



Effect of phytase supplementation of a plant-based diet on phosphorus and nitrogen bioavailability in sea bream *Sparus aurata*

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Abstract

To assess to what extent addition of phytase to a plant-based diet results in spatio-temporal changes of phytate, available P, soluble protein, total amino acids and the activity of the main digestive proteases in gilthead sea bream, fish were fed two plant-based diets with or without phytase. Stomach, proximal intestine and distal intestine contents were monitored for these parameters at 0, 1, 2, 4 and 6 h after feeding. A reduction ($P < 0.0001$) of the soluble P-IP6 in the stomach when phytase was added to the diet was observed. Within stomach, most of the total P-IP6 was precipitated (86%), possibly due to the low acidification capacity of the sea bream ($\text{pH} > 4$), but 57% of the dietary P-IP6 was dephosphorylated, suggesting that phytase could have the capacity to dephosphorylate insoluble IP6 at such pH. An increment (60%) ($P < 0.01$) in total gastric protease activity was observed by phytase addition, this being the first demonstration of the *in vivo* effect of IP6 on the pepsin activity in fish stomach. Gastric pH and residence time of the digesta inside the stomach are critical factors for an efficient phytase action and improve P and N bioavailability in plant-based diets used in fish aquaculture.

KEY WORDS: amino acids, digestive proteases, phosphorus, phytase, phytate, *Sparus aurata*

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Introduction

The development of the aquaculture industry has been associated with the use of fish meal as the main protein source in diets. However, due to its limited supply and high cost, there is a general interest to replace this animal protein source by plant-derived ingredients. For many years, fish nutritionists have investigated how to utilise plant proteins, because they are cheaper and more available than animal protein sources. Nevertheless, one of the major obstacles limiting their use in fish diets is the presence of different antinutritional factors, being one of them the phytate or IP6 (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate). Most of the phosphorus present in vegetable protein sources is bound to phytate (P-IP6) and due to its low digestibility (Cao *et al.* 2007) is excreted into the water, what may cause algal blooms, this being the major reason why the use of such proteins sources is restricted in sustainable farming (Baruah *et al.* 2004). Moreover, under the physiological conditions existing in the gut, IP6 chelates positively charged ions such as Ca^{2+} , Mg^{2+} , Zn^{2+} and Fe^{2+} , thus reducing their bioavailability in fish (Erdman 1979). In addition, IP6 also may form complexes with cationic groups of proteins and amino acids present in feedstuffs, reducing their digestibility in fish, poultry and pigs (Kumar *et al.* 2011). These IP6-protein complexes are insoluble and resistant to proteolytic digestion (Riche & Garling 2004). Also, there is evidence that IP6 may form complexes with the digestive proteases in fish (Kies *et al.* 2006; Morales *et al.* 2011), reducing their activity. The IP6 molecule can be hydrolysed by phytase, chemically known as *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate phosphohydrolase, rendering P available for absorption. The enzyme sequesters orthophosphate groups from the inositol ring of IP6 to produce free inorganic P along with

a chain of lower phosphoric esters (inositol pentaphosphate to inositol monophosphate) as intermediates (Baruah *et al.* 2004; Debnath *et al.* 2005). Most commercial microbial phytases act efficiently under the conditions present in the stomach, and several researchers have reported positive effect of phytase pretreatment of diets on P availability (Teskeredzic *et al.* 1995; Storebakken *et al.* 1998; xVanW-eerd *et al.* 1999; Cheng & Hardy 2003; Riche & Garling 2004; Yoo *et al.* 2005). Nevertheless, the results obtained in different *in vivo* and *in vitro* studies are contradictory (Lanari *et al.* 1998; Storebakken *et al.* 1998; Sugiura *et al.* 1998; Forster *et al.* 1999; Vielma *et al.* 2000; Glencross *et al.* 2004; Riche & Garling 2004; Sajjadi & Carter 2004; Denstadli *et al.* 2006; Kies *et al.* 2006; Morales *et al.* 2011).

The explanation for this heterogeneity probably rests on the variety of factors affecting phytase activity: gut pH, species-specific susceptibility to digestive proteases, differences in plant protein composition or ion content of plant ingredients; and hence IP6 dephosphorylation and protein bioavailability within fish gastrointestinal tract.

Therefore, the present study is designed to disclose the spatio-temporal dynamics of chemical variables directly related to phytase effects in the digestive tract of the species *Sparus aurata*: pH, contents of IP6, P, soluble protein and amino acids and protease activities.

Materials and methods

Experimental diets

Two experimental diets were formulated to contain around 420 g kg⁻¹ crude protein mainly provided by vegetable protein sources (Table 1). One of the diets contained 2500 FTU g⁻¹ dry diet of a bacterial 6-phytase from *Escherichia coli* expressed in *Pichia pastoris* (QUANTUMTM PHYTASE 2500 XT; AB Enzymes, Darmstadt, Germany). One FTU of phytase represented the amount of enzyme that liberates inorganic phosphorus from a 1.5 mM solution of sodium phytate at a rate of 1 µmol min⁻¹ at pH 5.5 and 37 °C. The diets also included 100 mg Cr₂O₃ kg⁻¹ as an inert marker and were manufactured using an electric pelleting machine which had a pelleting plate with 3 mm hole size, being stored at 4 °C until use.

