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Partial Substitution of Fishmeal by Meat and Bone Meal, Soybean Meal, and Squid Concentrate in Feeds for the Prawn, *Artemesia longinaris*: Effect on Digestive Proteinases

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Abstract

The effect of alternative protein sources on proteinase activity in the midgut gland of *Artemesia longinaris* was studied. Three isoproteic feeds were compared: a basal diet containing 48% fishmeal and 17% soybean meal (diet 1), the basal diet containing meat and bone meal as partial replacement of the fishmeal (diet 2), the basal diet containing additional soybean meal and a squid protein concentrate as partial replacement of the fishmeal (diet 3). Midgut gland extracts from the three treatments and wild prawns (control) were assayed to quantify proteinase activity. Specific inhibitors were used to identify proteinase classes by SDS-PAGE. Specific proteolytic activity was highest in the wild prawns and in prawns fed diet 2. Trypsin ac-

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tivity was higher in prawns fed the diets (1.9-2.6 absorbance/min/mg) than in wild prawns (0.6 absorbance/min/mg). Chymotrypsin activity was highest in prawns fed diet 2. Proteinase activity in samples on azocasein was inhibited by soybean trypsin inhibitor and Nα-p-tosyl-L-lysine chloromethyl ketone. Electrophoresis revealed at least six different bands with zones having caseinolytic activity in prawns fed the diets as compared to wild prawns, including four trypsins (16.6, 18.2, 21.9, and 25.1 kDa) and one chymotrypsin (53.7 kDa). Our findings indicate that proteolytic activity in *A. longinaris* adapts to the quality of the dietary protein and that fishmeal can partially be replaced by additional soybean meal in combination with squid protein concentrate.

Introduction

Artemesia longinaris, one of the most important prawn species in coastal fisheries in Argentina, is distributed along the South American coast from 23° to 43°S (Boschi and Gavio, 2005). Due to annual fluctuations in catches and to maintain a continuous market supply, it is important to establish the culture of *A. longinaris*. Culture of autochthonous or native species can serve as business diversification. High yields can be obtained due to their inherent physiological adaptation to local environmental conditions (Lemos et al., 2000). Understanding the nutritional requirements of a species is essential to ensuring profitable production and long-term sustainability in aquaculture.

Our group carried out growth experiments with *A. longinaris* under culture conditions (Fenucci et al., 1983), determined its nutritional requirements (Fernandez Gimenez and Fenucci, 2002; Romanos Mangialardo and Fenucci, 2002), and characterized its digestive proteinases in relation to the molting cycle (Fernandez Gimenez et al., 2002). However, no information is available on the relationship between feed composition and digestive enzymatic activity. Such information would help in formulating feeds that induce appropriate growth performance.

Fishmeal is the preferred protein source in aquafeeds because it is an excellent source of essential nutrients. However, limited availability and high demand make fishmeal a costly ingredient. The search for alternative low cost protein sources is increasing. Meat and bone meal, soybean meal, and squid meal may be used as protein sources for growing shrimps (Diaz et al., 1999; Divakaran et al., 2000; Medina Marti et al., 2005; Tan et al., 2005).

Meat and bone meal, meat scraps, and trimmings are the principal by-products of animal slaughterhouses. The quality of meat meal as a protein supplement depends on the production process as well as the raw material (Tacon and Akiyama, 1997). Soybean meal is the most important plant protein source currently used to supplement feeds for cultured shrimp but it cannot be used as the sole source of protein because it lacks certain essential amino acids and contains anti-nutritional factors such as lectins and proteinase inhibitors (Cordoba-Murueta and Garcia-Carreno, 2002). Squid protein has been used as a protein source in feeds with favorable responses in penaeids (Cruz-Ricque et al., 1987; Diaz et al., 1999). Squid may thus be of economic importance in areas where it can be obtained at low cost.

