recover phytin as a precipitate [43,51].

According to the conclusions of Chapter 1, the chelation of other metal ions with phytic acid cannot be avoided in the preparation of phytin, even with the addition of large amounts of calcium. In this experiment, the weight ratio of phosphorus, magnesium, zinc, and manganese in the phytin used in this study (200:117:1:2) is close to the weight ratio of these minerals in rice bran (200:85:0.6:1.5) [45]. In other words, the phytin used in this study is considered to reflect the mineral composition of the phytin in rice bran. Although the details of the production process are unknown, this result is highly consistent with our discussion of the natural phytin composition in Chapter 1.

Thus, in this experiment, the magnesium, zinc, and manganese doses were higher in the phytin group than in the other two groups. In addition, the phosphorus doses were higher in the SP and phytin groups due to phosphorus derived from phytic acid. Since the absorption rate of minerals generally decreases with increasing dosage, in this experiment, it was difficult to determine the effect of phytic acid on mineral absorption based on the apparent absorption rate alone, so the amount excreted in urine and the amount accumulated in the serum and organs were also used to determine the effect.

Nevertheless, in the case of magnesium, it is clear that phytic acid inhibited magnesium absorption, as the apparent absorption in the SP group was significantly lower

than in the control group with approximately equal intake (Table 5). Thus, phytic acid inhibited magnesium absorption, but there were no differences in the magnesium retention or organ concentrations among the three groups (Table 5). This means that when magnesium is supplied in sufficient amounts, as in the case of the AIN93G diet, magnesium homeostasis in the body is sufficiently maintained even when magnesium absorption is suppressed by phytic acid.

In the SP and phytin groups, femur zinc concentrations were significantly lower than in the control group (Table 6). We have observed that among serum and organs, bone zinc concentration is the most sensitive to change in dependence on decreased zinc absorption [53]. Although no clear difference was observed in the balance study (Table 5), these decreased femur concentrations suggest that phytic acid inhibited zinc absorption. In the balance study, no clear difference could be detected because the SP group with decreased zinc status is thought to have decreased endogenous fecal excretion, and the difference between the apparent and true absorption is larger than in the control group. The milder decrease in the zinc status in the phytin group may be due to some utilization of the zinc that was bound to phytin.

Furthermore, the serum copper concentrations were significantly lower in the SP and phytin groups than in the control group. This suggests that phytic acid may inhibit the absorption of copper in addition to magnesium and zinc.

For iron, the apparent absorption and serum and organ concentrations were lower only in the phytin group than in the control group (Tables 5 and 6). Since such a decrease was not observed in the SP group, the decreased iron utilization in the phytin group was most likely due to the effects of magnesium, zinc, and manganese bound to phytin. In this connection, it is known that the divalent metal transporter 1 (DMT1) acts on divalent iron

ions in addition to transporting manganese ions for uptake into the small intestine mucosal cells [54]. It could be supposed that the high amount of manganese contained in the ingestion of phytin may exacerbate the antagonistic effect of iron and manganese when bound to DMT1, leading to a decrease in iron absorption. A similar result, leading to reduced iron accumulation in organs when high manganese diets are administered to growing rats, has also been reported [55].

In the phosphorus analysis, we observed an increase in the serum inorganic phosphorus levels in the SP and phytin groups (Table 6) and a significant increase in the release of phosphorus from urine (Table 5). It indicates that phytic acid was partially hydrolyzed and releases inorganic phosphorus in the intestinal tract. In addition, magnesium and zinc bound to phytin were also presumed to be partially absorbed. These results indicate the chelating ability of metal ions is much lower after phytic acid hydrolysis [10], which facilitates the utilization of minerals in the intestine. However, the utilization of phytate in rats has not been well-understood. One study reported that phytin phosphorus utilization was similar to inorganic phosphorus when the vitamin D intake was adequate [56]. Another study has noted that phytic acid is hydrolyzed in the cecum and colon of rats by the gut flora [57]. Furthermore, a study on phytase in the rat small intestine indicated that the phytase activity was active in the duodenum yet still insufficiently hydrolyzed phytic acid [58]. This suggests that the hydrolysis of phytic acid in the gastrointestinal tract needs to be discussed further in future studies. The hepatic and splenic phosphorus levels were significantly increased only after rats were administrated phytin. The exact reason for this is unknown and it might be since the excessive magnesium intake affects the Na^+-K^+ dynamics in the blood, which in turn affects the amount of Na-dependent phosphate transporters located in the liver, resulting in increased

phosphorus accumulation in the organs.

In the present experiment, phytic acid clearly inhibited the absorption of magnesium and zinc, and may also have affected the absorption of copper. However, there was no effect on calcium absorption, which has been pointed out in the past [12]. In other words, phytic acid may affect the absorption of magnesium, but not calcium.

Neither soluble sodium phytate nor insoluble natural phytin had a major effect on the nutritional status of minerals when mineral intake was adequate. Despite changes in the content of zinc and magnesium in organs or metabolic balance, respectively, this can be considered as fluctuations in the values under the constant macroscopic nutritional state. Because minerals homeostasis is highly regulated *in vivo*, minor decreases in uptake can maintain homeostasis by enhancing absorption in the small intestine or reabsorption in the kidneys [59,60].

The effect on iron was observed only when phytin, which is phytic acid bound to magnesium, zinc, and manganese, was administered. The diversity in the results of studies examining the relationship between phytic acid and mineral absorption may be due to the diversity in the mineral composition of the phytic acid used.

Chapter 3

Nutritional availability of zinc contained in phytin in rats with adequate and low zinc status

Summary

In cereals and legumes, phytic acid is present as phytin bound with magnesium and zinc. In this study, the nutritional availability of zinc-containing phytin as a zinc source was compared between rats with an adequate zinc status and those with a low zinc status. Twenty-four male 4-week-old Wistar rats were divided into four groups (AZ, AZP, LZ, and LZP): the AZ group was fed a basal AIN93G diet containing adequate amounts of zinc (zinc concentration, 35.6 mg/kg), the AZP group was fed a phytin-supplemented basal diet (40.7 mg/kg), the LZ group was fed a low zinc diet (5.0 mg/kg), and the LZP group was fed a phytin-supplemented low zinc diet (10.2 mg/kg) for 4 weeks. The LZ group showed significantly lower feed intake, body weight, organ weights, organ and serum zinc concentrations, and serum alkaline phosphatase activity than the AZ group, indicating that they were moderately zinc-deficient. In the LPZ group fed the phytin-supplemented low zinc diet, the apparent absorption rate of zinc was lower than in the LZ group, but the amount of apparent absorption was higher than in the LZ group, and feed intake, body weight, and organ weights recovered to the same levels as in the AZ group. Organ and serum zinc levels in the LZP group were also significantly higher than in the LZ group. On the other hand, although the AZP group consumed more zinc than the AZ group, they excreted markedly more zinc in their feces, and the apparent amount of zinc absorption was significantly lower than that of the AZ group. In addition, serum and

femoral zinc concentrations and serum alkaline phosphatase activity were significantly lower in the AZP group than in the AZ group. These results indicate that zinc-containing phytin is utilized as a zinc source in the presence of a low zinc status, but inhibits zinc utilization when the zinc status is adequate.

Introduction

Zinc is the second most abundant essential trace element in the human body after iron, with approximately 90% of zinc stored in skeletal muscle and bone [59]. Zinc has been found to be necessary to maintain the function of over 300 enzymic reactions. Adult zinc intake shown in the 2019 Japanese National Health and Nutrition Survey [61] exceeds the estimated average requirement shown in the Dietary Reference Intakes for Japanese (2020) [62], with the exception of men over 75 years of age.

Phytic acid (myo-inositol hexaphosphate), which is abundant in grains and legumes, is a storage form of phosphorus in plants and is known to inhibit the absorption of minerals, including zinc, in the digestive tract in non-ruminant animals [47]. It was suggested that the stunting caused by zinc deficiency in Egyptian boys, in addition to low zinc intake, was related to the consumption of whole-grain bread, which contains high levels of phytic acid [9]. However, it is also considered that as long as the diet contains sufficient minerals, the effect of phytic acid intake from grains and legumes on mineral absorption is minor and negligible [26].

In Chapter 2, we conducted an animal nutritional experiment using different forms of phytic acid and preliminarily discussed the relationship between phytic acid and zinc absorption. Our previous studies showed that phytin from rice bran contains magnesium, zinc, and manganese [51,63]. This indicates that a significant portion of the zinc in grains and legumes is bound to phytic acid. Since zinc intake from grains and legumes accounts for about one-third of the total zinc intake in Japanese [61], it is important to investigate the nutritional availability of zinc in phytin. However, it is unclear whether the zinc bound to phytic acid has nutritional significance as a source of zinc. Although many animal studies have reported that phytate adversely affects the absorption of trace elements in

the diet [7,64] , no studies focusing on zinc bound to phytic acid have been reported to our knowledge. Here, we administered zinc-containing phytin to rats with an adequate or low zinc nutritional state to determine its effect on growth and the tissue zinc concentration, and conducted a one-week metabolic balance study.

Materials and methods

Reagents

Phytin derived from rice bran was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Analysis showed that the phytin used contained 19.2% phosphorus, 13.6% magnesium, 1.04% calcium, and 513 μg/g zinc. Ingredients of animal feed were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Animals and diets

Twenty-four 4-week-old male Wistar rats (mean ± SD of body weight, 81 ± 4 g) purchased from Shimizu Laboratory Supply Co. (Kyoto) were divided into four groups of equal body weight. Four kinds of diets (adequate zinc without phytin, adequate zinc with phytin, low zinc without phytin, and low zinc with phytin) based on the AIN93G formula [52] were prepared and fed to each group; the four dietary groups were named the adequate zinc group (AZ group), the adequate zinc-phytin-added group (AZP group), the low zinc group (LZ group), and the low zinc-phytin-added group (LZP group). Table 7 shows the composition of the experimental diets. The total zinc concentrations in the diets were measured as 35.6 ± 2.5 mg/kg (AZ group), 40.7 ± 1.3 mg/kg (AZP group), 4.97 ± 0.90 mg/kg (LZ group), and 10.2 ± 0.6 mg/kg (LZP group). The diets of the AZP and LZP groups contained more phosphorus and magnesium than those of the AZ and LZ

groups because of the addition of phytin-derived phosphorus and magnesium.

In order to administer the low zinc diet as early as possible, each group of rats was immediately fed each experimental diet without an acclimatization period. The rats were housed in a room at a room temperature of 22 ± 1°C with a 12-hour light/dark cycle (8:00-20:00). Body weight and food intake were recorded every two days. The feeding period lasted 28 days and rats were allowed access to tap water and experimental diets ad libitum. On day 23, each rat was transferred to an individual metabolism cage, and fecal samples were collected every two days for zinc balance studies. When the rearing period ended, blood was collected from the abdominal vena cava under deep anesthesia with isoflurane (MSD Animal Health, Tokyo, Japan) under non-fasting conditions, and the blood samples were centrifuged at 2,000 × g for 15 minutes to obtain serum. Organs were collected and weighed, frozen in liquid nitrogen, and stored at –30°C until analysis.

The experimental protocol followed the Guide for the Care and Use of Experimental Animals issued by the Prime Minister's Office of Japan and approved by the Animal Ethics Committee of Kansai University (Approval No.: 2203).

Analysis of metals

Portions of the liver, kidney, testis, spleen, femur, and serum collected as well as the experimental diets were digested using concentrated nitric acid. The digests were quantitatively diluted with ultrapure water and filtered through a 0.45-μm filter. The zinc concentration of the sample solution was determined using a flame atomic absorption spectrophotometer (AA-7000, Shimadzu Co., Kyoto, Japan) or inductively coupled plasma mass spectrometer (ICPMS-2030, Shimadzu Co., Kyoto, Japan). Phosphorus and magnesium in the experimental diets were measured by vanadomolybdate absorption

spectrophotometry [65] and flame atomic absorption spectrophotometry, respectively. In ICPMS analysis, ^{103}rhodium was used as an internal standard.

Blood examination

For a portion of the serum and whole-blood samples, biochemical testing was contracted to a clinical laboratory providing testing services (Japan Medical Laboratory Co., Kaizuka, Japan).

Statistical analysis

The groups in the animal study were divided by two factors: zinc concentration in the diet and presence or absence of phytin addition. However, because phytin contains zinc, the addition of phytin increases the zinc concentration in the diet. In other words, the two factors are not independent. Therefore, one-way analysis of variance (ANOVA) was used for statistical analysis, rather than two-way ANOVA. For some results, data were transformed into ranks and Kruskal-Wallis tests were performed. If the results of one-way ANOVA or Kruskal-Wallis tests were significant ($p < 0.05$), Tukey's multiple comparisons were performed to test for differences between the groups. Statistical software IBM SPSS Statistics 27 (IBM Japan, Ltd., Tokyo, Japan) was used for these analyses.

Results

Table 8 summarizes body and organ weights at the end of the rearing period, as well as total feed intake and feed efficiency during the rearing period. The LZ group, which received the lowest zinc diet, had significantly lower feed intake and lower body and organ weights, while the LZP group, which received zinc-containing phytin in addition to the low zinc diet, showed increased feed intake and similar body and organ weights as the AZ and AZP groups. Feed efficiency tended to be higher in the LZ group, which had the lowest feed intake.

Zinc concentrations in serum and organs are summarized in Table 9. The LZ group showed the lowest zinc concentrations in serum and all organs. The decrease in zinc concentration in the femur and serum was particularly marked. On the other hand, the LZP group, which ingested zinc-containing phytin, showed higher zinc concentrations in serum and all organs than the LZ group, especially in the liver, spleen, and testis, which were similar to those of the AZ group. The AZP group, which consumed more zinc than the AZ group due to the addition of phytin to the diet, had higher zinc concentrations in the liver than the AZ group, but zinc concentrations in the kidney, spleen, and testis were similar to those of the AZ group, and lower than those of the AZ group in the femur and serum.