Fish trial and sampling

Juveniles of *Sparus aurata* (58 ± 8 g) were obtained from a local hatchery and adapted to the assay conditions for

Table 1 Diet ingredients and diets composition

Ingredients (g kg ⁻¹)	Diet	
	Control	Control + Phytase
Fish meal (CP 650 g kg ⁻¹) ¹	200	200
Soya bean meal concentrate ²	100	100
Gluten meal ³	208	208
Pea meal concentrate ⁴	100	100
Broad bean meal concentrate ⁴	100	100
Fish oil ⁵	79	79
Sunflower oil ⁶	79	79
Vitamins + Minerals ⁷	15	15
Lysine + methionine	5	5
CMC ⁸	5	5
BHT ⁹	10	10
Sodium alginate	10	10
Soy lecithin	5	5
Squid hydrolysate	10	10
Maltodextrin	64	63
Chromium sesquioxide	10	10
Phytase ¹⁰	0	1
Proximate composition (%)		
Dry matter	93	93
Crude protein	42	42
Crude fat	20	20
Carbohydrates	28	28
Ash	3.1	3.1
Total P	11.8	11.8
P-IP6 before pelleting	1.4	1.4
P-IP6 after pelleting	1.4	1.2
Gross energy (Kcal kg ⁻¹)	3220	3220

¹ Supplied by Protazul 65, Pasaje Azul SA, Barcelona, Spain.

² Supplied by Hamblet Protein, Horsens, Denmark.

³ Supplied by Roquette, Laia España, Barcelona, Spain.

⁴ Supplied by Esteve Santiago SA, Valladolid, Spain.

⁵ Supplied by A.F.A.M.S.A., Vigo, Spain.

⁶ Supplied by Hacendado, Sevilla, Spain.

⁷ Vitamin premix (gr kg⁻¹ or IU kg⁻¹ dry diet): thiamin 40 mg, riboflavin 50 mg, pyridoxine 40 mg, calcium pantothenate 117 mg, nicotinic acid 200 mg, biotin 1 mg, folic acid 10 mg, cyanocobalamin 0.5 mg, choline chloride 2700 mg, inositol 600 mg, ascorbic acid 1000 mg, alpha tocopherol 250 mg, menadione 20 mg, cholecalciferol 2000 IU, ethoxyquin 100 mg, retinol acetate 5000 IU. Mineral premix (gr kg⁻¹ dry diet): calcium orthophosphate 1.6 g, calcium carbonate 4 g, ferrous sulphate 1.5 g, magnesium sulphate 1.6 g, potassium phosphate 2.8 g, sodium phosphate 1 g, aluminium sulphate 0.02 g, zinc sulphate 0.24 g, copper sulphate 0.20 g, manganese sulphate 0.08 g, potassium iodate 0.02 g.

⁸ Carboxymethyl cellulose.

⁹ Butylated hydroxytoluene.

¹⁰ Included in diet to reach 2500 FTU kg⁻¹ dry diet.

1 week prior to the assay. The set-up used for the trial consisted in a recirculating aquaculture system (RAS) that provided seawater under controlled temperature (24 ± 1 °C) and salinity (37 ± 1 g L⁻¹) to a series of

300-L glass fibre tanks. The flow rate was 8–9 L tank min⁻¹, and the renewal rate was 10–15% per day depending on the water quality values. The tanks were connected to a biofiltration unit made of three tanks with different biofilter substrates (rigid plastic mesh and moving plastic beds) and to a protein skimmer; a swirl separator was also part of the RAS as well as a sand filter. Photoperiod was set at 12L/12D. Prior to the feeding assay, gilthead sea bream juveniles were starved for 12 h. Each diet was provided to one group of 40 juveniles at 12 g kg⁻¹ body weight in dry matter. From that moment on, groups of eight fish were sampled at different moments; 0, 1, 2, 4 and 6 h after feeding. Fish were killed by immersion in ice-cold seawater with clove oil, weighed individually and frozen at -20 °C until dissection of the gastrointestinal tract.

For dissection, fish were only slightly thawed to assure that the digestive content remained as an easily removable pellet. The gut was dissected into three sections: stomach (S), proximal intestine including pyloric caeca (PI) and distal intestine (DI). The digestive content in each section was homogenized with either 2.0 mL (S) or 1.0 mL (PI and DI) of distilled water. The pH of the digestive contents was measured after centrifugation (14 000 g, 3 °C, 15 min) using a Crison pH25 pH meter, endowed with a microelectrode (CRISON 5208, Crison Instruments, Barcelona, Spain). Supernatants were kept frozen at -20 °C until analysis, while the precipitate was used to determine the content of chromium used as digestion marker in each portion of the fish gut.

Soluble protein, total P-IP6 and enzyme activities (total acid protease in S, and total alkaline protease, trypsin and chymotrypsin activities in PI and DI) were determined directly in the supernatants, while total amino acids and soluble P were determined after precipitating the soluble protein using TCA.

Chemical and enzymatic analysis

Chromium in the samples of digesta was determined by the method of Williams & David (1962) with minor modifications. In brief, after homogenization and centrifugation of the digestive contents, the precipitate was dried at 110 °C during 24 h. Dry samples were hydrolysed with concentrated nitric acid during 6 h at 150 °C. The mixture was cooled to ambient temperature and a second hydrolysis (150 °C, 12 h) was carried out after addition of 4 mL of a mixture comprising sodium molybdate, sulphuric acid and perchloric acid. The sample was after diluted in 50 mL

distilled water and the content of chromium determined colorimetrically at 350 nm.

P-IP6 was determined according to the indirect method of Haug & Lantzsch (1983) with some modification to adapt the assay to a microplate reader. In this method, 0.5 mL of sample was mixed with 1 mL of ferric solution [0.2 g of ammonium iron (III) sulphate 12.H₂O in 100 mL of 2 N HCl and made up to 1 L] in a 5 mL assay tube. The tubes were heated in a boiling water bath for 30 min. Once the tubes reached room temperature, the mixture was centrifuged (14 000 g, 15 min) and 100 µL of the supernatant was mixed with 150 µL of 2,2'-bipyridine solution into a 96-well microtiter plate. The decrease in iron determined colorimetrically (519 nm) is a measure of the phytic acid content. Released soluble P was determined by the phospho-molybdovanadate method (Engelen *et al.* 1994) adapted to a microtiter plate. The assay mixture consisted of 50 µL of sample and 100 µL of molybdovanadate reagent; after 10 min absorbance of the phospho-molybdovanadate complex was measured at 415 nm in a MULTISKAN EX ELISA microplate reader (Thermo Labsystems, Cheshire, WA, USA).

