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Phytate Determination in Various Cultivars of Korean Rice

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Abstract

To determine the amount of phytate in rice grains from various cultivars, two methods were employed and compared in respect of the accuracy and conveniency. Phytate in rice samples was extracted with HCl, and then the extracts were subjected to an anion-exchange column. Finally, the phytate in eluate was quantitated using two methods: one method is based on the complex formation between ferric ion and sulfosalicylic acid in the presence of phytate, and the other is the prior acid digestion of phytate sample, followed by the colorimetric determination of liberated phosphorus. Although two methods showed similar values of phytate in rice samples, the former method is simpler and more precise than the latter. Moreover, the former is more reliable for the samples with lower phytate levels. Especially, the dilution condition of rice sample before anion exchange column separation was important for the recovery of phytate in rice samples. Based on the former method, the amount of phytate in rice of various cultivars was estimated to range from 7.3 mg/g to 12.4 mg/g rice. This method would be useful for the determination of phytate in crop samples with a lower level of phytate, one of anti-nutrients in some agricultural plants.

Key words: phytate, rice, anti-nutrient

INTRODUCTION

Rice is one of the major crops cultivated throughout the world, especially in Asia. Most of the production is used in cooked form for human consumption as a protein source of adequate nutritional quality. However, the presence of a number of anti-nutritional factors in the rice seed meal has limited the application of rice as major foodstuff (1). One of the constituents, largely blamed for complexing with dietary essential minerals and rendering them poorly available to monogastric animals, is phytate (myo-inositol 1,2,3,4,5,6-hexaphosphate), one of major sources of phosphorus in plant seeds (2,3). For example, the reduced availability of zinc ions associated with plant protein diets is attributed primarily to the binding of zinc ions by phytate (3,4). Nonetheless, because of its chelating properties, inositol hexaphosphate has been successfully used to prevent renal stone formation (5,6). Additionally, phytate consumption in the diet is considered to have an anti-carcinogenic activity (7). Inositol compounds have also been found to play an important role as second messengers in intracellular signal transduction system (8).

Although qualitative and quantitative analyses for the estimation of phytic acid in plant stuffs such as legumes and cereals have been developed (9-14), the phytate ions in a different mineral and protein environment do not necessarily behave in exactly the same way for a given assay procedure. Therefore, one must be wary of directly applying to other foods the methodology developed for the determination of phytic acid in a particular plant product. In this regard, experimental conditions should be reevaluated for each foodstuff. High-performance liquid chromatography (HPLC) had been developed for phytate routine analysis in foodstuffs (14,15). However, this method may not be adequate for the determination of phytate in rice samples containing a low level of phytate ions. In addition, inductively coupled plasma atomic emission spectrometry (10) and 31P NMR (16) were introduced for the quantitative determination of phosphorus from phytate. But these methods demand a sophisticated technology inavailable in many laboratories, thus lacking in the conveniency. Although the colorimetric method, based on the precipitation of insoluble ferric phytate in acidic solution (9), and its digestion with concentrated H_2SO_4 and concentrated HNO_3 to liberate phosphorus (10), was reported, the digestion stage required careful monitoring to minimize losses due

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to incomplete or excessive digestion. Separately, a simple method to quantitatively assess total phytate content in food stuffs has been developed based on the interaction between ferric chloride and sulfosalicylic acid (9,13,17). In particular, the Wade method (9) has been reproducible for the rapid determination of phytate at low concentrations (8). Nonetheless, this method has not been used for the analysis of rice samples (1,13).

Here, we evaluated the usefulness of the new method in determining phytate content in rice samples, and examined the variation of phytate content among various cultivars of Korean rice seeds, which is not reported the phytate determination by Wade method (9).

MATERIALS AND METHODS

Rice materials

The five different rice cultivars (R1～R5) were harvested from National Crop Experimental Station, RDA, Suwon at 2003. And seven different rice cultivars were harvested from Chungnam Agricultural and Research Extention Services at 2004. Paddy rice was dehulled by laboratory scale huller (THU 35A, Satake, Japan, or SYTH-88, Ssangyoung, Korea), and then the brown rice was milled by laboratory scale whitener (MC-250, Satake, Japan or MC-90A, Tester, Korea), and then polished rice was finally obtained. After milling, the polished rice was stored at freezer (-70°C). Before experiment, the rice sample was ground by mill (Perten 3600, Perten Instrument Co., Sweden) and sieved by mesh (60 mesh, 250 μm), and then used for experimental analyses.

