Aquaculture 428–429 (2014) 195–202

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Effects of different protein hydrolysate products and levels on growth, survival rate and digestive capacity in Asian seabass (*Lates calcarifer* Bloch) larvae

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ARTICLE INFO

Article history:
Received 22 October 2013
Received in revised form 6 March 2014
Accepted 7 March 2014
Available online 27 March 2014

Keywords:
Asian seabass
Protein hydrolysate
Larval diet
Digestive enzymes
Gene expression

ABSTRACT

Larval rearing of Asian seabass is dependent on live food, similar to several other carnivorous fish species. The present study aimed to find suitable predigested protein sources for use in Asian seabass larval diets in order to reduce dependency on live feed. Fish muscle, squid mantle and soybean meal were hydrolyzed by Alcalase (alkaline enzyme), pepsin (acidic enzyme), and the combination of both enzymes producing a total of 9 protein hydrolysate (PH) products. The soybean meal was found least favorable for the larvae and omitted from the *in vivo* investigation. Fish muscle and squid mantle hydrolysate products were selected based on *in vitro* digestibility to replace fishmeal protein at 25% and 50% in isonitrogenous (50% protein) and isolipidic (20% lipid) diets. A diet with 0% PH was used as a control diet, and two other diets, one with minced mackerel muscle supplying protein, and a commercial diet were included as reference diets. The experimental diets were fed to larvae four times daily for 30 days from 17 days post hatch. Survival rate and growth performance were measured and enzyme activity and gene expression of stomach, pancreatic and brush border enzymes were determined. Larvae accepted all PH diets and the control diet well but did not accept the two reference diets which caused 100% mortality in the third week of the trial. In general, 25% PH inclusion increased larval growth compared to the 0% control group and increased specific activity of pancreatic and brush border enzymes. At 50% inclusion level, negative effects on growth performance and survival rate were observed. High levels of small peptides in the PH might have influenced digestive enzyme capacity as reflected in induced chymotrypsin activity but reduced mRNA level and specific enzyme activity of the brush border enzyme, leucine aminopeptidase. In conclusion, incorporation of 25% PH from either fish muscle or squid mantle treated with the Alcalase-pepsin enzyme combination improved digestive capacity and growth performance of the larvae with acceptable survival rate.

1. Introduction

Early weaning of fish larvae with microbound diets has been studied over the past three decades with the aim of shortening the live prey feeding period. Live feeds tend to vary in nutritional value and often carry high levels of pathogenic bacteria. Additionally, live feeds are associated with high rearing costs and labor requirements. Pre-digested protein, or protein hydrolysate (PH) containing small size peptides has been used as a protein source in fish larval diets with beneficial effects on maturation of digestive organs (Zambonino-Infante et al., 1997) and improved digestion and assimilation of nutrients (Tonheim et al., 2005). Pre-digested protein has been shown to improve growth of many marine fish species (Cahu et al., 1999; Kvåle et al., 2009). Protein hydrolysate has also been shown to improve the immune system in European seabass larvae (Kotzamanis et al., 2007) and large yellow croaker (Tang et al., 2008). According to Cahu et al. (1999), a moderate PH inclusion level (25%) in microbound diets may improve survival rate, growth and the onset of the adult mode of digestion, whereas excessively high inclusion levels (>50%) can lead to reduced larval growth (Cahu et al., 1999; Carvalho et al., 2004; Kolkovski and Tandler, 2000; Kvåle et al., 2002). However, Sovoie et al. (2006) observed no significant effects of 10–20% PH on growth and survival rate in newly-hatched spotted wolffish (*Anarhichas minor*).

The suitability and nutritional value of PH in larval diets depend on certain characteristics of the hydrolysate. Kotzamanis et al. (2007) suggested that the source and characteristics of the protein used, the

Abbreviations: PH, protein hydrolysate; FM, fish muscle; SM, squid mantle; SB, soybean meal; A, Alcalase; P, pepsin; C, combination of pepsin and Alcalase; DH, degree of hydrolysis; AG, amino acid group; FAA, free amino acid; dph, days post hatch; *ef-1α*, elongation factor-1α; *α-tub*, α-tubulin; *ubq*, ubiquitin; *pg*, pepsinogen; *try*, trypsinogen; *ctr*, chymotrypsinogen; *bal*, bile salt activated lipase; *amy*, amylase; *lap*, leucine aminopeptidase; *alp*, alkaline phosphatase; Cq, quantification cycle.

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http://dx.doi.org/10.1016/j.aquaculture.2014.03.004
0044-8486/

hydrolytic method, and the degree of hydrolysis may influence larval growth. A study in Asian seabass showed that the incorporation of acid digested fish meal at 45% into microbound diets improved larval growth (Nankervis and Southgate, 2006). However, effects of other types of protein hydrolysates and the optimum level of incorporation have not been explored.

The objective of the present study was to examine the effect of different PH products and incorporation levels on survival rate, growth and digestive capacity in Asian seabass larvae. Fish muscle (FM), squid mantle (SM) and full fat soybean meal (SB) were selected as protein sources for preparing PH using different enzymes: Alcalase, pepsin and a combination of the two. Alcalase is a commercial enzyme that has been widely used in the production of protein hydrolysates (Benjakul and Morrissey, 1997). Pepsin was selected in order to mimic the gastric digestion because most marine fish larvae lack a functional stomach during early larval stages, possibly limiting protein digestion (Rønnestad et al., 2003). *In vitro* protein digestibility using crude enzyme extracts from juvenile Asian seabass was determined for selection of the potential PHs. Subsequently, the best performing PHs were evaluated *in vivo* in a feeding trial. End point measurements included larval survival rate, growth, enzyme activity and gene expression of the main digestive enzymes.