Concentration of soluble protein was measured by the method of Bradford (1976) and total amino acids released from reaction mixtures were determined using the *o*-phthalaldehyde method (Church *et al.* 1983). Total acid protease activity in S was measured using the method of Anson (1938) using substrate haemoglobin (5 g L⁻¹) in 100 mmol L⁻¹ glycine-HCl buffer (pH 2.0). Total alkaline protease activity in PI and DI was evaluated using the method of Kunitz as modified by Walter (1984) using substrate casein (5 g L⁻¹) in 50 mmol L⁻¹ Tris-HCl buffer (pH 9.0). One unit of activity is defined as 1 µg of tyrosine released per min. Trypsin activity was measured using as substrate *N*α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) by the method of Erlanger *et al.* (1961) while chymotrypsin activity was measured using as substrate *N*-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA) using the method described by Ásgeirsson & Bjarnasson (1991). Total proteases, trypsin and chymotrypsin activities were expressed as total protease units present into gut portion at the different sampled time periods.

Calculations and statistical analysis

P-IP6, soluble P, soluble protein and total amino acids in each gut portion were expressed as µg g⁻¹ dry matter content. To determine the amount of dry matter (DM) in stomach, proximal and distal intestine sections, the following equation was used:

$$DM(\text{mg}) = (M_c \times M_d^{-1}) \times 100^{-1}$$

where M_c represents the total amount of inner marker determined in each gut portion (μg), and M_d represents the concentration of the inner marker in diets ($\mu\text{g mg}^{-1}$).

Basal intestinal levels at the time '0 h' of soluble protein, phosphorus and amino acids were subtracted to evaluate the effect of phytase only on the digestion product from dietary substrates.

The mean values are reported \pm the standard deviation of mean (SD) from eight replicates by treatment. After verification of the assumptions of normality and homoscedasticity, data were subjected to two-way ANOVA, where diet and time were the class variables, following the model:

$$Y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + e_{ijk}$$

where Y , the observed response; μ , the overall mean; τ_i , the effect of diet ($i = 1, 2$); β_j , the effect of time ($j = 1, 2, 3, 4, 5$); $(\tau\beta)_{ij}$, the effect of interaction between diet and time; and e_{ijk} , the random error.

When appropriate, Tukey's test was used for multiple comparisons of the means. Differences between means are significant at $P < 0.05$. All the analyses were performed using the STATGRAPHICS software package (STSC Software Group, Rockville, MD, USA).

Results

Gut pH

Time changes in pH measured in S, PI and DI are detailed in Fig. 1. Before feeding, fish showed a pH of 6.2 in S and 6.6 in both PI and DI. One hour after feeding, the pH in the S decreased to 5.4 and reached an average value of 4.5 at the end of the sampling period. No changes in pH throughout time were measured in the PI, while a significant alkalisation was detected in the DI, with a maximum average pH value of 7.2. The pH measured in the digesta in all segments was not significantly affected by phytase treatment (Table 2).

Phytate dephosphorylation within gastrointestinal tract

The temporal pattern of soluble P-IP6 in different portions of the gastrointestinal tract is detailed in Fig. 2. The phytase caused a significant effect on P-IP6 after the first hour of gastric digestion and by the end of the sampling period the concentration decreased from 0.2 to 0.05 mg g^{-1} DM.

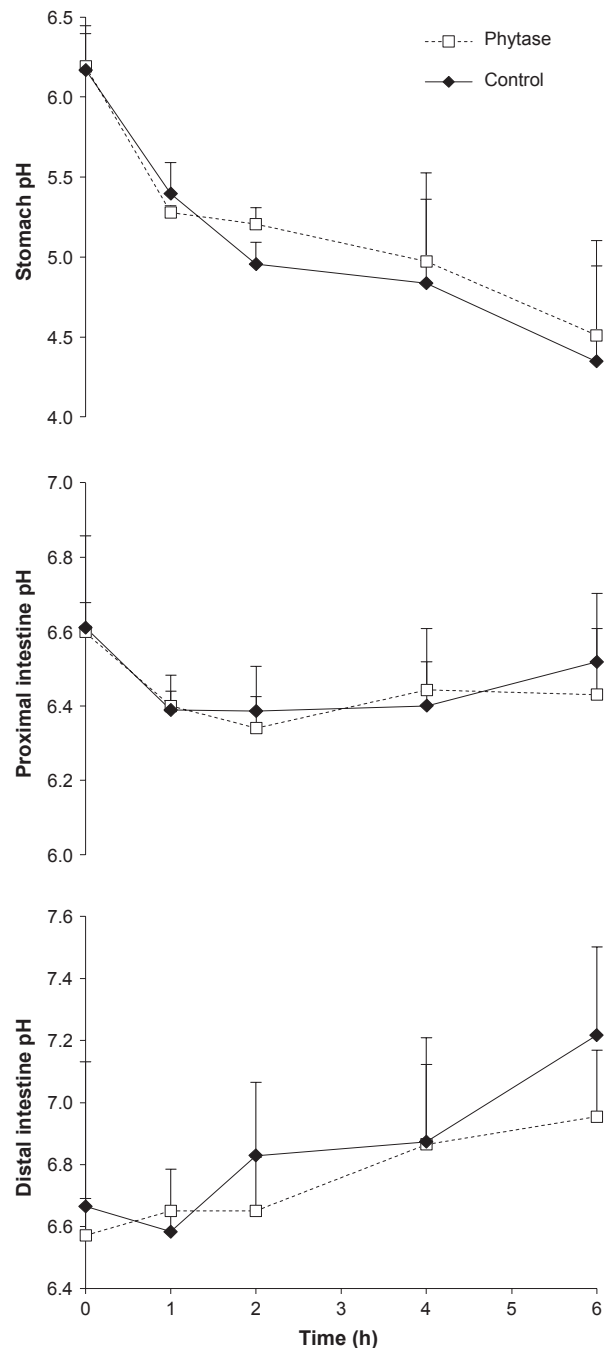


Figure 1 Temporal pattern of pH in different portions of the gastrointestinal tract (stomach, proximal and distal intestine) of juvenile gilthead sea bream. Solid and dashed lines represent 'control' and 'phytase' treatment, respectively. Bars indicate standard deviation of the mean ($n = 8$).