Table 10 shows the results of the zinc balance study. When comparing AZ and AZP groups, which have sufficient zinc in their diets, the zinc intake of rats in the AZP group was higher than in the AZ group due to the addition of phytin to the diet, as there was no difference in feed intake in the two groups. However, the AZP group excreted more zinc in the feces and had the lowest apparent absorption rate of the four groups. Furthermore, although the AZP group consumed more zinc than the AZ group, the amount of apparent

absorption was lower in the AZP group. On the other hand, the LZ group, which consumed the lowest amount of zinc, had the highest zinc absorption rate. The LZP group, which consumed more zinc than the LZ group due to the addition of phytin to the diet, had a lower apparent absorption rate than the LZ group, but a higher amount of apparent absorption.

Table 11 summarizes the results of blood biochemical tests. Significant differences between groups were observed in several items. There were several patterns in the appearance of differences. The first pattern was that the LZ group differed from the other three groups, with the LZ group having lower values for total protein and higher values for iron, transferrin saturation, and aspartate aminotransferase than the other three groups. The second pattern was that the AZ group had higher values than the other three groups for triacylglycerols, total cholesterol, and total lipids. The third pattern was that the AZP and LZP groups had higher values than AZ and LZ groups for phosphorus and magnesium. The zinc-containing enzyme alkaline phosphatase (ALP) showed specific changes, with the AZ group having the highest values, followed by the AZP group, while the LZ and LZP groups had much lower values. This pattern was similar to the pattern of intergroup variation in serum and femoral zinc.

Discussion

When casein or egg white is a primary protein source, the US National Research Council (US-NRC) recommends a diet containing approximately 12 mg/kg of zinc for weaning rats [66]. The LZ group in this study received a low zinc diet of about 5 mg/kg, which depended solely on the zinc contained in the casein used as a protein source. In the LP group, the rats showed reduced feed intake and growth retardation from the first week of feeding, and low organ weights and reduced organ and serum zinc concentrations were observed after the 4-week feeding period. In our previous study, feeding diets with a zinc concentration of 5.5 mg/kg, using egg white as the main protein source, caused a decrease in appetite and growth retardation [67] . In this experiment, relative testicular weight in the LZ group was not significantly lower than in the AZ group (data not shown). In other words, the LZ diet caused growth retardation, but did not lead to the reproductive dysfunction observed in severe zinc deficiency [68]. Furthermore, although food intake was reduced, the cyclic increase or decrease in food intake observed with severe zinc deficiency [69] was also not observed. Accordingly, it can be concluded that rats in the LZ group, which were fed diets with a zinc concentration of approximately 5 mg/kg, were indeed suffering from moderate zinc deficiency. However, since the feed efficiency of this group was higher than that of the other three groups, the growth retardation was not due to inadequate protein utilization in the body, but rather to an undernutritional state caused by a low feed intake, and the decreased concentrations of serum total protein and lipid-related components observed in the LZ group also reflect the state of undernutrition. Furthermore, the elevation in serum iron markers and aspartate aminotransferase activity observed in the LZ group should also be interpreted as a secondary effect of undernutrition.

The diet of the LZP group increased the zinc concentration to approximately 10 mg/kg with the addition of zinc-containing phytin. As a result, in the LZP group, the low levels of feed intake, body and organ weights, and serum parameters indicative of the nutritional status observed in the LZ group were restored to values comparable with those of AZ and AZP groups. In the balance study (Table 10), the amounts of apparent absorption of zinc in the LZP group was significantly higher than in the LZ group, clearly indicating that the zinc contained in the phytin was absorbed. These results show that a significant portion of the zinc contained in the phytin was effectively utilized in the LZP group.

On the other hand, although zinc concentrations in the organs of the LPZ group were elevated compared with the LZ group, zinc concentrations in the serum and femur as well as serum ALP activity were markedly lower than in the AZ and AZP groups, which had adequate zinc intake. Although the LZP group was not zinc-deficient to the point of loss of appetite, the zinc status may still be considered to be low. In an experimental study on induced zinc deficiency in rats, it was noted that when zinc was added stepwisely to a zinc-deficient diet (zinc content <1 mg/kg), recovery of body weight gain saturated at a dietary zinc concentration of 9 to 12 mg/kg [68]. Another study on zinc requirements in growing rats noted that less than 3 mg/kg of dietary zinc induces severe deficiency, from 3 to 6 mg/kg moderate deficiency, from 6 to 9 mg/kg mild deficiency, and from 9 to 15 mg/kg marginal deficiency [70]. These results are similar to the present experimental results.

The results of administering zinc-containing phytin in the presence of the zinc-sufficient condition differed significantly from the results of administering zinc-containing phytin under the zinc-deficient condition. That is, although the AZP group had

a higher zinc intake than the AZ group, there was little increase in organ zinc concentrations, and the femoral and serum zinc concentrations, as well as serum ALP, were lower than in the AZ group (Tables 9 and 11). In the zinc balance study, the AZP group excreted markedly more zinc in the feces than the AZ group, and absorbed less zinc (Table 10). These results indicate that when zinc-containing phytin is ingested when the zinc nutritional status is adequate, the inhibition of zinc absorption by phytic acid is marked. It is known that the expression of ZIP4, which is involved in zinc absorption in the small intestine, is markedly increased in the zinc-deficient state [71]. The different effects of zinc-containing phytin depending on the zinc nutritional status may mean that the inhibitory effect of phytate is less when expression of the transporter involved in zinc absorption is high, and the inhibitory effect of phytate is greater when the expression is low.

The concentration of serum lipid components was decreased in the AZP compared with AZ group (Table 11). Phytic acid can reduce the concentrations of serum lipids components has been reported in recent years [34], but the details of the mechanism are unclear. In this experiment, the addition of natural phytin affected lipid metabolism and changed the mineral composition of the diet, even without the use of bran and soy protein. The decrease in serum lipid concentrations appeared to be influenced by phytic acid as well as the nutritional status of zinc, and this part should be discussed further later (in Chapter 5).

The World Health Organization states that zinc is less available when the molar ratio of phytate to zinc exceeds 15 [72]. In fact, one study of dietary monitoring of young men showed that when the phytate to zinc molar ratio exceeds 15, zinc absorption is markedly inhibited and the zinc balance may even become negative [73]. Similar studies have been

conducted in rats and chicks, with both reaching similar conclusions [74,75]. However, in these experiments, the phytic acid concentration in the diet was high and the zinc concentration in the diet was also high, so it is likely that the inhibition of zinc absorption by phytic acid occurred more strongly than in reality. Furthermore, the phytic acid given was purified sodium phytate. However, sodium phytate does not occur naturally, and the phytin present in grains and legumes contains magnesium and zinc [63]. In other words, it would be unrealistic to discuss the effects of phytic acid in grains and legumes based on experiments using sodium phytate.

The molar ratio of phytic acid to zinc in the present study was 17 in the AZP group and 70 in the LZP group. If previous reports [72–75] are to be followed, inhibition of zinc absorption by phytic acid should be more pronounced in the LZP group. Indeed, the apparent zinc absorption rate of the LZP group was about 40%, which is lower than that of the LZ group (Table 10). However, this decrease in absorption was not sufficient to counteract the ameliorative effect of zinc-containing phytin on zinc-deficient rats. We hypothesize that the administration of zinc-containing phytin to zinc-deficient rats may cause the following series of changes in the gastrointestinal tract. First, when the zinc-containing phytin, or zinc phytate, enters the stomach, the low pH environment causes some of the chelated zinc to be released and the phytate itself becomes soluble. Next, when the zinc enters the duodenum and jejunum, the zinc in the digested material is efficiently absorbed from the small intestinal mucosa because the expression of ZIP4 is increased due to the zinc-deficient state [71]. Remaining unabsorbed zinc and endogenous zinc excreted from the gastrointestinal tract are bound to phytic acid in the ileum or large intestine and are not absorbed and excreted in feces. This results in a higher amount of zinc excretion in the feces in the LZP group and a lower apparent absorption rate. That is,

although inhibition of zinc absorption by phytic acid also occurs with low zinc nutrition, this inhibition occurs after most of the zinc has been absorbed in the duodenum or jejunum, so the symptoms associated with zinc deficiency may have been ameliorated in the LZP group.

Phytic acid has been considered one of the factors causing zinc deficiency since cases of zinc deficiency were reported by Prasad in Egypt [9]. In this Egyptian study, those who developed the deficiency were dependent on a diet consisting mainly of whole wheat bread and had little access to animal products. With such a diet, the source of zinc would have been wheat, and the zinc would likely have been zinc phytate. According to the results of the present experiment, in Egyptian boys with zinc deficiency, the phytin (zinc phytate) in whole grain bread did not inhibit zinc absorption, but rather should supplement zinc intake. Nevertheless, zinc deficiency could have occurred for the following reasons. Their routine diet was extremely unbalanced [9] and the intake of major nutrients was marginal to the requirements. Under such low nutritional conditions, the various defense mechanisms, involving increased protein synthesis that occurs in response to loading, often fail to operate. In other words, in the Egyptian case, low nutrition may have resulted in inhibition of zinc absorption by phytic acid as in the case of adequate zinc intake, without the increased expression of ZIP4 that should occur under low zinc conditions.

The purpose of this experiment was to qualitatively examine the potential of zinc-containing phytin to be a source of zinc, separately for low zinc status and zinc adequate status. The results, as summarized in Table 10, show that when the zinc status is low, the zinc in zinc-containing phytin functions to some extent as a source of zinc and improves the zinc status, but when the zinc status is adequate, the phytic acid has a strong effect

and acts in the direction of inhibiting zinc absorption. It has been believed that non-refined grains, even though they contain more zinc than refined grains, have a negative effect on the zinc status because phytic acid inhibits zinc absorption [9]. However, the present results suggest that intake of non-refined grains in low zinc status can improve the zinc status, even if the phytic acid intake is higher, because the zinc bound to phytic acid is available to some extent. On the other hand, intake of non-refined grains when the zinc status is adequate, even if zinc intake is increased, will result in less zinc absorption due to the greater effect of absorption inhibition by phytic acid. However, the decrease in zinc absorption due to phytic acid when the zinc status is adequate is small and does not cause a serious effect on zinc status.

In the present study, inorganic phosphorus and magnesium concentrations in serum were significantly increased in the AZP and LZP groups treated with phytin (Table 11). Similar increase in serum phosphorus concentration due to phytic acid administration was likewise observed in the experiment of Chapter 2 (Table 6). These suggest that a portion of phytic acid was hydrolyzed in the gastrointestinal tract, resulting in increased absorption of phosphorus and magnesium. Recently, it was reported that phytase secreted by the intestinal microflora improves zinc absorption in the large intestine [47]. On the other hand, it remains unclear whether phytase is secreted in the intestines of non-ruminant animals. Since phytate is the storage form of phosphorus in plants, phytates usually contain a high density of phosphorus [2,51]. The nutritional significance of phosphorus derived from phytic acid will be discussed in the next chapter.

Chapter 4

Effect of phytic acid administration on the growth, bone formation, and phosphatases in phosphorus-deficient rats

Summary

The fundamental importance of phosphorus for life is well recognized. Plant-based foods constitute the primary supply of phosphorus intake, with most phosphorus in these foods being found in the form of phytic acid. This research investigated the effects of phytic acid administered to rats in a phosphorus-deficient environment. Wistar rats were fed an AIN93G basal diet with gluten as the protein source and supplemented with 0.68% or 1.56% sodium phytate (total phosphorus content of 0.03%, 0.15%, 0.30%, respectively) for 4 weeks. Phytic acid administration substantially ameliorated the growth impairment caused by phosphorus deficiency. The serum phosphorus concentration reflected the phosphorus intake, with the highest values in the high phytic acid group, phytic acid group, and phosphorus-deficient group, in that order. Serum alkaline phosphates activity increased roughly twofold in the phosphorus-deficient group. Phytic acid intake substantially promoted the mineralization of rat femurs, and the calcium, magnesium, phosphorus, and zinc concentrations in femur increased in a phytic acid dose-dependent manner. These results indicate that the ability of rats in utilizing phytate appears to be related to the degree of phosphorus insufficiency. Additionally, phytic acid-bound phosphorus can be efficaciously assimilated and utilized by rats when phytate serves as the primary source of phosphorus intake in the diet.

Introduction

Phosphorus, a sixth abundant element in the human body, profoundly influences several essential biochemical processes including skeletal metabolism and nucleic acid synthesis, as well as energy metabolism through ATP production [76,77]. About 85% of total phosphorus is found bound to calcium and magnesium in hydroxyapatite crystals composed of bone and dental tissues while another 14% is distributed in soft tissues such as muscles and cell membranes, and approximately 1% exists extracellularly [62]. Phosphorus deficiencies have been reported to include hypophosphatemia, muscle weakness, growth arrest, and rickets [76,78–80]. Nonetheless, dietary deficiency in phosphorus is infrequent due to widespread distribution throughout food sources. Notably, based on data from the national health and nutrition survey in 2019, phosphorus intake amounts to around 1,007mg per day, primarily derived from grains and legumes at nearly 24% [61].

