Effects of Intestinal Microflora and Dietary Phytate on Intestinal Phytase Activity in Germfree and Conventionalized Rats

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Germfree and conventionalized rats were fed a basal or 2% phytate diet. On mucosal phytase activity, the intestinal microflora has no marked effect. Dietary phytate decreased phytase activity in mucosal homogenates, but this effect was not clear in the course of purification. In the germfree intestinal mucosa, the electrophoretic pattern showed two peaks of phytase activity which were different in divalent metal ion requirements. One of these purified phytases did not have any alkaline phosphatase activity.

Key words: phytase; dietary phytate; intestinal mucosa; germfree and conventionalized rats

Phytate (myo-inositol hexakisphosphate), which is usually found in cereals and legumes, is widely known as an antinutrient for essential minerals. However, the interaction among phytate, minerals and other dietary ingredients are not clear, and the dephosphorylations of phytate also occur in the digestive tract. In the growing mice and rats, almost 60% of dietary sodium phytate was hydrolyzed (22, 23) and mucosal phytase activity in the small intestine was the highest in the duodenum and the lowest in the terminal ileum (4, 6). This might suggest that the proximal part of the small intestine was the major region of phytate hydrolysis. Miyazawa and Yoshida (9) found that the the specific activity of phytase (EC 3.1.3.8 or 3.1.3.26) in the cecal contents and total activity of mucosal phytase in the proximal part of the small intestine were significantly higher in germfree (GF) than conventionalized (CV) rats, and suggested that intestinal microflora do not play a major role in phytate hydrolysis (8). On the other hand, Wise and Giburt (18) reported that phytate was hydrolyzed by intestinal microflora, based on studies in GF and CV rats. It is not clear which enzyme contributes to the hydrolysis process of dietary phytate in the digestive tract, intestinal or microbiological phytase. Yang et al. (20) found that phytate feeding increased the activity and amount of the 90 KDa subunit of intestinal phytase in weanling rats, and Lopez et al. (6) also showed that synthetic phytic acid and phytate in wheat bran enhanced intestinal phytase activity, whereas Roberts and Yudkin (14) reported that dietary phytate may have a decreasing effect on mucosal phytase activity. The difference in the effect of phytate feeding on intestinal phytase may be due to the presence of intestinal microflora which include microbiological phytase. We then examined the effect of phytate feeding on intestinal phytase with or without intestinal microflora using GF and CV rats. The present study was also undertaken to purify and characterize mucosal phytase from GF rat intestines fed a phytate-free diet, because most of previous studies (1, 2, 4, 6, 10, 13, 20, 21) on intestinal phytase were undertaken under conditions that had some effect of phytate feeding or intestinal microflora.

Male GF rats of the inbred Fischer strain (F344 / Yit) were obtained from the Yaskult Central Institute for Microbiological Research. Six of these 12 GF rats, which were 4 weeks of age, were conventionalized before the experimental diets were given. CV rats were obtained by orally inoculating the fresh cecal contents of conventional rats diluted with physiological saline solutions. Both GF and CV rats were divided into two groups of three rats each, and fed a phytate-free or 2% phytate-supplemented diet for 3 weeks. The rats were housed individually at constant temperature (22±2°C) and humidity (50±10%) with a 12-hour light/dark cycle. The composition of the diets was based on L-488F (19). Both diets were sterilized with 50 kGy of γ-irradiation with 60Co. The diets and deionized water were provided ad libitum throughout the experiment period.

