Revista Agrarian
ISSN: 1984-2538

Challenges for efficient use of phytase in fish nutrition

Desafios para utilização eficiente da fitase na nutrição de peixes

Cristiano Costenaro-Ferreira[1], Marco Aurélio Lopes Della Flora[2]

[1] Faculdade de Agronomia Elizeu Maciel (FAEM), Universidade Federal de Pelotas (UFPEL), Av. Eliseu Maciel s/nº., CEP 96010-900, Capão do Leão, RS, Brazil. Email: costenaro.cf@gmail.com.
[2] Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Av. Antônio Carlos 6627, CEP 31270-901, Belo Horizonte, MG, Brazil.

Recebido em: 10/06/2015 Aceito em:09/11/2015

Resumo: O fósforo é estocado nas plantas principalmente na forma de ácido fítico (IP6) e representa entre 50 e 80% do fósforo total presente nos ingredientes vegetais. Por ser indigestível pelos animais não ruminantes e permanecer carregado negativamente no pH fisiológico, liga-se a uma grande variedade de nutrientes, principalmente cátions, os quais são eliminados via fezes. Este fato torna necessária a suplementação de fósforo disponível o que aumenta os custos e problemas ambientais. Em vista disso, a fitase tem sido utilizada como forma de degradar a molécula de IP6 e aumentar o aproveitamento do fósforo indisponível. Esta enzima é usada com sucesso na alimentação de aves e suínos, porém controversa para peixes. Como toda enzima, a fitase possui uma faixa de temperatura e pH ótimos para atuar e desnatura quando submetida a extremos. Assim, o processamento da ração (extrusão) e as diversas condições anatômicas e fisiológicas das espécies de peixe desafiam sua integridade e atuação. Em vista disso, entender os principais aspectos envolvidos nos resultados obtidos com o uso da fitase na alimentação de peixes, conduz ao seu uso correto considerando as condições para sua atividade e os desafios impostos tanto pelo processamento quanto pela espécie de peixe.

Palavras-chave: Biodisponibilidade, desfitinização, eutrofização, fitato, fósforo

Abstract: Phosphorus is stored in plants majorly in the form of phytic acid (IP6), which represents 50–80% of the total phosphorus present in the vegetable ingredients. As it is indigestible by nonruminant animals and remains negatively charged in the physiologic pH, it binds to a large variety of nutrients, mainly cations, which are discarded via feces. This leads to the addition of available phosphorus, which increases the cost and environmental problems. Therefore, the utilization of phytase helps to break IP6 and increases the use of the unavailable phosphorus. Phytase is successfully used in poultry and pigs feeding, whereas for fish, the results are ambiguous. Phytase acts in an optimum range of temperature and pH and denatures when subjected to extremes conditions. Thus, feed processing (extrusion) and the anatomical and physiological conditions of the fish species challenge its molecular integrity and activity. Hence, understanding the main aspects based on the results obtained by the use of phytase in fish feeding leads to optimal utilization of this enzyme due to its ideal activity and the challenges induced by processing and the fish species.

Keywords: Bioavailability, dephytinisation, eutrophication, phytate, phosphorus

Introduction

Fish nutrition in intensive farming depends entirely on the artificial feed, and therefore, it should contain the suitable amount of the nutrients in a bioavailable form for the satisfactory growth of the cultivated species (Kestemont, 1995). The bioavailability is a very important factor, since a feed with suitable amount of nutrients, but in an unavailable form, leads to low absorption, poor growth, and higher nutrient excretion, which generates environmental problems (Lazzari & Baldisserotto, 2008; Ganga et al., 2015).

The use of alternative ingredients instead of conventional ones represents their lower cost and/or greater availability; however, although many vegetable ingredients show acceptable protein and energy digestibility, they have amino acid imbalance and a wide range of the antinutritional factors (saponins, tannins, phytate, protease inhibitors, lectins, glucosinolates, alkaloids, etc.) and indigestible compounds such as

Revista Agrarian
ISSN: 1984-2538

oligosaccharides and non-starch polysaccharides (Francis et al., 2001). Thus, it is necessary to inactivate or eliminate (by thermal, solvent, or enzymatic treatments) these factors to get a product that may compose the feed.

Phytate is the main form of phosphorus storage in the vegetable tissue and it is found in animals and vegetables as inositol mono-, di-, tri-, tetrakis-, and pentakisphosphate (Kohlmeier, 2003); however, inositol hexakisphosfate is only found in plants and is also known as *myo*-inositol hexakisphosphate, phytic acid, and phytate (Loewus & Murthy, 2000).

