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Nutritive value of squid and hydrolyzed protein supplement in shrimp feed

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Abstract

Though some protein sources like squid and protein hydrolysates are assumed as growth enhancers for shrimp, little is known about the biochemical basis of this phenomenon. Low, heat-dried squid (*Dosidicus gigas*) (SQ) and two commercial protein hydrolysates from fish (FH) and krill (*Euphasia* sp.) (KH) were assayed in feeding trials with *Penaeus vannamei*. Feeds were prepared with the tested proteins at 3%, 9%, and 15% of the total crude protein. A total of nine experimental feeds plus a commercial one as control (C32) were tried. Additionally, digestibility in vivo and in vitro was evaluated. Survival was not different among groups. Weight gain of shrimp and total and specific proteolytic activity for trypsin and chymotrypsin were affected by type and quantity of supplemented protein. In vivo and in vitro digestibilities were also influenced by the source and quantity of the protein supplement. Shrimp fed feed with FH at 3% protein supplementation grew more than those fed with higher supplementations. Groups fed SQ had similar results as those fed FH, and gained more weight when fed the lowest SQ quantity. SDS-PAGE showed a large concentration of small peptides in SQ, which may explain results similar to FH. KH enhanced shrimp growth at all supplementations and had a lower degree of hydrolysis (DH) than FH. SQ also demonstrated good growth performance, but better at the lower supplementation, probably because of the presence of small peptides and possibly free amino acids from protein hydrolyzed by endogenous enzymes in the squid mantle. We conclude that hydrolyzed protein is a good supplement for shrimp feeds, but it must meet specific requirements for adequate assimilation.

Keywords: Shrimp; Protein; Hydrolysate; Squid; pH-stat

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1. Introduction

One of the most expensive ingredients in feed production is protein. Different protein sources are used for commercial feed production to reduce costs. Manufactures may ignore whether some protein sources are appropriate in quantity and quality for adequate digestion and assimilation by the intended organism. An example is soybean protein, which is currently used to supplement feeds for cultured shrimp to reduce costs. However, it cannot be used as the sole source of protein because it is low in certain essential amino acids (Floreto et al., 2000) and there are antinutritional components present (Ezquerra et al., 1997a).

Discrepancies in the lowest protein concentration necessary to yield acceptable growth rates (usually called the optimum) of shrimp in previous studies are related to protein quality. In several studies, the recommended protein concentration in feed for penaeids ranges between 30% and 45% (Andrews and Sick, 1972; Balaz, 1973; New, 1976; Neal, 1980; Piedad-Pascual, 1990; Akiyama et al., 1991).

Some ingredients, such as protein hydrolysates, have been reported to possess healthy and nutraceutical properties and have been used as supplements to enhance food properties (Haard, 2001). Anggawati et al. (1990) used fish hydrolysates to supplement feeds for *Penaeus monodon*. They found that replacing 3% fish meal by fish hydrolysate was enough to enhance shrimp growth. However, they did not investigate the reasons supporting growth enhancement.

Investigations into fish larvae growth confirm that feeds with fish protein–hydrolysate supplement improve growth and digestive system development, being better at low concentration (Zambonino-Infante et al., 1997; Day et al., 1997; Cahu et al., 1999).

Squid meal is an alternative source of protein used as a growth-enhancement ingredient in shrimp feeds (Cruz and Guillaume, 1983). It is anticipated that the Mexican squid (*Dosidicus gigas*) posses a similar factor. This organism is an abundant resource in the Gulf of California, and has been recently incorporated into shrimp feeds. However, the product used in feed production is dried at a high temperature (direct flame) to convert it into meal., and the high temperature significantly reduces the nutritional value of the product (Córdova-Murueta and García-Carreño, 2001).

The objective of this research was to evaluate the effect of some alternative protein sources that have growth enhancement properties for shrimp, to gain knowledge of biochemical processes of digestion of protein, and to measure the effect of such protein sources on digestive enzyme activities of shrimp.

