Evaluation of a Beta-propeller Phytase for its Application in Aquaculture

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Abstract

The beta-propeller phytases are structurally different from the commercially available phytases, possess high thermal stability, an optimal temperature of 55 to 70°C, unique Ca^{2+}-dependent catalytic properties, a pH optimum close to 7, and exhibit activity within a range of pHs that is broader than those of the histidine acid phytases. In this work, the performance of FTEII, a new beta-propeller phytase, was compared with those of three commercial phytases in terms of thermostability at 99°C, resistance to proteolysis by digestive enzymes, and their effectiveness for phosphorus (P) release from two feed ingredients.

FTEII showed the highest thermostability with residual activity of 82±3 after 1.5 min treatment at 90°C. All phytases were resistant to shrimp digestive enzymes and to porcine trypsin, residual activities showed values higher than 60% in all cases. Total phosphorus released from each phytase-treated ingredient was time, temperature and type of ingredient dependent. FTEII treatments showed the higher levels of phosphorus release in both, soybean meal (68%) and pea (57%) at 50°C. Endogenous hydrolytic activity of both ingredients promoted a phosphorus release, up to 28% or 17% of total phosphorus in soybean meal or in pea protein concentrate, respectively. Unlike commercial phytases, FTEII is phytate specific; therefore it is possible to assume that most of the phosphorus released came only from phytate. FTEII offer an alternative as phytase additive for pelleted feeds and for releasing phosphorus under conditions suitable for digestive tracts of species grown in aquaculture.

Key words: *Phytase, Soybean meal, Pea protein*

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Introduction

Phytate (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate), the major storage form of phosphorus in plant-derived feedstuffs, is regarded as an antinutritional factor, since it forms insoluble complexes with proteins and a variety of nutritionally important metal ions such as calcium, zinc, magnesium and iron, and decreases phosphorus bioavailability (Greiner and Konietzny, 2006).

Phytate accounts for 60–90% of total phosphorus content in plants, principally in cereals and legumes, and it is considered to be an important reserve material in the germination and growth of plants (Vats *et al*., 2007). In cereals, phytate accounts for 60–80% of total phosphorus (Selle *et al*., 2007), in soybean (*Glycine max*), phytate represent a 57% of total organic phosphorus and 70% of total phosphorus (Beleia *et al*., 1993). Soybean meal accounts for 6.49 (5.7-6.94) g/kg of total phosphorus and 3.88 (3.54-4.53) g/kg of phytic phosphorus that represent 59.9% (53-68) of total phosphorus (Selle and Ravindran, 2007). Phytate in pea (*Pisum sativum*) is present in 0.75-0.94% (Carnovale *et al*., 1988).

Aquaculture nutrition has been trying to find suitable alternatives to fish meal. Under this context, soybean meal is the most promising plant protein source among the other ingredients, as alternatives to fish meal (Biswas *et al.*, 2007). Due to the presence of high content of phytate the use of soybean meal is often limited in aquaculture nutrition. The phytate phosphorus, which cannot be digested by crustaceans or fish species due to the lack of phytase enzyme, becomes a pollutant in the aquatic environment, which can be balanced by the use of exogenous phytase (Biswas *et al.*, 2007).

Legume seeds are an important source of dietary protein, with great potential for human and animal nutrition. The nutritive utilization of legumes can be negatively affected by their content of antinutritional factors such as R-galactosides, trypsin inhibitors, or phytic acid, which interfere with the digestive utilization of proteins and minerals by monogastric animals (Urbano *et al.*, 2007).

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8, EC 3.1.3.26, and EC 3.1.3.72) catalyze the release of phosphate from phytate, generating less-phosphorylated *myo*-inositol derivatives (Lei *et al.*, 2007).

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Simple-stomached species such as swine, poultry, and fish cannot utilize dietary phosphorus because their gastrointestinal tracts are deficient in enzymes with phytase activity (Lei *et al.*, 2007). Therefore, during the last two decades, these enzymes have been used as feed additives to enhance the utilization of plant-derived feedstuffs, improving the utilization of phytate-phosphorus in diets, so that inorganic phosphorus supplementation is unnecessary, reducing their manure phosphorus excretion to the environment (Lei *et al.*, 2007). While supplemental phytase has shown to improve utilization of calcium, zinc, and iron utilization by animals (Lei *et al.*, 2013), the digestibility response of copper or manganese is less consistent, and the ability of phytase to improve amino acid availability has been controversial (Lei *et al.*, 2013; Selle and Ravindran, 2007).

At present, phytases are classified into four major classes (Lei *et al.*, 2007; Mullaney *et al.*, 2007): histidine acid, beta-propeller, cysteine, and purple acid phytases. Most of the commercially available phytases are histidine acid phytases and possess catalytic activity in the pH range of 2.5 to 6. On the other hand, the beta-propeller phytases, also referred to as alkaline phytase, are structurally different from the histidine acid phytases, possess pH optimums close to 7, and exhibit activity within a range of pHs that is broader than those of the histidine acid phytases (Kerovuo *et al.*, 1998; Kim *et al.*, 1998; Ha *et al.*, 2000; Shin *et al.*, 2001). In addition, beta-propeller phytases are characterized to have a high thermostability, an optimal temperature of 55 to 70°C and exhibit unique Ca^2-dependent catalytic properties (Ha *et al.*, 2000; Oh *et al.*, 2004).

Although commercial production of phytases is currently focused on fungal histidine acid phytases from *Aspergillus* species (Lei *et al.*, 2007; Oh *et al.*, 2004), the second phytases generation, also histidine acid phytases but isolated from strains of *Escherichia coli* (AppA and AppA2), were identified as more effective phytases than the fungal PhyA (Lei *et al.*, 2013; Rodríguez *et al.*, 1999), with acidic pH optimum, higher catalytic efficiency, and pepsin resistance.