Phytic acid, a common compound in nature composed of inositol and orthophosphate, occurs often in grains and legumes serving as storage for phosphorus [2]. Considering the lack of phytase enzymes in the gut of animals, phytate-derived phosphorus has frequently been deemed impossible to digest, leading to reduced feed efficiency and waste of resources [2,6,7]. Nevertheless, there are reports of protein expression and activity of phytase in the mucosa of rat small intestine [58,81]. Recently, the interplay between phytic acid and gut flora has gained considerable interest, with some investigations suggesting that the colonic microbiota secrete phytase to hydrolyze the majority of phytic acid and facilitate the absorption of dietary micronutrients [82,83]. There is still a research gap in the mechanism of phytic acid breakdown in the animal intestine.

Our past studies found that the serum phosphorus level in rats modestly increases following uptake of diets supplemented with phytic acid [63,84]. This suggests an influence of phytic acid consumption on the nutritional state of phosphorus in rats. However, as many of the experimental feeds contained considerable amounts of phosphorus present in the proteins [7,63,82,84], experimentation solely utilizing phytic acid as a phosphorus source in analyses of its role in phosphorus metabolism remains unaccounted for.

The digestion and absorption of phosphorus mainly takes place in the gastrointestinal tract, under the influence of phosphatases (alkaline and acidic) [85–88]. Phosphatases are metal enzymes responsible for catalyzing phosphoric esters into inorganic phosphate, which plays a pivotal role in the formation of hard tissue [89–91]. For this study, various levels of sodium phytate were supplemented to phosphorus deficient basic feed using gluten as a protein source and the growth, blood examination, several mineral levels in soft tissues, degree of bone mineralization, and phosphatase status of the rats was assessed.

Materials and methods

Reagents and diets

Gluten derived from wheat (073-00575) and sodium phytate (099107) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Wheat gluten contains 76% crude protein and 0.168% phosphorus. The other ingredients for the animal feed were purchased from Oriental Yeast Co., Ltd.

The PD diet was a basal phosphorus-deficient diet (phosphorus 0.03%) prepared using wheat gluten as the protein source and replacing potassium dihydrogen phosphate in the AIN93G mineral mixture with potassium chloride. The other two diets (PA diet and HPA diet) were modified to a phytic acid diet with a low phosphorus concentration of 0.15% and a high-phytic-acid diet with an adequate phosphorus concentration of 0.30% by addition of sodium phytate, respectively. The composition of each diet is summarized in Table 12 Each diet had a protein concentration of 15.2% and was supplemented with lysine and threonine to prevent major differences in essential amino acid composition among diets.

Animals

The experimental protocol followed the Guide for the Care and Use of Experimental Animals issued by the Prime Minister's Office of Japan and approved by the Animal Ethics Committee of Kansai University.

Eighteen male Wistar/ST strain rats aged 4 weeks were divided into three groups with equal mean body weights and fed PD, PA, and HPA diets. The rats were allowed to access tap water and feed *ad libitum*. Water and feed intake and body weights were measured throughout the study period. After feeding for 4 weeks, the livers, kidneys,

femurs, femoral muscle, and blood were collected under isoflurane (Fujifilm Wako Pure Chemical Co., Tokyo) anesthesia. Some of the blood was centrifuged at 1,500 x g for 15 minutes to obtain serum. The livers and kidneys were frozen in liquid nitrogen and stored at –30˚C until analysis.

Analysis of metals

Portions of liver, kidney, femur, femoral muscle, and serum collected, as well as gluten were subjected to wet digestion using a mixture of HNO_3 and $HClO_4$ (5/1, v/v). The digests were diluted with ultrapure water and filtrated through a 0.45-μm filter. The standard metal element solutions and sample solutions were analyzed using an inductively coupled plasma mass spectrometer (ICPMS-2030, Shimadzu, Kyoto, Japan) and ^{103}rhodium was used as an internal standard. The inorganic phosphorus determination was performed using the vanadomolybdate method [65].

Blood examination

For a portion of the serum and whole-blood samples, biochemical testing was contracted to a clinical laboratory providing testing services (Japan Medical Laboratory Co., Kaizuka, Japan).

Statistical analysis

The effect of sodium phytate administration was examined by one-way analysis of variance, and when the effect was significant, a multiple comparison test using the Tukey method was performed. IBM SPSS Statistics 29 (IBM Japan, Ltd., Tokyo, Japan) was used as the software for statistical analysis; $p < 0.05$ was considered significant.

Result

The final body weights and organ weights, along with feed consumption and feed efficiency throughout the feeding period are summarized in Table 13. All these growth parameters were significantly higher in a dose-dependent manner after the administration of phytic acid.

The serum biochemical parameters are shown in Table 14. Serum inorganic phosphorus concentrations reflected the dietary phosphorus concentrations and were highest in the HPA group, PA group, and PD group, in that order. Serum alkaline phosphatase activity was significantly higher in the PD group compared to the other two groups that administered phytic acid. Serum calcium concentration waw significantly lower in the HPA group than the other two groups. On the other hand, the total cholesterol and total lipids were significantly lower, in the HPA group, compared to the PD group. Total iron binding capacity was significantly higher in the phytic acid administered PA and HPA groups than in the PD group, but serum iron was not significantly different among the three groups. Serum copper was higher in the PD group than the other two groups. No differences were observed among the groups for the other parameters.

Calcium, magnesium, phosphorus, iron, zinc, copper and manganese in liver, kidney and muscle are summarized in Table 15. In both the liver and kidneys, iron and manganese concentrations tended to decrease with the increasing phytic acid intake, and significantly higher accumulation observed in the PD group. In the muscle, the PD group had a higher calcium concentration and a lower phosphorus lower than the other two groups. The iron concentrations tended to be higher with the increasing phytic acid intake, with significant differences between the PD and HPA groups. The copper concentration was higher in the HPA group than the other two groups.

Table 16 shows the concentrations of calcium, magnesium, phosphorus, and zinc in the femur. All these were increased in a phytic acid dose-dependent manner; in other words, as dietary phosphorus concentration increased, there was a corresponding significant increase in calcium, magnesium, phosphorus, and zinc accumulation in the femur.

Discussion

In the present experiment, to ensure that the rats fell into the phosphorus-deficient state, gluten was employed as the protein source rather than casein, which is generally used in the AIN93G formulation [52]. This is due to the fact that casein is a typical example of phosphoprotein, wherein phosphoric acid is linked to multiple of the serine residues of the amino acids that compose the protein. In the standard refined feed of AIN93G, casein contributes almost half of the phosphorus content of the diet [52]. In a study comparing the effects of varying proteins in diet on rats, we discovered that gluten did not generate significant adverse effects on growth and health compared to casein [92]. Since the main aim of this study is to examine the utilization of phosphorus in phytic acid, it is necessary to ensure that the phosphorus from phytate was the predominant source of phosphorus supply to the diet, hence gluten, which is remarkably deficient in phosphorus, was incorporated in the diet components and lysine and threonine were added to meet the amino acid requirement.

The administration of phytic acid significantly improved growth rate and feed efficiency (Table 13). Low energy metabolism due to phosphorus deficiency was also improved. A comparable growth recovery was evident in an experiment in which phosphorus-deficient rats were supplemented with orthophosphoric acid for five days [78]. This suggests that phosphorus in the phytate form can be absorbed and utilized by rats in the phosphorus-deficient state akin to orthophosphate. In addition, in that study, although the relative weight of liver increased as we observed, the relative kidney weight remained unchanged. Conversely, in the present experiment, the relative kidney weight tended to decrease with elevated phytic acid concentration and was significantly lower in the HPA group than the other two groups (Table 13). We supposed that this could be a

compensatory renal hypertrophy instigated by chronic phosphorus deficiency in the DP and PA groups. It has been noted that after partial removal of kidneys, the remaining kidney enlarges to compensate for the fortified function [93]. The renal tubules are composed of renal tubular cells and are responsible for material exchange, including reabsorption of essential minerals from the raw urine [77]. In enduring phosphorus deficiency, under the joint influence of parathyroid hormone and FGF23, the renal phosphorus clearance is diminished and enhance phosphorus reabsorption to preserve phosphate homeostasis in the body. [94]. And persistent tubular reabsorption hyperfunction is likely to culminate in proliferation and hypertrophy of the urinary tubules [93]. Hence, this phenomenon dissipated in the HPA group with sufficient phosphorus provision.

The serum phosphorus concentration is typically employed as a critical indicator to evaluate the phosphorus nutritional status within the body [76]. In this experiment, severe hypophosphatemia was observed in the PD group, which was clearly a consequence of deficient phosphorus intake. In the PA and HPA groups, different degrees of rebound in serum phosphorus were observed according to the total phosphorus concentration in the feeds. In other words, according to the phytic acid levels, the phosphorus nutritional status of the rats was directly affected by the varying phytic acid concentration. Phytic acid is usually considered unavailable by non-ruminants [6,7]. However, the results in this experiment demonstrate that phytate not only ameliorates phosphorus deficiency but also supports the growth of rats as a resource of phosphorus intake.Comparing to our previous research conducted with feeds using the standard AIN93G formulation and combined with phytates, a particularly significant high phosphorus intake state was not observed [63,84]. The rationale behind this phenomenon remains unclear, but what is certain is that

it appears to occur solely in contexts where there are no other sources of phosphorus uptake that can be effectively utilized.

One study proposes that free phytate may penetrate the cell via pinocytosis *in vitro* [95]. However, the efficacy of phytate transportation by endocytosis is quite diminished, and even if absorbed in large quantities through the small intestine epithelial cells, phytate bearing six phosphate groups will accumulate in considerable quantities in the liver for decomposition, culminating in an elevation of phosphorus abundance in the liver. However, there was no increase in hepatic phosphorus in either the PA or HPA groups. Thus, the enhancement of phytate absorption and metabolism by endocytosis appears to be marginal.

The interaction of intestinal bacteria and phytic acid does not seem to be negligible. In this experiment, phosphorus deficiency is destined to considerably alter the composition of the gut flora, as phosphorus is a crucial element for the survival and proliferation of all organisms. Consequently, strains that can efficiently utilize phosphorus in the phytate form will possess the advantage of proliferation and gradually dominate the flora [83]. Despite this, the capacity of the colon to absorb the mineral remains inconclusive. Phosphate absorption in the colon appears to be physiologically irrelevant in most cases and is observed only under conditions of extremely high luminal phosphate concentration [76]. In a state of dietary phosphorus depletion, the expression of Npt2b protein in the intestine increase to enhance phosphorus absorption [87]. However, it was shown that Npt2b proteins are expressed mainly in the anterior half of the small intestine in rats; whereas their expression in the ileum is negligible [89]. Furthermore, phytate concentration in the HPA group was as high as 1.56%, and most trace element concentrations in the soft tissues and serum remained unchanged compared to the PD

group, with the reduction of stored iron in the liver but no change in the levels of serum iron and hemoglobin. This indicates that no trace element deficiency syndrome such as anemia occurred. The ability of phytic acid to chelate with divalent was greatly diminished by the hydrolysis, so that minerals absorption was improved. The nutrient absorption capacity of the cecum and colon is quite limited, and the small intestine is the most active area for all types of trace micronutrients. Hence, it can be inferred that the utilization of phytic acid in this experiment should be primarily focused on digestion and absorption in the small intestine.

Phytate solubilization is obligatory for its digestion in the intestine or direct absorption. Numerous studies have shown that phytates have a tendency to chelate with metal cations and precipitate in the gut [6,7]; however, our prior investigation demonstrated that the state of phytate in the intestine is rather intricate involving pH, ionic strength, coexisting substances in the diet, and even the pre-uptake status of phytate (solubility, degree of refinement, and mineral composition) [51]. Thus, in this research, purified and water-soluble sodium phytate was fed to rats, theoretically ensuring that phytate could persist appreciably soluble in the gastrointestinal tract.

Serum ALP may derive from various type of ALP secreted by tissues throughout the body, such as tissue non-specific ALP in bone, liver, and kidney, and intestinal alkaline phosphatase (IAP) in the small intestine [85]. In the PD group, serum ALP activity was significantly higher than the other two groups. There exist various explanations for the change in ALP activity, such as zinc deficiency [90], abnormal liver function [96], and disrupted bone metabolism [97]. As mentioned above, the high dosage of phytic acid did not significantly affect the nutritional status of the trace elements in this experiment, and no significant changes in serum zinc concentrations were observed in the three groups

(Table 14). Consequently, the fluctuations in serum ALP also did not appear to be caused by zinc deficiency resulting from phytic acid intake. Furthermore, no significant changes in aspartate aminotransferase and alanine aminotransferase activity were observed (Table 14), implying that ALP augmentation in the PD group is unlikely to originate from hepatic inflammation.

Femur formation was substantially enhanced in the phytate-administered PA and HPA groups compared to the PD group. Although many reports indicating that dietary phosphorus restriction increase bone mineral content [98], it is also recognized that bone mineralization is hindered and bone resorption is accelerated in the presence of hypophosphatemia [79]. It is essential to recognize that phosphorus plays an instrumental role in supporting skeletal mineralization and intermediary metabolism in all bodily tissues, and thus minor modifications in experimental conditions can directly affect the phosphorus nutritional status of experimental subjects, resulting in substantially disparate results. In the present experiment, rats in the PD group could obtain extremely meagre amounts of phosphorus from gluten (approximately 2.5 mg/d), which was about 20 times less than the total phosphorus uptake in the HPA group (about 44 mg/d). It is predictable that in this state of extreme phosphorus deficiency, the balance between bone formation and bone resorption would be completely disrupted. Expansion of hydroxyapatite requires sufficient calcium and phosphate outside the matrix vesicles [99]. Phosphorus intake from diet is far from sufficient to support the functioning of major organs such as the liver and kidneys and the synthesis and metabolism of ATP, so there would be minimal excess phosphorus available to participate in the formation of hydroxyapatite crystals in the femur, and therefore there would be no superfluous pyrophosphate around the bone tissue that would need to be broken down by the bone specific alkaline phosphatase

(BAP) in order to promote mineralization of the femur. Osteoclasts affected by parathyroid hormone will proliferate and continue to enhance the bone resorption effect to provide phosphorus to maintain soft tissue and extracellular fluid phosphate levels [80]. Lower phosphorus levels and higher calcium levels were observed in the muscle groups around the femur in the PD group (Tables 15 and 16). Hydroxyapatite in the femur was continuously broken down and phosphate was transported to the soft tissues to uphold phosphorus requirements, and the resulting calcium ions are reflected in increased skeletal muscle and serum calcium concentrations.