P-IP6 was detected in PI and DI at the first and second hour after feeding, respectively, and the concentration was maintained constant (0.4 mg g^{-1} DM) during digestion. No

Table 2 Main effects of phytase and digestion time on the pH and bioavailability of P, protein and amino acids in the different sections of the digestive tract of sea bream

Dependent variable	Digestive tract section	P-value	
		Phytase	Time
pH	S	NS	<0.0001
	PI	NS	0.0245
	DI	NS	<0.0001
Soluble P-IP6	S	<0.0001	0.0006
	PI	NS	NS
	DI	NS	0.0148
Soluble P	S	<0.0001	NS
	PI	<0.0001	0.0002
	DI	NS	0.0073
Soluble protein	S	NS	<0.0001
	PI	<0.0001	0.0005
	DI	NS	0.0057
Amino acids	S	NS	NS
	PI	<0.0001	0.0173
	DI	0.0385	0.0007

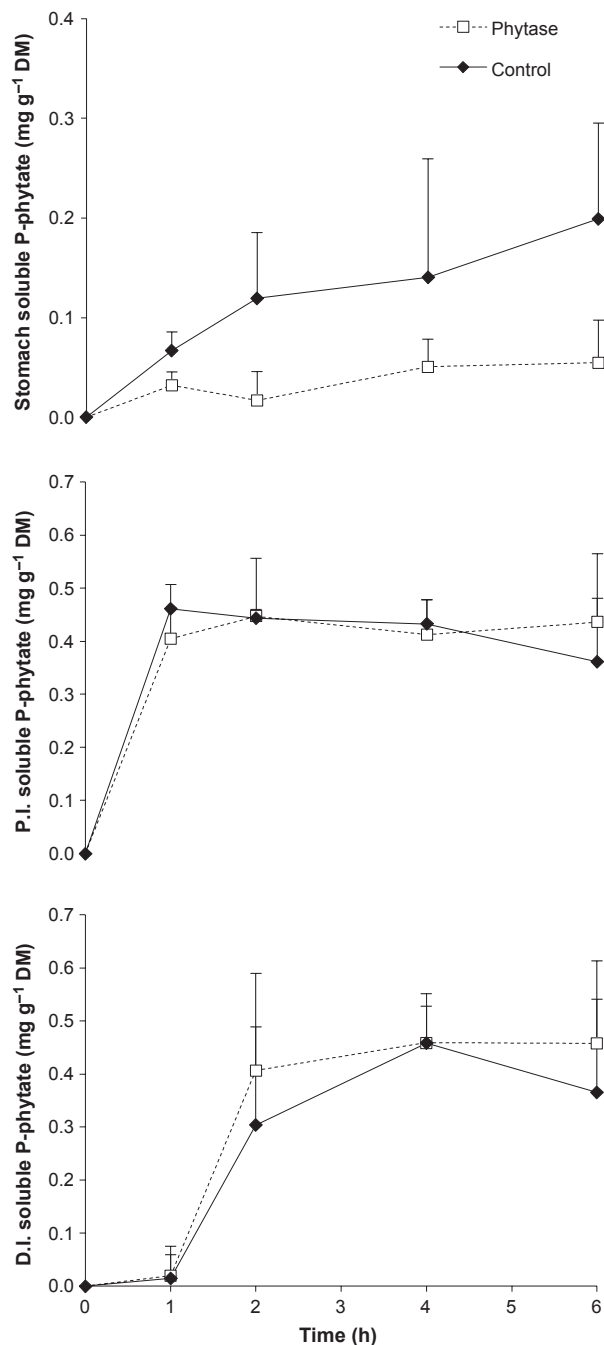
differences in soluble P-IP6 at the intestine were detected between treatments. A significant increase in soluble P was measured at S and PI of fish fed on the diet containing phytase, although no differences were observed in DI (Fig. 3).

Solubilization and hydrolysis of dietary protein

The changes in the amount of soluble protein measured along digestion are shown in Fig. 4. A similar pattern was obtained for fish fed with both the control and phytase diets; while the amount of soluble protein at the stomach showed a significant reduction with time, a significant increase was measured at the PI and the DI. The presence of phytase in the feed was correlated to significantly higher values of soluble protein at the PI (Table 2). On the other hand, the presence of phytase did not influence the amount of free amino acids released in the stomach, but significantly increased their amount at the PI; 50 mg g⁻¹ DM during the first two hours of digestion (Fig. 5).

Changes in the enzymatic activity of digestive proteases

The effects of both phytase and digestion time on the activity of the digestive proteases are resumed in Table 3. Fish fed on any of the diets showed a significant reduction in the activity of both stomach and intestinal proteases with time, although in this latter case, a sharp increase was detected 4 h after feeding in DI. No significant effect of the phytase on the activity of proteases was evidenced.

**Figure 2** Temporal pattern of soluble P-IP6 in different portions of the gastrointestinal tract of juvenile gilthead sea bream. Bars indicate standard deviation of the mean ($n = 8$).