The beta-propeller bacterial phytases from *Bacillus* species are an alternative to histidine acid phytases enzymes because of their high thermostability, calcium-phytate complex substrate specificity, pH profile, and proteolysis resistance (Kerovuo *et al.*, 2000; Kim *et al.*, 1999; Oh *et al.*, 2004). In contrast to the histidine acid phytases, *Bacillus* phytases are

specific for phytate. Therefore, important phosphate compounds other than phytate are not hydrolyzed by the *Bacillus* phytases and remain available for animal uptake.

Recently several beta-propeller phytases, one from *Bacillus subtilis* (Guerrero-Olazarán *et al.*, 2010) and others that were designed by a structure-guided consensus approach (Viader-Salvadó *et al.,* 2010) were produced in the methylotrophic yeast *Pichia pastoris.* In this work, one of these beta-propeller phytases (FTEII), designed to have high thermostability and activity over a broad range of pH (Viader-Salvadó *et al.,* 2010), was evaluated for their performance as feed additive. The performance of this new phytase was compared with three commercial phytases (histidine acid phytases) in terms of thermostability at 99°C, resistance to proteolysis by digestive enzymes, and their effectiveness for phosphorus (P) release from two feed ingredients.

Materials and methods

FTEII and commercial phytase preparations.

FTEII preparation was obtained from cell-free culture medium recovered from a 5-L bioreactor culture of a recombinant *P. pastoris* strain (Viader-Salvadó *et al.,* 2013). Cell-free culture medium was concentrated 20-fold and diafiltrated by ultrafiltration as described elsewhere (Viader-Salvadó *et al.,* 2010). This enzyme preparation was analyzed by Bradford protein and enzymatic activity assays and used for all assays.

Commercial phytases used as reference were Natuphos® (5,000 FTU/g, granulate, BASF), Allzyme®SSF (1000 FTU/g, granulate, Alltech, Inc), both histidine acid phytases classified as 3-phytase from *Aspergillus niger*, and Ronozyme®P (5,000 FTU/g, granulate, NOVOZYMES/DSM), histidine acid phytase classified as 6-phytase from *Peniophora lycii.* All commercial phytases were resuspended at 10 mg/mL in acetate buffer (360 mM sodium acetate, 100 µg/mL Tween 20, 1 mM $CaCl_2$, pH 5.5). The suspensions were clarified by centrifugation at 6900 g for 30 min. The clear supernatants were fractioned and stored at -20°C until their use.

Enzymatic activity assays

For FTEII, phytase activity was measured in a reaction mixture containing the enzyme preparation, 1.6 mM sodium phytate, 100 mM Tris- HCl buffer (pH 7.5), and 1 mM $CaCl_2$, with incubation at 37°C for 30 min. The reaction mixture was stopped by adding an equal volume of 15% trichloroacetic acid, and the inorganic phosphate released was measured using the ascorbic acid method (Guerrero-Olazarán *et al.,* 2010). For commercial phytases, enzyme activity were measured in acetate buffer (360 mM sodium acetate, pH 5.5, without $CaCl_2$) instead of Tris-HCl buffer. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 µmol of phosphate per min from sodium phytate under the assay conditions.

Thermostability. Thermostability of each phytase preparation was evaluated by measuring phytase activity after incubation at 99°C for 1.5 or 5 min in 360 mM sodium acetate (pH 5.5) with 5 mM $CaCl_2$ for FTEII or without $CaCl_2$ for commercial phytases. Residual activity was calculated as phytase activity measured after heat treatment and expressed as a percentage (Viader-Salvadó *et al.*, 2010).

Phytase susceptibility to digestive enzymes. Susceptibility to shrimp (*Litopenaeus vannamei*) digestive enzymes was tested by incubating a mixture containing the phytase preparations at 0.08 U/mL and 1X (0.045 U *N*-α-benzoyl-DL-arginine-*p*-nitroanilide [BAPNA]/mL of trypsin), 10X, or 40X shrimp digestive enzyme extract in presence of 1 mM $CaCl_2$, 100 mM Tris-HCl (pH 7.5) at 37°C for 30 min, followed by phytase activity measurement to test for residual phytase activity. Similar assays were performed with 0.12 (1X) and 1.20 (10X) U/mL porcine trypsin (Sigma-Aldrich, St. Louis, MO) instead of the shrimp digestive enzymes. Trypsin activity determinations were carried out evaluating amidase activity in a reaction mixture containing porcine pancreas trypsin or shrimp digestive enzymes, 1 mM BAPNA as the substrate, 20 mM Tris-HCl (pH 7.6), and 20 mM $CaCl_2$ at 37°C. The amount of *p*-nitroaniline released was monitored for 3 min by measuring the increase in absorbance at 405 nm (ε=8,270 M^{-1} cm^{-1}).

Phosphorus release from feed ingredients. Phosphorus release was evaluated for soybean meal (0.38% of phytic phosphorus and 0.65% of total phosphorus, Natural Proteins, NL, Mexico.) and pea protein concentrate (0.58% phytic phosphorus and 0.89% of total phosphorus, Prestige protein R400, Parheim Foods Ltd. Saskatoon, Canada). All phytases were used in a concentration of 1600 or 800 U/kg of ingredient. Both ingredients (250 mg) were treated with each phytase in a reaction mixture containing water (pH 6.3, 250 mg ingredient:500 μL water). Each ingredient suspension-water-phytase and controls (ingredient without enzymes) were incubated with constant stirring at 250 rpm, different temperatures (30, 40 and 50°C) and times (0 to 12 h). The enzymatic reaction was stopped with 9.5 mL of 15% TCA, the supernatant recovered by two successive centrifugations (8000 g, 30 min and 14000 g, 15 min) and phosphate released was determined as a percentage of total phosphorous reported for each ingredient, 6.5 g/kg for soybean meal and 8.9 g/kg for pea protein concentrate.