2. Materials and methods

2.1. Growth trials

P. vannamei were obtained from the rearing ponds at CIB near La Paz, B.C.S., Mexico. Biozimes (Vancouver, B.C., Canada) donated the freeze-dried krill-hydrolysate (*Euphasia* spp.), and Protein Recovery (Warrenton, OR, USA) provided the liquid fish protein hydrolysate (made mainly from Pacific whiting and bottom fish). Squid meal was prepared

from fresh giant squid (*D. gigas*), acquired from a seafood supplier in Guaymas, Sonora, Mexico. The mantle was cut into small pieces and dried in a low-temperature dryer with air circulation at 50 °C for 12 h.

A commercial shrimp feed Silver Cup™ (by El Pedregal., Mexico, under license of Sterling H. Nelson & Sons) labeled as 35% protein was used as the basal feed. The repelleted commercial feed, containing 2% gelatin as binder, was used as the control. The test feeds were prepared by replacing a protein-equivalent amount of the basal mixture with squid meal (SQ), fish hydrolysate (FH), or krill hydrolysate (KH) at 3%, 9%, and 15% on a total crude protein basis (Table 1). To pellet the feeds, 2% gelatin was added as a binder and the mixed material put through a meat grinder with a 2-mm die hole. The pellets were dried for 6 h at 50 °C. Proximate analysis of feeds and protein ingredients were done by official methods (A.O.A.C., 1990), energy was determined in an adiabatic calorimeter (TAR™, model 1261). Amino acid analysis was made on feeds and experimental protein sources by reverse-phase HPLC using an *O*-ophthaldialdheyde precolumn derivatization (Jones et al., 1981).

Growth trials were performed for 54 days in triplicate 70-l plastic aquaria provided with an air supply through air stones. Eight *P. vannamei* (2.5 g initial weight) were stocked in each aquarium. Filtered seawater (32 ‰) was used with a daily water exchange of 80%. Temperature was maintained at 28 °C ($\pm$ 0.5). The daily rations for each experimental unit were estimated by the relation, $Y = 11.74 - 6.79 \log 10X$, where Y is percentage of biomass and X is the individual average weight (Teichert-Coddington and Rodriguez, 1995). The rations were fed as two equal portions at 0900 and 1700 h daily. Weekly adjustments were made using the average weight and survival data from each experimental unit. Feed consumption was recorded daily to determine feed conversion ratio (FCR), which was calculated by dividing the total amount of feed consumed by total weight gain.

2.2. Apparent digestibility

The apparent digestibility coefficient (ADC) of protein in feeds was measured in vivo. The same feed formulas were prepared as above, except for the addition of 10 g of chromic oxide (Cr_2O_3) per kg of feed.

After the growth trial, shrimp were fed feeds containing chromic oxide. Feces were collected 2 days later and continued for 7 days. Feces were collected 2 h after feeding by

Table 1
Percentage of ingredients used to prepare the experimental feeds

Feed *	C32	F3	F9	F15	K3	K9	K15	SQ3	SQ9	SQ15
Gelatin	2	2	2	2	2	2	2	2	2	2
Base meal	98	91	75	59	91	75	59	91	75	59
Krill hydrolysate	0	0	0	0	5	15	25	0	0	0
Fish hydrolysate	0	4	12	20	0	0	0	0	0	0
Squid meal	0	0	0	0	0	0	0	5	14	23
Filler (corn starch)	0	3	11	19	2	8	14	2	9	16

* Names explained in Table 2.

siphoning from the tank bottom, using screening to eliminate feed particles from the feces. Feces were gently rinsed with distilled water to eliminate excess salts. Evaluation of chromic oxide in dried feeds and in lyophilized feces was performed by wet-acid digestion, as described by Olvera-N. et al. (1993). The ADC was estimated using the equation of Fenucci et al. (1980).

$$\text{ADC} = \%\ \text{of protein digestibility} = 100 - \left(100 \times \frac{I_a}{I_h} \times \frac{P_h}{P_a}\right)$$

where I_a = % Cr_2O_3 in feeds; I_h=% Cr_2O_3 in feces; P_a=% protein in food; P_h=% protein in feces.

