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Review paper

EXOENZYMES IN AQUAFEEDS WITH PARTICULAR REFERENCE TO MICROBIAL PHYTASE: A REVIEW

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Summary

The global aquaculture industry has come under increasing pressure to optimize production efficiency while reducing environmental loadings. These new demands, which have been accompanied in certain countries by legislation, have stimulated the development of elite low output dietary formulations. Several feed manufacturers have developed diets with modified energy: protein ratios, which reduce environmental phosphorus loadings with minimum impact upon fish growth. However, problems relating to end product quality have materialized following application of these high lipid diets and it is clear that alternative strategies must be developed. In particular, there remains an urgent need to replace the expensive fishmeal component of aquafeeds. One approach might be to supplement diets with exogenous enzymes (exoenzymes) that enhance the value and utility of alternative, low grade proteins while reducing ecological impacts. This paper briefly reviews the literature relating to experimental exoenzyme aquafeeds, with specific reference to phytase.

Key words: *phytase, lipase, larval rearing, enzymes, diet*

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INTRODUCTION

In recent years the animal feedstuffs industry has turned its attention to the potential that supplemental hydrolytic (exo)enzymes have as a method of assisting digestion and absorption of low-grade formulations and reducing environmental pollution (review: Walsh *et al.*, 1993). In several laboratory and field-based trials, remarkable success has been achieved in enhancing the performance characteristics of commercially important terrestrial species. For example, Ritz *et al.* (1995), reported that supplementation of turkey poult diets with amylase both increased feed conversion efficiency and growth rates. Others have reported success in the enzymatic control of phosphorus excretion in cattle, increased survival and performance in weanling pigs and enhanced digestibility of barley-based diets with the addition of b-glucanase (Walsh *et al.*, 1993). These, and similar studies, have been provoked by changes in national agenda concerning animal production and environmental protection. In many countries legislation has been approved to ban or decrease the farming of pigs on environmental grounds (*e. g.*, Singapore, Taiwan) and as a means of ensuring reduced environmental phosphorus loading (*e. g.*, Holland and the US). Similar restrictions are now in place (*e. g.*, Denmark), or going through the approval process, with respect to aquaculture. A natural progression in the field of dietary enzyme supplementation will likely follow, therefore, with aquafeeds. Indeed, several studies have already been undertaken using enzyme addition to fish feeds. In particular, two areas of importance to aquaculture production have been considered: larval rearing and reduced environmental loading of phosphorus. The following provides a brief overview of various studies with fish that have employed feed exoenzymes.

EXOENZYMES AND LARVAL FISHES

Fish larvae are generally considered to differ markedly from their adult counterparts with respect to the complexity of their digestive systems. When compared to adults, a notable feature of the gut of hatching and post-yolk-sac larvae is a relatively low enzyme activity (*e. g.*, Pedersen *et al.*, 1987; Moyano *et al.*, 1996; Kolkovski *et al.*, 1997). Following first feeding, however, enzyme activity increases until transformation to the juvenile stage (Figure 1). These ontogenetic changes in enzymatic activity result due to an increasing capacity of the liver, pancreas and mucosal epithelium to produce enzymes (Govoni *et al.*, 1986) and/or reflect heightened presence of exogenous enzymes. Wunder (1936) postulated that exoenzymes, contributed by prey organisms, assisted fish during the digestive process. This theory, which was later supported by Schäperclaus (1961), thereby implied that the addition of enzymes to food would increase digestibility. Jancarik (1964), in contrast, put forward the idea that, rather than adding to the endogenous load of enzymes, food-borne exoenzymes worked to activate endoenzymes. On