All the analytical determinations were carried out at least three times (coefficient of variation less than 5%). All results were compared between groups using analysis of variance and Tukey's multiple comparisons with a significance level of $P < 0.05$

Results

Thermostability. FTEII showed the highest thermostability with residual activities of 82±3 and 63±3% after 90 s and 300 s of heat treatment, respectively, as is shown in Figure 1. Natuphos, and Ronozyme showed the same residual activities of 30 and 16%, after 90 s and 300 s of heat treatment, respectively, while Allzyme showed residual activities of 45 and 30%, after the same treatment. While FTEII lost only 18% activity after 90 s of heat treatment, Natuphos and Ronozyme, both lost 70% of initial activity and Allzyme lost 55% of its initial activity.

By increasing the treatment time, FTEII lost only a 37% of its initial activity, while commercial enzymes lost until 84% of their initial activity. Allzyme showed to be the most heat stable phytase among the commercial phytases tested.

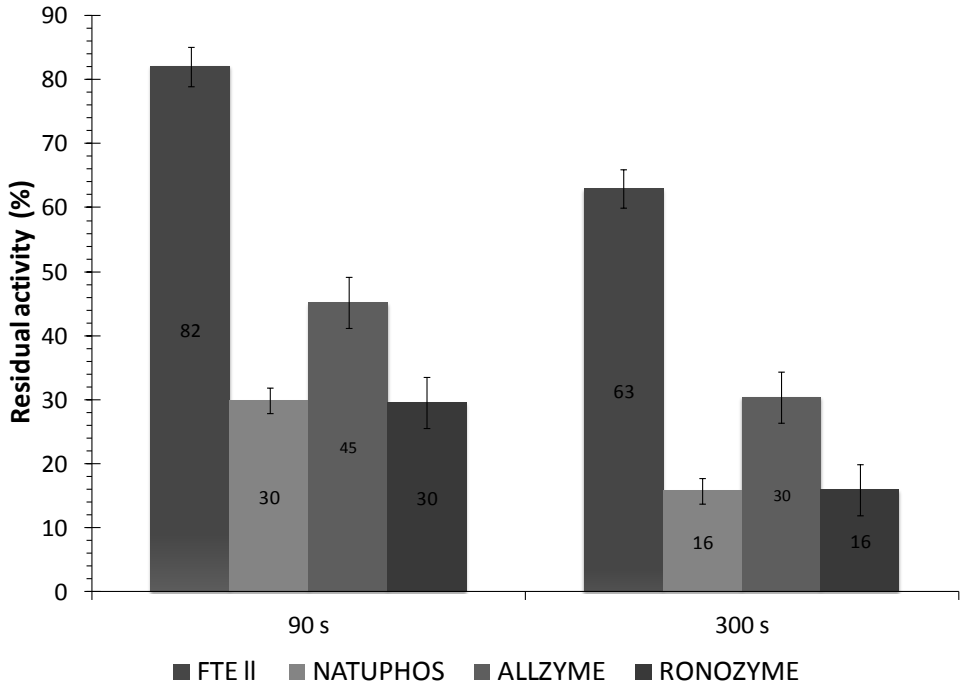

Fig. 1. Residual activities of FTEII after 90 and 300 seconds of heat treatment at 99°C and pH 5.5. FTEII heat treatment was in presence of 1 mM $CaCl_2$. Values represent the means for at least three independent determinations (coefficient of variation, <5%).

Phytase susceptibility to digestive enzymes. All phytases were resistant to shrimp digestive enzymes and to porcine trypsin. Residual activities showed values higher than 60% in all cases (Fig. 2 and 3). The residual activities of FTEII after shrimp digestive enzyme treatment were higher than 67% (Fig. 2). After 30 min of shrimp digestive enzymes treatment, Allzyme showed residual activities that were higher than 100% (Fig. 2). Natuphos residual activity increases were observed when the protease concentration was increased from 1X to 40X (Fig. 2), while FTEII residual activity increased 13% when the protease concentration was increased from 1X to 10X. However, a 33% depletion of residual activity was observed after 40X shrimp digestive enzyme treatment of FTEII (Fig. 2).

After 30 min of porcine trypsin treatment, residual activities of all phytases were higher than 95% (Fig. 3). Residual activity increases were observed when the protease concentration was increased from 1X to 10X, 16% for FTEII and 12% for Natuphos, while residual activity for Allzyme decreased in a 10%. While FTEII and Natuphos showed

similar performance under trypsin treatment, Allzyme showed less stability when the trypsin concentration was increased.

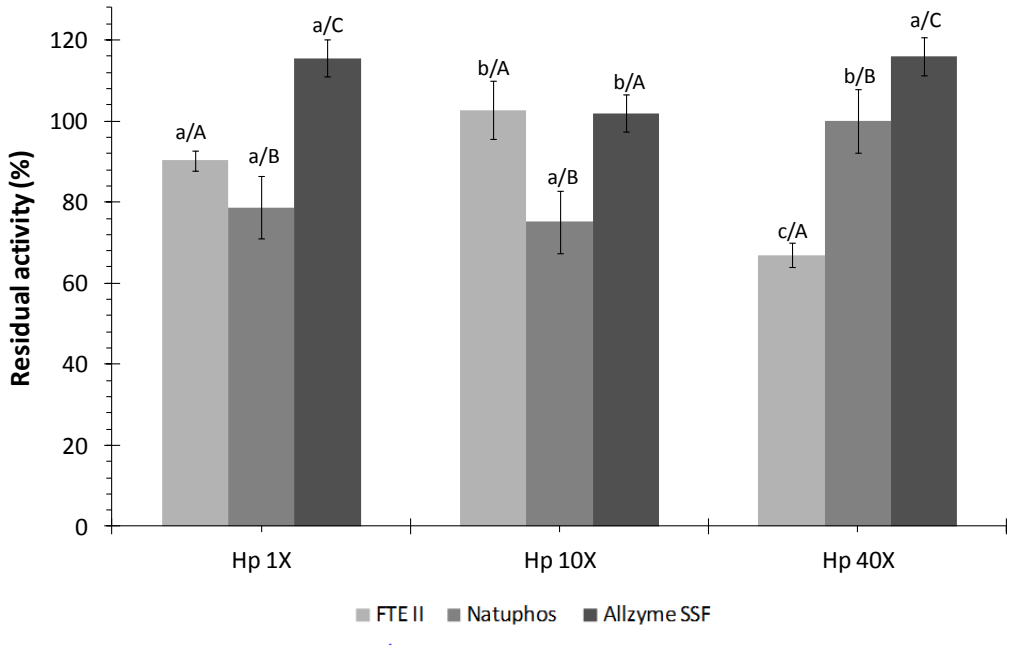

Fig. 2. Residual phytase activities of FTEII, Natuphos and Allzyme preparations after shrimp digestive enzyme (Hp) treatment. The data are the means ± the standard deviations of at least three independent assays. Different lowercase, differences for the effect of the protease treatment concentration on each phytase, $P < 0.05$. Different uppercase, differences among phytases at the same protease treatment concentration, $P < 0.05$.