The rats were anesthetized with ether and sacrificed by decapitation at 7 weeks of age. The whole small intestine was removed and flushed with cold distilled water, and each small intestine was divided into three segments of equal length. In these 3 intestinal segments,
the proximal part (= including duodenum) was subjected
to enzymatic analysis and the mucosa was scraped away
from the underlying muscle layers with a glass slide.
The scraped mucosal tissue was diluted 1:10 (volume
basis) with distilled water and homogenized by AL-
PHA-TORN homogenizer (Nigorikawa-Rika Kogyo
Co., Tokyo, Japan), separately for each rat. After the
enzyme activities were examined in mucosal homo-
genates, equal amounts of homogenate for three rats in
the same experimental group were combined and cen-
trifuged at 10,000 × g for 30 min to separate the soluble
and particulate fractions. The particulate fraction was
suspending in isolation buffer (20 mm Tris-HCl buffer,
PH 7.4) and placed on ice. Then precooled n-butanol
was added slowly with stirring until the n-butanol
concentration reached 28.6%. After centrifugation at
2,000 × g for 30 min, the aqueous phase was separated
and dialyzed overnight against isolation buffer. All
procedures were carried out 4°C unless stated other-
wise.
Disk PAGE was employed to purify mucosal phytase,
using 5.0% acrylamide as the separating gel (PH 8.9),
2.5% acrylamide as the stacking gel (PH 6.8), and Tris-
glycine (PH 8.4) as the electrophoresis buffer. The
sample obtained from GF rats fed phytate-free diet was
used, because there were not any effects of dietary
phytate or intestinal microflora on mucosal phytase
activity. Electrophoresis was performed in a cold-room
at 4°C. The separating gel was cut into 2 mm pieces,
and the enzymes were extracted with isolation buffer.
Phytase and alkaline phosphatase (ALPase) activities
of extracts associated with mobility were measured,
since some researchers considered that phytase and al-
kaline phosphatase are the same protein (4, 17, 21) but
others (2, 12, 13) did not. Fractions containing phytase
activity were collected as peak I and II.
The activities of phytase and ALPase were deter-
mined by the methods of Rao and Ramakrishnan (13)
except for the use of β-glycerophosphate instead of so-
dium p-nitrophenyl phosphate as the substrate for
ALPase activity, and the concentration of substrates
according to Davies and Flett (4). The substrates for
phytase and ALPase activity were sodium phytate
(Sigma Chemical Co., St. Louis, USA) and β-glycer-
ophosphate (Tokyo Chemical Industry Co., Tokyo, Ja-
p). For determination of phytase activity, the reac-
tion mixture consisted of 40 mm Tris-succinate buffer
(pH 7.4), 0.83 mm sodium phytate, 1 mm MgCl₂, and
homogenate in a final volume of 3.0 ml or extracted
enzyme solution in a final volume of 0.5 ml. For deter-
mination of alkaline phosphatase activity, the reaction
mixture consisted of 40 mm Tris-HCl buffer (pH 9.0),
5 mm β-glycerophosphate, 1 mm MgCl₂, and homoge-
genate in a final volume of 3.0 ml or extracted enzyme
solution in a final volume of 0.5 ml. Effects of oxalate,
which has been reported as an intestinal phytase in-
hibitor, and L-phenylalanine, which is known as an in-
testinal ALPase inhibitor, on enzyme activities were
examined at the concentration of 80 mm and 50 mm,
respectively. Incubation was carried out at 60°C for
phytase, because of much higher specific phytase ac-
tivity at 60°C than at 37°C (16), and at 37°C for ALPase
for 10 min. Protein concentrations of the mucosal ho-
 mogenate were measured by the method of Lowry et
al. (7). One unit of enzyme activity was defined as the
amount liberating 1 μmol of inorganic phosphate from
the substrate per min. Specific enzyme activities in the
homogenates were analyzed statistically by the 2-way
ANOVA and the difference between the means was
considered significant at p < 0.05.
The effects of Zn²⁺ and Mg²⁺ on phytase activity of
the individual peak were determined, and the relative
activities were expressed as % of the activities com-
pared to those of non-ionic controls. The concentra-
tion of Zn²⁺ was decided according to the reports by Rao
and Ramakrishnan (13) and Haru et al. (5). Aliquots
of separating gel extract were incubated with 0.3 mm ZnCl₂
for 3 hours at room temperature and then assayed as
described above. Final concentrations of Zn²⁺ in the
assay mixture were 0.05 mm. Addition of MgCl₂ to the
substrate was at a concentration of 1 mm.
Specific activities of intestinal phytase and ALPase
in GF and CV rats fed diets with or without phytate are
summarized in Table 1. In the mucosal homogenates,
no significant effect of intestinal microflora on either
phytase or ALPase activities was recognized in the com-
parison of GF and CV rats. This result disagreed with a
finding by Miyazawa and Yoshida (9) that the total ac-
tivity of mucosal phytase in the proximal part of the
small intestine was higher in GF compared with CV
rats. Dietary phytate, 2% in the diet, decreased phytase
activity in the mucosal homogenate. ALPase activity
was not affected by dietary phytate. Yang et al. (20)
 purified the 70 kDa and 90 kDa subunits, which had
phytase activity, from rat intestinal mucosa, and the 90
kDa subunit was not detected at birth but appeared when
rats were forcibly weaned and fed laboratory chow.
Lopez et al. (6) also reported the enhancement of intes-
tinal phytase by 0.5% dietary phytate, and the induc-
tion of phytase activity was greater in feeding of syn-
thetic sodium phytate than of wheat bran, which con-
tains the same amount of phytic acid. The effects of
dietary phytate on intestinal phytase are conflicting, ei-
Table 1. Mucosal enzyme activities in the proximal part of the small intestinal tracts in germfree and conventionalized rats.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Homogenate</th>
<th>Aqueous phase (n-butanol treatment)</th>
<th>Specific activities¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Oxalate²</td>
</tr>
<tr>
<td>Phytase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germfree rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytate-free diet</td>
<td>0.078 ± 0.007⁴</td>
<td>2.74</td>
<td>0.46 (16.9%)³</td>
</tr>
<tr>
<td>Phytate-supplemented diet</td>
<td>0.048 ± 0.007*</td>
<td>2.13</td>
<td>0.46 (21.6%)</td>
</tr>
<tr>
<td>Conventionalized rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytate-free diet</td>
<td>0.068 ± 0.015</td>
<td>2.78</td>
<td>0.56 (20.0%)</td>
</tr>
<tr>
<td>Phytate-supplemented diet</td>
<td>0.042 ± 0.008*</td>
<td>2.43</td>
<td>0.46 (18.9%)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germfree rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytate-free diet</td>
<td>3.41 ± 0.18</td>
<td>24.0</td>
<td>9.92 (41.2%)</td>
</tr>
<tr>
<td>Phytate-supplemented diet</td>
<td>2.94 ± 0.28</td>
<td>17.5</td>
<td>7.67 (43.9%)</td>
</tr>
<tr>
<td>Conventionalized rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytate-free diet</td>
<td>3.12 ± 0.39</td>
<td>20.6</td>
<td>8.21 (39.9%)</td>
</tr>
<tr>
<td>Phytate-supplemented diet</td>
<td>2.57 ± 0.29</td>
<td>18.4</td>
<td>8.17 (44.5%)</td>
</tr>
</tbody>
</table>