In decapod crustaceans, proteolytic enzymes synthesized in the midgut gland play a key role in the assimilation of food protein (Muhlia-Almazan et al., 2003). The capacity of an animal to obtain nutrients from a particular food source is largely determined by the profile and activity of its digestive enzymes; however digestive responses to specific nutrients appear to vary widely among species (Furne et al., 2005). It is possible to predict the ability of a species to utilize nutri-

ents by analyzing its digestive enzyme profile. Trypsin and chymotrypsin are the most abundant proteolytic enzymes in the midgut gland of decapods and are responsible for 60% of protein digestion (Lemos et al., 2000).

Earlier research shows that *A. longinaris* fed diets containing fishmeal, meat and bone meal, soybean meal, and squid protein concentrate had similar growth rates and good protein digestibility (Fernandez Gimenez et al., in press). The current study was conducted to determine the effect of partial replacement of fishmeal by these alternative protein sources in diets for *A. longinaris* under laboratory conditions.

Materials and Methods

Feed and feeding trials. *Artemesia longinaris* prawns (1.3±0.51 g) were obtained from a commercial fisherman in the coastal waters of Mar del Plata, Argentina (38°S). They were kept in 150-l glass aquaria with continuous aeration and a photoperiod of 11 h:13 h dark. Temperature was 18°C, pH 7, salinity 31 ppt, and ammonium concentration ≤0.2 mg/l. Sea water was filtered to 5 μm and exchanged at a daily rate of 50%.

Three dry pelletized feeds were prepared (Table 1). Feed ingredients were obtained from a local feed manufacturer, mixed, cold pelleted (<50°C) by extrusion to obtain 3-mm diameter pellets (Fenucci and Zein Eldin, 1976), and oven-dried for 24 h at 50°C. Feed formulations were based on the chemical compositions of the by-products to obtain isoproteic and isolipidic diets. The chemical compositions of the formulated feeds were confirmed by proximate analysis (AOAC, 1997). The feeds were tested in triplicate groups of eight randomly chosen prawns. All groups were daily fed *ad libitum*.

Prawn sample preparation. At the end of the experiment, prawns from each aquarium were counted and weighed. Midgut glands were removed from decapitated specimens. Samples from the same treatment were pooled and stored at -20°C. The pooled samples were homogenized in chilled distilled water and centrifuged for 30 min at 10,000 x *g* at 4°C. The supernatant was kept at -20°C until used as an enzyme extract.

Proteinase analysis. Soluble protein in crude extract was measured by the method described by Bradford (1976), using chicken egg white albumin as the standard. Specific proteinase activity was determined using enzyme extracts and 1% azocasein in 50 mM Tris-HCl, pH 7.5 (Garcia-Carreno, 1992).

Trypsin activity was measured with Nα-benzoyl-DL-arginine p-nitroanilide (BAPNA) as the substrate (Erlanger et al., 1961). BAPNA (1mM) was dissolved in 1 ml of dimethylsulfoxide (DMSO) and made to 100 ml with Tris HCl, pH 7.5, containing 20 mM CaCl2. Enzyme extracts (5 μl) were added to 0.75 ml of the substrate solution at 37°C and absorbance at 410 nm was recorded for 10 min.

Chymotrypsin activity was evaluated using 0.1 mM Suc-Ala-Ala-Pro-Phe-p-NA (SAPNA) in 0.1 M Tris HCl, pH 7.5 containing 10 mM CaCl2. Enzyme extracts (5 μl) were mixed with 0.75 ml of the substrate solution and absorbance at 410 nm was recorded for 5 min (del Mar et al., 1979).

Specific proteinase, trypsin, and chymotrypsin units of activity were expressed as the change in absorbance per min per mg protein (abs/min/mg). Each assay included blanks and commercial enzymes (1 mg/ml) as internal controls. Assays were run in triplicate.