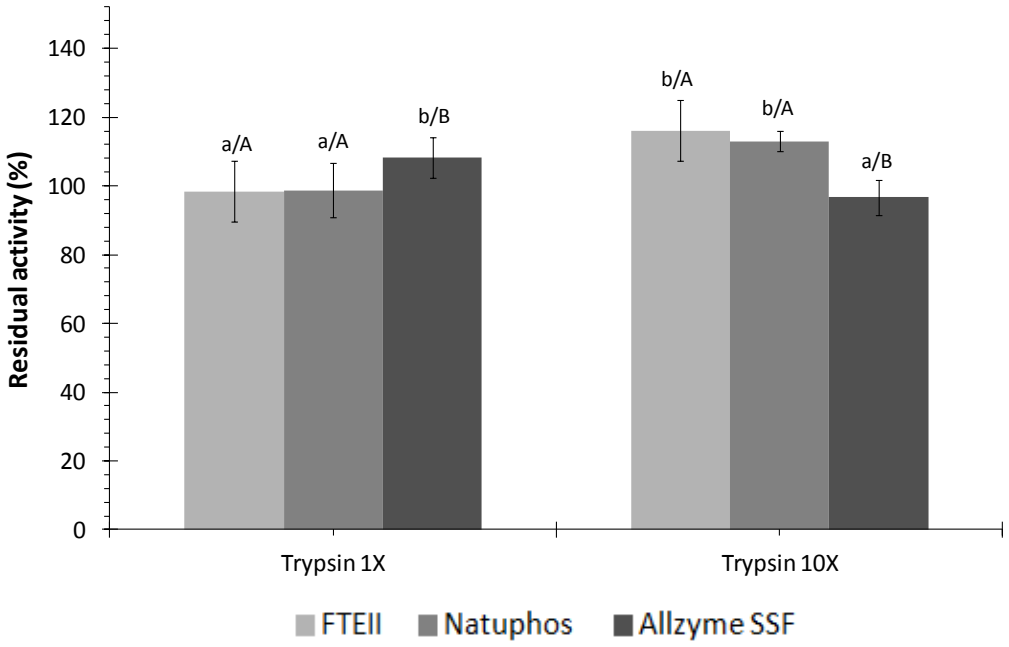

Fig. 3. Residual phytase activities of FTEII, Natuphos and Allzyme preparations after trypsin treatment. The data are the means ± the standard deviations of at least three independent assays. Different lowercase, differences for the effect of the protease treatment concentration on each phytase, $P<0.05$. Different uppercase, differences among phytases at the same protease treatment concentration, $P<0.05$.

Phosphorus release from feed ingredients. Figure 4 shows the phosphorus release kinetics from soybean meal for FTEII treatments at different temperatures. The average rate of phosphorus release was higher at 40°C (0.37 g kg^{-1} h^{-1}) than treatments at 30 and 50°C (Table 1). After 12 h incubation the highest value of phosphorus released was observed at 50°C with 4.38± 0.09 g/kg (67.5±1.3% from total phosphorus) followed by the treatment at 40°C with 4.29± 0.09 g/kg (66.2±1.4% from total phosphorus). The phosphorous released at the end of the treatment at 30°C was 12 and 14% lower than those at 40 and 50°C, respectively (Table 1).

The average rate of phosphorus release for controls (0.14 g kg^{-1} h^{-1}) was in average 56% lower than those showed by FTEII treatments. The phosphorus released from total phosphorus for controls at 12 h incubation was 27% in average, representing 40% of phosphorus released from the soybean meal treated with FTEII phytase (Table 1).

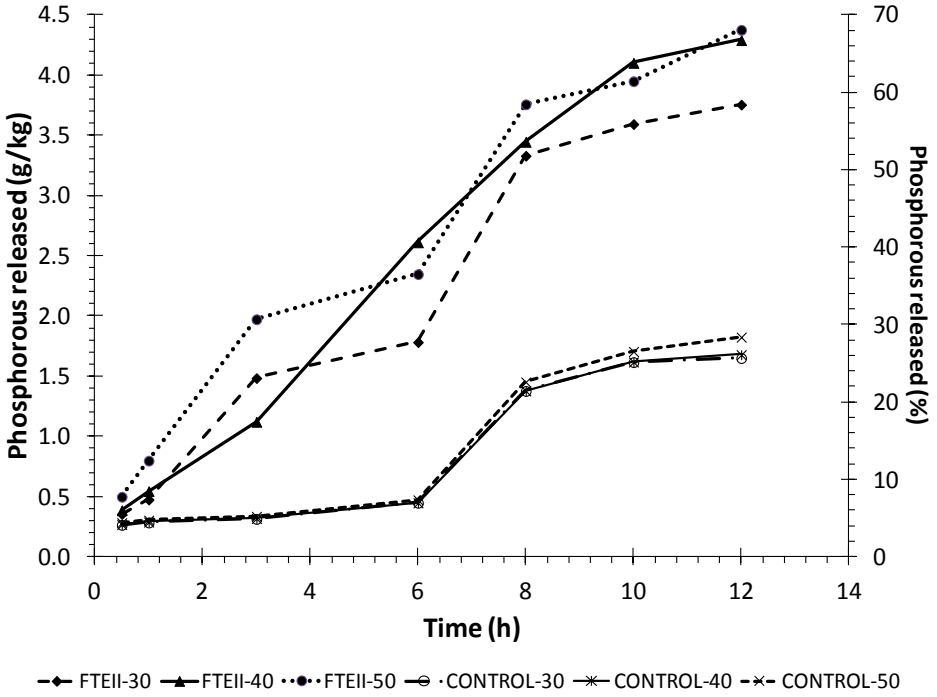

Fig. 4. Phosphorus release kinetics from soybean meal. Treatments with 1600 U/kg of FTEII at 30, 40 and 50°C. Controls were treatments without enzyme. The data are the means ± the standard deviations of at least three independent assays (coefficient of variation, <5%).