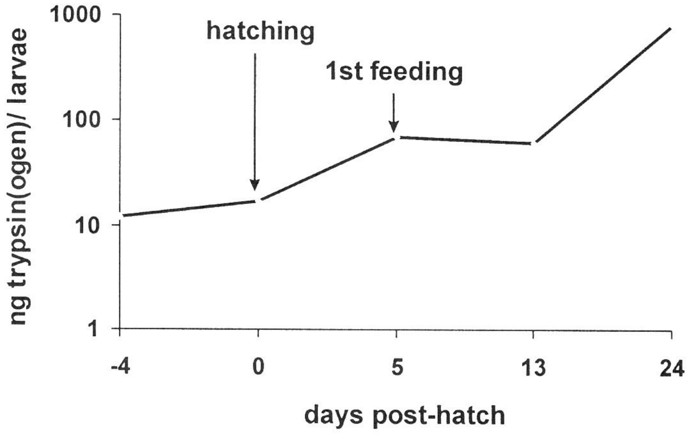

Figure 1. Evolution of trypsin(ogen) activity in larval herring Clupea harengus. *Animals were maintained with 200* Acartia tonsa L^{-1} *as prey organisms (from the data of* P e d e r s e n *et al., 1987).*

Slika 1. Razvoj aktivnosti tripsin(ogene) kod ličinki haringe C l u p e a h a r e n g u s. *Životinje su hranjene s 200* Acartia tonsa *L kao grabežljivcima (*P e d e r s e n *et al., 1987)*

the other hand, K u r o k a w a *et al.* (1998) suggest that, for larval Japanese sardine at least, exoenzymes play no role in protein digestion.

One feature of the larval fish gut that has received considerable comment (*e. g.*, W a t a n a b e, 1981), is the ability of the second segment to absorb intact proteins. It is believed that this mechanism represents a discrete device by which larval fish maximize protein absorption (T e s k e r e d z i c *et al.*, 1998). Alternatively, the ability to absorb macromolecules may represent an accessory method by which larval animals resorb endogenous protein derived from digestive enzymes (*e. g.* see S e g n e r and R ö s c h, 1992). In fact the latter hypothesis has some credence. H o f e r and N a s i r U d d i n (1985), argued that if secreted proteins (enzymes) were lost *via* the faeces, this would compromise larvae with respect to protein balance. Further support for the supposition that the larval gut may resorb endogenous enzyme protein comes from the experimental findings of P e d e r s e n and colleagues (P e d e r s e n *et al.*, 1987; P e d e r s e n and H j e l m e l a n d, 1988). The results of these authors indicate that larval herring invest significant resources, in terms of protein, for the production of digestive enzymes (Figure 1), although fecal loss of trypsin(ogen) was found to be negligible. P e d e r s e n and H j e l m e l a n d (1988), also speculated that lumenal enzymes, rather than being resorbed, might be reused, following reversible binding to the gut epithelium.

Recent innovations in the field of larval fish rearing include the development of microdiets that are employed during the crucial transition stage from yolk-sac to first feed larvae. A major problem of microdiets though, is the high levels of mortality associated with their use. The limited success attained with micro or dry diets is believed to be affiliated with the larvae's incompletely developed digestive system and reduced digestive capacity (Dabrowski and Culver, 1991; Kolkovski *et al.*, 1993). The mortality problem is, however, generally overcome when microdiets are supplemented with live foods, especially when the latter have been fortified (*e. g.*, see Koven *et al.*, 2001). Thus, a prerequisite for the culture of many species of larval fishes is the co–culture of live feeds (*e. g.*, microalgae, rotifers and *Artemia* at various stages of development). This requirement increases the costs of maintaining a hatchery, even though the use of live food does not, necessarily, secure high survival or good larval growth. Inferior larval growth rates during cultivation may reflect poor nutrient content of proffered food, a restricted feed intake by the larvae, incorrect prey size range and/or problems with food palatability (see: Kolkovski *et al.*, 1993; Zambonino Infante and Cahu, 1994; Rosenlund *et al.*, 1997). Equally likely is that better survival and growth experienced with larvae fed on live food results due to the digestive enzyme "supplements" being utilized by the fish gut (*e. g.*, Dabrowski and Glogowski, 1977; Dabrowska *et al.*, 1979).