A study demonstrated a notable inverse correlation between phosphorus consumption and serum BAP activity [100]. It has also been found that rats fed low phosphorus diets show a decrease in bone-type ALP activity [91]. Consequently, it can be inferred that in a phosphorus-deficient condition, BAP activity should also be decreased in rats. Taken together, the above analysis of ALP status suggests that the most direct cause of the increase in serum ALP activity seems most plausibly attributed to the activation of IAP, which is associated with the breakdown of phosphate compounds in the gut. Phytic acid is an inositol hexaphosphate whose structural features of a monophosphate bond conform to ALP's prerequisite for an enzymatic substrate. Remarkably, the hydrolysis of phytic acid has predominantly been discussed in conjunction with the phytase of acid phosphatase, yet few studies have explored the relationship between ALP and phytic acid, even though IAP is a marker enzyme in the small intestine. This may be due to the fact that the pH in small intestinal is weakly acidic to neutral, whereas ALP is more active in alkaline surroundings, and that in alkaline environments phytate is typically considered to be in a precipitated state and incapable of engaging in biochemical reactions. In fact, there is no alkaline environment in the living

organism (pH 10-11) in which ALP is optimally active, but ALP is still highly involved in biochemical reactions throughout the body [86]. Moreover, as stated previously, the solubility of phytates is highly variable, and a significant amount of phytates may be dissolved in the chyme. A study reported that various forms of phosphatidylinositol (1-6) can be hydrolyzed as ALP substrates [101]. Therefore, if phytate can be hydrolysed by ALP, then under the conditions of this experiment, i.e., phosphorus deficiency and active IAP, phytic acid could indeed be viewed as an excellent source of phosphorus consumption.

There is much controversy regarding the role of intestinal phytase. However, those findings reveal that phytase activity is extremely low and does not efficiently hydrolyze phytic acid into orthophosphate [58,81]. When micronutrients become deficient, mechanisms are usually developed to enhance the absorption of the corresponding micronutrients. However, due to the absence of phosphorus deficiency trials, it remains uncertain whether a mechanism exists to enhance phytase secretion *in vivo* when solely phytic acid is present as a source of phosphorus intake.

Furthermore, during bone resorption, osteoclasts secrete large amounts of tartrate-resistant acid phosphatases (TRAPs), which is implicated in the degradation of hydroxyapatite to promote bone resorption [88]. TRAPs secreted by osteoclasts follow the bloodstream to various tissues and involved in the hydrolysis of various phosphate esters and anhydrides [88]. Secretion of TRAPs has also been observed in soft tissues such as the liver and small intestine [102]. To date, no studies related to phytate and acid phosphatase in animals have been reported. However, from the present results, there is a considerable possibility that TRAPs may be involved in the hydrolysis of phytic acid.

Chapter 5

Effect of phytic acid administration on the zinc concentration, uric acid biosynthesis, and serum lipid components in rats

Summary

The effects of different doses of phytic acid intake on the absorption of several minerals including zinc, iron, copper, calcium, magnesium, manganese and molybdenum, the biosynthesis of uric acid, and the serum lipid components were examined. Weaning male Wistar rats were fed a basal AIN93G diet or the basal diet supplemented with 0.5% or 1.0% sodium phytate for 4 weeks. Phytic acid administration reduced the serum and femur zinc levels in a dose-dependent manner but did not affect the liver and kidney zinc levels. In addition, iron and calcium in the liver and manganese concentrations in the liver and kidney were significantly reduced by phytic acid administration. In phytic-acid-administered rats, the molybdenum concentration and xanthine oxidase activity in the liver and the serum uric acid decreased in a dose-dependent manner. In addition, phytic acid administration also reduced the serum lipid components including triacylglycerol and total cholesterol.

Introduction

Phytic acid (inositol hexaphosphate) is a major phosphorus storage form present in plant tissues, such as seeds, and is often present as phytin, which is a mixed salt of calcium and magnesium [2]. Therefore, a high intake of beans and unrefined grains leads to a high intake of phytic acid. Phytic acid strongly chelates to many metal ions, which may inhibit their intestinal absorption [50]. In particular, it was believed that a large intake of phytic acid causes zinc deficiency, as Egyptian boys who had growth inhibition due to zinc deficiency ate whole grain breads high in phytic acid on a daily basis [9]. However, zinc deficiency caused by phytic acid occurs only in the case of insufficient zinc intake, and it is not necessary to worry about zinc deficiency associated with phytic acid when the zinc intake is sufficient [26].

In recent years, there have been increasing reports that phytic acid intake has a positive effect on maintaining good health [34]. Epidemiological studies and animal experiments have shown that phytic acid is effective in preventing neurodegenerative diseases [22], renal stones [103], several types of cancer [21,104,105], and fatty liver [106]. Furthermore, it has been reported that phytic acid lowers the serum uric acid concentration [25,33].

Thus, phytic acid is now expected to be a functional ingredient that has a positive effect on health, rather than an antitrophic factor that inhibits mineral absorption. However, many studies examining the effects of phytic acid intake are one-sided, and few examine both positive and unfavorable effects at the same time. In this study, in order to re-evaluate the health effects of phytic acid, phytic acid was administered to rats, and the effects on the concentrations of several minerals including zinc, as well as the serum uric acid and lipid components were examined at the same time. In addition, the effects of

phytic acid administration on the liver xanthine oxidase (XOX) activity involved in the production of uric acid [107] and the concentration of molybdenum, which is an essential component of XOX [108], were also investigated.

Materials and methods

Animals and diets

The experimental protocol followed the Guide for the Care and Use of Experimental Animals issued by the Prime Minister's Office of Japan and was reviewed and approved by the Animal Ethics Committee of Kansai University (Approval No. 2005).

Eighteen 4-week-old male Wistar rats (SHIMIZU Laboratory Supplies Co., Kyoto) were divided into 3 groups. One group (control group) was fed a basal AIN93G diet [52], and the other two groups (0.5PA group and 1.0PA group) were fed the basal diet containing 0.5 or 1.0% sodium phytate (Sigma-Aldrich, St. Louis), respectively. Sodium chloride was added to the diets of the control group and the 0.5PA group in order to equalize the sodium intake of each group. During the feeding period, the animals ingested the diets and water (tap water) *ad libitum*. After feeding for 4 weeks, the liver, kidney, femur, and blood were collected under isoflurane (Fujifilm Wako Pure Chemical Co., Tokyo) anesthesia. The blood was centrifuged at 1500 x g for 15 minutes to obtain the serum. The livers, kidneys and femurs were frozen in liquid nitrogen and stored at –30˚C until analysis.

Analysis of metals

Approximately 1 g of liver, kidney, and femur were heated with 5 mL of nitric acid until there were no solids. The obtained solution was filtered through a 0.45 μm filter, and the concentrations of zinc, iron, copper, manganese, molybdenum, calcium, and magnesium were determined using an atomic absorption spectrophotometer (AA-7000, Shimadzu, Kyoto) or an inductively coupled plasma mass spectrometer (ICPMS-2030, Shimadzu, Kyoto). In the analysis with ICPMS, ^{45}scandium was used as an internal

standard. The serum zinc and copper levels were measured using a commercial kit (Metalloassay LS, Metallogenics Co., Chiba).

Assay of hepatic xanthine oxidase (XOX) activity

About 1 g of liver was homogenized with 9 mL of saline. The homogenate was centrifuged at 8000 x g for 20 minutes and the XOX activity in the supernatant was measured as follows [109]. To 200 μL of the homogenate, 800 μL of 0.5 mM xanthine solution and 3 mL of 0.1 M Tris-HCl buffer (pH 7.4) were added, and the mixture was incubated for 20 minutes at 37˚C. After the incubation, 1 mL of 30% perchloric acid was added and centrifuged, and the uric acid contained in the supernatant was determined by high performance liquid chromatography (HPLC). The condition of HPLC was as follows: equipment, LC-20Ai (Shimadzu, Kyoto); column, Develosil ODS-HG (4.6 mm φ x 250 mm, Nomura Kagaku, Seto); mobile phase, 20 mM sodium phosphate buffer (pH 3.0)/acetonitrile=99/1 (v/v); column oven, 30˚C; flow rate: 1.0 mL/minute; detection, absorbance at 292 nm (SPD-20A, Shimadzu, Kyoto). The activity of XOX that produces 1 μmol of uric acid per minute was defined as 1 unit. Protein in the supernatant of the liver homogenate was determined by Lowry's method [110].

Blood examination

Uric acid, triacyl glycerol (TAG), total lipid (TL), total cholesterol (TCHOL), HDL-cholesterol (HDL-CHOL) concentrations as well as other relevant biochemical parameters were measured with an automatic biochemistry analyzer (Olympus AU5431; Olympus Co., Tokyo) by Japan Medical Laboratory Co. (Kaizuka).

Statistical analysis

For each measurement, the differences among the groups were tested by one-way ANOVA followed by Tukey-Kramer's multiple comparison test. GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego) was used as the statistical analysis application.

Results

The body weights and organ weights at the end of the feeding period, as well as feed consumption and feed efficiency during the feeding period are summarized in Table 17. There was no difference in growth among the experimental groups.

Table 18 shows the zinc concentrations in the serum and organs of each experimental group. There was no difference in the zinc concentrations in the liver and kidney among the experimental groups, but the zinc concentrations in the serum and femur were significantly lower in the two groups administered phytic acid than in the control group. Increased phytic acid doses tended to lower the serum and femoral zinc levels.

Table 19 summarizes the concentrations of several metals in the serum, liver, and kidney. As with zinc, the concentration of some metals was reduced by phytic acid administration. Concentrations of iron and calcium in the liver and manganese in the liver and kidneys were significantly reduced by phytic acid administration.

Figure 3 shows the serum uric acid concentration, liver XOX activity, and molybdenum concentration. All of these three parameters were significantly lower in a dose-dependent manner after the administration of phytic acid.

Table 20 summarizes the concentrations of the serum lipid components (TAG, TL, TCHOL, HDL-CHOL) in each group. Phytic acid administration significantly reduced the values for all the measured components. However, unlike the serum and femoral zinc or serum uric acid, no further decrease was observed when the dose of phytic acid was increased.

The serum biochemical parameters and hemoglobin concentrations are shown in Table 21. Serum urea nitrogen levels tended to be increased in the 0.5 PA group. On the other hand, no significant difference was observed in complete blood count.

Discussion

In this experiment, the serum and femur zinc levels were significantly reduced in rats fed diets supplemented with phytic acid (Table 18). This suggests that phytic acid inhibited the absorption of zinc. However, the zinc concentration in the liver and kidney did not change even after the administration of phytic acid, and a decrease in food intake and growth inhibition associated with zinc deficiency did not occur (Table 17). In conjunction with our discussion of phytic acid in the previous chapters, we can conclude that regardless of its form (soluble phytate or natural phytin) phytic acid does have the ability to inhibit zinc absorption. Yet, after various validations [63,84], we noticed that this absorption impediment doesn't result in zinc deficiency. This is probably because the AIN93G used as the basal diet contains 30 μg/g of zinc, which is sufficient for growth. And high intake of water-soluble phytates also does not lead to marginal zinc deficiency as the stabilization of the zinc pool in the body is not disturbed [59].

The concentrations of some minerals other than zinc in serum and organs also decreased (Table 19), and were observed mainly in the 1.0PA group. It can be inferred that phytic acid also inhibited the absorption of minerals other than zinc, and this effect appears to be enhanced with increasing phytic acid intake. Similarly to zinc, reduced absorption of these minerals did not lead to the manifestation of a severe deficiency. The relationship between phytic acid and minerals was also explored in Chapter 2 and it was suggested that the manganese co-existing in phytin affected iron absorption. The fact that the changes in serum inorganic phosphorus were not significant may indicate that phytic acid hydrolysis was low in this experiment, and then the metal chelating power of phytic acid would have been largely retained. For example, in the case of iron, administration of phytic acid decreased the iron concentration in the liver, but did not decrease the

hemoglobin concentration or the transferrin saturation rate of serum (Table 21). That is, because the iron concentration in the basal AIN93G diet was sufficiently high, the decrease of iron absorption by phytic acid did not lead to the development of iron deficiency.

It has been reported that the intake of phytic acid reduces serum uric acid in humans [25,33]. In the present study as well, it was confirmed that the serum uric acid concentration was decreased in the rats to which phytic acid was administered (Figure 3). For the decrease in serum uric acid caused by phytic acid, a mechanism has been proposed in which the intestinal absorption of a purine base that is the source of the serum uric acid is reduced because phytic acid inhibits the conversion of nucleic acids in the diet to purine bases [111]. However, in the present study, rats were fed a nucleic-acid-free diet. Therefore, the decrease in the serum uric acid concentration cannot be explained by the decrease in the amount of purine base absorbed. It has been reported that phytic acid itself inhibits the XOX activity involved in uric acid production [112], but in Chapter 4 we also showed that phytic acid is difficult to be taken up efficiently by cytosis, it is unlikely that it will reach a concentration that inhibits the XOX activity in the liver.