Table 1. Average rate of phosphorus release and total phosphorus released values from different treatments of soybean meal.

Treatment	ARPR (g kg^{-1} h^{-1})			TPR (g/kg)			TPR (%)		
				TPRSC (g/kg)			TPRSC (%)		
	30°C	40°C	50°C	30°C	40°C	50°C	30°C	40°C	50°C
Control	0.14	0.14	0.15	1.65±0.05	1.68±0.06	1.83±0.06	25.5±0.8	25.9±0.9	28.1±0.9
FTEII 1600	0.32	0.37	0.34	3.76±0.07	4.29±0.09	4.38±0.09	57.9±1.1	66.2±1.4	67.5±1.3
	0.17	0.23	0.18	2.11±0.07	2.61±0.09	2.55±0.09	32.4±1.9	40.3±2.3	39.3±2.2
Natuphos		0.33			3.84±0.12			59.1±1.9	
		0.20			2.26±0.12			34.8±3.1	
Allzyme		0.34			2.39±0.32			61.2±2.1	
		0.21			2.11±0.07			36.8±4.1	
FTEII 800		0.28			3.46±0.02			53.3±0.3	
		0.15			1.88±0.02			28.9±0.5	

ARPR: average rate of phosphorus release.
TPR: total phosphorus released after 12 h incubation.
TPRSC: total phosphorus released subtracting control after 12 h incubation.
The data are the means ± the standard deviations of at least three independent assays.

Average rates and kinetics of phosphorus release from pea protein concentrate at different temperatures for FTEII treatments are shown in Table 2 and Figure 5. Unlike soybean meal, in pea protein concentrate the average rates of phosphorus release was higher at 50 °C (0.37 g kg^{-1} h^{-1}) than at 40°C (0.35 g kg^{-1} h^{-1}) and at 30°C was lower (0.26 g kg^{-1} h^{-1}). After 12 h incubation, the highest phosphorus released was observed at 50°C with 5.05± 0.11 g/kg (56.7±1.2% from total phosphorus). The treatment at 30°C showed a lower percentage of phosphorus release at the end of the treatment, 22 and 28% lower than those at 40 and 50°C, respectively (Figure 5 and Table 2).

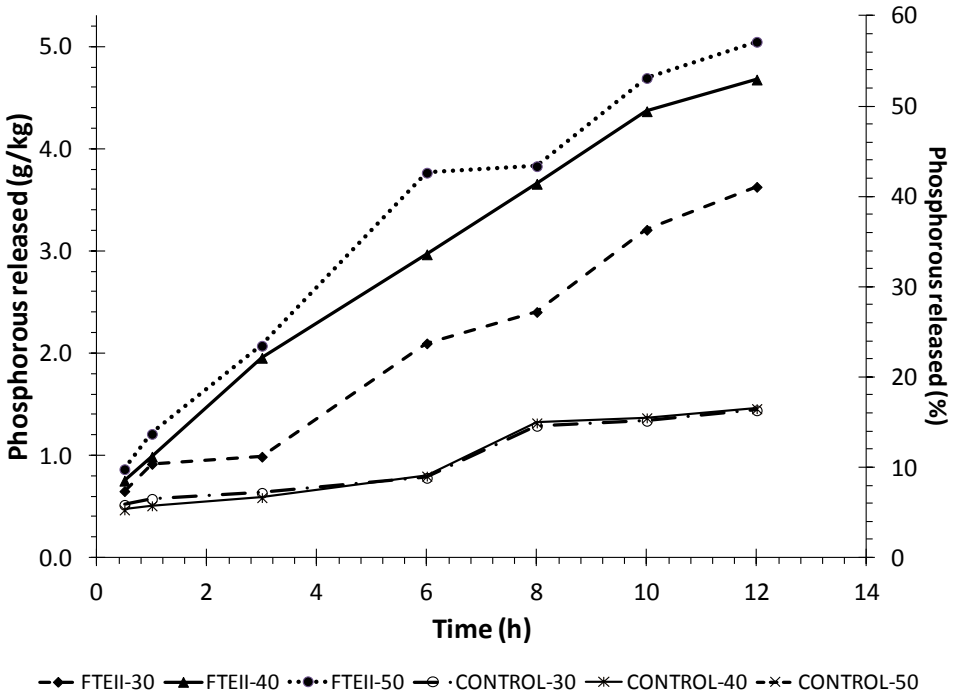

Fig. 5. Phosphorus release kinetics from pea protein concentrate. Treatments with 1600 U/kg of FTEII at 30, 40 and 50°C. Controls were treatments without enzyme. The data are the means ± the standard deviations of at least three independent assays (coefficient of variation, <5%).

The average rate of phosphorus release for controls in pea protein concentrate treatment was only 27% (0.09 g kg^{-1} h^{-1}) of those obtained by FTEII treatment (0.33 g kg^{-1} h^{-1}). After 12 h of incubation, the control of pea protein concentrate treatment released up to 17% of total phosphorus (1.54±0.05 g/kg) representing an average value of 33% of phosphorus released from pea protein concentrate treated with FTEII phytase (Table 2).

Despite the percentage of phosphorus released from pea protein concentrate was lower than those released in soybean meal, the amount of phosphorus released in pea protein concentrate was higher than in soybean meal, reaching up to 5.05 g/kg after 12 h incubation.