Evidence to support the importance of exoenzymes in the digestive process of larval fish has been presented, among others, with a series of studies with turbot (*Scophthalmus maximus*, Bothidae). Thus, Munilla-Moran *et al.* (1990), examined the development of the digestive system and various enzyme activities in 3–day–old larvae and their prey items. The results from these studies indicated that first feeding turbot larvae were equipped with a fully functional digestive system. However, exoenzymes, derived from prey organisms, were considered to play an important role in the digestive process, particularly for esterase (89–94% of total) and protease (43–60% total). Moreover, Munilla-Moran and colleagues (1990), concluded that the contribution of exoenzymes to total digestive capacity was not only significant, but also increased in importance with fish age. These and similar findings (*e. g.*, Lauff and Hofer, 1984; Dabrowski and Glogowski, 1977), have led to the proposal that future use of dry larval fish diets should be associated with supplementary exogenous proteases to assist in protein digestion (Gatesoupe *et al.*, 1997; Kolkovski *et al.*, 1997). Nevertheless, while there is little doubt that replacement of live prey with inert food remains an important goal for the controlled culture of a number of (particularly marine) species, progress in this field will rely heavily upon an increased understanding of larval digestive physiology and behaviour. Clearly, the potential role that exoenzymes play is intriguing but their relative importance, ‹remembering that several larval teleosts (marine and freshwater) can be reared exclusively on microdiets›, will only come to light with further research effort.

EXOENZYMES AND ADULT DIETS

A limited number of studies have considered digestive enzyme supplements for adult and juvenile animals. In an interesting study, Rodger *et al.* (1995), incorporated mammalian pancreatic enzymes (4g/kg feed), into feed as a measure to treat Atlantic salmon (*Salmo salar*, Salmonidae), for pancreas disease. However, following feeding (0.6–0.9% body weight day^{-1}) for 41 days, no reduction in mortality associated with the disease, or growth benefit was recorded. Likewise, Carter *et al.* (1992), who fed Atlantic salmon on rations containing α-amylase and subsequently monitored individual food consumption and growth rates over a number of weeks, recorded no significant differences in growth performance, appetite, or food conversion efficiencies between treated and control feeds. In a later study (Carter *et al.*, 1994), in which growing Atlantic salmon were fed a soybean meal-based feed supplemented with a protease and carbohydrase, fish were observed to return superior appetite, growth performance and feed conversion efficiencies when compared against control fish. Moreover, salmon fed the enzyme supplemented, soybean meal-based diets, achieved similar food conversion efficiencies to fish fed on standard fish meal-based diets. Together, the studies of Carter and colleagues indicate that dietary addition of enzymes to grower diets might be worthwhile where the digestibility of, for example, the protein component, is reduced relative to fish meal-based feeds. Pre-treatment of a soybean residue, derived from the production of soy milk, with papain, has also been shown to improve growth performance of carp (*Cyprinus carpio*, Cyprinidae) when compared to animals fed untreated diets (Wong *et al.*, 1996). Papain treatment of the soybean diet reduced water turbidity. Also with carp, addition of a mixture of amylase, protease, β-glucanase, β-glucosidase and cellulase (1.5 g kg^{-1} as Polizyme® to fingerling diets was found to increase growth and whole body protein levels (Bogut *et al.*, 1994).

The studies of Samuelsen *et al.* (2001), examined the potential for using supplemental dietary microbial lipase as a means to optimize the use of dietary lipids and, perhaps, to alter end product quality characteristics of rainbow trout. This attempt at producing "designer fish" — that is fish that express quality characteristics of interest to consumer and processor, without impacting growth performance — however, had limited impact in terms of altering fillet proximate composition or fillet yields. However, closer examination of the fillet revealed that changes were associated with lipase treatment with regard to monounsaturated fatty acid profiles. Although the study of Samuelsen *et al.* (2001) did not evaluate possible sensorial changes to the eating quality of the fillet, they suggested that dietary enzyme supplementation might represent one means through which fillet quality characteristics could be manipulated. Clearly this area of study demands further, more rigorous investigation.

