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Testing feeds and feed ingredients for juvenile pink shrimp *Farfantepenaeus paulensis*: in vitro determination of protein digestibility and proteinase inhibition

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Abstract

Protein is the major component for shrimp feeds and must be nutritionally appropriate and readily digestible. In vitro protein digestibility of feeds and feed ingredients, and their inhibitory effect on the digestive proteases of juvenile *Farfantepenaeus paulensis* were examined. The analysis included animal (four types of fish meal, meat meal and blood meal) and plant (soybean meal and wheat flour) ingredients. Commercial shrimp feed from different sources were also assessed. The degree of hydrolysis of feedstuff with shrimp enzymes was determined through the pH-stat routine as well as monitored in SDS-PAGE over time. Enzyme inhibition of shrimp enzymes was verified by spectrophotometric and substrate-SDS-PAGE assays. Brazilian fish meal, meat meal and wheat flour presented higher digestibility either by in vitro degree of hydrolysis as in SDS-PAGE. Intermediate digestibility was found for Super Prime Chilean and Argentinean fish meals. Least digestible ingredients were soybean meal and blood meal that exhibited high inhibitory effects on shrimp proteinases. Commercial shrimp feeds also differed in terms of digestibility and inhibitory effects. The routine may be employed for quality control of feedstuffs for shrimp culture, being potentially applicable to different segments of the shrimp culture industry such as feed suppliers, feed manufacturers and farmers.

Keywords: Shrimp; Digestibility; Protease; Feed; Ingredient

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1. Introduction

Shrimp comprises about 80% of total global crustacean aquaculture production; corresponding to 1.27 million metric tons in 2001 (FAO, 2003). Under current culture practices, at least 2 million metric tons of feed would be required to sustain this yield, representing up to 60% of total variable costs of shrimp culture (Sarac et al., 1993). Protein is the major component in shrimp feeds and a growth-limiting nutrient further than denote the most expensive ingredient in aqua feeds. Besides the price, availability of high quality protein ingredients is also a problem for feed producers. Shrimp feeds still rely on fish meal as a key protein source despite its recognized problems of price and availability (Forster et al., 2003). The search for alternative protein sources of high nutritional quality has been focus of several segments involved in aquaculture (Tacon and Barg, 1998). On the other hand, increasing ingredient and feed manufacturing costs concurrent with stationary and sometimes decreasing shrimp market price, demands reduction in production costs to maintain profitability (Tacon and Akiyama, 1997). Thus, feed protein must then fulfill the nutritional requirements of the farmed species (e.g. amino acid composition) but also be readily digestible, under a cost-effective perspective.

In addition to fish meal, shrimp feeds usually contain standard ingredients such as soybean meal and wheat flour (Lan and Pan, 1993). Though alternative protein ingredients have also been considered for feed formulation (Sudaryono et al., 1999; Mendoza et al., 2001; Forster et al., 2003), protein digestibility of feedstuffs mostly depend on the type and quality of the raw material and processing strategy (Pike and Hardy, 1997; Rique-Marie et al., 1998; Francis et al., 2001), that may oscillate considerably in regional terms (Garcia-Carreño et al., 1997; Swick, 2002). Therefore, shifts in ingredient properties may be reflected in a variable quality of commercial shrimp feeds (Cuzon et al., 1994; Talavera, 2003; Coutteau, 2004).

The present study aims to evaluate digestibility of feeds and feed ingredients for the pink shrimp *Farfantepenaeus paulensis*. The species constitute one of the major fishery resources of Southern Brazilian coast and has been cultured mainly during the 1980s and 1990s. Exhibiting higher carnivorous habits compared to currently reared *Litopenaeus vannamei* (Brazilian production in 2003: ca. 90,000 t), culture of *F. paulensis* was drastically decreased due to the lack of adequate feeds to sustain profitable growthout in ponds. Recently, this species has been considered a potential alternative for sub-tropical and temperate areas. Protein digestibility of feeds and ingredients was assessed in vitro by pH-stat reaction and digestion of protein fractions in SDS-PAGE gels, by using hepatopancreas enzymes from *F. paulensis*. Additionally, inhibitory effects of feedstuff was verified by analysis in substrate-SDS-PAGE and spectrophotometric assays.

2. Material and methods

2.1. Experimental animals, feeds and ingredients

Juvenile *F. paulensis* were raised from postlarva IX–XI (molts) or PL 26 (days after metamorphosis) in 12,000 mt circular tanks including sand bottom, salinity 34 ± 0.5‰.

Shrimp were fed commercial feed containing 40% protein from 0.01 to 2.0 g shrimp wet weight and 35% protein after 2.0 g shrimp wet weight. After 236 days culture period, hepatopancreas (digestive gland) of 7.9 ± 3.0 g wet weight shrimp were excised and immediately stored in liquid nitrogen (-180 °C) for enzyme assays. Animals were kept unfed for 45 min before hepatopancreas extraction.