A low percentage of phosphorus released from pea protein concentrate is likely justified by a high content of phytate-phosphorus and total phosphorus in this ingredient (5.76 and 8.9 g/kg, respectively) compared to the content of phytate-phosphorus and total phosphorus in soybean meal (3.88 and 6.49 g/kg, respectively). Furthermore, endogenous hydrolytic activity was higher in soybean meal (up to 28% of phosphorus released) than pea protein concentrate (up to 17% of phosphorus released); therefore, FTEII was more efficient to release phosphate in pea protein concentrate than in soybean meal. FTEII showed values of phosphorus released 3.7 % lower in soybean paste than pea protein concentrate at 30°C, 18.79 % lower at 40°C and 27.4 % lower at 50°C.

While the hydrolysis under FTEII treatment in soybean meal was more efficient at 40°C than 50°C, in pea protein concentrate was more efficient at 50°C than 40°C, consistent with the average rates of phosphorus release and levels of phosphorus released only by FTEII activity (without endogenous hydrolytic activity). The hydrolysis at 30°C was less efficient in both ingredients, where pea protein concentrate showed the lowest phosphate release efficiency.

Table 2. Average rate of phosphorus release and total phosphorus released values from different treatments of pea protein concentrate.

Treatment	**ARPR (g kg^{-1} h^{-1})**			**TPR (g/kg)**			**TPR (%)**		
				TPRSC (g/kg)			**TPRSC (%)**		
	30°C	**40°C**	**50°C**	**30°C**	**40°C**	**50°C**	**30°C**	**40°C**	**50°C**
Control	0.09	0.09	0.10	1.44±0.05	1.46±0.05	1.54±0.05	16.2±0.5	16.4±0.5	17.3±0.6
FTEII 1600	0.26	0.35	0.37	3.63±0.07	4.68±0.09	5.05±0.11	40.8±0.8	52.6±1.0	56.7±1.2
	0.17	0.26	0.27	2.19±0.07	3.22±0.09	3.51±0.11	24.6±1.3	36.2±1.5	39.4±1.8
Natuphos		0.35			4.59±0.11			51.6±1.2	
		0.27			3.26±0.11			36.6±1.8	
Allzyme		0.33			4.53±0.11			50.9±1.2	
		0.26			3.20±0.11			35.9±1.8	
FTEII 800		0.26			3.87±0.06			43.4±0.9	
		0.18			2.53±0.08			28.4±1.5	

ARPR: average rate of phosphorus release.
TPR: total phosphorus released.
TPRSC: total phosphorus released subtracting control.
The data are the means ± the standard deviations of at least three independent assays.

The phosphorous release performance of FTEII compared to commercial phytases performance was tested at 40°C using a treatment with 1600 U/kg. Treatment with FTEII at 800 U/kg was also evaluated to determinate the concentration effect.

The highest average rate of phosphorus release for soybean meal was observed for FTEII treatment at 1600 U/kg (0.37 g kg^{-1} h^{-1}), followed by Allzyme and Natuphos treatment (0.34 and 0.33 g kg^{-1} h^{-1}, respectively), while treatment at 800 U/kg with FTEII showed an average rate of phosphorus release of 0.28 g kg^{-1} h^{-1}, corresponding to a 25% from the value obtained by FTEII treatment at 1600 U/kg.

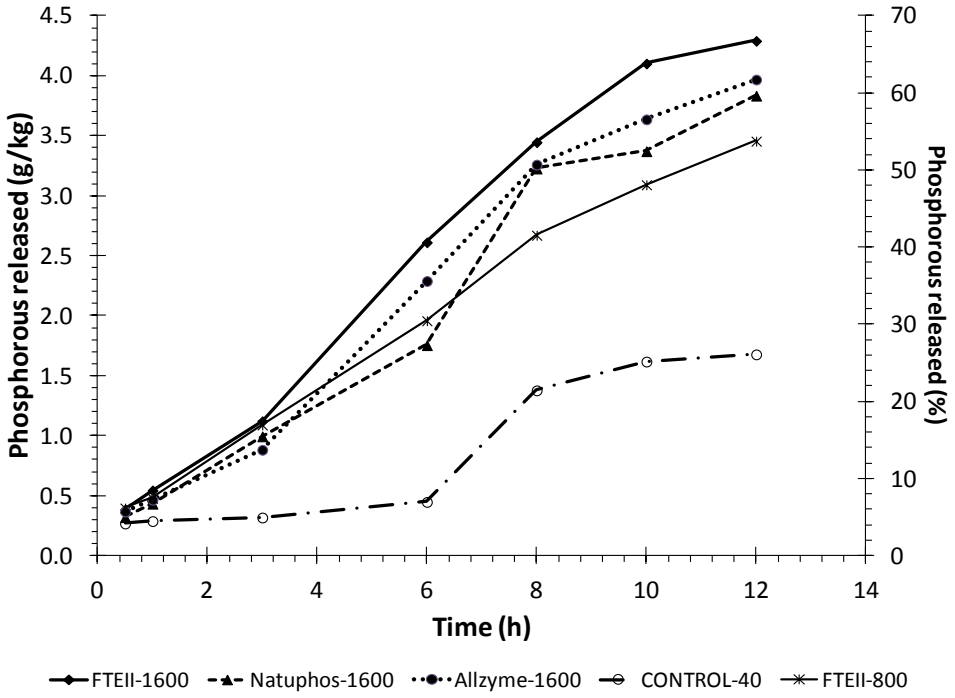

Fig. 6. Total phosphorus release kinetics from soybean meal. Treatments with 1600 or 800 U/kg of FTEII, Natuphos and Allzyme at 40°C. Controls were treatments without enzyme. The data are the means ± the standard deviations of at least three independent assays (coefficient of variation, <5%).

After 12 h incubation, FTEII treatment at 1600 U/kg showed the highest phosphorus released value (4.29±0.09 g/kg) corresponding to 66.2% of total phosphorous present in soybean meal, while Natuphos and Allzyme released 59.1 and 61.2%, respectively (Table 1).