We measured the liver XOX activity and molybdenum concentration, and found that both decreased depending on the dose of phytic acid (Figure 3). Since molybdenum has a strong affinity for phosphate [113], it is quite possible that the phosphate group of phytic acid binds to molybdenum in the intestinal tract in phytic-acid-administered rats, and the absorption of molybdenum is reduced. It is considered that as a result of phytic acid inhibiting molybdenum absorption, the biosynthesis of XOX, which is a molybdenum-containing enzyme, decreased, and the production of uric acid decreased. In this regard, we have observed that a decrease in the liver molybdenum concentration leads to a

decrease in the XOX activity in rats administered tungsten, which antagonizes molybdenum *in vivo* [114].

Administration of phytic acid reduced the serum lipid components in addition to serum uric acid (Table 19). The mechanism by which phytic acid lowers the serum lipid content is unknown. Some studies have hypothesized that the decrease in serum lipid levels is a secondary effect of phytic acid intake affecting minerals nutritional status [23,24]. In other words, this has been linked to malnutrition. We have demonstrated that phytic acid intake within a certain amount does not cause malnutrition due to mineral deficiencies. In addition, similar decreases in serum lipids have been found in Chapter 3 using natural phytin [84], and in conjunction with the results of this experiment, we are more certain that this is an effect from phytic acid itself.

In this experiment, no change was observed in the serum inorganic phosphate concentration of the rats to which phytic acid was administered (Table 21), and it can be estimated that phytic acid is hydrolyzed to a lesser extent. This may seem to contradict the experimental results in Chapters 2 and 3. Indeed, from past experiments, we've noticed that the utilization of phytic acid is greatly influenced by the current nutritional status. The uncertainty surrounding the status of phytic acid *in vivo* is causing this outcome.

We can attribute the lipids-lowering effect of phytic acid to the following two aspects. On one hand, when phytic acid is hydrolyzed to a lesser extent retaining a strong binding capacity, at which point phytic acid is hardly absorbed, it is most likely that phytic acid interfered with the absorption of several dietary components including lipids and carbohydrates in the gastrointestinal tract [15,115]. On the other hand, when phytate is hydrolyzed to a higher degree, binding capacity is low and free minerals and inositol are released. At this point, minerals that would otherwise be difficult to utilize may enhance

nutritional status and improve lipid metabolism [116]. Moreover, inositol, once considered a member of the vitamin B complex, is very easily absorbed and has been shown to improve dyslipidemia [117].

Conclusion

Phytic acid, which is the ester of six phosphoric acid molecules bound to inositol, is present as phytin bound to several minerals in cereals and legumes. Until recently, dietary phytic acid has been recognized as an anti-nutritional substance that precipitates minerals such as zinc and inhibits mineral absorption. Nevertheless, recent research has indicated that phytic acid intake improves serum lipid and uric acid levels and functions in disease prevention and treatment. Most studies assessing the impact of phytic acid intake have been conducted from a biased perspective, discerning it either as an antinutrient or functional component; hence, a need exists for a reevaluation of phytic acid consumption from a neutral standpoint. This study utilized both insoluble phytin, which bonds with several minerals, and the soluble sodium phytate often incorporated into nutritional supplements, to probe the effects of phytic acid on mineral nutrition and its functional attributes, such as reducing serum uric acid, primarily through nutritional experiments employing rats.

Chapter 1 described the effect of preparation methods on the mineral composition of phytin. Phytin was prepared from rice bran and wheat bran by several methods. When calcium salt was added during neutralization, most of the phytic acid was recovered as magnesium phytate or calcium phytate, respectively. Furthermore, when ethanol was added to precipitate, phytic acid was recovered without these minerals. This means that phytic acid prepared from rice bran and other materials has different mineral compositions depending on the preparation method. The animal studies on phytic acid that have been conducted so far do not mention the minerals bound to the phytic acid used, and it was considered necessary to reassess this aspect.

Chapter 2 described the effect of sodium phytate and phytin on mineral nutritional

status. Rats were fed either water-soluble sodium phytate or insoluble phytin bound with magnesium, zinc, and manganese as in rice bran. Decreases in tissue or serum concentrations of several trace elements in phytic acid-administered rats. Decreased iron absorption in the phytin group was not observed in the sodium phytate group. In rats fed sodium phytate or phytin, which have a higher intake of phosphorus than the control group, a tendency for increased phosphorus uptake was observed. Furthermore, rats fed phytin with higher zinc intake had higher serum and femur zinc concentrations than rats given sodium phytate. These findings indicate that the inhibition of iron absorption, which has been considered to be an effect of phytic acid, may be due to the minerals bound to phytic acid, and that part of the phosphoric acid in phytic acid and zinc bound to phytic acid may be available.

Chapter 3 described the effect of zinc-containing phytin on zinc nutrition. To examine the effect of phytin containing zinc, a constituent of zinc present in rice bran and wheat bran, on zinc nutrition, rats with adequate or low zinc status were administered zinc-containing phytin. In rats with adequate zinc status, inhibition of zinc absorption by phytic acid was observed, but did not lead to zinc insufficiency. On the other hand, in rats with low zinc status, administration of zinc-containing phytin eliminated the growth inhibition caused by low zinc status and improved zinc nutritional status. This indicates that zinc bound to phytic acid is actively utilized as a zinc source in cases of zinc deficiency. It has been believed that rice bran and wheat bran may cause severe low zinc status because they contain phytic acid, but rather, they have a great potential to alleviate zinc insufficiency.

Chapter 4 described the significance of phytic acid as a phosphorus source. To examine the availability of phosphorus in phytic acid, sodium phytate was administered

in a dose-increasing to phosphorus-deficient rats. Growth inhibition and impaired bone formation brought about by phosphorus deficiency were greatly improved by sodium phytate administration. Since phosphatase activity was significantly increased in phosphorus-deficient rats, it was concluded that phosphate bound to inositol is released and effectively utilized as a phosphorus source by the increase in phosphatase activity during phosphorus deficiency.

Chapter 5 describes how phytic acid functions to reduce serum uric acid levels. Rats with adequate mineral nutritional status were administered sodium phytate in a dose-increasing manner. Phytic acid inhibited the absorption of some minerals, but not strong to induce deficiency. On the other hand, sodium phytate administration decreased serum uric acid levels in a dose-dependent manner. Liver molybdenum concentration and xanthine oxidase (XOX) activity, a molybdenum-containing enzyme, were also decreased in a dose-dependent manner by phytic acid administration. It is clear that the decrease in serum uric acid reported previously with phytic acid administration was caused by the decrease in XOX activity associated with the inhibition of molybdenum absorption by phytic acid.

The relationship between phytic acid and micronutrients is very complex, a fundamental understanding of the concentrations and molecular species of nutrients and other chemicals in the diet will lead to a better understanding of the interaction between micronutrients and phytic acid. It can be concluded that the relationship between phytic acid and minerals varies according to mineral nutritional status. In other words, when mineral nutritional status is adequate, phytic acid inhibits the absorption of some minerals, but to a lesser extent and does not lead to a decrease in mineral nutritional status. On the other hand, it was demonstrated in rats lacking zinc or phosphorus that phytate can be

used as a mineral source when mineral nutritional status is low. Furthermore, it was shown that inhibition of molybdenum absorption was engaged in the serum uric acid-lowering effect of phytic acid. Hence, it can be concluded that phytic acid is a promising functional ingredient.

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Tables and figures

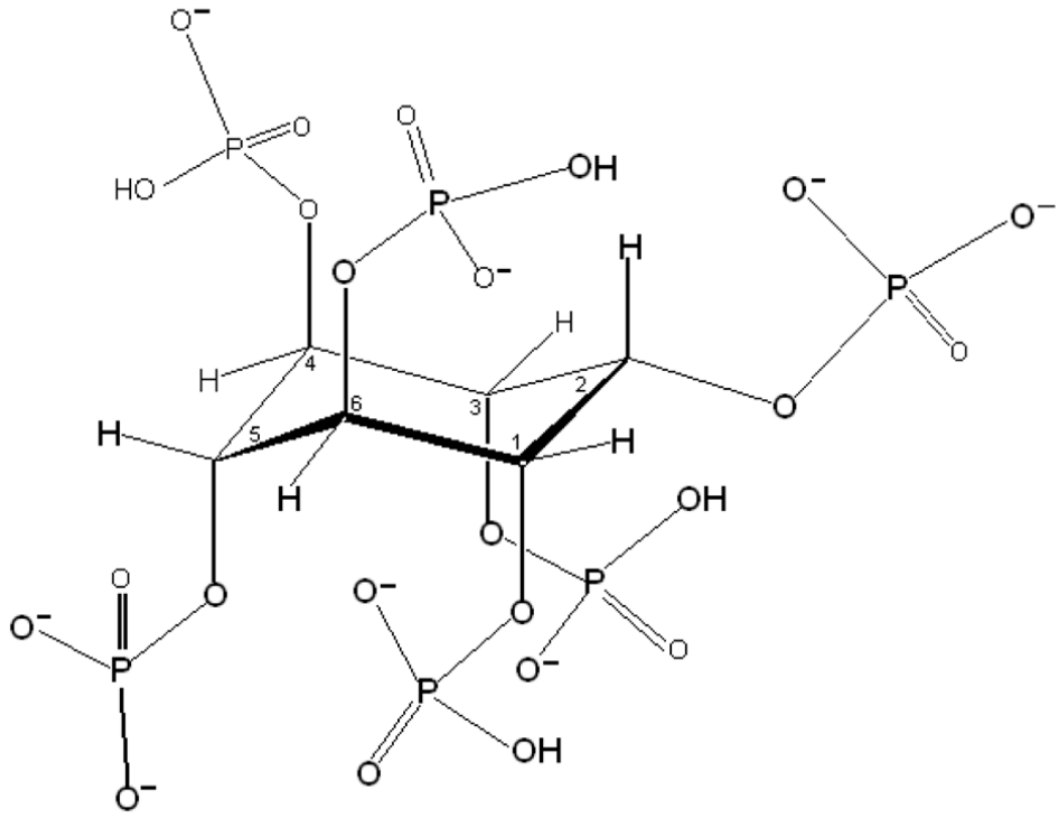

Figure 1 myo-inositol-1,2,3,4,5,6-hexakisphosphate [2]

Table 1 Mineral composition in bran in Chapter 1

Minerals	Measured value (mg/g)*		Reported value (mg/g)	
	Rice bran	Wheat bran	Rice bran**	Wheat bran***
P	21.2 ± 0.2 (66.5)	7.10 ± 0.40 (69.8)	20.0 (68.6)	10.1 (58.6)
Mg	9.84 ± 0.15 (30.8)	2.19 ± 0.08 (21.5)	8.5 (29.2)	6.11 (34.8)
Ca	0.49 ± 0.01 (1.5)	0.55 ± 0.02 (5.4)	0.35 (1.2)	0.73 (4.2)
Mn	0.21 ± 0.01 (0.7)	0.15 ± 0.02 (1.5)	0.15 (0.5)	0.11 (0.6)
Zn	0.08 ± 0.01 (0.2)	0.06 ± 0.01 (0.6)	0.06 (0.2)	0.07 (0.4)
Fe	0.08 ± 0.01 (0.2)	0.12 ± 0.01 (1.2)	0.08 (0.3)	0.11 (0.1)
Total	31.9 ± 0.3	10.2 ± 0.5	29.14	17.23

Values in parentheses are percentages (%) of the total (P+Mg+Ca+Mn+Zn+Fe) amount.

* Values are means ± SD (n=3).

** Listed value in the Standard Tables of Food Composition in Japan (eighth revised edition) [45].

*** Quoted from US food database [46].

Table 2 Mineral composition of phytin produced from rice bran or wheat bran by several methods in Chapter 1

Minerals	Produced from rice bran (mg/g)				Produced from wheat bran (mg/g)			
	Alkali addition	Calcium salt addition	Magnesium salt addition	Ethanol precipitation	Alkali addition	Calcium salt addition	Magnesium salt addition	Ethanol precipitation
P	84.4 ± 7.5	118.5 ± 1.5	148.6 ± 9.9	97.3 ± 3.8	80.5 ± 6.6	108.9 ± 0.5	140.3 ± 3.3	101.1 ± 16.2
	(61.8)	(40.1)	(54.9)	(77.1)	(56.8)	(36.3)	(51.8)	(94.5)
Mg	47.3 ± 4.3	7.29 ± 0.40	117.3 ± 3.4	24.9 ± 1.4	35.8 ± 2.5	3.13 ± 1.47	113.5 ± 4.3	1.05 ± 0.14
	(34.7)	(2.5)	(43.1)	(19.7)	(25.2)	(1.0)	(42.2)	(1.0)
Ca	2.64 ± 0.26	167.4 ± 12.6	2.42 ± 0.07	1.91 ± 0.27	17.5 ± 1.0	178.7 ± 0.6	10.3 ± 0.6	1.76 ± 0.16
	(1.9)	(56.8)	(0.9)	(1.5)	(12.3)	(59.7)	(3.8)	(1.6)
Mn	1.25 ± 0.13	1.17 ± 0.06	0.80 ± 0.07	0.77 ± 0.07	4.30 ± 0.36	6.37 ± 0.40	1.79 ± 0.02	0.16 ± 0.02
	(0.9)	(0.4)	(0.3)	(0.6)	(3.0)	(2.1)	(0.7)	(0.1)
Zn	0.43 ± 0.03	0.44 ± 0.03	0.61 ± 0.01	0.23 ± 0.07	1.49 ± 0.13	1.02 ± 0.05	1.35 ± 0.16	0.05 ± 0.01
	(0.3)	(0.1)	(0.2)	(0.2)	(1.1)	(0.3)	(0.5)	(< 0.1)
Fe	0.45 ± 0.06	0.22 ± 0.04	1.32 ± 0.12	1.08 ± 0.04	2.21 ± 0.16	1.53 ± 0.20	2.42 ± 0.07	2.78 ± 0.89
	(0.3)	(0.1)	(0.5)	(0.9)	(1.6)	(0.5)	(0.9)	(2.6)
Total	136.5 ± 12.3	294.1 ± 12.8	271.2 ± 9.8	126.2 ± 5.2	141.8 ± 10.3	300.0 ± 5.5	269.9 ± 0.8	106.9 ± 17.2

For each mineral, the upper values are the means ± SD (n=3) of the content (mg/g), and the lower values in parentheses are the percentages (%) of the total (P+Mg+Ca+Mn+Zn+Fe) amount.