Commercial shrimp feeds and feed ingredients were tested in vitro for protein digestibility and proteinase inhibition. Feed ingredients were obtained from a local feed manufacturer and included animal (fish meal, drum-dried blood meal, meat meal) and plant (defatted soybean meal, wheat flour) protein sources. Different quality fish meals were tested: Super Prime Chilean, Brazilian, Argentinean and Mexican Standard. Casein (Sigma) was employed as positive control since it is digested by penaeid proteinases (Jiang et al., 1991; Le Moullac et al., 1996; Lemos et al., 1999). Ingredients were analyzed for nitrogen (A.O.A.C., 1984) and non-protein nitrogen (Stone et al., 1989) contents. Total protein percentage in ingredients was obtained by multiplying $N \times 6.25$. Commercial shrimp feeds from Brazilian and Mexican aquafeed manufacturers were obtained from local suppliers. Moisture (gravimetrically), nitrogen (A.O.A.C., 1984) and energy (adiabatic calorimetry) contents were determined in feeds.

2.2. Enzyme preparation

Approximately 1.2 g of shrimp hepatopancreas, corresponding to six midgut glands, were homogenized in 530 μl chilled 10 mM Tris–HCl buffer at pH 7.5 and enzyme extracts were obtained after centrifugation at $10{,}000 \times g$ for 30 min at 4 °C. After lipid elimination, total soluble protein was determined in the supernatants (Bradford, 1976). Total proteinase activity in extracts was measured by the rate of hydrolysis of 1% azocasein in 50 mM Tris buffer, pH 7.5 (Garcia-Carreno, 1992). Triplicate 10 μl extracts were mixed with 0.5 ml substrate solution at 25 °C. The reaction was stopped 10 min later by the addition of 0.5 ml 20% trichloroacetic acid (TCA) and the mixture was centrifuged for 5 min at $6500 \times g$. The supernatants were separated from the undigested substrate, and the absorbance for the released dye was recorded at 440 nm. The rate of absorbance change over time was calculated by the difference from reactions stopped at zero (negative controls) and 10 min. Total proteinase activity was expressed as units of change in absorbance per min per mg protein of the enzyme extract (ΔAbs min^{-1} mg^{-1} protein).

2.3. Protein digestion in SDS-PAGE

The digestion of feeds and feed ingredients by *F. paulensis* proteinases was detected over time in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The water-soluble protein fraction of feeds and feed ingredients was assayed with hepatopancreas extract and the product of digestion was observed in SDS-PAGE over time. Feeds and ingredients were ground and dissolved in 1:3 (w/v) solution of 0.1 N NaOH for 6 h at room temperature (23–25 °C) following pH setting to 8.0. Protein extracts were placed in ice-cold bath prior to analysis. Two-hundred microliters protein extracts were assayed with 230 μl enzyme extract that corresponded to four activity units (ΔAbs min^{-1} mg^{-1} protein) (see Section 2.2) at 25 °C, following hourly sampling

between 0 and 6 h. Samples of the protein plus enzyme reaction were immediately ice stored (4 °C) prior to loading into SDS-PAGE. The electrophoresis was carried out using 12% acrylamide (Laemmli, 1970) and 0.1% SDS. Samples were loaded in a temperature-controlled (4 °C) vertical electrophoresis device (Hoefer, San Francisco, CA). One gel was used to each feed or ingredient and included molecular mass standards. After electrophoresis, gels were fixed and stained by immersion in a solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250. After staining for at least 24 h, gels were de-stained with same solution without Coomassie dye and scanned. Protein digestion could be observed by the disappearance of protein bands stained by Coomassie blue over time. Since Coomassie is unable to stain peptides of less than 3 kDa (Bradford, 1976), the absence of certain protein bands in SDS-PAGE was indication of protein polypeptide shortening over time.

2.4. Detection of proteinase inhibition

Inhibitory effects of ingredients and feeds on digestive proteinase activities of *F. paulensis* were quantified and characterized. Feeds and ingredients were ground and dissolved in a 1:2 (w/v) solution with distilled water. After protein determination (Bradford, 1976), 20 μl of the supernatant was assayed with 10 μl of hepatopancreas extract, corresponding to 0.0146 mg protein of each reaction component. The mixture was assayed with 0.5% azocasein in 50 mM Tris–HCl pH 8.0 as substrate. Distilled water was used instead of ingredient/feed extracts as control. Commercial proteinase inhibitors were also assayed with shrimp enzymes: 10 mM TLCK (trypsin inhibitor) in 1 mM HCl pH 3.0, 100 mM PMSF (serine proteinase inhibitor) in isopropanol, 250 μM SBTI (soybean trypsin inhibitor) in distilled water. Six replicates were analyzed per treatment that included blanks without enzyme extract. Total proteinase activity was quantified following the procedures described in Section 2.2.