The FTEII treatment at 800 U/kg released 53% of total phosphorous (3.46±0.02 g/kg), 19% less than those released by FTEII treatment at 1600 U/kg.

Subtracting the endogenous hydrolytic activity (phosphorus released by the control), FTEII at 1600 U/kg and 40°C released 40.3±2.3 % (2.61±0.09 g/kg) of total phosphorous present in soybean meal, Natuphos 34.8±3.1 % (2.26±0.12 g/kg) , Allzyme 36.8±4.1 % (2.11±0.07 g/kg) and FTEII at 800 U/kg released 28.9±0.5 % (1.88±0.02 g/kg)

These results demonstrate a higher hydrolytic efficiency of FTEII on soybean meal compared to the commercial phytases tested.

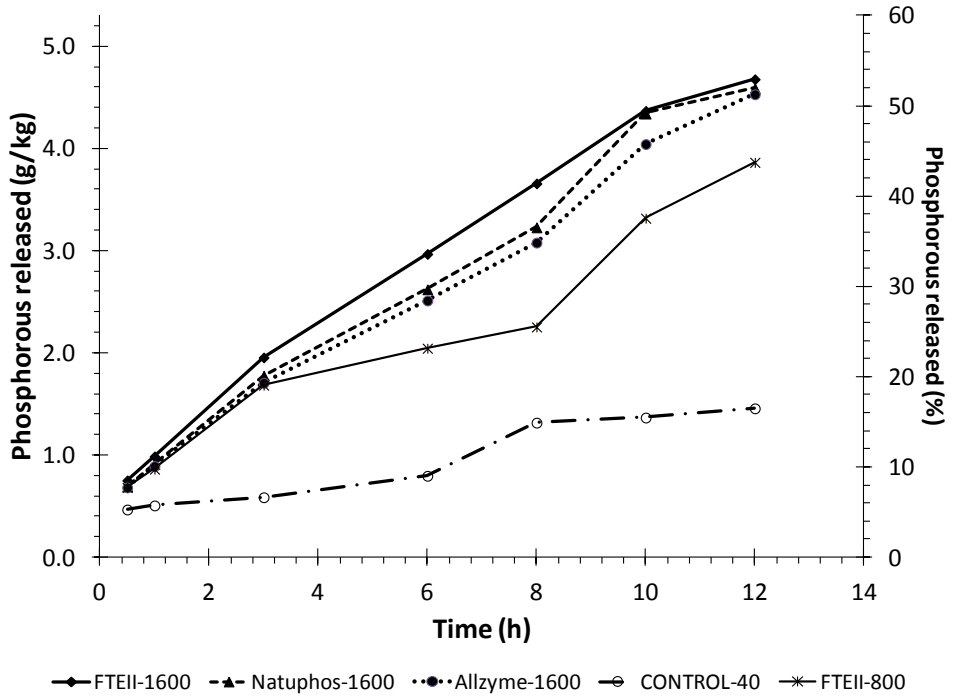

Fig.7. Phosphorus release kinetics from pea protein concentrate. Treatments with 1600 or 800 U/kg of FTEII, Natuphos and Allzyme at 40°C. Controls were treatments without enzyme. The data are the means ± the standard deviations of at least three independent assays (coefficient of variation, <5%).

Figure 7 shows the phosphorus release kinetics of pea protein concentrate treated at 40°C with FTEII, Natuphos, or Allzyme at 1600 U/kg, or FTEII at 800 U/kg. The average rate of phosphorus release for pea protein concentrate treated with FTEII at 1600 U/kg or Natuphos showed a value of 0.35 g kg^{-1} h^{-1}, higher than those obtained by Allzyme and FTEII at 800 U/kg treatments (Table 2).

The highest phosphorus release was observed in FTEII treatment at 1600 U/kg, with 4.68± 0.09 g/kg (52.6±1%). Natuphos and Allzyme showed similar performance but lower than FTEII treatment at 1600 U/kg. FTEII treatment at 800 U/kg released 43% of total phosphorous, 17% less than those released at 1600 U/kg.

Subtracting the percentage of phosphorus released by the control, FTEII at 1600 U/kg released 36.2±1.5 % (3.22 ±0.09 g/kg) of total phosphorous present in pea protein concentrate, while Natuphos, Allzyme and FTEII at 800 U/kg released 36.6±1.8 % (3.26 ±0.11 g/kg), 35.9±1.8 % (3.20 ±0.11 g/kg) and 28.4±1.5 % (2.53±0.08 g/kg) respectively. These results demonstrate a similar hydrolytic efficiency of all phytases tested in pea protein concentrate.

Discussion

Recently, FTEII, a new beta-propeller phytase, was designed and produced in *P. pastoris* (Viader-Salvadó *et al*., 2010, 2013). Based on their pH profiles, the beta-propeller phytases may have potential as feed additives for animals with neutral-basic digestive tracts and in the pretreatment of plant-derived ingredients used in animal diets. In this paper we have compared the FTEII performance against three commercial phytases in terms of thermostability, resistance to proteolysis by digestive enzymes, and their effectiveness for phosphorus release from two feed ingredients, soybean meal and pea protein concentrate.

In terms of thermostability, FTEII showed higher thermostability under the same denaturation conditions than commercial phytases tested, with 82% residual activity, in comparison to only 30% for Ronozyme, a second generation phytase, provided with technology to increase thermostability.

The results showed that FTEII have an intrinsic thermostability, allowing support thermal treatment at broad time intervals. This property is important for phytases, since they would be able to resist the high temperatures encountered in the feed-pelleting process used in commercial fish diets (Wang *et al*., 2009). Most of commercial phytases are thermally unstable, resulting in loss of activity at processing temperatures of >80°C, temperatures over 87°C during steam pelleting of feed results in a reduction of the phytase activity by more than half (Wang *et al*., 2009).