Due to phytic acid (IP6) complex, positively charged elements such as proteins and minerals decrease its bioavailability for nonruminant animals; thus, it is considered as one of the main antinutrients present mostly in vegetable ingredients (Francis et al., 2001). In order to overcome these negative effects, it is necessary to add the phytase enzyme in the feed to break IP6.

Phytase supplementation may increase feed intake, nutrients uptake, and energy utilization, besides improving the growth of fish and shrimps (Fox et al., 2006; Nwanna et al., 2008; Wang et al., 2009); however, some results are contradictory and the effect is associated with the application form of phytase and the species studied.

Thus, this review aims to discuss, from related literature, the benefits, barriers, and challenges of phytase use in fish nutrition.

Phytate

Phosphorous is mainly stored in plants in the form of IP6 (Figure 1), known as inositol hexakisphosphate or phytate when in salt form, with formula $C_6H_{18}O_{24}P_6$, which represents 50–80% of the total phosphorus found in vegetable ingredients (Francis et al., 2001). Moreover, IP6 has a strong chelating power of di- and trivalent minerals (Ca^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+}, Fe^{2+} and Fe^{3+}), proteins, amino acids, starch, and lipids that remain unavailable for nonruminants (Yoon et al., 1983; Francis et al., 2001).

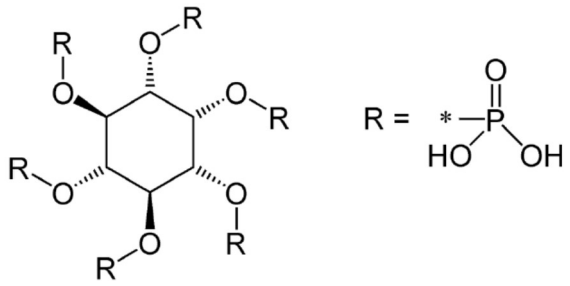

Figure 1. Chemical structure of phytic acid (Kumar et al., 2012).

Organic minerals are important as trace minerals sources, but due to IP6 complex, they are indigestible and are not hydrolyzed by nonruminants; therefore, phosphorus supplementation is given to avoid its deficiency (Yao et al., 2011; MA et al., 2014). Phytate in vegetable based diet also decreases zinc bioavailability that results in its low digestibility coefficient and low level in the blood and tissues of Atlantic cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*) (Kousoulaki et al., 2010; Prabhu et al., 2014). Due to the low digestibility of the IP6 complex, a large amount of phosphorus and other nutrients of the diet are discharged in water and this can lead to eutrophication (Baruah et al., 2004).

There are evidences of the interaction of the phytate and lipids, called lipophytin, which form a complex of Ca/Mg–IP6 with lipids, peptides, and derivatives. The Ca–IP6 complexes may be involved in the formation of metallic soaps in intestinal lumen, which would decrease the energy utilization from lipids (Leeson, 1993).

Khan & Ghosh (2013) studied the effect of crescent levels of IP6 on intestinal amylase and protease activity of three stomachless fish: rohu (*Labeo rohita*), catla (*Catla catla*), and mrigal (*Cirrhinus mrigala*). They reported that the enzyme activities decreased with the increase of IP6 concentration and that mrigal was most sensitive to IP6 while catla was least affected.

Thus, IP6 interaction decreases both nutrient bioavailability and enzyme activity that affect digestion, absorption, and growth. Likewise, IP6 effect depends on its amount in the diet, food habit of the fish, and the presence or absence of stomach in fish species (Kumar et al., 2010; Khan & Ghosh, 2013).

Phytase

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Phytase is an enzyme found in nature worldwide. It is produced by seeds (Afify et al., 2011), fungi (Nagashima et al., 1999; Wyss et al., 1999a; Wang et al., 2007), bacteria (Wyss et al., 1999b; Garrett et al., 2004), yeast (Promdonkoy et al., 2009; Sthal et al., 2014) and in lower amount by small intestine of fish (Huang et al., 2009), poultry (Maenz & Classen, 1998) and pigs (Selle et al., 2009).

For poultry and pigs, endogenous phytase activity is discarded, because limestone is used as a calcium source in their diet, and this characteristic changes the intestinal pH to ranges beyond the threshold of the enzyme action; besides, more Ca–IP6 complex is formed (Selle et al., 2009).