Inhibition of shrimp enzymes by ingredient/feed/inhibitor solutions were also detected and characterized in substrate-SDS-PAGE (Garcia-Carreño et al., 1993). Commercial inhibitors were assayed with hepatopancreas extract in order to identify trypsin, chymotrypsin and serine proteinases corresponding to active bands in SDS-PAGE (Garcia-Carreño and Haard, 1993). The mixture of each ingredient/feed/inhibitor solutions with hepatopancreas extract was loaded into the electrophoresis device (4 °C) according to Section 2.3. After electrophoresis for protein separation, gels were immersed in 3% casein in 50 mM Tris at pH 7.5, for 30 min at 5 °C, allowing the substrate to diffuse into the gel at low enzyme activity. The temperature was then raised to 25 °C for 90 min for the digestion of the protein substrate by the active fractions. Gels were next washed in water, and immediately fixed and stained by immersion in a solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250. After staining at least 24 h, gels were de-stained with the same solution without Coomassie dye. Clear zones on the blue background indicated proteinase activities and could be compared to molecular mass standard bands. The 0.1% concentration of SDS does not affect proteinase activity in PAGE (Garcia-Carreño et al., 1993). At this SDS concentration, proteins are negatively charged, they have the same charge per unit of length (Walker, 2000) and therefore the mobility is due to the weight of the protein. Gels for inhibition detection and protein

digestion over time (Section 2.3) were scanned and digitally analyzed by Kodak Digital Science 1D analysis software v. 3.0 to determine band weights and the degree of inhibition of ingredient/feeds/inhibitors tracks in relation to control.

2.5. In vitro protein digestibility

Digestibility of the protein fraction of feeds and feed ingredients was evaluated by the degree of protein hydrolysis using *F. paulensis* hepatopancreas enzymes (Dimes and Haard, 1994; Ezquerra et al., 1997). Ground feeds or ingredients (0.08 g protein per assay) were stirred with distilled water in the hydrolysis vessel for approximately 1 h to dissolve the soluble protein fraction. The pH of the mixture was continuously adjusted to 7.9 with 1 N NaOH to facilitate complete solubilization of protein. Protein hydrolysis by shrimp enzymes was determined by the pH-stat titration (Pedersen and Eggum, 1983) using a 718 Stat Titrino (Metrohm, Switzerland) with the adequate software for data logging (Metrodata Menu Program 718 STAT TitrinoPC). Prior to starting the reaction, the pH was automatically adjusted to 8.0. The hydrolysis was started by the addition of 4.0 units of enzyme activity (see Section 2.2) of the hepatopancreas extract, pH 8.0. Nitrogen was bubbled to avoid air contact during hydrolysis. Assays were carried in triplicate and temperature was maintained at 26 ± 0.3 °C using a jacket reaction vessel and a circulated bath. After 1 h reaction, the degree of protein hydrolysis (DH%) was calculated as (Córdova-Murueta and Garcia-Carreño, 2002):

$$\mathrm{DH\%} = \left(B \times N_B \times 1.4 \times \left[\frac{(S\%/100)}{8} \right] \right) \times 100,$$

where B = ml of standard alkali (0.1 N NaOH) consumed to maintain the reaction mixture at pH 8.0; N_B = normality of the titrant; $S\%$ = protein content in the reaction mixture expressed as %.

For comparison purposes, the degree of hydrolysis of feeds and ingredients was measured using a mixture of commercial enzymes (Satterlee et al., 1979): 1.6 mg/ml trypsin type IX from porcine pancreas (Sigma T-0134); 3.1 mg/ml chymotrypsin from bovine pancreas (Sigma C-4129); 1.3 aminopeptidase from porcine intestinal mucosa (Sigma P-7500); 7.95 mg/ml pronase type XIV from *Streptomices griscus* (Sigma P-5147). All enzymes were dissolved in distilled water and adjusted to pH 8.0. Activity of commercial enzymes mixture was determined with 1% azocasein (Section 2.2) and a volume corresponding to 4.0 activity units was employed to determine the degree of hydrolysis.

2.6. Statistical analysis

The performance of feeds and ingredients was compared by one-way ANOVA followed by Tukey's multicomparison test after checking for normality and equal variance of data. The degree of hydrolysis between shrimp enzymes and commercial enzymes was compared by t-test considering the same requisites. Differences were considered significant at $P < 0.05$ (Zar, 1984).

3. Results

Ingredients of different composition were tested. Protein contents varied from 13.1 to over 90% in wheat flour and blood meal, respectively (Table 1). Fish meal presented 63.3–69% CP and intermediate values occurred for meat (43.9% CP) and soybean (46.8% CP) meals. The highest quantity of non-protein nitrogen was verified for Brazilian fish meal (2.9%) followed by Super Prime Chilean (1.8%) and Argentinean fish meal (1.2%). Less than 1% non-protein nitrogen was found in Mexican fish meal (0.7%), wheat flour (0.4%) and soybean meal (0.3%). Blood meal showed the lowest content of 0.1%. Commercial feeds also showed different protein content (Table 2). The highest value was registered in Mexican SC (46.6%) followed by Brazilian (38.3%) and Mexican PI (36.8%) (Table 2). In contrast, gross energy content was higher in Mexican PI than Mexican SC and Brazilian revealing a higher lipid content. Moisture was about 6% in Mexican feeds SC and PI reaching a higher value in Brazilian feed.