To evaluate the contribution of individual proteinases to the total activity of the midgut gland, each sample was incubated with specific inhibitors and the residual activity was evaluated using

Table 1. Composition of formulated feeds.

	Diet		
	1	*2*	*3*
Ingredient (g/100 g dry feed)			
Fishmeal (66% crude protein)[1]	48	27	27
Meat and bone meal (61% crude protein)[2]	-	23	-
Soybean meal (43% crude protein)[3]	17	17	23
Squid protein concentrate (86% crude protein)[4]	-	-	10
Manioc starch	20	20	22
Wheat	9.5	7.5	12.5
Fish oil	2	2	2
Fish soluble	2	2	2
Lecithin	0.5	0.5	0.5
Cholesterol	0.5	0.5	0.5
Vitamin supplement[5]	0.5	0.5	0.5
Proximate composition (% dry matter)			
Dry matter	99.55	99.47	99.60
Crude protein	39.30	39.80	37.20
Total lipid	8.30	7.80	7.00
Ash	6.23	5.94	5.20

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[3] Melrico S.A., Argentina
[4] Diaz et al., 1999
[5] mg per kg vitamin premix: cholecalciferol 35, thiamin 163, rivoflavin 156, pyridoxine 213, calcium pantothenate 250, biotin 250, niacin 500, folic acid 25, B_{12} HCL 20, ascorbic acid Rovimix STAY C 781, menadione 34, inositol 300, choline chloride 200, α-tocopherol acetate 1750, vitamin A acetate 180 (Laboquimica S.R.L., Argentina).

azocasein as the substrate (Garcia-Carreno and Haard, 1993). Phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor (SBTI) were used as inhibitors of proteinases belonging to the serine class. Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK) were used as the specific inhibitors of trypsin and chymotrypsin, respectively. Activity in inhibition assays was reported as a percentage of inhibition and activity measured in the absence of the inhibitor was considered 100%.

Proteinase composition and molecular weight were studied after sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE), as per Laemmli (1970).

All reagents and standards were of analytical reagent grade.

Statistical analysis. Results were analyzed by ANOVA and Student's *t* test to find differences among means. Data are expressed as means±SD. Arc sine transformation to percentages

was applied. Pearson's rank correlation coefficient was used to identify significant correlations between soluble protein and specific enzyme activity. In all cases, significance was set as $p<0.05$ (Sokal and Rohlf, 1995).

Results

The highest proteinase activity in the midgut gland extracts was observed in wild prawns and prawns fed diet 2 (Table 2). Soluble protein and specific enzymatic activity were not significantly correlated with each other (Table 3). Comparison of the percent inhibition using different inhibitors in test tube assays provided information about the class of proteinase enzymes (Table 4). Electrophoresis revealed at least six different bands with zones having caseinolytic activity in prawns fed the diets as compared to wild prawns (Table 5).

Discussion

Prawns fed diets 1 and 3 exhibited similar patterns of low enzymatic activity. Chymotrypsin activity in *Penaeus vannamei* is influenced by the protein source (Le Moullac et al., 1996; Rivas-Vega et al., 2006). In mammals, there is a positive correlation between trypsin and chymotrypsin

Table 2. Soluble protein and specific enzymatic activity (absorbance/min/mg) in the midgut gland of *Artemesia longinaris* fed different formulated feeds.

		Specific enzymatic activity (abs/min/mg)		
Treatment	*Soluble protein (mg/ml)*	*Proteinase*	*Trypsin*	*Chymotrypsin*
Diet 1 (n = 21)	6.8±1.42	0.3±0.06[a]	2.1±0.22[b]	1.3±0.15[a]
Diet 2 (n = 18)	5.0±0.85	0.4±0.04[b]	2.6±0.73[b]	2.9±0.18[b]
Diet 3 (n = 20)	5.2±0.75	0.3±0.04[a]	1.9±0.27[b]	1.6±0.43[a]
Wild (n = 20)	6.0±0.01	0.4±0.05[b]	0.6±0.60[a]	1.5±0.12[a]

Values are means of triplicate assays±SD. Different superscripts in the same column indicate statistical difference ($p<0.05$).