PHYTIC ACID PHOSPHORUS AND PHYTASE

Phosphorus (P) is ubiquitous in fish, being the second most abundant mineral after calcium. Approximately 85% of body phosphorus is found in bony tissues, where it is maintained as calcium phosphate and calcium hydroxyapatite. As well as its important role in skeletal tissues P is, in the form of phospholipids, structurally important with respect to cell membranes. P plays a critical role during respiration, muscular contraction, cell division and other energy transforming processes (Lall, 1991). In fish, P may be absorbed directly from the water in a dose–dependent manner; although the main site of uptake (gills or gut) remains to be established. However, since the quantities of P in most natural water columns would likely be inadequate to meet requirements (Table 1), there is an absolute requirement for dietary P, particularly with respect to ensuring normal bone mineralization processes. P deficiency leads to a number of pathological states (Table 1), including increased mortality, sluggishness, decalcification and enhanced visceral lipid accumulation. It is important to note that most P compounds are generally only soluble in acids such that differences in assimilation are apparent between gastric and agastric species. The latter has important consequences during the formulation of diets since, even though a foodstuff may contain adequate amounts of P, quantitatively speaking, there may still exist a requirement for supplementation (Table 2).

Table 1. Examples of the impact of phosphorus deficiency upon various species of cultured teleost and reported ranges of phosphorus requirement.

Tablica 1. Primjeri utjecaja nedostatka P kod različitih vrsta riba i njihove potrebe za P

DEFICIENCY SYMPTOM Simptomi nedostataka	SPECIES Vrste	P REQURIEMENT (% of diet) Potrebe P (% u hrani)
reduced growth rates	common carp	0.6–0.7
smanjeni prirast	red sea bream	0.7
	rainbow trout	0.5–0.8
	Japanese eel	0.3
reduced feed conversion		0.6–1.0
smanjena konverzija	**Atlantic salmon**	
	rainbow trout	
	common carp	0.4–0.8
	channel catfish	
dysfunction of skeletal tissue	Atlantic salmon	
disfunkcija koštanog tkiva	rainbow trout	
	red sea bream	
	common carp	0.4–0.6
	tilapia	

Table 2. Examples of the bioavailability of phosphorus to Atlantic salmon, channel catfish and common carp from various feedstuffs and mineral supplements employed by the aquaculture industry (data from Lall, 1991).

Tablica 2. Primjeri iskoristivosti P kod atlantskog lososa, kanalnog somića i šarana iz raličitih sastojaka i minerala u hrani koja se rabi u akvakulturi (Lall, 1991)

FEEDSTUFF	SALMON	CATFISH	CARP
casein	92	90	97
brewer's yeast	79	—	93
menhaden meal	87	39	
wheat middlings	32	28	
soybean meal	36	~40	
phytate	0	0	~20
$CaH_4(PO_4)$. H_2O	90	94	94
$CaHPO_4$	72	65	46

A major concern with respect to aquaculture is the environmental loading of P, which is considered a point source of pollution. Many examples are now available regarding hypernutrification and eutrophication events associated with fish farm effluents (*e. g.,* Bergheim *et al.*, 1991). The two major fish farm contributors of P to the environment are uneaten feed and fecal material. There is increasing pressure upon animal producers, both on economic and environmental grounds, to increase utilisation of low-grade ingredients while, at the same time, reducing environmental impact. However, even though a number of strategies have been emplaced by the aquaculture industry to reduce environmental loading of nutrients (see: McLean *et al.*, 1996; McLean and Rønsholdt, 1997), P burdens still remain of critical concern, both to the aquafeed and production sectors.

Several methods have been examined in attempts to reduce P outputs. These include the formulation of fish diets with enzymatically-treated fish-meals (Jacobsen and Børresen, 1995), and de-boned meals. In the studies of Jacobsen and Børresen (1995), enzyme treatment reduced fishmeal P concentrations by almost a half when compared to traditional production methods. When employed in aquafeeds, the enzyme treated fish meal-based diets had a 40% measured reduction in P when matched against feeds produced with standard low temperature meals. However, during 4 week feed trials with 60g rainbow trout, inferior growth rates were recorded for animals fed feeds composed of enzyme-treated fish meal. In production level trials (100g trout grown to 200g), however, no differences in performance between groups was observed; although poorer feed conversion ratios were observed in enzyme-treatment groups.

Many authors (*e. g.,* Tacon, 1995), have called for the replacement of expensive fish meal-based feeds with alternative, plant-based protein sources.