The digestion of the protein fraction of ingredients and feeds by shrimp enzymes could be detected over time in SDS-PAGE. Meat meal, Brazilian and Argentinean fish meals showed almost complete digestion after 2 h (Fig. 1). Protein bands were not distinguishable in times zero and 1 h in Brazilian, Super Prime Chilean, Argentinean fish meals and meat meal. Mexican fish meal produced bands lower than 14.4 kDa that were present after 1 h digestion. Blood meal generated two intense bands of 15.3 and 28.7 kDa at time zero (Fig. 1). The 28.7 kDa disappeared after 2 h while the 15.3 kDa band persisted during the whole experimental period (6 h), reducing its intensity after 2 h. Plant ingredients (soybean meal and wheat flour) showed bands that were not digested over the 6 h experimental period (Fig. 2). Soybean meal showed a higher number of protein bands not digested by shrimp proteinases compared to other ingredients and feeds. Bands exhibited a dynamic pattern over time, with the vanishing of some and the appearance of new bands. Twelve bands heavier than 20.1 kDa were detected at 0 and 1 h. After 2 h reaction, the intensity of bands of 94.3, 88.7 and 78.7 kDa was decreased, and new bands of 92.7, 85.3, 50.5 and 45.2 kDa appeared. Such bands continued present in the zymogram until 5 h reaction. At 6 h, bands remained at 115.3, 108.0, 65.8, 58.1, 32.6, 29.7, 27.5, 24.5 and 19.4 kDa. The digestion of wheat flour did not result in band separation by SDS-PAGE. At zero and 1 h, most proteins were retained above 97 kDa and some bands were distinguishable at 55.8,

Table 1
Nitrogen, non-protein nitrogen and protein content in feed ingredients tested for *F. paulensis*

Feed ingredient	Total nitrogen (%)	Non-protein N (%)	Protein ($N \times 6.25$)
Super Prime Chilean fish meal	11.0 (0.12)	1.8	69.0 (0.75)
Brazilian fish meal	10.3 (0.02)	2.9	64.2 (0.13)
Argentinean fish meal	10.1 (0.08)	1.2	63.3 (0.53)
Mexican Standard fish meal	10.5 (0.03)	0.7	65.5 (0.18)
Blood meal	14.6 (0.03)	0.1	91.1 (0.18)
Meat meal	7.0 (0.25)	1.3	43.9 (2.92)
Soybean meal	7.5 (0.01)	0.3	46.8 (0.06)
Wheat flour	2.1 (0.01)	0.4	13.1 (0.06)
Casein (reference)	14.4	–	90.0

Results expressed as mean (S.D.).

Table 2
Moisture, total nitrogen, protein and gross energy content in shrimp feeds tested for *F. paulensis*

Feed	Moisture	Total N (%)	Protein (%)	Energy (kJ g^{-1})
Brazilian feed	9.2 (0.29)	6.1 (0.47)	38.3 (2.92)	17.03 (1.21)
Mexican feed SC	6.5 (0.06)	7.5 (0.02)	46.6 (0.1)	18.8 (0.04)
Mexican feed PI	6.9 (0.06)	5.9 (0.03)	36.8 (0.2)	19.4 (0.05)

Results expressed as mean (S.D.).

50.3, 31.7, 26.4, 16.3, and 14.3 kDa (Fig. 2). After 2 h protein was accumulated in the range between 14.4 and 20.1 kDa, persisting over 6 h. Commercial feeds also exhibited undigestible proteins for 6 h. Mexican feed PI displayed bands greater than 30.0 kDa at zero and 1 h. After 2 h, pronounced bands of 122.7 kDa (top of the gel), 40.0 and 35.3 kDa were observed until the end of the experiment. A higher range of band weights (16.1, 18.6, 22.1, 39.7, 46.0, 67.7, 87.0 and 131.3 kDa) was detected in Mexican feed SC at zero and 1 h. After 2 h, most bands (16.1, 18.6, 39.7, 46.0, 67.7 and 87.0 kDa) were still present. Gel image analysis indicated a reduction of more than 80% in the sum of band intensities after 2 h digestion in Brazilian and Argentinean fish meals, meat meal and wheat flour.

Most ingredients and feeds presented inhibitory effect on *F. paulensis* proteinase activity (Fig. 3). Specific inhibitors SBTI (trypsin), PMSF (serine proteinase) and TLCK (trypsin) showed a high degree of inhibition, 68%, 52%, 34% in relation to the control, respectively. Soybean meal exhibited the highest proteinase inhibition among feeds and ingredients with 38% inhibition. Intermediate inhibition was verified in Brazilian feed (20%), Brazilian fish meal (20%), Mexican feed PI (18%) and wheat flour (17%). Super Prime Chilean fish meal (9.7%), meat meal (9.3%), Argentinean fish meal (9.3%) and Mexican feed SC (7%) presented lower inhibitory effect. No significant inhibition was detected for Mexican standard fish meal and blood meal in relation to control ($P < 0.05$).

Zymograms of substrate-SDS-PAGE also revealed inhibitory effects of ingredients and feeds on shrimp proteinase activity (Fig. 4). *F. paulensis* displayed 8 active bands between 15.9 and 45.2 kDa as seen in the control track. Several of these bands of different molecular weight were completely or partially inhibited. A quantitative analysis of the

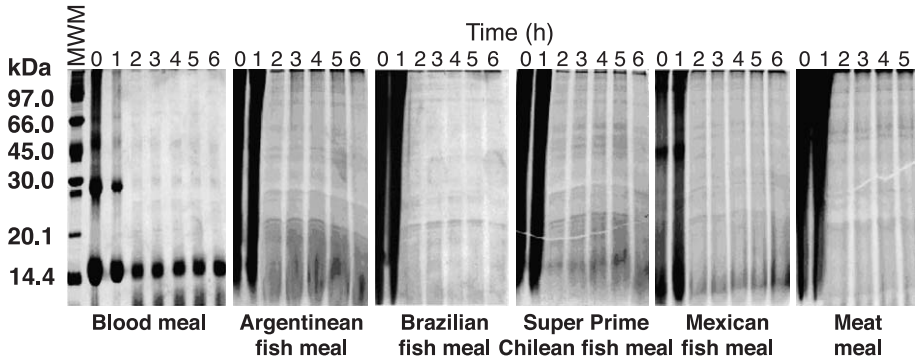

Fig. 1. SDS-PAGE zymograms of different types of animal protein incubated with *F. paulensis* digestive enzymes over time (more details in Material and methods). MWM = molecular weight markers.