Phytase supplementation by spraying or encapsulation are approaches used to overcome the lack of thermostability of phytases, however some reports indicate that encapsulation of microbial phytases tended to diminish its ability to liberate phosphorus, likely due to a reduced interaction between the enzyme and dietary phytate-phosphorus (Vandenberg *et al*., 2011), and phytase spraying on feed pellets cannot hydrolyze phytate efficiently *in vitro* (Wang *et al*., 2009).

Phytase C from *B. subtilis*, a beta-propeller phytase, has been shown to be resistant to papain, pancreatin, and trypsin, even under conditions of calcium depletion. Nevertheless, this phytase has been found to be susceptible to pepsin (Kerovuo *et al.*, 2000), an effect that was explained by the denaturation of the enzyme at low pH, making it more susceptible to pepsin. In the present work, we tested the susceptibility of FTEII and two commercial phytases to shrimp (*L. vannamei)* digestive enzymes. All phytases were resistant to shrimp digestive enzymes (residual activities of 60 to 100%) and to the exposure to porcine trypsin (activity above 95%). In addition, all phytases showed increased residual activities when the concentration of the protease preparations were increased from 1X to 10X or 1X to 40X. This result may be due to conformational changes occurring on the phytases because of the hydrolysis of some peptide bonds. An increase in residual activity (30%) due to exposure to proteolytic enzymes (pepsin) has been previously described for the phytase r-AppA from *E. coli* (Rodríguez *et al.*, 2000). Authors concluded that stable polypeptides of r-AppA with phytase activity were generated after pepsin treatment.

In vitro methods can be helpful to understand the mode of action of feed enzymes under standardized conditions and to predict the nutrient digestibility of feedstuffs. In this work we have evaluated the effectiveness for phosphorus release from two ingredients treated with FTEII and two commercial phyatses *in vitro* conditions.

The total phosphorus released from each ingredient treated included the phosphorus released by the endogenous hydrolytic activity (phosphorus released by control treatment), because it represents the actually available phosphorus. However to evaluate the hydrolytic efficiency of tested phytases, it is necessary to consider the phosphorus released from controls.

Total phosphorus released from each ingredient treated with FTEII phytase was time, temperature and type of ingredient dependent. The average rate of total phosphorus release

was temperature and ingredient dependent, which was affected by a synergic effect of FTEII and endogenous hydrolytic activities. Endogenous hydrolytic activity of both ingredients promoted a phosphorus release up to 28% or 17% of total phosphorus in soybean meal or in pea protein concentrate, respectively, with an average rate of phosphorus release higher at 50°C in both ingredients.

While in soybean the highest average rate of phosphorus release under FTEII treatment was at 40°C, in pea protein concentrate was at 50°C. FTEII was more efficient to release phosphorus in pea protein concentrate than in soybean meal at 40 and 50°C, consistent with the higher average rates of phosphorus release and levels of phosphorus released determined at these temperatures and considering only the FTEII activity. The same effect was observed at 40°C in both commercial phytases tested. Higher availability of substrate in pea protein concentrate than in soybean meal or inhibitors in soybean meal may be considered to be the cause of this effect.

While FTEII demonstrated be more efficient regarding its hydrolytic activity in soybean treatment than commercial phytases ($P<0.05$), showing the highest average rate of phosphorus release, in pea protein concentrate all phytases showing similar hydrolytic activity.

The reduction to half concentration of FTEII showed a reduction of average rate of phosphorus release in 25%, indicating an enzyme concentration dependent response and that an excess of substrate concentration is present in both ingredients.

FTEII treatments showed the highest levels of phosphorus released in both, soybean meal (68%) and pea protein concentrate (57%) at 50°C after 12 h incubation. On the other hand, FTEII treatments showed higher levels ($P<0.05$) of phosphorus released, compared to commercial phytases treatment at 40°C after 12 h incubation in both ingredients.

The percentage of phosphorus released from pea protein concentrate was lower than those released in soybean meal; however the amount of phosphorus released in pea protein concentrate was higher than in soybean meal. This result can be justified by the high content of phytate and total phosphorus in pea protein concentrate (5.76 and 8.9 g/kg) compared to the content in soybean meal (3.88 and 6.49 g/kg).

The phytate content in different raw materials have different characteristics influencing their hydrolysis and subsequent release of phosphorus. Thus, the efficacy of phytase

depends on the phytate solubility, which varies among vegetable feedstuffs and affects the degree of enzymatic degradation and their utilization by the animal (Pointillart, 1993).

Phytase pretreatment feed ingredients has been studied *in vitro* assays (Frias *et al.*, 2003; Urbano *et al.,* 2003), *in vivo* with some aquatic species (Vielma *et al*., 2002; Nwanna *et al.*, 2003; Wang *et al*., 2009, Denstadli *et al*., 2011; Fortes-Silva *et al.,* 2011) and it has demonstrated to be effective on reduction of phytate-phosphorus, increase of phosphorus bioavailability, reduction of phosphorus excretion and, some cases, it has demonstrated to be effective on specific growth rate (SGR), digestibility and retention efficiencies of nutrients and energy. Under these considerations phytase pretreatment of feed ingredients could be an effective method for supplementation phytase as feed additive.

In this work we demonstrate a higher hydrolytic efficiency of FTEII on soybean meal compared to the commercial phytases tested, and a similar hydrolytic efficiency of all phytases tested in pea protein concentrate. Considering the global results, FTEII demonstrates a better performance in phosphorus release than commercial phytases under the tested conditions, further unlike commercial phytases, FTEII is phytate specific; therefore it is possible to assume that most of phosphorus released comes only from phytate.

FTEII is a beta-propeller phytases requiring calcium for activity and stability, and exhibiting maximum activity at pH between 6.0 and 9.0, and is suitable for animals with neutral digestive tracts. The stability of FTEII in a high temperature range is another important and useful characteristic for their application as animal feed additives, because the process of pelleting uses steam at high temperatures. FTEII offer an alternative as phytase additive for pelleted feeds and for releasing phosphorus under conditions suitable for digestive tracts of species grown in aquaculture.

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