Discussion

Gut pH during digestion

Phytic acid is probably one of the most studied antinutritional factors in animal nutrition due its effects on avail-

ability of phosphorus and other divalent minerals such as Ca^{2+} and Mg^{2+} as well as on proteins (Selle *et al.* 2009). Proton dissociation sites on the phytic acid molecules have variable pKa values, increasing the charge of the molecule with the increment of the pH (Evans *et al.* 1982). This implies that changes in pH, which take place in the different sections of the gastrointestinal tract, have a great importance on the binding capacity of IP6.

In the present study, the pH measured at the stomach decreased from 6.2 (before feeding) to 4.5 after 6 h of digestion, with the greatest reduction taking place 1–2 h postfeeding. These values were in agreement with those obtained by Yufera *et al.* (2004) or Márquez *et al.* (2011), although a lower stomach pH has been also reported in this species (Deguara *et al.* 2003; Nikolopoulou *et al.* 2011). Nevertheless, such differences may depend on several factors like the fish size, food transit rate or buffering capacity of the ingredients used in the diets.

Gut pH, soluble phytate and phytase activity

The solubility of IP6 is largely pH dependent, being more soluble at low pH and precipitating in the form of IP6–Ca at pH above 4.0 (Grynspan & Cheryan 1983). As the stomach pH measured in the present study was higher than 4, a great part of the total P–IP6 present in the diet was precipitated (86%), this reducing its susceptibility to be hydrolysed by phytase. However, while after six hours of digestion, the reduction in soluble P–IP6 in the stomach of fish fed on the feed including phytase was only $0.15 \text{ mg g}^{-1} \text{ DM}$ (100 g kg^{-1} P–IP6 total in diet), the increase in soluble P was $0.83 \text{ mg g}^{-1} \text{ DM}$ (70 g kg^{-1} total P in diet). Therefore, it is suggested that phytase could have dephosphorylated not only the soluble fraction of P–IP6, but also part of the insoluble IP6 ($0.68 \text{ mg g}^{-1} \text{ DM}$). In fact, the amount of total soluble P released in the stomach of fish fed on the phytase treatment was equivalent to a dephosphorylation of 57% of the dietary P–IP6. In contrast, no significant effect of the enzyme on the solubilisation of P–IP6 was detected at the intestine, where a constant concentration of nearly $0.4 \text{ mg g}^{-1} \text{ DM}$ (28% dietary P–IP6) was maintained along the digestion time. This absence of differences could be explained taking into account that the amount of soluble P–IP6 should be the result of a dynamic equilibrium, modulated by pH and the concentration of divalent cations, between the insoluble IP6 precipitated as an inorganic salt (Grynspan & Cheryan 1983) or complexed with proteins (Kies *et al.* 2006) and the enzymatic dephosphorylation of IP6 by phytase. Despite [P–IP6]

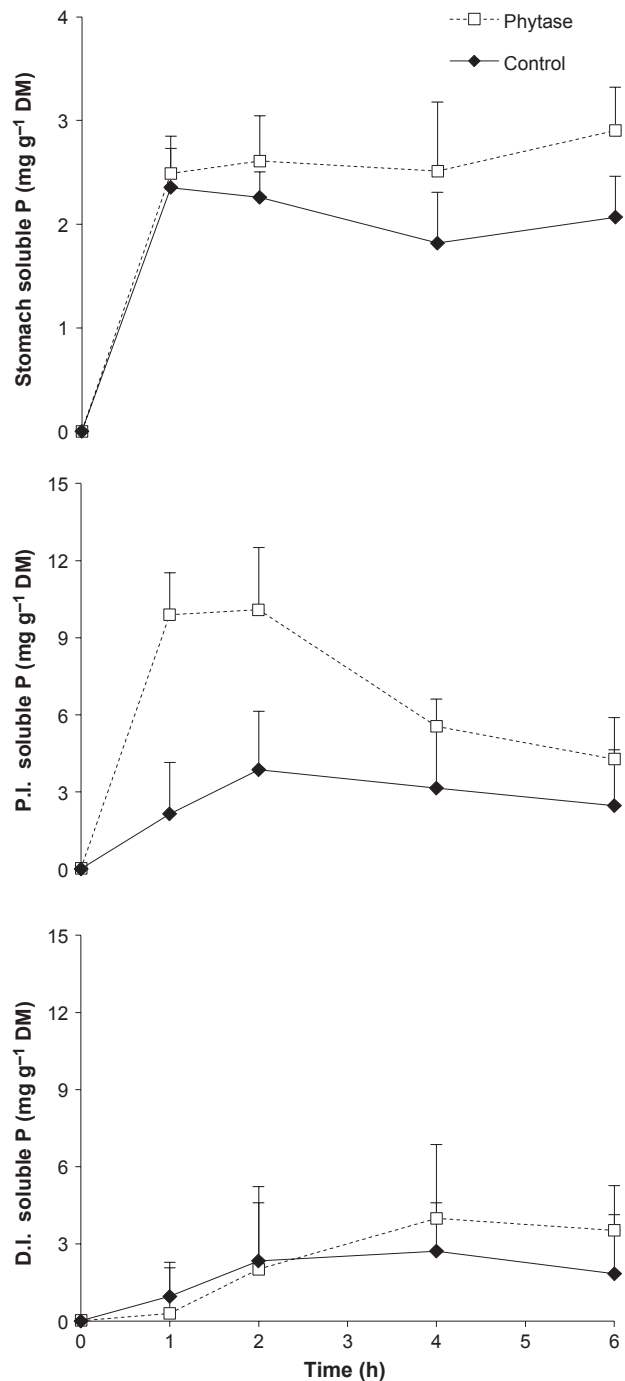


Figure 3 Temporal pattern of soluble P in different portions of the gastrointestinal tract of juvenile gilthead sea bream. Bars indicate standard deviation of the mean ($n = 8$).