2.3. Proteolytic activity

Shrimp were placed in iced seawater (0 °C) for anesthesia and dissection 1 day after feeding ceased. Midgut glands were extracted individually by homogenization in 530 μl of distilled water. Enzyme extracts were obtained by centrifugation for 30 min at 10,000 × *g* and 4 °C. The supernatant was decanted and stored at − 20 °C until used. Soluble protein was determined using bovine serum albumin as standard by the Bradford method (Bradford, 1976). Total proteolytic activity of extracts was evaluated using the methodology of García-Carreño and Haard (1993), with 1% azocasein as substrate in Tris–HCl 50 mM buffer (pH 7.5) at 25 °C. The amount of released peptides was evaluated spectrophotometrically at 366 nm in a Perkin Elmer spectrophotometer model Lambda Bio20. All assays were done in triplicate.

Trypsin (EC 3.4.21.4) activity was measured spectrophotometrically at 410 nm using 0.1 mM benzoyl-Arg-*p*-nitroanilide (BAPNA, Sigma B-4875), in 50 mM Tris–HCl, pH 7.5, 20 mM $CaCl_2$ as substrate at 37 °C. Quantifications were done following the progress of the reaction in a Perkin Elmer spectrophotometer (model Lambda Bio 20) set. The activity was obtained using the formula: (Abs_{410} ml of reaction volume)/(8800 mg protein) (García-Carreño et al., 1994). Chymotrypsin (EC. 3.4.21.2) activity was measured at 25 °C, using 0.1 mM succinyl-Ala_Ala-Pro-Phe-*p*-nitroanilide (SAPNA, Sigma S-7388) in 50 mM Tris–HCl, pH 7.5, 20 mM $CaCl_2$ at 37 °C, using the same method and formula used for trypsin activity. The activity is expressed in units of activity for shrimp hepatopancreas by multiplying the activity/ml by the volume (ml) of the hepatopancreas extract.

Statistical differences were evaluated using ANOVA and multiple comparison test LSD (Less Significant Differences, Statgraphics Plus, v. 6.1) when differences were found. Protein fractions from enzyme extracts were separated by 12% SDS-acrylamide gel electrophoresis (SDS PAGE), and zymograms for endopeptidase activity were performed, using 12% S-SDS polyacrylamide gel electrophoresis (S-SDS-PAGE) according to García-Carreño et al. (1993).

Enzymatic activity was determined in the squid meal., fish, and krill hydrolysates, and in enzyme extract by in vitro assays including S-SDS PAGE. The extracts of the krill hydrolysate and the squid meal were prepared by stirring 2.5 g of each ingredient with 25 ml of distilled water for 15 min at 25 °C and centrifuging at 10,000 × *g* for 5 min. Because

the fish hydrolysate was a liquid product, 25 ml was taken and centrifuged, as were the other two ingredients. When proteolytic activity was found, the samples were incubated 6 h at 50 °C to determine if the proteolytic activity was resistant to the treatment. Samples were taken every hour and analyzed in S-SDS PAGE.

2.4. In vitro digestibility

The degree of protein hydrolysis of the experimental feeds was evaluated by the pH-stat method, using enzyme extracts from shrimp hepatopancreas, as described in Ezquerra et al. (1997b). A 718 Stat Titrino (Metrohom Ion Analysis, Switzerland) with computer interface, using the Metrodata Menu Computer Program 718 STAT TitrinoPC was used. Feeds were ground using a mortar and pestle, and then the appropriate amount of feed was weighed to yield 0.08 g of protein. The feed was stirred with distilled water in the hydrolysis vessel, adding enough water to give 10 g of substrate mixture, including the amount of enzyme (substrate + water + enzyme = 10 g). The pH of this mixture was adjusted to 7.9, using a solution of 1 mol/l NaOH and stirred for one h to facilitate complete solubilization of protein and stabilization of the pH. Prior to starting the reaction, the pH was automatically raised to 8.0 by the pH-stat by adding 0.1 mol/l NaOH. To start the reaction, 2.5 units of enzyme (pH adjusted to 8.0) were added. All assays were done in triplicate. The reaction mixture was maintained at 28 °C, using a jacketed reaction vessel and a circulating water bath. The degree of hydrolysis (DH) was calculated using the formula (Navarrete del Toro, 1999):

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

where B is the amount in ml of solution 0.1 mol/l NaOH consumed to maintain the reaction mixture at pH 8.0; N_B is the normality of the NaOH solution; S% is the amount of protein expressed as % in the reaction mixture. The degree of hydrolysis of casein, using shrimp enzymes was performed with the same procedure as for feed.