In contrast, several varieties of fish species exhibit different characteristics due to distinct food habits, anatomies, and physiologies. For example, Nile tilapia can break about 50% of the IP6 by endogenous enzyme activity (Kumar et al., 2012).

Phytase is an acid phosphatase that removes phosphate groups from IP6 (Figure 2) and generates *myo*-inositol and inorganic phosphates (Mitchell et al., 1997). In practice, however, only a little percentage of the phosphorus is released, because the enzyme breaks the IP6 partially (Kemme et al., 2006).

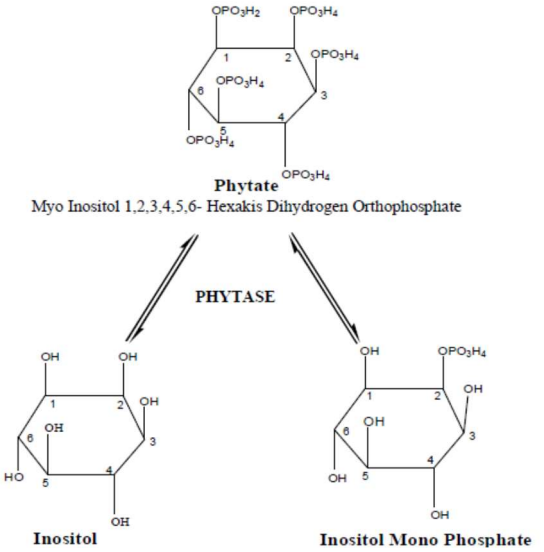

Figure 2. Phytase action on phytate (Kumar et al., 2012).

Phytase can be separated in two groups according to the phosphate position of phosphate group that is released. Microorganism phytase, in particular, fungal (international nomenclature E.C. 3.1.3.8), usually breaks the phosphate group of the C1 and C3 carbons from IP6, and is called *myo*-inositol hexakisphosphate 3-phosphohydrolase. Plant phytase (E.C. 3.1.3.26) acts first in C6 and is called *myo*-inositol hexakisphosphate 6-phosphohydrolase (Wyss et al., 1999b; Lei & Porres, 2003).

Phytase exhibit some differences in the ability to release phosphate groups. Complete release of these groups may lead to delivery of inositol and six inorganic phosphates (Pi) (Garrett et al., 2004); whereas their partial release as phytase from *Bacillus subtilis* generates inositol 3-phosphate (IP3) and three Pi (Kerovuo et al., 2000).

Phytase activity is measured as FTU (*units of phytase activity*) where 1 FTU corresponds to the activity of phytase that releases, from IP6, 1µmol of inorganic phosphorus per minute under standardized conditions (pH 5.5 and 37°C; Wyss et al., 1999a).

Recently, knowledge regarding phosphorus pollution and loss of nutrients has contributed to the crescent use of the phytase in animal feeding because most of the nutrients are in the feed, and just unavailable. Thus phytase usage contributes in environmental sustainability.

Application methods

Many studies have been conducted in order to find the best form of phytase application, with an aim to facilitate the process and not to reduce the enzyme activity. Extruded diet is used worldwide and phytase may be added in the feed before or after the extrusion process.

When phytase is added before the extrusion process, protein denaturation may occur due to high temperature (Wyss et al., 1998). Alternatively, phytase application by aspersion after extrusion, avoids thermal denaturation, and its action collides with the anatomy and physiology of the species, which must offer ideal conditions for phytase action (temperature and pH); however, its application changes according to the production source (as will be discussed).

As fishes are cold-blooded, these organisms did not offer the ideal temperature for most commercial phytase activity. Furthermore, the acid pH of the stomach and basic pH of the intestine of stomachless fish are challenges to phytase activity. Considering this, Nwanna & Schwarz (2007) reported that crescent levels of phytase when added to the diet of common carp (*Cyprinus carpio*, a stomachless fish) before

pelleting (60°C), did neither enhance the growth nor increased the phosphorous levels in bones. The authors concluded that carp intestine did not provide suitable conditions for enzyme activity and that vegetable ingredients need to be pretreated for the enzyme activity independent of the anatomical and physiological conditions of fish.

Thus, Nwanna et al. (2008) confirmed the benefits of vegetable ingredients' pretreatment with phytase (4000 FTU) before pelleting (60°C), reporting higher growth and mineral deposition in the scales of common carp in relation to the diet with the same enzyme activity, but without incubation.

Alternatively, Yan et al. (2002) verified, after analysis of IP6 in the stomach content of channel catfish (*Ictalurus punctatus*), that dietary phytase was operating even under low pH and pepsin conditions. This increased the phosphorous deposition in bones, despite the growth not being affected.