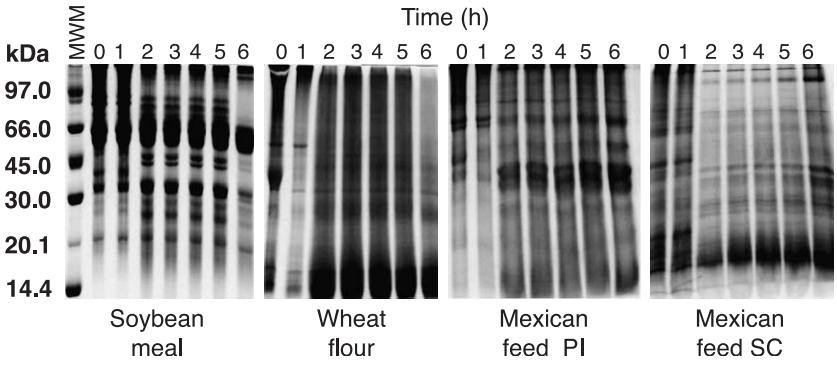

Fig. 2. SDS-PAGE zymograms of different types of plant and shrimp feed protein incubated with *F. paulensis* digestive enzymes over time (more details in Material and methods). MWM = molecular weight markers.

degree of band inhibition in zymograms is presented in Table 3. Trypsin inhibitor TLCK affected low molecular weight bands of 15.9, 18.1, 20.1, 22.6 kDa with 100%, 63%, 36% and 12% inhibition in relation to the control, respectively. On the other hand, PMSF

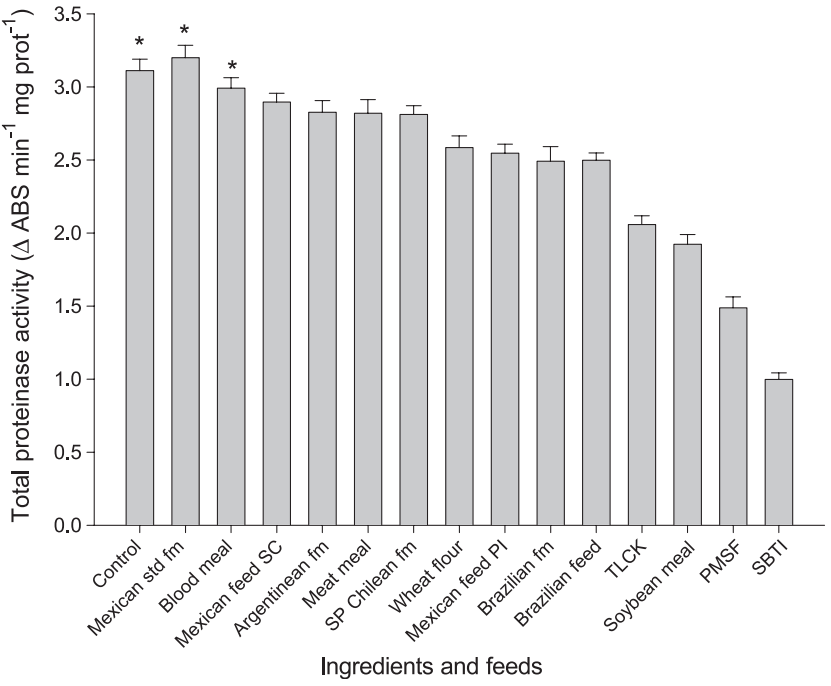

Fig. 3. Residual proteinase activity of *F. paulensis* digestive enzymes assayed with different ingredients, shrimp feeds and specific inhibitors. Abbreviations: std = standard, fm = fish meal, SP = Super Prime, TLCK = trypsin inhibitor, PMSF = serine proteinase inhibitor, SBTI = soybean trypsin inhibitor. Bars exhibiting asterisks are not significantly different (P>0.05).

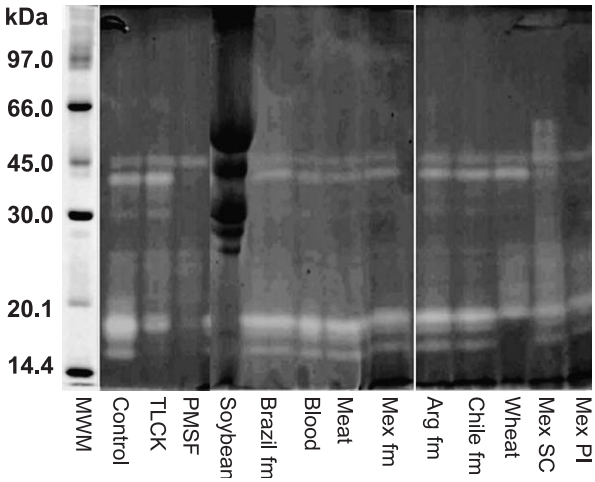

Fig. 4. Substrate-SDS-PAGE showing proteinase activity bands assayed with different ingredients, shrimp feeds and specific inhibitors. Abbreviations as in Fig. 3. MWM = molecular weight markers.