Table 3. Correlation matrix of soluble protein and specific enzyme activity coefficients in midgut glands of the prawn, *Artemesia longinaris*.

	Total activity		*Trypsin*		*Chymotrypsin*	
	r	*p*	*r*	*p*	*r*	*p*
Protein	-0.782	0.218	-0.144	0.856	-0.829	0.171
Total activity	1	-	-0.492	0.508	0.332	0.668
Trypsin			1	-	0.567	0.433
Chymotrypsin					1	-

Table 4. Effect of specific inhibitors on proteinase activity of *Artemesia longinaris* midgut glands using azocasein as the substrate.

Treatment	*Soybean trypsin inhibitor*	*Percent inhibition*		
		Phenylmethylsulfonyl fluoride	*Nα-p-tosyl-L-lysine chloromethyl ketone*	*N-tosyl-L-phenyl-alanine chloromethyl ketone*
Diet 1 (n = 21)	49.8±1.94[a]	ND	59.3±4.12[a]	ND
Diet 2 (n = 18)	41.6±1.66[b]	ND	36.8±3.11[b]	ND
Diet 3 (n = 20)	76.9±2.37[c]	14.3±7.39[a]	81.7±11.66[a]	ND
Wild (n = 20)	28.6±3.62[d]	25.5±4.93[a]	75.9±10.89[a]	ND

ND = no inhibition detected
Values are means of triplicate assays±SD. Different superscripts in the same column indicate statistical difference (p<0.05).

Table 5. Protein fractions of proteinase activity bands in SDS-PAGE of midgut gland extracts of *Artemesia longinaris*.

Molecular weight (kDa)	*Activity*	*Inhibition by*	*Enzyme*
53.7	+	S, P	Chymotrypsin
34.7	+	S	Serine proteinase
25.1	+	S, T	Trypsin
21.9	+	S, T	Trypsin
18.2	+	S, T	Trypsin
16.6	+	S, T	Trypsin

S = soybean trypsin inhibitor; P = phenylmethylsulfonyl fluoride; T = Nα-p-tosyl-L-lysine chloromethyl ketone

activities, due to the activation of chymotrypsinogens for the trypsin. In crustaceans, this pattern is apparently different, since there is no correlation between the activities of these enzymes (Rivas-Vega et al., 2006). This coincides with our results.

Diet 2 induced the highest proteinase, trypsin, and chymotrypsin activities, indicating an ability of the prawn to compensate for low quality dietary protein. Digestive activity can increase in crustaceans given feed with low nutritional quality (Le Vay et al., 2001).

Soybean meal can replace up to 44% of the fishmeal in diets for *A. longinaris* with no adverse effect on growth or survival (Medina Marti et al., 2005). Shrimp fed diets containing ≥25% meat and bone meal as a replacement for fishmeal had a lower growth rate (Diaz and Fenucci, 2002;

Tan et al., 2005). Squid meal improved larval growth in *P. vannamei* (Le Moullac et al., 1994) and enhanced trypsin activity in adults (Le Moullac et al., 1996). Growth improved in *P. vannamei* fed squid protein at various levels (Cruz-Rique et al., 1987). In diets for the red shrimp, *Pleoticus muelleri*, inclusion of at least 2.5% squid protein improved growth and survival (Diaz et al., 1999).

Thus, it seems that *A. longinaris* can modulate enzymatic secretions according to the type of ingested protein and that the nutritional imbalance caused by the use of soybean meal in feeds for prawns can be compensated by the inclusion of squid protein concentrate. Proteolytic enzymatic activity in the midgut gland of *A. longinaris* can be influenced by dietary protein quality and the inclusion of fishmeal in commercial feeds for prawns can be minimized without affecting production.

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