solubility within the intestine, P concentration in the proximal intestine was significantly higher in fish fed the phytase diet during the first two hours. The positive effect of phytase to increase P digestibility has been extensively studied in different fish species, either with stomach (Riche &

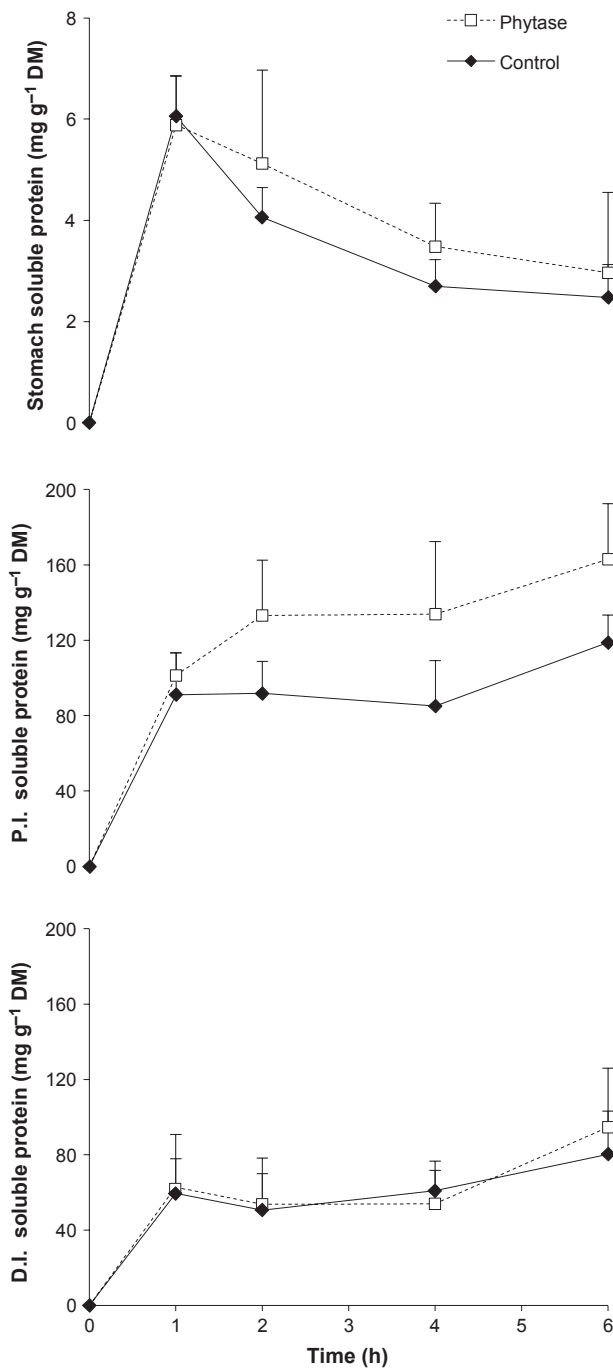


Figure 4 Temporal pattern of soluble protein in different portions of the gastrointestinal tract of juvenile gilthead sea bream. Bars indicate standard deviation of the mean ($n = 8$).

Brown 1996; Vielma *et al.* 1998, 2004; Sugiura *et al.* 2001; Sajjadi & Carter 2004) or without it (Baruah *et al.* 2007; Nwanna 2007). However, the effect of phytase on protein solubility and hydrolysis within the gastrointestinal tract has not yet been studied in depth.

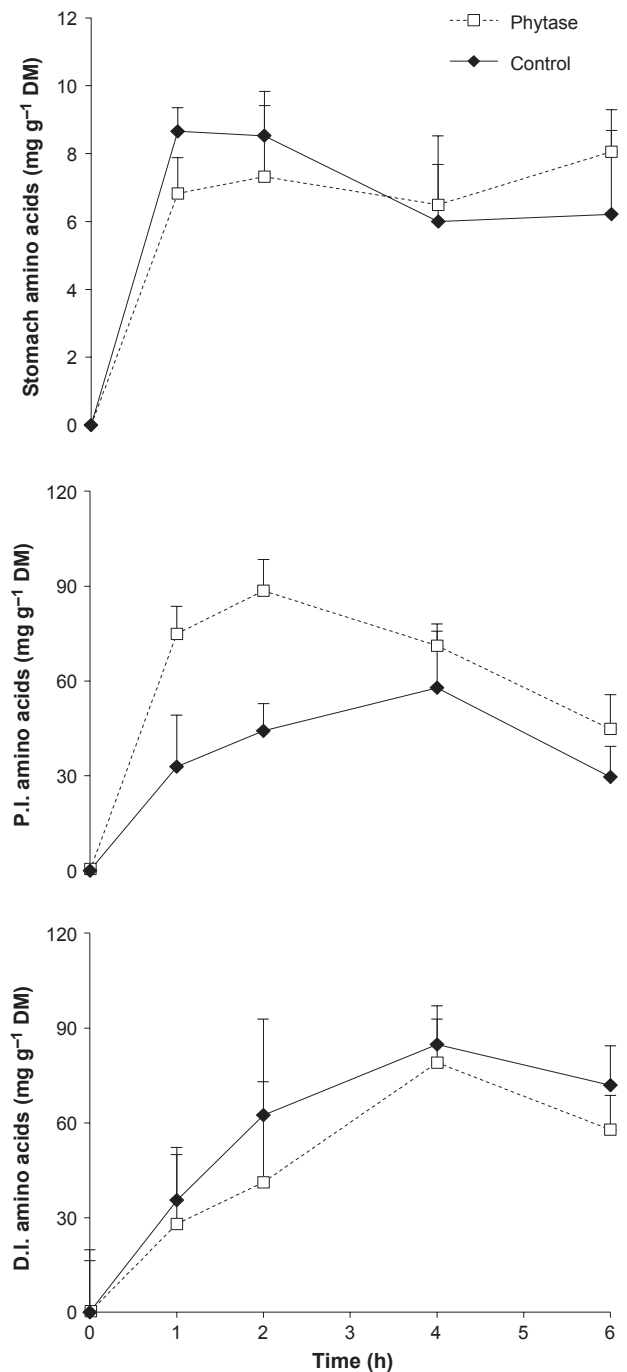


Figure 5 Temporal pattern of total amino acids released in different portions of the gastrointestinal tract of juvenile gilthead sea bream. Bars indicate standard deviation of the mean ($n = 8$).