(serine proteinase inhibitor) responded on bands between 15.9 and 35.3 kDa at 4–100% inhibition. Soybean meal produced the highest inhibition among ingredients and feeds bearing uniform 100% inhibition for all bands. Moreover, dense protein bands with no activity were detected between 20.1 and 66.0 kDa (Fig. 4). In terms of number of bands inhibited, blood and meat meals also affected the eight bands detected but in a different degree compared to soybean meal (Table 3). Brazilian fish meal and Mexican feed SC inhibited seven bands while Mexican, Argentinean and Super Prime Chilean fish meals and Mexican feed PI had effect on six bands. Wheat flour inhibited only three bands. The pattern of band inhibition varied among ingredients and was classified as inhibition on low (15.9 and 18.1 kDa), intermediate (20.1–29.3 kDa) and high (35.3 and 45.2 kDa)

Table 3
Quantitative analysis of the degree of inhibition (%) of synthetic inhibitors and feedstuff on *F. paulensis* proteinases detected in substrate-SDS-PAGE (Fig. 3)

Band	MW (kDa)	Control	TLCK	PMSF	Soy	Brz fm	Blood	Meat	Mex fm	Arg fm	Chi fm	Wheat	Mex SC	Mex PI
1	45.2	0	0	0	100	0	41	29	2	0	0	0	0	0
2	35.3	0	0	100	100	42	57	62	37	18	5	0	81	84
3	29.3	0	0	100	100	64	93	98	63	0	0	8	100	42
4	22.6	0	12	21	100	100	61	100	0	30	18	0	33	100
5	21.9	0	0	4	100	100	100	96	100	64	58	0	28	0
6	20.1	0	36	100	100	100	29	100	0	100	100	0	100	100
7	18.1	0	63	97	100	52	48	61	25	44	54	74	85	68
8	15.9	0	100	100	100	49	53	49	53	37	49	100	70	71

Soy = soybean meal; Brz fm = Brazilian fish meal; Blood = blood meal; Meat = meat meal; Mex fm = Mexican standard fish meal; Arg fm = Argentinean fish meal; Chi fm = Super Prime Chilean fish meal; Wheat = wheat flour; Mex SC and Mex PI = Mexican feeds.

molecular weight bands (Table 3). Lower weight bands were mainly inhibited by soybean, wheat, Mexican SC and Mexican PI. Effects on intermediate weight bands were verified in soybean, blood, meat and Brazilian fish meal. Heavier proteinase bands were affected by soybean, blood, Mexican Feeds SC and PI.

Digestibility measured by the degree of hydrolysis (DH) using both shrimp and commercial enzymes varied among feeds and feed ingredients (Fig. 5). Casein resulted in the highest DH with both shrimp and commercial enzymes. For the remaining feeds and ingredients different DH values were found depending on the enzyme source used. With shrimp (*F. paulensis*) enzymes, Brazilian fish meal, meat meal and wheat flour were not significantly different ($P>0.05$) and presented higher DH (4.2–4.8%) among feeds and ingredients. Next, Brazilian feed, Mexican feed PI and Super Prime Chilean fish meal were significantly lower ($P<0.05$) registering DH values above 3.0% (Fig. 5). A third homogeneous group ($P>0.05$) with 1.9–2.5% DH was composed by Argentinean fish meal, soybean meal and Mexican feed SC. Mexican standard fish meal did not differ from soybean meal and Mexican feed SC showing 1.6% DH. The lowest significant DH ($P<0.05$) was recorded for blood meal (0.7%).

Assays using the mixture of commercial enzymes resulted in different results compared to shrimp enzymes. Ten of the twelve substrates tested showed significant differences