Reagents

Calcium and sodiums phytates were obtained from Sigma Chemical Co. Dowex 1 (AG 1-X4 resin, 100～200 mesh) was purchased from Bio-Rad laboratories. 1-Amino-2-naphthol-4-sulfonic acid, sodium molybdate, sodium sulfite, and anhydrous 5-sulfosalicylic acid dihydrate were obtained from Hayashi Pure Chemical Industries, Ltd, Japan, Kokusan Chemical Werks, Ltd, Shimakyu's Pure Chemicals, Ltd, and Junsei Chemical Co., Ltd, respectively. Other reagents including iron (III) chloride were of analytical grade. Polished rice or soybean grains were milled, and passed through a 0.7 mm sieve to produce the rice powder.

Separation of phytate from seed samples

Acid extraction of phytate from powder of rice or soybean was carried out by slightly modifying the procedure of Oberleas and Harland (12). Each seed powder (2 g) was extracted with 40 mL of 2.4% HCl (0.65 M), which proved to be efficient in extracting phytate from crops.

Anion-exchange purification of phytate

In order to avoid the effect of inorganic phosphorous and other interfering compounds, the purification of phytate sample through anion-exchange resin is necessary (12). For this purpose, glass barrel Econo-column (0.7 × 15 cm; Bio-Rad laboratories) containing 0.5 g AG 1-X4 anion exchange resin was employed. In order to ensure the saturation of resin chloride form, the columns should be washed first with 0.7 M NaCl (15 mL) followed by distilled water (30 mL). After 1 week or 3 experiments, the resin should be replaced. First, the 2.4% HCl extract was centrifuged (1,000 rpm × 10 min), and then 10 mL of supernatant was diluted to 20 mL (rice) or 50 mL (soybean) with distilled water. Ten mL of the diluted sample was accurately applied onto the column (0.5 g), and then the column was eluted with 15 mL of 0.1 M NaCl to remove inorganic phosphate ions. Finally, phytate was eluted with 10 mL of 0.7 M NaCl.

Determination of phytate by Wade method

The eluate (100 μL) from anionic exchange column was transferred into the test tube (10 mL), to which 0.25 mL of Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$; 0.3% sulfosalicylic acid, stable) and 0.65 mL of D.W. were included (9,13). The mixture was stirred on the vortex mixer (>5 sec), and then centrifuged at room temperature (8,000 rpm × 10 min). Finally, the absorbance of the supernatant was read at 500 nm. The difference of absorbance between the control (250 μL Wade reagent + 750 μL distilling water) and each phytate sample was expressed as a practical value of absorbance of each sample. For the preparation of standard phytate curve, 100 μL of each standard phytate (100～600 μg/mL) in distilled water was added to 250 μL of Wade reagent, and then the mixture was diluted to 1 mL with distilled water.

Calculation

From the standard curve, the amount of phytate in the eluant obtained from the column is determined as A (mg/mL). Actual amount (mg/g sample) is estimated as follows: amount of phytate (mg/g sample)=A × 2 × 40/2, where A, phytate mg/eluant mL; 2, dilution factor before ion exchange separation; 40/2, extraction solvent (40 mL) / sample weight (g). Data are expressed as means (n=6 determinations) ± SD.

Determination of phytate by acid digestion method

Determination of phytate by acid digestion was performed by the modification of AOAC method (10). The phytate sample (1.0 mL) from ionic resin column was digested with 0.5 ml of sulfuric acid (95%) and 3 mL of nitric acid (65%) under hood at medium heat temperature (>100°C) until the thick yellow vapor dis-