After the pH-stat assay using the shrimp enzymes, the DH of feed was evaluated using a mixture of commercial enzymes: 1.6 g/l trypsin type IX from porcine pancreas (Sigma T-0134 FW); 3.1 g/l chymotrypsin from bovine pancreas (Sigma C-4129); 1.3 g/l amino-peptidase from porcine intestinal mucosa (Sigma P-7500); 7.95 g/l pronase type XIV from *Streptomices griscus* (Sigma P-5147). All enzymes were dissolved in distilled water and pH adjusted to 8.0 with 0.1 mol/l NaOH. Enzymatic activity was measured with 1% azocasein at 25 °C. Enzyme equivalent to 2.5 activity units of enzyme was used for these pH-stat assays.

ANOVA and multiple comparison tests using the LSD test (Statgraphics Plus v. 6.1) on the final weight of shrimp were used to determine the best performance of each experimental ingredient in feed compared with the control. Also a two-way ANOVA was conducted to determine if growth was affected by the source and quantity of each supplemental protein. Correlation analysis (Statgraphics Plus v. 6.1) was done on the DH of feeds against the ADC obtained from the in vivo assays to evaluate the relation between the two variables.

3. Results

Compositions of feeds and protein sources on a dry weight basis are listed in Table 2. There were no significant differences in protein content and energy in joules in the feeds ($P > 0.05$).

3.1. Amino acid composition and concentration

Akiyama et al. (1991) published the minimum recommended g of amino acids per 100 g of feed to support adequate growth for penaeid. The experimental protein ingredients analyzed had adequate concentration of all the essential amino acids, except KH for Phe and His, and SQ for Phe (Table 3). The control feed had higher concentration of all the essential amino acids. The F15, K3, and K15 feeds were a little low (4% and 8%) in methionine. Tryptophan was not evaluated.

Table 4 shows the proximate composition of shrimp muscle. All the experimental groups had higher protein content in muscle tissue and the highest was the group fed with 9% krill hydrolysate (K9).

3.2. Growth trials

Evaluation of the final weight indicates that, except for F15, SQ9, and SQ15, all the experimental groups performed better than the control group. Feeds supplemented with the lowest amount of the experimental protein ingredients yielded the best growth. Experimental groups with the highest value were SQ3, F9, and K9 (Table 5, $P < 0.001$).

Table 2
Composition of feeds given to each shrimp group, and experimental sources of protein as dry basis expressed in %

Feeds and experimental ingredients * *	Protein	Lipid	Ash	Crude fiber	Joules
C32	40.6	11.4	14.1	0.8	19.3
F3	41.5	11.1	14.2	0.9	18.4
F9	40.0	10.9	13.6	0.7	18.4
F15	39.0	10.7	14.5	1.0	18.0
K3	42.4	12.7	13.2	1.0	18.8
k9	42.8	10.6	13.2	1.3	18.8
k15	42.2	9.7	12.6	2.2	18.8
SQ3	42.4	11.4	13.5	0.8	19.7
SQ9	43.6	10.5	11.9	0.4	20.1
SQ15	44.4	11.0	10.3	0.8	19.7
KH	58.5	11.7	18.6	2.7	nd *
FH	45.3	13.8	23.4	0.0	nd
SQ	68.0	4.7	6.2	0.0	nd

* nd = Not determined.

** C32 = Control feed; K3 = 3% krill hydrolysate; K9 = 9% krill hydrolysate; K15 = 15% krill hydrolysate; F3 = 3% Fish hydrolysate; F9 = 9% fish hydrolysate; F15 = 15% fish hydrolysate; SQ3 = 3% squid; SQ9 = 9% squid, SQ15 = 15% squid; FH = Fish hydrolysate; KH = Krill hydrolysate; SQ = Squid meal.