In addition, in a study performed by Wang et al. (2009), it was observed that when soybean meal was pretreated with phytase before including it in the diet, lesser amount of enzyme was necessary to obtain better performance in relation to phytase aspersion on the ready diet of rainbow trout (*O. mykiss*).

Thus, phytase can be applied before or after extrusion; when applied before extrusion, it can be incubated with vegetable ingredients or just mixed with them. Therefore, the ambiguous results indicate that is necessary a greater understanding about phytase that will be used to verify if the organism of studied fish offers the minimum requirements for its action or needs to be incubated with the vegetable ingredients to not waste enzyme, already, only with pretreatment phytase activity does not depend on fish anatomy.

Temperature

Phytases are thermolabile and have an optimum temperature of activity from 45 to 65°C (Greiner & Konietzny, 1999); thus, they usually cannot resist high temperatures of feed extrusion process ranging from 80 to 200°C (Goelema, 1999) because they denature when temperature exceeds 90°C (Wyss et al., 1998). Thus, they can be applied to the liquid form after pelleting; however, this increases the production cost (Garrett et al., 2004).

To prevent this problem, many researchers have performed studies aiming to produce phytase resistant to high temperatures, to mix it with the ingredients before extrusion in order to reduce activity loss (Garrett et al., 2004; Zhang et al., 2007).

Nevertheless, despite efforts to produce a thermostable enzyme, Rodriguez et al. (2000) found that to achieve 45% of maximum activity at 80°C/20 min with a phytase produced by transgenic *Pichia pastoris*, there was a decrease in its activity at temperatures below 37°C. Moreover, Nagashima et al. (1999) reported that *Aspergillus niger* phytase (strain SK-57) remained active at temperatures from 0 to 30°C, but only 30% activity was observed at 50°C. Unlike pigs and poultry, that maintain body temperature around 37 and 41°C, respectively, fish have a large variation depending on the environment where they are farmed.

Atlantic salmon (*Salmo salar*) at 8°C (Denstadli et al., 2007), Nile tilapia at 28°C (Xie et al., 2011), and silver catfish (*Rhamdia quelen*) at 18–28°C (Fracalossi et al., 2004) are examples of the wide temperature range at which the phytase must act. Furthermore, the diet of the poultry and pigs, in general, does not come in contact with the water and can be powdered; whereas, for the fish, when ingredients pretreatment is not performed, the enzyme must support extrusion process and act on fish body temperature.

Phytase activity in gastrointestinal tract: pH and proteases action

Phytase is pH dependent and it has an ideal value for maximum action. When pH deviates from the ideal point, its activity is decreased. It can be classified in two groups according to its optimum pH: acid (pH 5) and alkaline (pH 8) phytase (Baruah et al., 2007).

Tomschy et al. (2012) reported that the optimum pH for *A. fumigatus* (strain Q27L) phytase is 6.5, whereas Wyss et al. (1999a) reported that for the same producing species, but wild type strain, there was more than 80% of activity in the pH from 4.0 to 7.3.

In a study performed *in vivo* by Yan et al. (2002), it was verified that *A. niger* phytase was breaking IP6 in the stomach of channel catfish (*I. punctatus*) within the first 2 h after providing diet with 0, 500, 1000, 2000, 4000, or 8000 FTU.

Phytase is a protein, and therefore, it is subjected to gastrointestinal protease action; some *in vitro* and *in vivo* studies were performed to evaluate protease resistance. Wang et al. (2007)

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ISSN: 1984-2538

described that phytase produced by *P. pastoris* with *A. fumigatus* genes resists pepsin even after 2 h of incubation with crescent levels of the protease, but it does not resist trypsin. Equivalent results were reported for *Yersinia rohdei*, where its phytase improved the utilization of phytate phosphorus in animal feed, especially in swine feed. Moreover, the attractive biochemical characteristics allow the *Y. rohdei* phytase to be supplemented at a low level to yield sufficient activity. The phytase also hadhigh relative activity (80%) and strong resistance to pepsin at pH 1.5 (Huang et al., 2008). Kerovuo et al. (2000) reported the opposite, noting that *B. subtilis* phytase resisted trypsin but not pepsin.

A study performed *in vitro* showed that transgenic *P. pastoris* phytase was resistant to pepsin and not to trypsin; however, its activity was lesser at pH 4.0 than at pH 6.0 (Rodriguez et al., 2000). Considering that pepsin in the stomach is associated with low pH, and trypsin in the intestine is associated with high pH, it is possible to deduce that this phytase will not be efficient *in vivo*.