appeared, approximately 2 or 3 min after boiling started. Heating was continued for another 5 min at medium heat, then for another 5 min at low temperature. After the container flask was cooled down, 10 mL of distilled water was included to dissolve the residue for 10 min, if necessary, under the heating at low temperature. This procedure was repeated two times or more. The solution (25~30 mL), after cooling down, was transferred to 50 mL vol. flask, to which 2 mL of molybdate solution (2.5% ammonium molybdate in 1 M H_2SO_4), and 1 mL of sulfonic acid reagent (0.16 g 1-amino-2-naphthol-4-sulfonic acid, 1.92 g Na_2SO_3, and 9.6 g $NaHSO_3$ in 100 mL vol. flask) were included. Finally, the solution was diluted to 50 mL with distilled water, and after 15 min stirring, the absorbance was read at 640 nm. The phytate amount was determined according to the following equation; Phytate, mg/g sample=mean $K \times A \times 20/(0.282 \times 1{,}000)$, where A, absorbance of solution; average P (μg)/A; phytate, 28.2% P; 20, sample extraction factor (extraction of 2 g sample with 40 mL acid); 0.282, P/phytate; 1,000, weight ratio factor (mg/g); mean K, Table 1.

Preparation of standard phosphate curve

Standard phosphate solution (80 μg/mL) was prepared by including dried desiccated K acid phosphate (0.35 g) and 10 N H_2SO_4 (10 mL) in H_2O (500 mL), and diluting the mixture to 1 L with H_2O. Aliquot (1, 3 and 5 mL) of standard phytate solution was taken into 50 mL vol. flasks, to which 20 mL of D.W., 2 mL of molybdate solution and 1 mL of sulfonic acid solution were included. Finally, the mixture was diluted to 50 mL with distilled water, and after 15 min stirring, the solution was read at 640 nm. The mean K was calculated as Table 1.

RESULTS AND DISCUSSION

Although the acid digestion method (10) has been used for the determination of phytate in samples of crops, this method requires a complete removal of phosphate ions present in the sample (18), and a long time digestion of the phytate samples with strong acids, thus posing a limitation of convenience. Such a disadvantage of acid digestion may be encountered with plant samples containing a low level (<1%) of phytate because of the loss of phytate during the acid digestion procedure. Recently, another method, Wade procedure, was introduced for the simple determination of phytate ions in plant samples containing a low level of phytate (13). This method is based on the theory that the absorbance of ferric ion sulfosalicylic acid complex at 500 nm is decreased in the presence of phytate, which stoichiometrically chelate with the ferric ions. Here, the method was slightly modified to conveniently determine phytate in rice samples. When the decrease in absorbance of the complex at 500 nm was plotted against phytate concentration, a good relationship was obtained with a coefficient (r^2) of 0.999 (Fig. 1). The standard curve prepared with purified calcium phytate was similar to that obtained with sodium phytate.

Table 1. The mean K for phytate determination

Std P solution, mL	μg P	A	K, amount (P)/A
1.0	80	0.1713	466.93
3.0	240	0.5053	474.93
5.0	400	0.8367	478.09

Mean K=473.32.

Based on this standard curve of phytate, the optimal time for the acid (2.4% HCl) extraction of phytate from rice powder sample was determined. As shown in Fig. 2, the extraction of rice powder sample with was maximally achieved within 1 hr, indicating that the acid extraction of phytate was readily achieved under the condition used.

Next, we examined the effect of pH on the separation of phytate on anion-exchange column. When the solution of standard phytate dissolved in distilled water was adjusted to pH 1.0 or 6.0, and then applied on the anion exchange column, the final yield after column separation was almost the same between pH 1.0 and pH 6.0 with a recovery of more than 95%, indicating a negligible effect of pH (pH 1 or 6) on the recovery of phytate in the column separation (Table 1).

In the subsequent experiment, the pH of rice extract for the recovery of phytate in the column separation, the