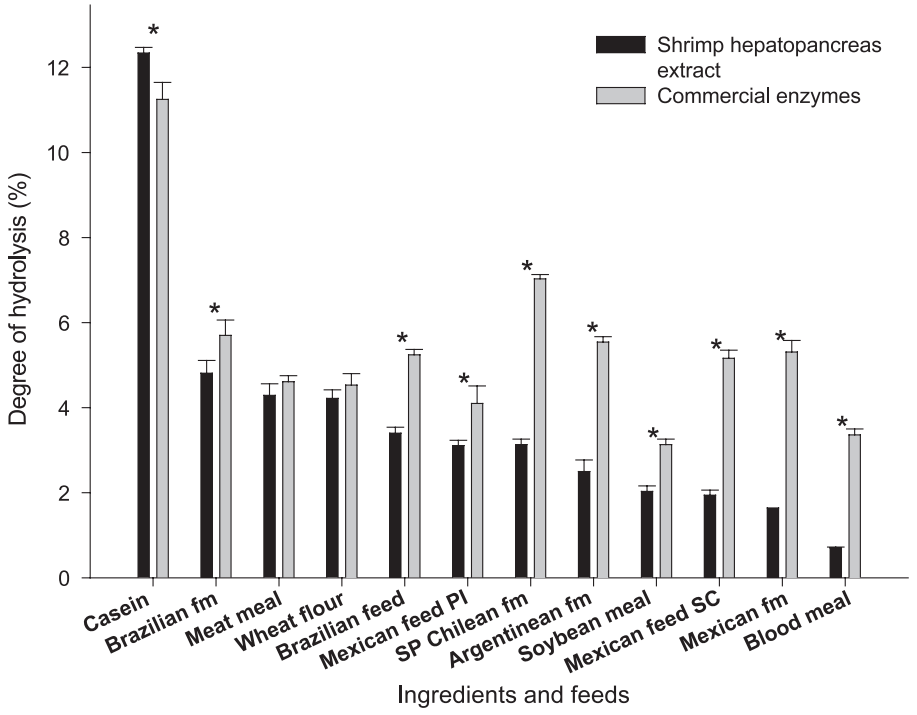

Fig. 5. In vitro digestibility measured by the degree of hydrolysis (%) of different ingredients and shrimp feeds with *F. paulensis* enzymes and commercial enzymes. Abbreviations as in Fig. 3 (more details in Material and methods). Bars in a same ingredient or feed showing asterisks are significantly different ($P<0.05$).

($P < 0.05$) in DH depending on the enzyme source. Higher DH values were observed using commercial enzymes in blood meal (4.8 times), Mexican standard fish meal (3.2 times), Mexican feed SC (2.7 times), Argentinean and Super Prime Chilean fish meal (2.2 times) (Fig. 5). As an exception, casein exhibited lower DH with commercial enzymes than with shrimp enzymes. Contrasting to shrimp enzyme assays, higher DH with commercial enzymes were observed in fish meals Super Prime Chilean, Brazilian, Argentinean and Mexican standard compared to the remaining ingredients and feeds.

4. Discussion

Results of SDS-PAGE, degree of hydrolysis (DH%), and inhibition indicated significant variation in the digestibility among feeds and ingredients, and a pronounced inhibitory effect of some of them on shrimp enzymes. Studies on inhibition of feedstuff on shrimp proteases are still scarce in the literature (Garcia-Carreño et al., 1997) though its importance for the selection of adequate ingredients for feed formulation. Furthermore, more than 30% of shrimp feed protein is derived from plant seed meal that may contain protease inhibitors (Francis et al., 2001). The inhibition by TLCK and PMSF was used to characterize proteinase bands in SDS-PAGE. Active bands inhibited with both TLCK and PMSF were considered trypsin, while the inhibition by only PMSF was evidence of chymotrypsin. Low molecular weight trypsins (15.9 and 18.1 kDa) and higher weight chymotrypsins (29.3 and 35.3 kDa) were detected confirming previous observations on adult *F. paulensis* (Lemos et al., 1999). Accordingly, trypsin bands were mainly inhibited by soybean meal, wheat flour and Mexican feeds (68–100% inhibition, Table 3). In contrast, chymotrypsin activity was affected by soybean meal, blood meal, meat meal and Mexican feeds at 81–100% inhibition. Since trypsin (and possibly chymotrypsin) is responsible for most shrimp protein digestion (Galgani et al., 1984, 1985; Tsai et al., 1986), present data should be considered when using these feedstuffs for *F. paulensis* formulated feeds, particularly soybean meal that exhibited high inhibition either in gel (Table 3, Fig. 4) and tube assays (Fig. 3).

Protein pattern over time in SDS-PAGE (Figs. 1 and 2) and in vitro digestibility (DH%) (Fig. 5) reflected the availability of feed and ingredient protein to be digested by *F. paulensis* digestive enzymes. Protein pattern in gel over time revealed an adequate analysis to determine digestibility coinciding with DH% values for most feedstuff tested. Higher DH% of Brazilian fish meal, meat meal and wheat flour corresponded to important reduction (>80%) in protein bands intensity after 2 h digestion of such ingredients. On the other hand, poorly digestible feedstuff (low DH%) as soybean meal, blood meal and Mexican feed SC displayed persistent protein bands in SDS-PAGE after 6 h digestion, a period longer than reported for shrimp digestion (Dall et al., 1990; Ceccaldi, 1997).

The pH-stat routine for determination of in vitro protein digestibility with enzymes from the target species has demonstrated adequate to check quality of shrimp feed ingredients as fish meal and soybean meal, as well as alternative plant sources (Ezquerra et al., 1997, 1998; Garcia-Carreño et al., 1997; Cruz-Suarez et al., 2000). The present in vitro digestibility results provided a ranking of protein quality for ingredients and feeds