Gut pH, protein solubility and phytase activity

Several *in vitro* studies suggest that the way IP6 interacts with proteins depends mainly on the pH environment (Okubo *et al.* 1976; Wise 1983; Konishi *et al.* 1999; Kies

Table 3 Effect of phytase treatment on the enzymatic activity of the main digestive proteases at different times after feeding

		Time (hours)				P-value	
Enzymatic activity (U × 1000)	Treat.	1	2	4	6	Treat.	Time
Stomach							
Total protease ¹	C	1.30 ± 0.29	0.48 ± 0.22	0.34 ± 0.11	0.26 ± 0.17	0.0044	<0.0001
	Phy	1.33 ± 0.31	0.77 ± 0.25	0.57 ± 0.27	0.44 ± 0.25		
Proximal intestine							
Total protease	C	0.58 ± 0.13	0.55 ± 0.07	0.28 ± 0.08	0.19 ± 0.08	NS	<0.0001
	Phy	0.53 ± 0.20	0.45 ± 0.16	0.44 ± 0.14	0.15 ± 0.07		
Trypsin ²	C	6.75 ± 1.97	4.02 ± 0.92	1.91 ± 1.69	0.95 ± 0.48	NS	<0.0001
	Phy	5.55 ± 2.45	3.48 ± 1.35	2.99 ± 1.54	0.475 ± 0.48		
Chymotrypsin ³	C	54.47 ± 8.55	48.08 ± 6.04	31.69 ± 14.00	21.61 ± 7.74	NS	<0.0001
	Phy	44.47 ± 14.54	40.71 ± 7.28	36.98 ± 12.07	16.12 ± 5.65		
Distal intestine							
Total protease	C	0.14 ± 0.05	0.27 ± 0.11	0.39 ± 0.11	0.27 ± 0.09	NS	<0.0001
	Phy	0.14 ± 0.06	0.21 ± 0.08	0.61 ± 0.19	0.19 ± 0.12		
Trypsin	C	1.31 ± 0.58	2.56 ± 1.24	3.44 ± 1.61	1.87 ± 0.99	NS	<0.0001
	Phy	1.07 ± 0.54	2.02 ± 1.15	4.79 ± 2.18	0.73 ± 0.24		
Chymotrypsin	C	16.27 ± 4.79	28.33 ± 15.22	40.67 ± 14.00	23.09 ± 8.27	NS	<0.0001
	Phy	14.21 ± 6.94	18.99 ± 8.18	58.08 ± 23.81	17.66 ± 14.46		

¹ Total protease units: 1 U = 1 µg tyrosine min⁻¹.

² BAPNA units: 1 U = 1 µM p-nitroanilide min⁻¹.

³ SAPNA units: 1 U = 1 µM p-nitroanilide min⁻¹.

et al. 2006; Morales *et al.* 2011). At low pH values placed below the isoelectric point of most proteins, binary protein–IP6 complexes are formed by salt-like linkages with the α-NH2 terminal group and the ε-NH2 group of lysine, the imidazole group of histidine, and the positively charged guanidyl group of arginine (Cosgrove 1966). As pH increases, approaching to the isoelectric point of proteins, ternary complexes IP6–divalent cation–proteins are formed. These complexes are formed *de novo* in the small intestine between negatively charged protein carboxyl groups and the IP6 via a cationic bridge, usually Ca⁺² (Anderson 1985). The relative importance of these ternary complexes is difficult to assess, as the equilibrium between IP6–mineral or IP6–cation–protein complexes depends on the concentration of the cation (Champagne 1988). However, it seems that the formation of IP6 ternary complexes do not result in a significant reduction in protein availability (Selle *et al.* 2012).

In the present study, the presence of phytase in the diet did not significantly affect solubilisation of dietary protein in the stomach, while a significant increase was observed in the proximal intestine. According to the Cosgrove (1966) and Rajendran & Prakash (1993), once IP6 binary complexes are formed they could be protected by a shield of aggregated protein, being less susceptible to hydrolysis by phytase. However, taking into account the values of IP6 dephosphorylation measured in the stomach (Fig. 2), it is

suggested that phytase may disrupt a fraction of the binary protein–IP6 complexes, although it was not possible to prove this hypothesis measuring the uncomplexed protein in the soluble fraction, as under the pH of the stomach almost all proteins were precipitated (Csonka *et al.* 1926; Kies *et al.* 2006). In contrast, this measurement could be taken at the higher pH in the proximal intestine, and the preventive effect of phytase on the formation of protein–IP6 complexes was evidenced by an increased amount of soluble protein.

Several studies reported a significant negative effect of the IP6 on protein digestibility in different fish species, such as Atlantic salmon *Salmo salar* (Sajjadi & Carter 2004), rainbow trout *Oncorhynchus mykiss* (Spinelli *et al.* 1983; Sugiura *et al.* 2001) and tilapia *Oreochromis niloticus* (Riche & Garling 2004). Also, several *in vitro* studies evidenced that phytase not only improves solubility of the dietary protein under certain pH, but also prevents the negative effect of IP6 binding on protein hydrolysis (Ravindran 1995; Kies *et al.* 2006; Morales *et al.* 2011). The present study partially agreed with these reports and besides the increase in soluble protein, a higher release of amino acids in the proximal intestine of the fish fed on a diet including phytase during the initial two hours of digestion was evidenced. Such effect was not observed in the distal portion of the intestine, being the differences found probably due to the important

variations in the relative concentration of the products of protein hydrolysis and ions present in the digesta in each gut section.