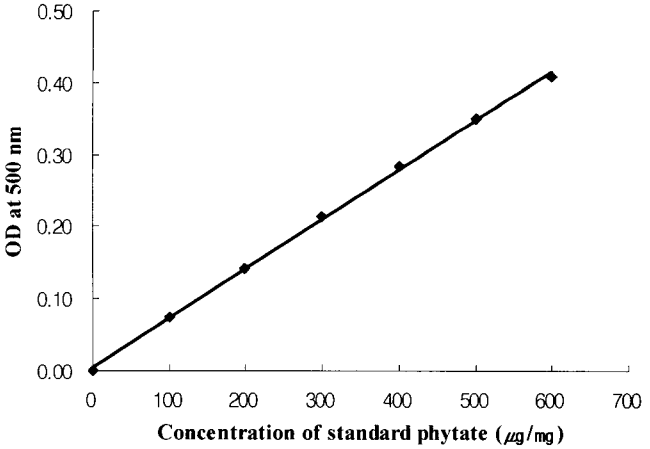

Fig. 1. Standard curve for the phytate determination according to Wade method. A standard solution of sodium phytate (100~600 μg/mL) was prepared by dissolving the sodium phytate in distilled water, and the aliquot (100 μL) of each concentration was added to Wade reagent (250 μL) in 10 mL test tube, and then the final volume was adjusted to 1 mL with distilled water.

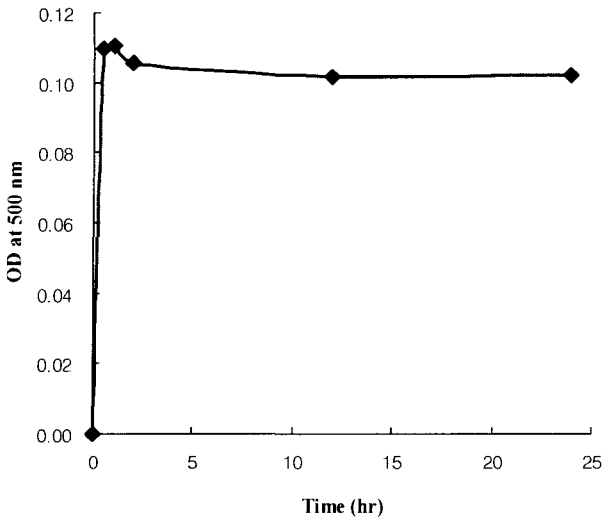

Fig. 2. Time-dependent effect on extraction of phytate from rice sample. Rice powder sample (2 g) was extracted with 40 mL of 2.4% HCl for various times (0.5~24 hr). Then, after 2-fold dilution, the mixture was centrifuged (5,000 rpm, 10 min), and the supernatant was applied onto exchange column for the separation of phytate as described in Methods.

extract from the rice (Ilpoom cultivar), was subjected to pH adjustment (pH 6.0), and then the sample was applied on the anion exchange column. Table 2 indicates that the recovery after pH adjustment (pH 6.0) was lower than that without pH adjustment; 6.91±0.079 mg/g (no pH adjustment) vs. 6.53±0.22 (pH 6.0). This might be somewhat different from the effect of pH on the recovery of standard phytate on the anion exchange column (Table 2). This difference might be ascribed to the effect of NaCl produced from the pH adjustment of rice extract with NaOH. Thus, the pH adjustment of the rice extract

Table 2. Effect of pH on the recovery of standard phytate on anion exchange column

pH	Yield (%)
1.0	97.35±0.62
6.0	95.70±0.93

The solution of standard phytate (0.15 mg/mL), dissolved in distilled water, was adjusted to pH 1.0 or 6.0, and then the solution (1 mL) was applied onto the anion exchange column. The final yield after column separation was determined as described in Fig. 2.

led to the decrease in the recovery of phytate. Instead, the effect of sample dilution on the recovery of phytate in the column separation was examined, since the dilution of rice sample with distilled water might contribute to the reduction in concentration of salt, which might interfere with phytate binding to the exchange column (13).

As shown in Table 3, the direct loading of the rice sample onto the column resulted in a sharp reduction of the recovery, indicating that some compounds, such as salts, in crude rice sample might interfere with the phytate recovery. Alternatively, this might be caused by the limited capacity of ionic resin column (0.5 g). In contrast, the 5-fold dilution resulted in the slightly lower recovery of phytate, compared to 2-fold dilution. This might be explained by the notion that the 5-fold dilution of rice sample led to a lower recovery due to non-specific adsorption of phytate to column or resin. Thus, it seems that the recovery of phytate on ionic exchange column may depend on the concentration of phytate or salts in the sample. This might be consistent with the previous reports that the sample containing less than 1% phytate should be diluted less than 25-fold (13).