based on the capacity of shrimp proteases to hydrolyze peptide bonds. Casein, a purified protein, was used as positive control and showed effectively digested by *F. paulensis* enzymes as observed for other penaeid species (Akiyama et al., 1989; Jiang et al., 1991; Le Moullac et al., 1996). The analysis of different type fish meals as potential ingredients for *F. paulensis* indicated Brazilian fish meal more digestible than the higher-valued Super Prime Chilean fish meal. The reason may be related to the freshness of raw material used in the production of fish meal (Pike and Hardy, 1997). While premium quality fish meals are obtained from fresh whole fish under adequate drying temperature and time of exposure (Romero et al., 1994), the raw material used for Brazilian fish meal is mostly by-product of industrial fish processing for human consumption. Accordingly, less fresh fish meal exhibited higher digestibility possibly due to hydrolysis during decomposition of the raw material (Cruz-Suarez et al., 2000). Among other animal sources tested, meat meal showed highly digestible (Fig. 5) being a promising ingredient for *F. paulensis* as previously verified for *L. vannamei* (Forster et al., 2003). Additional studies on other nutritional characteristics (e.g. essential amino acids) of locally available meat meal would enable complementary evidences for the use of meat meal in shrimp feeds. In spite of its highest protein content among ingredients, blood meal showed poorly digestible as denoted by the DH% and the presence of indigestible protein bands in SDS-PAGE (Fig. 1). It could be then considered the least adequate animal protein source for *F. paulensis* among the ingredients tested.

Though employed as a major ingredient in shrimp feed, soybean meal exhibited reduced digestibility and a high degree of protease inhibition for *F. paulensis*. The present analytical results (protein pattern in SDS-PAGE, DH%, inhibition in substrate-SDS-PAGE) can be considered as sufficient evidence of the presence of trypsin inhibitor (Synder and Kwon, 1987) impairing protein digestion by shrimp digestive enzymes. The occurrence of trypsin inhibitor can be attributed to insufficient heat during processing of soybean meal (Swick, 2002). Moreover, the digestibility is normally reduced in non-dehulled soybean meal by the presence of high cellulose levels, and seems to be case of soybean meal available in Brazil (Swick, 2002). Since ingredients tested were obtained from feed manufacturers, the poor digestibility verified for soybean meal should be reason to increase quality control of this feedstuff prior to use in shrimp feeds. Though the tolerance to trypsin inhibitors may vary depending on the cultured species (Francis et al., 2001), the potential quality variation of local soybean meal should be regarded as an inadequate feature in the development of feeds for *F. paulensis*. However, properly processed soybean meal has been found to be highly digestible in in vivo studies with other shrimp species and the present findings may be reflective of the source presently used. In contrast, wheat flour was confirmed as a fairly digestible ingredient (Lee and Lawrence, 1997) as to corroborate its wide use in shrimp feeds (Lan and Pan, 1993). The use of wheat flour in shrimp feeds may depend on adequate processing to avoid the occurrence of trypsin inhibitor potentially present in uncooked raw material (Tacon and Akiyama, 1997).

Differences in protein digestibility among feeds tested may accompany the observed variation in composition of commercial shrimp feeds (Cuzon et al., 1994; Talavera, 2003; Coutteau, 2004). The verified variation in feed digestibility as its inhibition potential may be attributed to shifts in ingredient type and quality used for feed

formulation. Recently, soluble protein content has been determined as complementary quality criterion (Coutteau, 2004) that may be related to its availability and digestibility of feed protein (Lan and Pan, 1993). Since the pH-stat reaction for mixed proteins can provide a fairly estimate of digestibility (Grabner and Hofer, 1985), these early data can be followed by regular analysis on the quality of different types of feeds (e.g. manufactured, on-farm-made).

An objective and reliable method to determine protein digestibility of feeds and ingredients is highly desirable for the development of shrimp diets (Lee and Lawrence, 1997). Single or multi-enzyme in vitro digestibility tests with enzymes from mammals or microorganisms have worked well for warm-blooded animals but may not be appropriate in simulating shrimp digestion for several reasons (Dimes and Haard, 1994). Most of the proteases employed in such assays are not present in shrimp digestive system, some of them acting on different pH from shrimp digestive gland (e.g. pepsin digestion test; A.O.A.C., 1984). Shrimp digestive enzymes may differ in catalytic properties from homologous enzymes of mammals as verified for fish (Dimes and Haard, 1994) as suggested by significant different digestibilities found with *F. paulensis* versus commercial enzymes (Fig. 5). Furthermore, protein digestion includes hydrolysis by many different enzymes, each having specific action on different parts of the polypeptide (Gauthier et al., 1982), and thus in vitro assays should comprise a complete pool of enzymes corresponding to those found in shrimp hepatopancreas (Dimes et al., 1994).

The present routine for determination of protein digestibility in feedstuffs is potentially applicable to different segments of the shrimp culture industry such as ingredient suppliers, feed manufacturers and farmers. Ingredient suppliers may include species-specific digestibility data in their product specifications sheets. Feed manufacturers may benefit of applying this routine due to its input to major processes that define feed production cost as feed formulation, quality control and industrial support (research and development). Farmers could also employ specific digestibility data to evaluate commercially available feeds as well as in developing on-farm made diets. Continued research is necessary to optimize and expand the routine for shrimp nutrition. For obvious reasons, data on digestibility of alternative vegetable and non-marine animal feedstuffs are highly expected and will require further confirmation by in vivo performance under cultivation. Testing protein digestibility of prepared feeds also shows promising for feed quality control. An expansion of method's predictive capacity is suggested by experimental relationships of in vitro protein digestibility data to growth, net growth efficiency, nitrogen efficiency and feed stability. Then, the next step would be applying research findings to pond field conditions.

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