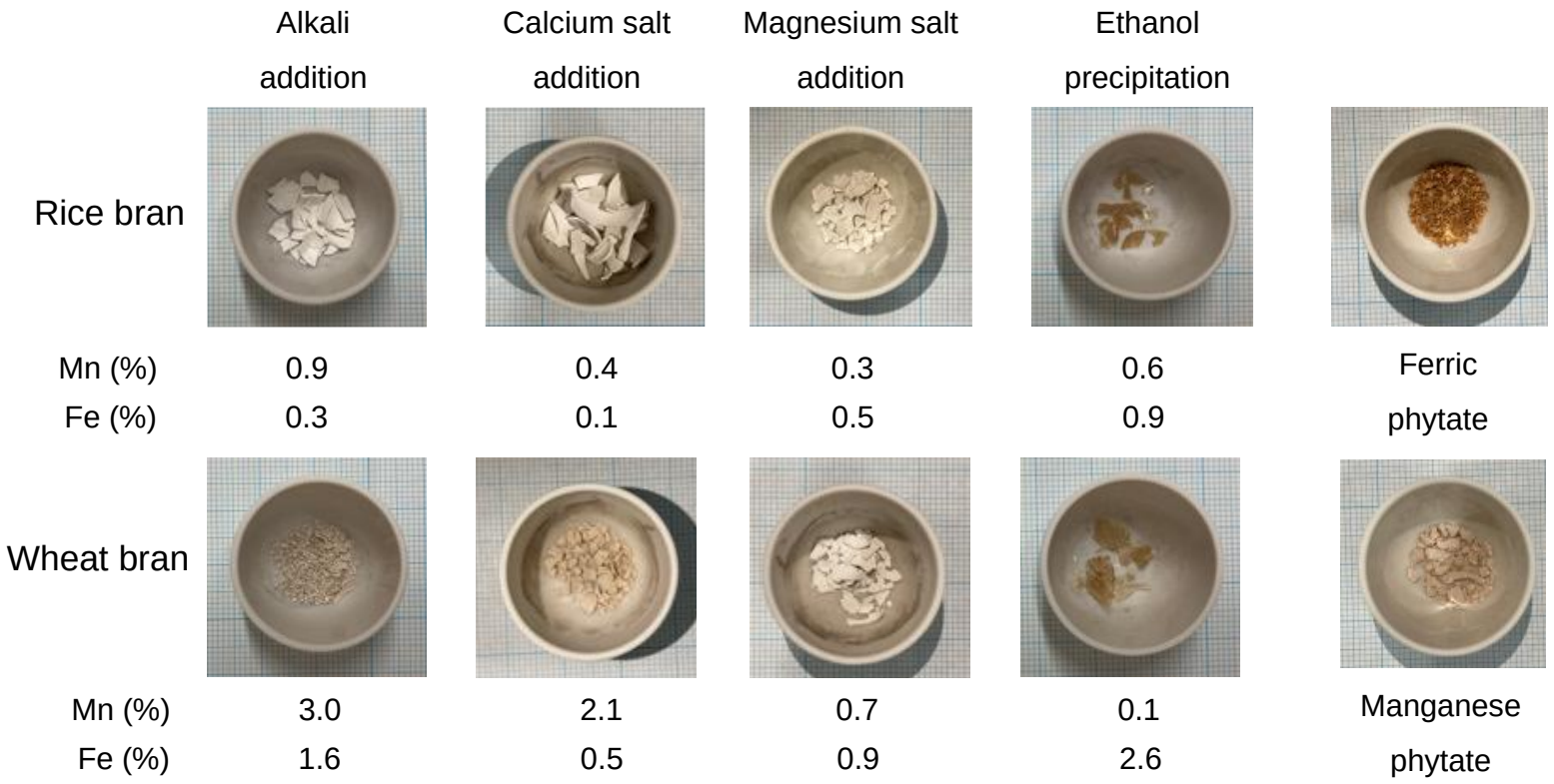

Figure 2 Phytin products produced by different sources and method in Chapter 1

Table 3 Measured mineral concentrations (μg/g) of diets in animal experiment performed in Chapter 2

Minerals	Control group	SP group	Phytin group
Calcium	4980 ± 172	4894 ± 48	5048 ± 37
Magnesium	525 ± 30	512 ± 11	1694 ± 28
Phosphorus	3158 ± 107	5114 ± 33	5156 ± 121
Iron	37.1 ± 0.1	41.9 ± 0.2	38.9 ± 1.2
Zinc	41.3 ± 1.0	43.8 ± 1.5	50.9 ± 1.7
Copper	6.0 ± 0.2	6.3 ± 0.5	6.3 ± 0.8
Manganese	8.9 ± 2.5	9.4 ± 0.6	30.5 ± 3.5

Values are means ± SD of triplicate measurements.

Table 4 Body weights, feed intake and water consumption in animal experiment performed in Chapter 2

	Control group	SP group	Phytin group
Body weight (g)	281.5 ± 4.5^{a}	299.1 ± 5.0^{b}	294.1 ± 4.5^{ab}
Feed intake (g/d)	16.7 ± 0.3	17.3 ± 0.2	17.8 ± 0.6
Water consumption (mL/d)	18.1 ± 1.2	18.9 ± 0.85	17.2 ± 1.0

Values are means ± SEM (n=6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$). No significant differences were observed among groups for the items without a superscript in the mean.

Table 5 Balance of several minerals in animal experiment performed in Chapter 2

	Control group	SP group	Phytin group
Diet intake (g/d)	21.8 ± 0.4	23.4 ± 0.4	23.2 ± 1.0
Calcium			
Intake (mg/d)	108.7 ± 2.0	114.5 ± 2.1	116.9 ± 5.2
Fecal excretion (mg/d)	48.6 ± 2.2	52.7 ± 1.2	56.2 ± 4.4
Apparent absorption (mg/d)	60.1 ± 1.8	61.8 ± 1.4	60.7 ± 3.4
Apparent absorption (%)	55.4 ± 1.7	54.0 ± 0.8	52.1 ± 2.6
Urinary excretion (mg/d)	0.85 ± 0.11	0.60 ± 0.03	0.87 ± 0.12
Retention (mg/d)	59.3 ± 1.8	61.2 ± 1.5	59.8 ± 3.4
Retention (%)	54.6 ± 1.6	53.4 ± 0.8	51.4 ± 2.6
Magnesium			
Intake (mg/d)	11.46 ± 0.20^{a}	11.98 ± 0.19^{a}	39.2 ± 1.6^{b}
Fecal excretion (mg/d)	3.40 ± 0.15^{a}	5.30 ± 0.14^{b}	23.0 ± 1.6^{c}
Apparent absorption (mg/d)	8.06 ± 0.17^{b}	6.68 ± 0.17^{a}	16.3 ± 1.3^{c}
Apparent absorption (%)	70.4 ± 1.1^{c}	55.7 ± 1.0^{b}	41.5 ± 3.1^{a}
Urinary excretion (mg/d)	5.46 ± 0.29^{b}	4.43 ± 0.12^{a}	11.5 ± 0.8^{c}
Retention (mg/d)	2.60 ± 0.27	2.24 ± 0.14	4.72 ± 1.93
Retention (%)	22.8 ± 2.5	18.7 ± 1.1	11.6 ± 4.7
Phosphorus			
Intake (mg/d)	68.9 ± 1.1^{a}	119.7 ± 2.2^{b}	119.4 ± 4.2^{b}
Fecal excretion (mg/d)	18.0 ± 1.0^{a}	38.5 ± 0.5^{b}	45.9 ± 3.3^{b}
Apparent absorption (mg/d)	50.9 ± 1.0^{a}	81.2 ± 1.8^{b}	73.5 ± 3.8^{b}
Apparent absorption (%)	70.4 ± 1.1^{c}	67.8 ± 0.6^{b}	61.6 ± 2.0^{a}
Urinary excretion (mg/d)	15.1 ± 1.0^{a}	41.8 ± 1.1^{c}	29.9 ± 1.4^{b}
Retention (mg/d)	35.8 ± 1.4	39.4 ± 1.1	43.6 ± 3.6
Retention (%)	52.0 ± 2.0^{b}	32.9 ± 0.6^{a}	38.5 ± 2.5^{a}
Iron			
Intake (μg/d)	810 ± 15^{a}	980 ± 15^{b}	901 ± 38^{b}
Fecal excretion (μg/d)	610 ± 31^{a}	731 ± 11^{ab}	782 ± 49^{b}
Apparent absorption (μg/d)	200 ± 17^{b}	249 ± 11^{b}	119 ± 38^{a}
Apparent absorption (%)	24.8 ± 2.3^{b}	25.4 ± 0.8^{b}	13.4 ± 4.2^{a}

Table 5 Balance of several minerals in animal experiment performed in Chapter 2

	Control group	SP group	Phytin group
Zinc			
Intake (μg/d)	901 ± 16[a]	1025 ± 16[b]	1179 ± 49[c]
Fecal excretion (μg/d)	615 ± 32[a]	717 ± 10[ab]	894 ± 60[b]
Apparent absorption (μg/d)	286 ± 23	318 ± 11	285 ± 49
Apparent absorption (%)	31.8 ± 2.7	31.0 ± 0.9	24.2 ± 3.8
Copper			
Intake (μg/d)	131 ± 2[a]	147 ± 2[b]	146 ± 6[b]
Fecal excretion (μg/d)	117 ± 4	123 ± 3	125 ± 8
Apparent absorption (μg/d)	14 ± 4	24 ± 2	21 ± 7
Apparent absorption (%)	10.9 ± 3.1	16.3 ± 1.3	14.4 ± 4.4
Manganese			
Intake (μg/d)	194 ± 4[a]	220 ± 3[a]	706 ± 30[b]
Fecal excretion (μg/d)	172 ± 10[a]	191 ± 10[a]	624 ± 41[b]
Apparent absorption (μg/d)	22 ± 6	29 ± 2	82 ± 33
Apparent absorption (%)	11.6 ± 3.2	13.3 ± 0.8	11.5 ± 4.3

Values are means ± SEM (n=6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$). No significant differences were observed among groups for the items without a superscript in the mean.

Table 6 Mineral concentrations in the serum and organs in animal experiment performed in Chapter 2

	Control group	SP group	Phytin group
Calcium			
Serum (mg/dL)	10.3 ± 0.1	10.5 ± 0.1	10.6 ± 0.1
Liver (μg /g)	44.1 ± 0.3	44.7 ± 1.5	44.4 ± 1.0
Kidney (μg/g)	79.8 ± 0.8^{ab}	84.2 ± 3.9^{b}	74.2 ± 1.1^{a}
Spleen (μg/g)	45.6 ± 1.2	49.2 ± 1.5	49.7 ± 1.2
Magnesium			
Serum (mg/dL)	1.83 ± 0.02	1.78 ± 0.02	1.83 ± 0.02
Liver (μg /g)	194 ± 1	199 ± 5	203 ± 1
Kidney (μg/g)	194 ± 2	191 ± 2	195 ± 2
Spleen (μg/g)	204 ± 2	201 ± 1	207 ± 2
Phosphorus			
Serum (mg/dL)	7.5 ± 0.2^{a}	8.6 ± 0.1^{b}	8.5 ± 0.2^{b}
Liver (mg/g)	1.02 ± 0.07^{a}	1.08 ± 0.08^{a}	1.75 ± 0.18^{b}
Kidney (mg/g)	1.47 ± 0.04	1.50 ± 0.04	1.57 ± 0.11
Spleen (mg/g)	0.52 ± 0.02^{a}	0.44 ± 0.03^{a}	0.68 ± 0.02^{b}
Femur (mg/g)	27.1 ± 1.4	28.9 ± 1.3	31.4 ± 2.7
Iron			
Serum (μg/dL)	190 ± 11^{ab}	215 ± 11^{b}	168 ± 15^{a}
Liver (μg/g)	62.7 ± 4.6^{b}	63.9 ± 5.9^{b}	39.4 ± 5.9^{a}
Kidney (μg/g)	43.1 ± 1.7	41.7 ± 0.6	40.6 ± 2.2
Spleen (μg/g)	147 ± 7^{b}	170 ± 8^{b}	113 ± 5^{a}
Femur (μg/g)	35.1 ± 1.2	31.7 ± 1.3	29.0 ± 2.3
Zinc			
Serum (μg/dL)	100 ± 5^{b}	64 ± 2^{a}	85 ± 7^{b}
Liver (μg/g)	21.7 ± 0.8	22.1 ± 1.0	24.8 ± 0.6
Kidney (μg/g)	26.4 ± 0.7^{b}	22.8 ± 0.5^{a}	24.2 ± 0.8^{ab}
Spleen (μg/g)	18.5 ± 0.2^{b}	17.1 ± 0.3^{a}	17.6 ± 0.5^{ab}
Femur (μg/g)	124 ± 2^{b}	97 ± 3^{a}	109 ± 5^{a}
Copper			
Serum (μg/dL)	95 ± 4^{b}	80 ± 3^{a}	77 ± 1^{a}
Liver (μg/g)	3.60 ± 0.10	3.39 ± 0.20	3.66 ± 0.22
Kidney (μg/g)	10.7 ± 1.2	11.5 ± 1.2	10.1 ± 1.9
Spleen (μg/g)	0.65 ± 0.04	0.54 ± 0.04	0.62 ± 0.03

Table 6 Mineral concentrations in the serum and organs in animal experiment performed in Chapter 2

	Control group	SP group	Phytin group
Manganese			
Serum (μg/dL)	0.89 ± 0.03	0.78 ± 0.03	0.87 ± 0.04
Liver (μg/g)	2.27 ± 0.11	2.10 ± 0.08	2.37 ± 0.06
Kidney (μg/g)	0.97 ± 0.02	0.95 ± 0.01	1.00 ± 0.01
Spleen (μg/g)	0.19 ± 0.01	0.18 ± 0.01	0.20 ± 0.01

Values are means ± SEM (n=6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$). No significant differences were observed among groups for the items without a superscript in the mean.