To validate the accuracy and precision of Wade method for the determination of phytate ions in rice samples, we assessed the phytate content in rice extract, 2-fold diluted, to which no or known amounts of standard phytate were added. Table 4 shows that a good recovery was found in all cases with recovery values of >95%. These results indicate that the conditions used here are suitable for assessing total phytate in rice samples.

From this, the level (6.9~16.9 mg/g) of phytate in the rice sample can be determined with a confidence by the Wade method. In a separate study, the phytate content in rice was also determined using the acid digestion method, and the values were compared to those obtained using Wade method. As shown in Table 5, there was no significant difference of values (7.3~11.1 mg/g) between two methods. However, the recovery after acid digestion in the digestion container was limited to 85% despite repeated experiments. Moreover, compared to the

Table 3. Effect of dilution on recovery of rice phytate

Dilution fold	Sample A (R3)	Sample B (R4)	Sample C (R5)
5-folds	11.05±2.20	8.60±1.32	7.58±1.62
2-folds	11.97±0.29	9.24±0.50	8.31±0.65
1-fold	5.67±0.03	5.38±0.30	-

Each rice powder of three cultivars (Ilpoom cultivar) was extracted with 2.4% HCl, diluted with distilled water by 1-fold, 2-folds or 5-folds, and the recovery after anion exchange column separation was determined as described in Fig. 2. -, Not determined.

Table 4. Validation of precision and accuracy of the phytate assessment procedure

Phytate added (%)	Total phytate content (%)	Phytate assessed (%)	Recovery (%)
0	0.691	0.691	100
0.5	1.191	1.181±0.001 (3)	99.1±1.1
1.0	1.691	1.643±0.001 (7)	96.9±1.3

Standard phytate amounts (0.5 or 1.0%) were added to a crude rice sample containing 0.691% phytate. The mixture was centrifuged and then the supernatant, after 2-fold dilution, was applied onto exchange column.

Table 5. Comparison between Wade method and acid digestion method

Method	Amount (mg/g)				
	R1	R2	R3	R4	R5
Wade method	7.28±0.58	7.58±0.36	11.05±2.20	8.61±1.32	7.58±1.62
Digestion method	7.25±1.76	7.48±1.05	11.06±0.49	8.35±0.29	7.41±1.04

For the determination of phytate in rice powder sample (R1~R5), acid digestion method and Wade method were carried out as described in experimental methods. Values were expressed as mg amount of phyate per g powder of rice from different cultivars.

Wade method, the acid digestion method was more cumbersome, and took a longer time; the final step of Wade method took 30 min, while that of the digestion method took more than 6 hr.

When the amount of phytate was determined under optimal condition of Wade method, the amount of phytate among rice samples from seven different cultivars ranged from 9.5 mg/g to 12.4 mg/g rice (Fig. 3).

In an independent study, where the amount of phytate in soybean samples were determined using Wade method, the phytate amount in soy bean ranged from 19.4 mg/g to 22.4 mg/g rice (data not shown). In comparison, the level of phytate in rice was approximately twice lower than that (~18 mg/g) in soybean, but higher than that (2 mg/g) in wheat flour (13). Based on the above, the Wade method is suggested to be a chosen method for the determination of phytate in crops. Especially, it would be useful for the determination of phytate in rice samples with a lower phytate level with a confidence. Thus, it could be employed to quantitatively assess the phytate, one of antinutrients in some agricultural plants.

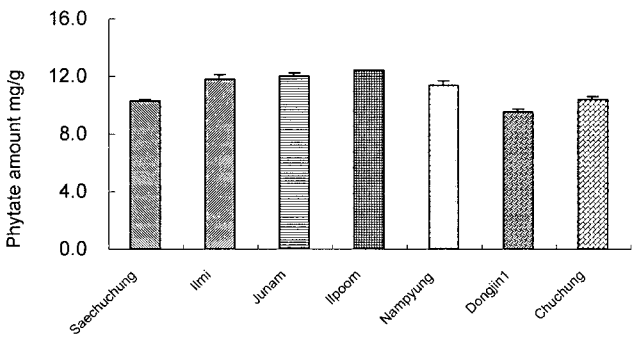

Fig. 3. Amount of phytate in Korean rice of seven different cultivars. The amount of phytate was determined according to Wade method as described in experimental methods ($p<0.05$).

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