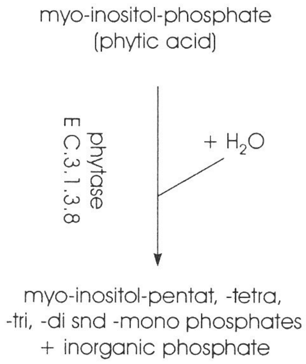

Figure 2. Summary diagram of the mechanism of action of phytase in the release of phosphorus from phytic acid.

Slika 2. Sumarni prikaz mehanizma aktivnosti fitaze u otpuštanju P iz fitinske kiseline.

One of the major problems underlying the application of plant sources for feedstuffs, however, is the poor availability of P. In soybean meal for example, only 36–40% of the 0.62% total P is available for absorption (Table 2). This poor availability results because most of the P is stored in the form of the hexaphosphate ester of myoinositol, or phytic acid (Figure 2). The latter situation has many important consequences with respect to the production of aquafeeds, not least because the dietary P requirement for optimal growth, for example, in trout of 50–200g is circa 0.5% (Rodehutscord and Pfeffer, 1995a) and for carp, around 0.7% (Kim *et al.*, 1998; Table 1). Use of soybean meal as an alternative protein source for trout feed, therefore, requires supplemental P which increases dietary costs; even though sufficient P is present in the diet. Another consequence of using plant proteins, such as soybean meal, is that phytic acid can bind to minerals in the gut, thereby making them unavailable, while reducing digestibility of proteins (Conneely, 1992). Thirdly, as the P moiety of phytic acid is not absorbed by the gut, the net amount of P which may pass into the environment will be greater relative to fishmeal–based diets. Another important aspect of phytic acid is that it has been reported to adversely affect the stability of trypsin and to interfere with the activation of trypsinogen (Caldwell, 1992).

However, it has been known for some time that the addition of fungal enzymes to plant–based diets can overcome the preceding obstacles, since they are able to release P from the hexaphosphate ester of myoinositol core by hydrolysis (Nelson *et al.*, 1968; Figure 2). A number of phytases have been demonstrated to dislodge orthophosphate from integral parts of the phytic acid molecule. Of significance in this regard has been the phytase enzyme produced by *Aspergillus ficuum*. The high specific activity and thermostability of this enzyme is of importance due to the processes that it would have to endure during feed production (Mayer and McLean, 1995). There also exist a number of commercial sources of *Aspergillus* phytase, generally produced by recombinant DNA technologies. However, in selecting the origin of phytase, attention must be given to optimal conditions for the preparation, since it is known that the activity of certain phytases are pH dependent (Shäfer *et*

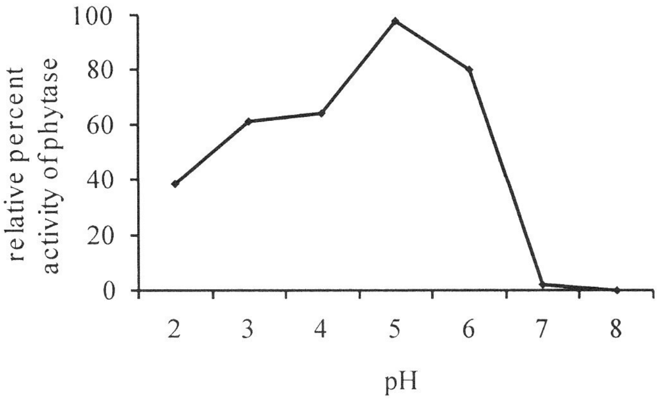

Figure 3. The pH dependent nature of the activity of an Aspergillus *phytase (from Hoppe, 1992).*
Slika 3. Ovisnosti pH o aktivnosti Aspergillus *fitaze (Hoppe, 1992)*

al., 1995; Figure 3). Irrespective of the pH lability of phytase, the activity of the enzyme would not be hindered during the storage of treated feeds. Hence, it is likely that the phytase would release phytic acid P from food pellets before use. Since studies have demonstrated improved retention of phosphorus from phytase treated plant-based feeds (Brown, 1993; Rodehutscord and Pfeffer, 1995b; Schäfer *et al.*, 1995), reasonable indications are available to suggest that phytase activity is not totally nullified in the gut. This may occur due to enzyme protection within the chyme/chyle. In other cases, phytase may simply be employed as a pre-treatment for feed constituents; that is phytic acid bound phosphorus may be pre-released during soybean meal processing prior to incorporation into aquafeeds.