Table 7 Composition of experimental diets in animal experiment performed in Chapter 3

Ingredients (g/kg)	AZ	AZP	LZ	LZP
Corn starch	397.486	387.486	397.486	387.486
Casein	200	200	200	200
Gelatinized corn starch	132	132	132	132
Sucrose	100	100	100	100
Soybean oil	70	70	70	70
Cellulose	50	50	50	50
AIN93G mineral mixture	35	35	–	–
Zn-free AIN93G mineral mixture	–	–	35	35
AIN93 vitamin mixture	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
L-Cystine	3	3	3	3
tert-Butylhydroquinone	0.014	0.014	0.014	0.014
Zinc-containing phytin	–	10	–	10
Zinc content (mg/kg) *	35.6 ± 2.5	40.7 ± 1.3	5.0 ± 0.9	10.2 ± 0.6
Phosphorus content (mg/kg)*	3145 ± 32	4998 ± 82	3186 ± 115	5224 ± 188
Magnesium (mg/kg)*	579 ± 32	1877 ± 11	530 ± 41	1867 ± 5

*, Actual measured values (means ± SD for 3 determinants).

Table 8 Body weight, feed intake, and organ weights in animal experiment performed in Chapter 3

	AZ	AZP	LZ	LZP
Final body weight (g)	295 ± 7^{b}	292 ± 5^{b}	252 ± 4^{a}	282 ± 6^{b}
Body weight gain (g/28 d)	215 ± 6^{b}	211 ± 5^{b}	172 ± 4^{a}	200 ± 5^{b}
Feed intake (g/28 d)	432 ± 6^{b}	428 ± 12^{b}	327 ± 6^{a}	424 ± 11^{b}
Feed efficiency*	0.498 ± 0.002^{b}	0.492 ± 0.004^{b}	0.524 ± 0.001^{c}	0.472 ± 0.001^{a}
Liver weight (g)	13.59 ± 0.83^{b}	11.25 ± 0.37^{a}	10.92 ± 0.45^{a}	12.05 ± 0.32^{ab}
Kidney weight (g)	2.04 ± 0.03^{b}	2.02 ± 0.04^{ab}	1.85 ± 0.03^{a}	2.05 ± 0.07^{b}
Spleen weight (g)	0.60 ± 0.03^{a}	0.57 ± 0.02^{a}	0.51 ± 0.03^{a}	0.55 ± 0.01^{a}
Testis weight (g)	2.75 ± 0.22^{b}	2.74 ± 0.05^{b}	2.15 ± 0.06^{a}	2.83 ± 0.05^{b}

*, Feed efficiency calculated as (body weight gain)/(feed intake).

Values are means ± SEM (n = 6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Table 9 Organ zinc concentrations in animal experiment performed in Chapter 3

Organs	AZ	AZP	LZ	LZP
Liver (μg/g)	22.6 ± 0.6^{b}	25.5 ± 0.4^{c}	19.9 ± 0.7^{a}	22.2 ± 0.7^{ab}
Kidney (μg/g)	26.1 ± 0.2^{c}	26.0 ± 0.2^{c}	16.9 ± 0.2^{a}	19.1 ± 0.2^{b}
Spleen (μg/g)	18.9 ± 0.4^{b}	18.3 ± 0.1^{b}	15.9 ± 0.5^{a}	18.0 ± 0.3^{b}
Testis (μg/g)	22.1 ± 0.3^{b}	21.7 ± 0.2^{b}	18.7 ± 0.4^{a}	21.3 ± 0.1^{b}
Femur (μg/g)	121.8 ± 2.4^{d}	114.4 ± 1.5^{c}	31.5 ± 1.1^{a}	50.5 ± 1.9^{b}
Serum (μg/dL)	124.2 ± 4.9^{d}	104.3 ± 3.2^{c}	17.8 ± 2.9^{a}	38.2 ± 4.5^{b}

Values are means ± SEM (n = 6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Table 10 Zinc balance in animal experiment performed in Chapter 3

	AZ	AZP	LZ	LZP
Intake (μg/d)	773.1 ± 25.4^{c}	871.5 ± 21.5^{d}	72.9 ± 2.7^{a}	212.0 ± 5.2^{b}
Fecal excretion (μg/d)	565.3 ± 28.3^{c}	722.9 ± 5.0^{d}	16.6 ± 0.5^{a}	119.9 ± 4.9^{b}
Apparent absorption (μg/d)*	207.8 ± 11.6^{d}	148.6 ± 22^{c}	56.2 ± 2.7^{a}	92.1 ± 2.6^{b}
Apparent absorption (%)	27.1 ± 2.0^{b}	16.8 ± 2.2^{a}	76.3 ± 1.5^{d}	43.4 ± 1.2^{c}

Values are means ± SEM (n = 6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

*, Data were converted to ranks and then statistical tests were performed.

Table 11 Blood biochemical examination in animal experiment performed in Chapter 3

	AZ	AZP	LZ	LZP
Serum				
Total protein (mg/dL)	5.2 ± 0.1^{b}	5.1 ± 0.1^{ab}	4.9 ± 0.1^{a}	5.1 ± 0.1^{ab}
Triacylglycerol (mg/dL)	144 ± 17^{b}	83 ± 12^{a}	89 ± 13^{a}	91 ± 7^{a}
Total cholesterol (mg/dL)	93 ± 7^{b}	84 ± 3^{ab}	68 ± 6^{a}	78 ± 6^{ab}
Total lipids (mg/dL)	423 ± 29^{b}	315 ± 13^{a}	286 ± 16^{a}	312 ± 12^{a}
Aspartate aminotransferase (IU/L)	65 ± 1^{a}	64 ± 1^{a}	74 ± 3^{b}	67 ± 3^{ab}
Alanine aminotransferase (IU/L)	29 ± 2^{a}	28 ± 1^{a}	29 ± 1^{a}	26 ± 2^{a}
Alkaline phosphatase (U/L)	99.5 ± 3.6^{c}	83.4 ± 4.7^{b}	40.1 ± 4.0^{a}	48.5 ± 0.7^{a}
Inorganic phosphorus (mg/dL)	8.1 ± 0.1^{a}	9.3 ± 0.2^{b}	7.5 ± 0.1^{a}	8.9 ± 0.2^{b}
Magnesium (mg/dL)	1.9 ± 0.03^{a}	2.1 ± 0.03^{b}	1.9 ± 0.04^{a}	2.1 ± 0.02^{b}
Iron (μg/dL)	235 ± 26^{ab}	193 ± 9^{a}	294 ± 18^{b}	197 ± 11^{a}
Transferrin saturation (%)	47.3 ± 4.8^{ab}	42.6 ± 1.5^{a}	64.7 ± 4.7^{b}	40.5 ± 1.6^{a}
Whole blood				
Hemoglobin (g/dL)	13.0 ± 0.1^{a}	13.4 ± 0.1^{a}	13.0 ± 0.1^{a}	13.1 ± 0.1^{a}
Hematocrit (%)	41.0 ± 0.3^{a}	42.1 ± 0.3^{a}	41.0 ± 0.2^{a}	41.2 ± 0.4^{a}

Values are means ± SEM (n = 6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Table 12 Composition of experimental diets in animal experiment performed in Chapter 4

Ingredients (g/kg)	PD (P 0.03%)	PA (P 0.15%)	HPA (P 0.30%)
Wheat Gluten [1)]	200	200	200
Gelatinized corn starch	132	132	132
Corn starch	395.886	394.358	385.586
AIN93G mineral mixture [2)]	35	35	35
Sucrose	92.20	86.92	86.92
AIN93 vitamin mixture [3)]	10	10	10
L-Lysine (HCl)	13.7	13.7	13.7
L-Threonine	1.2	1.2	1.2
Sodium phytate [4)]	-	6.804	15.575
Cellulose powder	50	50	50
Soybean oil	70	70	70
TBHQ	0.014	0.014	0.014
Phosphorus contents (mg/kg)			
Wheat gluten	337	337	337
Sodium phytate	-	1163	2663
Total	337	1500	3000

[1)] Protein content is 76% and phosphorus content is 0.168%.

[2)] Prepared by removing KH_2PO_4 from the AIN93G mineral mix and adding KCl to equalize the potassium concentration.

[3)] Choline bitartrate contained.

[4)] Phosphorus content is 17.1%.

Table 13 Body weight, feed intake, and organ weights in animal experiment performed in Chapter 4

	PD	PA	HPA	ANOVA
Final body weight (g)	136 ± 7^{a}	226 ± 5^{b}	260 ± 5^{c}	< 0.001
Body weight gain (g)	57 ± 7^{a}	148 ± 5^{b}	181 ± 4^{c}	< 0.001
Total feed intake (g)	232 ± 19^{a}	376 ± 2^{b}	410 ± 12^{b}	0.004
Feed efficiency (g/g)	0.25 ± 0.04^{a}	0.39 ± 0.02^{ab}	0.42 ± 0.02^{b}	0.033
Liver weight (g)	4.49 ± 0.33^{a}	8.33 ± 0.28^{b}	9.80 ± 0.42^{c}	< 0.001
(Relative weight) *	3.30 ± 0.13^{b}	3.68 ± 0.06^{b}	3.77 ± 0.11^{b}	0.012
Kidney weight (g)	1.24 ± 0.08^{a}	1.90 ± 0.07^{b}	1.96 ± 0.05^{b}	< 0.001
(Relative weight) *	0.91 ± 0.02^{b}	0.84 ± 0.02^{a}	0.76 ± 0.06^{a}	< 0.001

Values are means ± SEM (n = 6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

* Relative weights are calculated in g per 100g body weight.

Table 14 Blood examination in animal experiment performed in Chapter 4

	PD	PA	HPA	ANOVA
Total protein (mg/dL)	5.3 ± 0.1	5.3 ± 0.1	5.5 ± 0.1	0.069
Total lipids (mg/dL)	210 ± 7^{b}	196 ± 4^{ab}	180 ± 8^{a}	0.019
Triacylglycerol (mg/dL)	25 ± 2	20 ± 2	20 ± 3	0.297
Total cholesterol (mg/dL)	74 ± 3^{b}	71 ± 2^{ab}	64 ± 2^{a}	0.037
Urea nitrogen (mg/dL)	12 ± 2^{a}	14 ± 1^{ab}	18 ± 1^{b}	0.019
Creatinine (mg/dL)	0.17 ± 0.01	0.19 ± 0.01	0.2 ± 0.02	0.224
Aspartate aminotransferase (IU/L)	89 ± 4	83 ± 5	84 ± 8	0.818
Alanine aminotransferase (IU/L)	48 ± 5	36 ± 1	42 ± 3	0.092
Alkaline phosphatase (IU/L)	787 ± 66^{b}	531 ± 29^{a}	403 ± 28^{a}	< 0.001
Inorganic phosphorus (mg/dL)	3.3 ± 0.1^{a}	5 ± 0.4^{b}	6.3 ± 0.3^{c}	< 0.001
Calcium (mg/dL)	12.6 ± 0.2^{b}	12.3 ± 0.2^{b}	11.3 ± 0.1^{a}	< 0.001
Magnesium (mg/dL)	2.2 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	0.051
Zinc (μg/dL)	123 ± 9	126 ± 4	113 ± 5	0.273
Copper (μg/dL)	77 ± 4^{b}	61 ± 1^{a}	59 ± 3^{a}	< 0.001
Iron (μg/dL)	228 ± 39	164 ± 16	257 ± 18	0.068
TIBC (μg/dL)	401 ± 26^{a}	466 ± 9^{b}	495 ± 11^{b}	0.004
Hemoglobin (g/dL)	12.8 ± 0.8	13 ± 0.3	13.3 ± 0.5	0.825
Hematocrit value (%)	39.9 ± 1.9	39 ± 1	38.4 ± 0.7	0.727

Values are means ± SEM (n = 6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Table 15 Organ mineral concentrations animal experiment performed in Chapter 4