2. Materials and methods

2.1. Protein hydrolysate preparation

A local marine food fish, the Japanese scad (*Decapterus maruadsi*), and longfin squid (*Loligo pealeii*) were purchased from the market, placed on ice and transported to the Department of Aquatic Science, Prince of Songkla University, Hat Yai. Upon arrival, both the FM and SM were filleted, chopped to 3 cm lengths and ground using a meat grinder (Super grinder, National, MK20NR, Japan). The SB (from Lee Feed Mill Public Company Limited) was ground (Super blender, National, MXT 2GN, Taiwan) and autoclaved at 121 °C, 15 psi for 15 min in order to inactivate heat-labile anti-nutritional factors. One hundred grams of each protein source was packed into polyethylene bags and stored at −20 °C until used. As shown in Table 1, the prepared FM, SM and SB were hydrolyzed using two different proteolytic enzymes: (P) pepsin (P7000, from porcine stomach mucosa; Sigma, St. Louis, MO, USA), and (A) alkaline protease (Alcalase 2.4L®, Sigma, Novozymes, Bagsvaerd, Denmark), as well as a combination of pepsin and alkaline protease (C). For PH treated with enzymes P and A, thawed samples were homogenized (Multiquick, Braun, MR400HC, Spain) in 0.1 M HCl (pH 3.0) and 0.2 M Tris–HCl (pH 8.0), respectively at a ratio of 1: 2 (w/v) for 2 min at ambient temperature. The enzyme concentration and hydrolysis time required to obtain the 50% degree of hydrolysis (DH) for each PH product was optimized prior to the processing. For the (C) type PH produced using the combination of both enzymes, pepsin hydrolysis was initiated at pH 3, and subsequent alkalinization to pH 8 using 6 N NaOH prior to Alcalase hydrolysis. After hydrolysis, the enzymes were inactivated by incubating the reaction mixture at 95 °C for 20 min. In the case of PH treated with enzyme P, the hydrolysate was neutralized by 6 N NaOH prior to inactivation. After heat treatment the mixtures were centrifuged at 7400 ×*g* at 4 °C for 20 min and the supernatants were collected. All products were dried at 60 °C overnight, ground thoroughly into powdered hydrolysate and stored at −20 °C for later incorporation into diets.

2.2. Analysis of PH products

2.2.1. Chemical composition

Proximate composition of native protein sources, FM, SM, SB and protein hydrolysate products were measured. Moisture, protein, fat and ash were determined according to AOAC (1990): dry matter by drying in an oven at 105 °C, ash by combustion at 550 °C in a muffle furnace for 3 h, protein content using the Kjeldahl method (Kjeldahl apparatus, Gerhardt, Germany) and fat using dichloromethane extraction (Soxtec System HT6, FOSS TECATOR, Sweden).

2.2.2. Protein solubility

Protein solubility was determined with the nitrogen solubility index (NSI) following the procedure of Morr (1985) which is calculated as follows:

$$\mathrm{NSI}(\%) = 100 \times \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}}.$$

2.2.3. Degree of hydrolysis

The DH was calculated as a proportion (%) of free amino groups (AG) released after hydrolysis with respect to total AG in each sample (Adler-Nissen, 1979; Benjakul and Morrissey, 1997). The DH was calculated as follows:

$$\mathrm{DH} = [(L_t - L_0)/(L_{max} - L_0)] \times 100.$$

L_t amount of α-AG released at time t
L_0 amount of α-AG in original homogenate
L_{max} total α-AG in original homogenate obtained after acid hydrolysis (6 N HCl at 110 °C for 24 h)

2.2.4. The molecular weights of soluble protein fractions in hydrolysate products

The molecular weights of soluble protein fractions were analyzed by gel filtration chromatography. Ten milligrams of dry hydrolysate powder were dissolved in 0.05 M sodium acetate (Merck, Darmstadt, Germany) buffer (pH 5.0), centrifuged at 7840 ×*g* (KUBOTA 3500,

Table 1
Conditions for production of different protein hydrolysate products at 50% DH.

Protein sources	Hydrolyzed enzyme	Enz. conc.[a] (units g protein^{-1})	pH	Temp. (°C)	Time (h)
Fish muscle	Pepsin[b] (FMP)	6.7×10^4	3.0	37	10
	Alcalase[c] (FMA)	0.18	8.0	50	1
	Pepsin/Alcalase (FMC)	1.2×10^4/0.02	3.0/8.0	37/50	10/1
Squid mantle	Pepsin (SMP)	8.0×10^4	3.0	37	10
	Alcalase (SMA)	0.04	8.0	50	1
	Pepsin/Alcalase (SMC)	1.9×10^4/0.01	3.0/8.0	37/50	10/1
Soybean meal	Pepsin (SBP)	11.2×10^4	3.0	37	10
	Alcalase (SBA)	0.15	8.0	50	2
	Pepsin/Alcalase (SBC)	1.4×10^4/0.01	3.0/8.0	37/50	10/2

[a] Enzyme concentration per reaction mixture containing 30 g of fish muscle and squid mantle and 10 g of soybean meal.
[b] Pepsin from porcine stomach mucosa 439 units mg solid^{-1}.
[c] Alcalase 2.4 Anson unit g^{-1}.