A number of studies have now been undertaken with dietary phytase inclusion in plant-based fish feeds. These include those undertaken with salmonids (Teskeredzic *et al.*, 1995; Ketola, 1994; Brown, 1993; Rodehutscord and Pfeffer, 1995a; Cain and Garling, 1995; Lanari *et al.*, 1998; Storebakken *et al.*, 1998; Vielma *et al.*, 1998; Forster *et al.*, 1999), channel catfish (Eya and Lovell, 1997; Li and Robinson, 1997; Jackson *et al.*, 1996), common carp (Schäfer *et al.*, 1995), seabass (Oliva-Teles *et al.*, 1998), and striped bass (Hughes and Soares, 1998). Most experiments to date have compared the performance of animals receiving partial substitution of a fish meal-based diet with and without phytase-treated soybean meal (*e. g.,* Rodehutscord and Pfeffer, 1995b; Schäfer *et al.*, 1995; Jackson *et al.*, 1996; Lanari *et al.*, 1998); although some authors have also included fish meal-based diets too, for direct contrasting (*e. g.,* Storebakken *et al.*, 1998). Addition of phytase to soybean meal-based diets for trout, carp, seabass and channel catfish resulted in decreased fecal P and concomitant increased P digestibility and retention (Ketola, 1994; Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995a; Schäfer *et al.,* 1995; Li and Robinson, 1997; Oliva-Teles *et al.*, 1998). In striped bass, Hughes and Soares (1998), reported increased scale and vertebral P concentrations, while Jackson *et al.* (1996), found that, for channel catfish, supplemental phytase increased appetite and reduced feed conversion efficiencies. *In toto*, the preceding experiments confirm that addition of phytase improves the biological value of soybean concentrates and, concomitantly significantly reduces environmental P loading. With increased research effort, further substantial reductions in P excretion from aquaculture are likely possible. For this to be realized, however, an enhanced understanding of P requirements, an enlightened selection of feed ingredients and careful attention to the impact of the addition of phytase must be gained.

The potential offered by exoenzymes in the aquafeed sense is great. When compared to the animal feed industry, there have been few studies, using a very narrow range of enzymes, with fish. Those trials which have been completed show great promise for future successful application. Such supplements could potentially place a different meaning to the term "least–cost" dietary formulation since this food biotechnology may increase the variety of products available in the larder for use — with the emphasis upon economy. The ability to target and incorporate a symphony of enzymes into aquafeeds, which may be active at all, or different levels of the gut, and capable of performing one defined, or several tasks, will also open doors to a more sustainable and environmentally sound industry.

Sažetak

EGZOENCIMI U HRANI ZA RIBE S POSEBNIM OSVRTOM NA FITAZU: PREGLED

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Kompletna industrija akvakulture nalazi se pod povećanim pritiskom glede optimalizacije efikasne proizvodnje, dok se u isto vrijeme smanjuju mogućnosti iskorištavanja akvatorija. Ovi, novi zahtjevi koji su u nekim zemljama povezani s legislativom, potaknuli su razvoj niskih iskorištenosti hranidbenih formulacija. Nekoliko proizvođača razvilo je hrane s modificiranom energijom: količina bjelančevina koja smanjuje nakupljanje P u okolišu s minimalnim utjecajem na rast riba. No, problemi koji se odnose na kvalitetu gotovoga proizvoda materijalirizirani su uporabom hrana s visokim postotkom lipida i jasno je da mora biti razvijena alternativna strategija, napose stoga što ostaje urgentna potreba za zamjenu skupog ribljeg brašna kao komponente u hrani za ribe. Jedna od mogućnosti mogla bi biti dodatak egzogenih enzima (egzoenzima) koji povećavaju vrijednost i iskorištenost nisko vrijednih bjelančevina. U ovom je radu dan kratak prikaz literaturnih podataka koji se odnose na eksperimentalne hrane s egzoenzimima, s posebnim osvrtom na fitazu.

Ključne riječi: fitaza, lipaza, uzgoj ličinaka, enzimi, hrana

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