Thus, as the phytase production is intended for nonruminants, it must be able to prevent the denaturation at low pH, under the action of pepsin in the stomach, or alkaline pH, under trypsin in the intestine, since the optimum pH is widely variable depending not only on the producing organism but also on the source strain (Wyss et al., 1999a).

Stimulators and inhibitors

There are elements that act on phytase activity and operate as stimulators or inhibitors, and this understanding helps to choose a phytase that is not inhibited by compounds of the diet. For example, Kerovuo et al. (2000) reported that it was necessary to add calcium chloride in all stages of purification to maintain *B. subtilis* phytase activity and when EDTA was added, there was loss of activity; therefore, Ca^{2+} was a cofactor. Wyss et al. (1999a) described that *A. fumigatus* phytase was inhibited by many ions and EDTA was a stimulator, whereas *A. niger* enzyme was neither affected by ions nor by EDTA. Concerning *P. pastoris* phytase, it was slightly stimulated by K^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}, while Zn^{2+}, Cu^{2+}, Fe^{2+}, and Al^{3+} were inhibitors (Wang et al., 2007). Thus, it is remarkable that stimulatory and inhibitory elements depend on the producer organism.

Substrates

Besides the IP6, phytase can break a wide variety of compounds that have phosphorus in the molecule. Wyss et al. (1999a) evaluated the phytase activity from different microorganisms on p-nitrophenyl phosphate, phenyl phosphate, fructose 1,6-bisphosphate, fructose 6-phosphate, glucose 6-phosphate, ribose 5-phosphate, alpha-glycerophosphate, beta-glycerophosphate, 3-phosphoglycerate, phosphoenolpyruvate, AMP, ADP, and ATP. They found that *A. fumigatus* phytase showed higher activity on p-nitrophenyl phosphate, phenyl phosphate, and fructose 1,6-bisphosphate (>110 FTU) compared to their own IP6 (30 FTU). The authors also reported that *Escherichia coli* and *A. niger* phytase exhibited 800 and 100 FTU of activity on IP6 and <50 and <20 FTU on the others substrates, respectively.

Wang et al. (2007) reported that phytase of *P. pastoris* with *A. fumigatus* (strain WY-2) gene showed 2700 FTU on IP6 that was hydrolyzed faster than other phosphorylated compounds, which had ≤2% of the total enzyme activity.

The effects of low specific phytases can be observed in animal feeding. Wang et al. (2009) evaluated inclusion levels of phytase in rainbow trout (*O. mykiss*) diet and verified that apparent digestibility coefficient of the lipid decreased with its addition. Instantly, the authors suggested that phytase might have partially inactivated the lipase, but the fact was unclear.

Riche & Garling Jr. (2004) replaced herring meal by untreated or pretreated soybean meal (5000 FTU) in Nile tilapia diet. They concluded that untreated soybean meal might replace the herring meal more (20–25%) than the incubated meal (10%) to not decrease the growth.

Since, (i) in the research performed by Wang et al. (2009), 60% of the diet was composed of soybean meal, which has oil rich in lecithin (Penci et al., 2010); (ii) acids phosphatase that act on phospholipids are present with phytase because purification process is not totally efficient (Konietzny et al.,1994); (iii) phospholipids are important emulsifiers that aid in the intestinal lipid absorption (Fontagné et al., 2000); and that (iv) the variety of substrates broken by phytase may be large, depending on the producer organism; there are evidences that phospholipids are degraded, and therefore, they decrease digestion and lipids absorption when pretreated with high phytase levels.

Considerations

Phytase is successfully used in poultry and pigs feeding, whereas for fish, the results are ambiguous; and this is associated to the factors inherent to the enzyme, diet processing, and the several fish species' anatomy and physiology.

The most practical method of application is to mix the enzyme before extrusion process; however, the phytases that support high temperatures do not act in the low temperature of fish organism. In addition, when phytase is applied after processing, fish physiology must provide the minimum requirements for its action, which are specific of the producer microorganism strain.

Finally, when it is incubated with the ingredients before processing, lower amount of enzyme is necessary and the IP6 hydrolysis occurs under controlled conditions, close to the optimum and independent of the fish anatomy.

Independent of the application method, it is very important to know the properties of the used phytase in order to ensure its minimum efficiency and the appropriate nutrient availability.

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