Effect of phytate and phytase on peptic activity

Several *in vitro* assays have demonstrated that protein binded to IP6 is refractory to pepsin digestion (Inagawa *et al.* 1987; Ravindran 1995). Vaintraub & Bulmaga (1991) described a reduction of approximately 90% in the ability of pepsin to hydrolyze bovine serum albumin in the presence of IP6, and by 65% in the cases of haemoglobin, casein and 11S soya protein. They noticed that maximal reductions were obtained at pH 2–3, being not so evident at pH 4.0–4.5, this being probably due to the decreased solubility and structural changes of proteins following aggregation with IP6. Similarly, Kies *et al.* (2006) observed that the addition of phytase increased pepsin hydrolysis of the IP6–protein complexes formed in soya bean meal at pH 2–3. Another factor to consider when assessing the ability of IP6 to inhibit pepsin digestion is the degree of dephosphorylation of the molecule, as the more pronounced effect is obtained for IP5 and IP6, whereas IP1 and IP2 does not seem to inhibit pepsin activity, as demonstrated by Knuckles *et al.* (1989) using casein and bovine serum albumen as substrate.

It is noteworthy that the reduction in the hydrolysis of dietary protein due to the presence of IP6 can be attributed not only to the formation of low digestible binary complexes with substrate proteins, but also in an indirect way to its binding to digestive proteases. Morales *et al.* (2011) demonstrated that incubation of IP6 in a concentration equivalent to that present in soya bean meal with extracts prepared with either pure pepsin or rainbow trout stomach, produced a significant reduction in their soluble protein contents (68% and 99%, respectively) and as a consequence, in their protease activity (82% and 58%, respectively). In the present study, the activity of gastric protease measured in fish fed on the phytase diet was around 60% higher than that of fish receiving the control diet (Table 3). To our knowledge, this is the first evidence of the effect of IP6 on the pepsin activity in fish stomach measured *in vivo*.

Such a positive effect of phytase could be supported by a probable lower concentration of gastric phytate in that treatment (Figs 2a and 3a) together with the ability of phytate to precipitate fish pepsins (Morales *et al.* 2011), not only pepsin substrates as reported by Vaintraub & Bulmaga (1991). Gastric hypersecretions triggered by a shortage of digestion products when pepsin-resistant proteins are rendered by IP6 complexation (Selle *et al.* 2012) cannot be

excluded in the case of pepsin in the present study, but it does not seem to apply to the gastric acid secretion as no effect of phytase on gastric pH was detected and, on the other hand, diets were designed to show similar buffering capacities. The possible effect of the binary IP6–protein binding and its effect on gastric hypersecretions and protective mucin outputs, which would increase endogenous amino acid flows not only in fish but also in other monogastriacs, requires further research.

Effect of phytate and phytase on the activity of intestinal proteases

On the other hand, no significant differences in the activity of intestinal proteases between fish receiving phytase were detected (Table 3). The possibility that IP6 may inhibit trypsin and other alkaline digestive proteases is an important but unresolved issue. A reduction in the activity of trypsin at pH 7.5 by IP6 was measured *in vitro* by Singh & Krikorian (1982). As both trypsinogen and trypsin require calcium ions to maintain their functionality, they hypothesized that the possible competitive binding of Ca^{+2} by IP6 may partially reduce the activity of the enzyme. This hypothesis was later confirmed by Caldwell (1992), who reported negative effects of phytic acid bound Ca^{+2} on the activation of trypsinogen and the stability of trypsin. More recently, Morales *et al.* (2011) reported a 14% reduction in the activity of rainbow trout trypsin in the presence of IP6, but no effect on chymotrypsin.

Most studies in terrestrial animals performed *in vivo* have failed to demonstrate inhibition of trypsin due to the formation of ternary complexes protein–cation–IP6 (Reddy *et al.* 1988; Knuckles *et al.* 1989; Vaintraub & Bulmaga 1991). In the case of fish, Sajjadi & Carter (2004) did not find any effect of including 8.2 g IP6 kg^{−1} diet on the trypsin activity of Atlantic salmon (*Salmo salar*). In contrast, Denstadli *et al.* (2006) observed that the cumulative trypsin activity in the chyme at the pyloric intestine was significantly lowered in this species when fed a high dose of IP6 (20.7 g kg^{−1}), although this effect was not evident when fish were fed on diets with a content of IP6 more similar to that present in plant-based diets (4.7 g IP6 kg^{−1}). At present, studies suggest that the formation of ternary IP6–protein complexes does not significantly affect the activity of intestinal proteases, although *in vitro* evidence of the negative effect of IP6 on the solubility and residual activity of intestinal proteases, not only from mammals but also from fish, would suggest possible compensatory mechanisms of intestinal proteases secretion.

Conclusions

The present study demonstrates that the changing conditions existing along the fish gastrointestinal tract have an important effect both on the capacity of phytase to dephosphorylate the native IP6 present in plant-based diets and on the interactions between IP6 and protein digestion. The average proportion of IP6 dephosphorylation within fish digestive tract (around 60%) is still far from a total utilization of the dietary IP6-P, indicating that more research is needed to improve plant P utilization. The results suggest also that total gastric protease activity in fish is reduced around 60% as consequence of IP6 binary protein-IP6 complexation. However, fish intestinal proteases are not affected by the enzymatic dephosphorylation of the native IP6 in a plant-based diet.

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