¹ Mucosal homogenates of the proximal part of each small intestine were combined for three rats.
² Concentrations of sodium oxalate and t-phenylalanine were 50 and 80 mmol/l, respectively.
³ µmol of inorganic phosphorus released from the substrate per min per mg of protein.
⁴ Means of three rats ± standard error.
⁵ The relative activities with respect to controls.
* Significantly different from phytate-free diet group at p < 0.05 according to two-way analysis of variance.

The inhibition or the induction. The dietary phytate seems to induce a part of mucosal phytase in the immature intestine (20), and there might be other inhibiting mechanisms on phytase activity in the mature intestinal tract. Roberts and Yudkin (14) reported that 5% sodium phytate added to the diet decreased the specific activity of phytase in the proximal small intestine of growing rats. They also reported that the effect of 1% dietary phytate was similar to that of the addition of 5% phytate, but less pronounced. Our previous study (16), which examined the effect of the same amount (1%) of dietary phytate on intestinal phytase, agreed with our results. However, the decreasing effect of dietary phytate on phytase activity that was recognized in the homogenates was not clear in the aqueous phase after n-butanol treatment (Table 1). This result might be caused by dialysis of the aqueous phase against isolation buffer, so that inhibitors in the homogenate, such as phytate, were carried away from the enzyme solution. Thus, the decreased intestinal phytase activity in phytate-fed rats is due to the inhibiting effect of dietary phytate, not to the degradation of phytase protein itself. On the other hand, the concentrations of minerals in enzyme solution, which were the homogenate or the aqueous phase after n-butanol treatment, may have an effect on phytase activity, since there have been some reports (4, 14) about the decreased mucosal phytase activity and the reduced availability of dietary minerals, due to the addition of phytate. Davies and Flett (4) found that Zn⁺⁺ was required for the greatest phytase activity after Mg⁺⁺, in the case of the minerals being removed from mucosal fractions. Since two-week feeding of sodium phytate, even at a level of 1% in the diet, caused a significant decrease in Zn content in rat bone (15), marginal Zn deficiency might have occurred in the intestinal mucosa when 2% or 5% phytate was added to the diets. Moreover, amount of total dietary phosphate may have an influence along with the effect of dietary phytate on mucosal phytase, because a greater degradation of phytate was observed in a marginal phosphorus diet compared with an adequate phosphorus diet (10). Further investigations into the interaction between dietary phytate and minerals in the digestive tract are required to determine the reason why dietary phytate decreased the intestinal phytase activity.