	PD	PA	HPA	ANOVA
Calcium				
Liver (μg/g)	66.4 ± 3	58.9 ± 3.7	58.4 ± 1.8	0.133
Kidney (μg/g)	108 ± 6	97 ± 2	104 ± 3	0.166
Muscle (μg/g)	153 ± 24^{b}	96 ± 6^{a}	87 ± 4^{a}	0.017
Magnesium				
Liver (μg/g)	247 ± 4	238 ± 6	244 ± 3	0.424
Kidney (μg/g)	198 ± 7	199 ± 4	195 ± 9	0.898
Muscle (μg/g)	301 ± 7	317 ± 6	318 ± 5	0.107
Phosphorus				
Liver (mg/g)	3.53 ± 0.08	3.43 ± 0.07	3.46 ± 0.08	0.651
Kidney (mg/g)	2.87 ± 0.03	2.95 ± 0.03	2.92 ± 0.05	0.324
Muscle (mg/g)	2.56 ± 0.04^{a}	2.75 ± 0.06^{b}	2.74 ± 0.02^{b}	0.016
Zinc				
Liver (μg/g)	29.8 ± 1.4	29.2 ± 0.4	28.2 ± 1.0	0.520
Kidney (μg/g)	22.5 ± 1.1	23.3 ± 0.4	23.2 ± 0.8	0.771
Muscle (μg/g)	6.14 ± 0.5	5.46 ± 0.37	5.91 ± 0.43	0.549
Iron				
Liver (μg/g)	136 ± 6^{b}	105 ± 7^{a}	89.9 ± 5.7^{a}	< 0.001
Kidney (μg/g)	68.2 ± 3.8^{b}	59.1 ± 3.7^{ab}	54.3 ± 2.2^{a}	0.028
Muscle (μg/g)	13.7 ± 1.5^{a}	16.3 ± 0.9^{ab}	19.1 ± 1.2^{b}	0.022
Copper				
Liver (μg/g)	4.64 ± 0.37	4.11 ± 0.3	4.47 ± 0.28	0.495
Kidney (μg/g)	4.16 ± 0.37	4.15 ± 0.28	4.36 ± 0.27	0.864
Muscle (ng/g)	645 ± 62^{a}	660 ± 60^{a}	968 ± 83^{b}	0.007
Manganese				
Liver (μg/g)	2.61 ± 0.13^{b}	2.23 ± 0.07^{ab}	2.01 ± 0.13^{a}	0.005
Kidney (μg/g)	1.25 ± 0.06^{b}	1.16 ± 0.05^{ab}	1.03 ± 0.04^{a}	0.030
Muscle (ng/g)	118 ± 10	118 ± 6	114 ± 11	0.936

Values are means ± SEM (n = 6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Table 16 Femoral mineral concentrations in animal experiment performed in Chapter 4

	PD	PA	HPA	ANOVA
Calcium (mg/g)	45 ± 2^{a}	77 ± 4^{b}	123 ± 7^{c}	< 0.001
Magnesium (mg/g)	0.64 ± 0.03^{a}	1.18 ± 0.05^{b}	1.94 ± 0.11^{c}	< 0.001
Phosphorus (mg/g)	17.9 ± 0.7^{a}	31 ± 1.5^{b}	49.5 ± 2.9^{c}	< 0.001
Zinc (μg/g)	79 ± 4^{a}	103 ± 4^{b}	122 ± 4^{c}	< 0.001

Values are means ± SEM (n = 6). Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Table 17 Body weight, feed intake, and organ weights in animal experiment performed in Chapter 5

	Control	0.5PA	1.0PA
Body weight gain (g)	206 ± 4	212 ± 3	201 ± 5
Feed intake (g)	428 ± 3	442 ± 13	415 ± 6
Feed efficiency (g/g)	0.69 ± 0.01	0.69 ± 0.01	0.69 ± 0.01
Liver weight (g)	11.2 ± 0.3	10.6 ± 0.4	10.8 ± 0.4
(Relative weight) *	(3.92 ± 0.1)	(3.62 ± 0.14)	(3.83 ± 0.15)
Kidney weight(g)	2.1 ± 0.03	2.08 ± 0.02	1.96 ± 0.02
(Relative weight) *	(0.73 ± 0.01)	(0.71 ± 0.01)	(0.70 ± 0.01)
Femur (g)	0.64 ± 0.03	0.63 ± 0.04	0.67 ± 0.04
(Relative weight) *	(0.22 ± 0.01)	(0.21 ± 0.01)	(0.24 ± 0.01)

Values are means ± SEM (n = 6).

* Relative weights are calculated in g per 100g body weight.

Table 18 Tissue zinc concentrations in animal experiment performed in Chapter 5

	Zinc concentration		
	Control	0.5PA	1.0PA
Serum (μg/mL)	2.94 ± 0.20^{b}	2.38 ± 0.10^{a}	2.20 ± 0.05^{a}
Liver (μg/g)	22.9 ± 0.7^{a}	23.7 ± 1.0^{a}	24.8 ± 0.5^{a}
Kidney (μg/g)	24.7 ± 0.6^{a}	24.6 ± 0.4^{a}	23.0 ± 0.6^{a}
Femur (μg/g)	109.2 ± 6.1^{b}	82.8 ± 7.6^{a}	68.8 ± 5.2^{a}

Values are means ± SEM (n=6).

[a,b)] Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Table 19 Several mineral concentrations in the serum, liver, and kidney in animal experiment performed in Chapter 5

	Control	0.5PA	1.0PA
Iron			
Serum (μg/mL)	2.93 ± 0.21	2.74 ± 0.16	2.51 ± 0.13
Liver (μg/g)	76.1 ± 4.5^{b}	58.4 ± 5.2^{a}	60.5 ± 3.5^{ab}
Kidney (μg/g)	46.9 ± 1.3	45.2 ± 1.2	42.5 ± 1.1
Copper			
Serum (μg/mL)	2.34 ± 0.26^{b}	1.56 ± 0.10^{a}	1.87 ± 0.05^{a}
Liver (μg/g)	3.35 ± 0.11^{ab}	3.52 ± 0.13^{b}	3.08 ± 0.08^{a}
Kidney (μg/g)	8.62 ± 0.67	7.66 ± 0.21	7.93 ± 0.97
Calcium			
Serum (μg/mL)	107 ± 2	106 ± 2	109 ± 2
Liver (μg/g)	53.4 ± 2.5^{b}	46.1 ± 2.1^{ab}	38.8 ± 1.2^{a}
Kidney (μg/g)	82.9 ± 6.5	81.4 ± 2.0	85.1 ± 4.3
Magnesium			
Serum (μg/mL)	18.9 ± 0.5^{a}	18.3 ± 0.2^{a}	18.3 ± 0.4^{a}
Liver (μg/g)	267 ± 7^{a}	247 ± 7^{a}	244 ± 7^{a}
Kidney (μg/g)	227 ± 5^{b}	215 ± 4^{b}	193 ± 4^{a}
Manganese			
Liver (μg/g)	2.36 ± 0.08^{b}	1.97 ± 0.06^{a}	1.74 ± 0.08^{a}
Kidney (μg/g)	0.76 ± 0.01^{b}	0.84 ± 0.02^{b}	0.59 ± 0.01^{a}

Values are means ± SEM (n=6).

$^{a,b)}$ Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Table 20 Serum lipid components in animal experiment performed in Chapter 5

Components	Control	0.5PA	1.0PA
TAG (mg/dL)	71 ± 12^{b}	42 ± 6^{a}	54 ± 2^{ab}
TL (mg/dL)	305 ± 19^{b}	226 ± 8^{a}	252 ± 7^{a}
TCHOL (mg/dL)	86 ± 6^{b}	71 ± 4^{a}	74 ± 5^{a}
HDL-CHOL (mg/dL)	56 ± 3^{b}	47 ± 1^{a}	45 ± 2^{a}

Values are means ± SEM (n=6).

[a,b)] Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

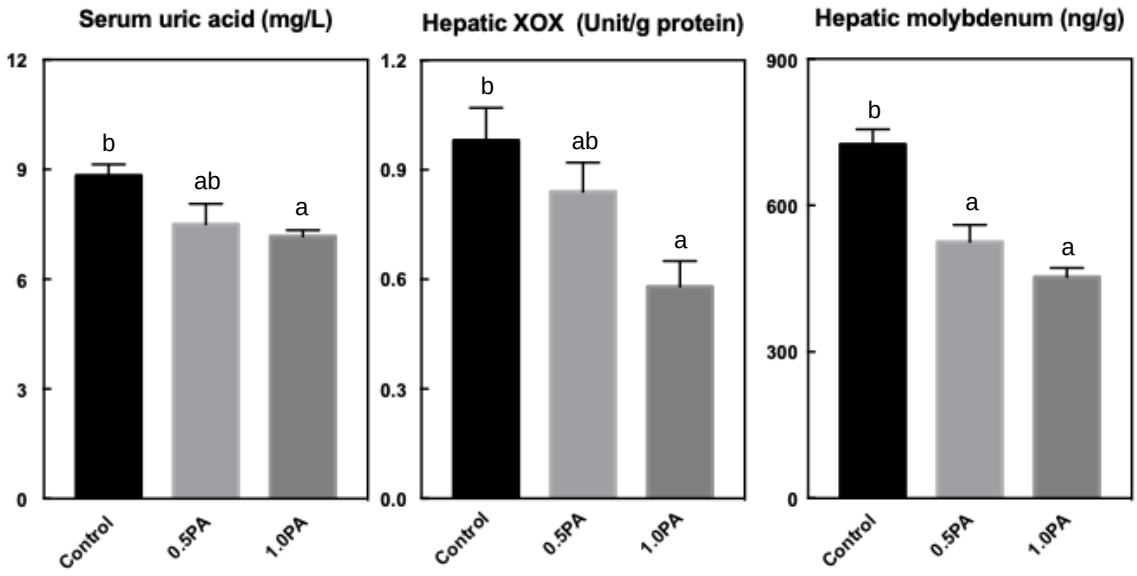

Figure 3 Effect of phytic acid administration on the serum uric acid concentration, hepatic XOX activity, and hepatic molybdenum concentration

Heights of the bars and vertical lines indicate means and SEM (n=6), respectively.

[a,b)] Heights of bars in the same frame not sharing a common superscript differ significantly ($p < 0.05$)

Table 21 Serum biochemical parameters and hemoglobin concentration in animal experiment performed in Chapter 5

	Control	0.5PA	1.0PA
Total protein (mg/dL)	5.3 ± 0.1	5.4 ± 0.1	5.4 ± 0.1
Albumin (mg/dL)	3.5 ± 0.1	3.6 ± 0.1	3.6 ± 0.1
Aspartate aminotransferase (IU/L)	75.8 ± 2.3	70.2 ± 3.2	71.5 ± 1.5
Alanine aminotransferase (IU/L)	41.8 ± 2.2	38 ± 2	43.7 ± 1.3
Urea nitrogen (mg/dL)	17 ± 1^{ab}	20 ± 1^{b}	14 ± 1^{a}
Creatinine (mg/dL)	0.22 ± 0.01	0.22 ± 0	0.22 ± 0.01
Inorganic phosphorus (mg/dL)	8.1 ± 0.2	8.7 ± 0.2	8.1 ± 0.2
Sodium ion (mEq/L)	139 ± 1	139 ± 1	139 ± 1
Chloride ion (mEq/L)	100 ± 1	102 ± 1	101 ± 1
Erythrocyte (10^6/μL)	706 ± 5	726 ± 15	707 ± 6
Hemoglobin (g/dL)	13.8 ± 0.1	13.5 ± 0.3	13.5 ± 0.1
Hematocrit value (%)	40.6 ± 0.2	39.7 ± 1	39.5 ± 0.3

Values are means ± SEM (n=6).

[a,b)] Means in the same row not sharing a common superscript differ significantly ($p < 0.05$).

Reports on this study

Research papers

1. Z. JIN, R. HOSOMI, K, FUKUNAGA, M. YOSHIDA
   Effect of Phytic Acid Administration on the Zinc concentration, Uric Acid Biosynthesis, and Serum Lipid Components in Rats
   Metallomics Research, 2021, Vol.1, No.1, reg-26-32 (Chapter 5)
2. Z. JIN, R. HOSOMI, K, FUKUNAGA, M. YOSHIDA
   Effect of Sodium Phytate and Phytin on the Absorption and Organ Concentration of Several Minerals in Rats
   Trace Nutrients Research, 2022, Vol.39, 1-7 (Chapter 2)
3. Z. JIN, R. HOSOMI, K, FUKUNAGA, M. YOSHIDA
   Nutritional Availability of Zinc Contained in Phytin in Rats with Adequate and Low Zinc Status
   Metallomics Research, 2023, Vol.3, No.3, reg-1-8 (Chapter 3)
4. Z. JIN, M. YOSHIDA
   Effects of differences in manufacturing methods and raw materials on the mineral composition of bran-derived phytin
   Trace Nutrients Research, 2023, Vol.40, 81-86 (Chapter 1)

Conference presentations (in Japan)

- 日本微量元素学会

  2020 年　第 31 回学術集会　金梓聞、細見亮太、福永健治、吉田宗弘
  食餌中のフィチン酸がラットの体内ミネラル濃度に及ぼす影響

  2022 年　第 33 回学術集会　金梓聞、細見亮太、福永健治、吉田宗弘
  低亜鉛給餌時におけるラットの成長及び血清生化学検査値に及ぼす亜鉛含有フィチンの影響

- 日本微量栄養素学会

  2022 年　第 39 回学術集会　金梓聞、細見亮太、福永健治、吉田宗弘
  フィチン酸ナトリウムまたはフィチンの投与がラットのミネラルの体内分布と出納に及ぼす影響

  2023 年　第 40 回学術集会　金梓聞、吉田宗弘
  製造方法と原料の違いがブラン由来フィチンのミネラル組成に及ぼす影響

- 日本栄養・食糧学会

  2023 年　第 77 回大会　金梓聞、細見亮太、福永健治、吉田宗弘
  リン欠乏ラットにおけるフィチン態由来リンの生体利用性（論文第四章）

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