Inhibitory effects of sodium oxalate, which was reported as an intestinal phytase inhibitor by Rao and Ramakrishnan (13), on phytase and ALPase activities were observed in both GF and CV rats (Table 1). The relative activities of phytase with sodium oxalate to the
control were 16.9–21.6%, though phytase is reported to be completely inhibited by sodium oxalate (13). The relative activities with respect to controls were 39.9–44.5% for ALPase. L-Phenylalanine, which is known as an intestinal ALPase inhibitor, did not inhibit phytase activities, but activated them to 128–140% of the control levels. The effects of L-phenylalanine on the phytase activity were also different in some reports (2, 21). Bitar and Reinhold (2) demonstrated that L-phenylalanine inhibited phytase activity competitively; however, Yong et al. (21) reported that L-phenylalanine did not affect phytase activity at all, and we found no inhibiting effect while relative activity with L-phenylalanine was 128–140%. Yong et al. (21) suggested that binding of L-phenylalanine induced some conformational change in the active site of only ALPase of a single enzyme protein that also had an active site of phytase. There is no clear reason for the phytase-activating effect of L-phenylalanine at present.

The electrophoretic pattern of the aqueous phase (n-butanol treatment) obtained from the mucosal homogenate of GF rats fed a phytate-free diet is shown in Fig. 1. Two peaks (I and II), which have phytase activities, were recognized and the peak II did not have an alkaline phosphatase activity. Bitar and Reinhold (2) also found two zones of activity for phytase in the intestinal mucosa of chickens by elution from DEAE-cellulose, and suggested phytase was distinct from ALPase. Yang et al. (21), however, demonstrated a single protein band from rat intestinal mucosa on PAGE, which had the two active sites. The possible involvement of phytase in the phosphatase reactions of intestinal brush border membrane of rats at acid pH was suggested by Kaneko et al. (11). The disagreement among these reports and our present study might be due to the differences in animals used, such as species, strain, age or diet adaptation, as well as microbial status, GF or CV. It is necessary to conduct further investigation on intestinal phytase and its enzyme structure, since we found there was an intestinal phytase that did not have an ALPase activity under the GF condition.

The effects of ions (Zn²⁺ and Mg²⁺) added separately or together on activities of phytase (peaks I and II) are shown in Fig. 2. The relative increases in activities of peak I and II due to the addition of these metal ions were different. When Zn²⁺ alone was present, relative phytase activity for peak I and II were 64% and 94%. Phytase activity was considerably greater when Mg²⁺ alone was present in peak I, and both Zn²⁺ and Mg²⁺ in peak II. Zn²⁺ has been shown to inhibit phytase activity (3, 21) and it might be because of the strong chelating effect of Zn²⁺ to the substrate, phytate. The activity was moderately inhibited by up to 2 mM ZnCl₂, but strongly inhibited at higher concentrations (21). We also found the lower concentration of Zn²⁺, even 0.05 mM Zn²⁺,
inhibited phytase activity of peak I, but no inhibition was observed in peak II.

In conclusion, the results of our study are consistent with the view that intestinal microflora have no remarkable effect on the mucosal phytase activity of the proximal part of the small intestine. The present study also showed dietary phytate may decrease the phytase activity in intestinal mucosa, but this effect was not clear in the course of purification. Furthermore, we found a phytase which does not show ALPase activity in the intestinal mucosa of GF rats. The presence of several enzymic proteins, which contribute dephosphorylations of phytate, may be possible in the digestive tract. Thus, further investigations are required to determine the hydrolysis of dietary phytate.

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REFERENCES