Table 3
Amino acid composition of the feeds and protein ingredients tested expressed as g/100 g of protein

AA	R†	FH**	KH	SQ	C32	F3	F9	F15	K3	K9	K15	SQ3	SQ9	SQ15
Arg	5.8	7.4^{b}*	6.9^{a}	0.9^{g}	8.2^{c}	9.3^{e}	8.6^{d}	7.5^{b}	7.6^{b}	10.8^{g}	7.2^{b}	9.1^{e}	9.7^{f}	9.7^{f}
His	2.1	2.1^{b}	1.5^{a}	2.0^{b}	3.2^{h}	3.0^{fg}	3.1^{g}	2.6^{c}	2.7^{d}	3.3^{h}	2.8^{de}	2.5^{c}	3.3^{h}	2.9^{ef}
Ile	3.5	4.5^{ab}	4.8^{cfg}	7.4^{j}	4.9^{gh}	4.7^{cdef}	5.0^{f}	4.4^{a}	4.7^{cde}	5.7^{i}	4.6^{abc}	4.7^{def}	5.4^{h}	5.2^{g}
Leu	5.4	6.5^{b}	7.3^{cd}	5.5^{a}	8.3^{g}	8.6^{h}	8.3^{g}	7.7^{ef}	7.5^{de}	9.1^{i}	7.2^{c}	7.9^{f}	9.5^{j}	9.7^{j}
Lys	5.3	8.7^{cd}	8.1^{bc}	8.0^{bcd}	8.0^{bc}	9.5^{ef}	10.2^{f}	6.4^{a}	7.3^{bc}	9.3^{de}	7.4^{bc}	7.2^{b}	8.9^{de}	8.7^{cd}
Met	2.4	3.1^{i}	2.5^{ae}	3.3^{j}	2.5^{cd}	2.4^{bc}	2.6^{ef}	2.3^{b}	2.2^{a}	2.8^{g}	2.3^{b}	2.6^{f}	3.0^{h}	2.7^{g}
Phe	4.0	4.0^{b}	3.9^{b}	3.1^{a}	5.3^{f}	4.8^{de}	5.3^{fg}	4.6^{c}	4.8^{de}	5.5^{g}	4.6^{cd}	4.6^{cd}	5.5^{fg}	4.9^{e}
Ala	–	8.8^{g}	6.2^{a}	11.2^{h}	7.5^{bc}	8.1^{de}	8.9^{g}	7.6^{cd}	7.2^{bc}	8.8^{g}	7.4^{bc}	7.0^{b}	8.5^{ef}	7.6^{cd}
Asp	–	10.6^{de}	8.9^{a}	15.7^{j}	11.5^{fg}	10.8^{e}	11.5^{g}	11.2^{f}	10.1^{c}	12.1^{h}	9.9^{b}	10.3^{cd}	12.8^{i}	11.3^{fg}
Glu	–	16.3^{c}	12.2^{b}	11.1^{a}	19.8^{gh}	18.1^{e}	19.5^{fg}	19.4^{f}	17.8^{de}	20.4^{i}	16.4^{c}	17.5^{d}	21.2^{j}	20.1^{hi}
Gly	–	7.6^{ab}	7.1^{ab}	12.6^{e}	7.9^{abc}	11.3^{d}	10.4^{d}	8.2^{abc}	7.8^{ab}	12.8^{e}	8.2^{abc}	8.5^{bc}	8.9^{c}	7.3^{a}
Ser	–	3.1^{c}	2.6^{a}	7.2^{h}	3.4^{d}	3.9^{e}	3.9^{e}	4.1^{f}	2.8^{ab}	3.5^{d}	3.0^{bc}	3.5^{d}	4.5^{g}	4.5^{g}
Thr	3.6	3.9^{a}	3.9^{a}	5.3^{h}	4.4^{df}	4.2^{bc}	4.4^{cd}	4.2^{b}	3.8^{a}	5.1^{g}	4.2^{b}	4.7^{e}	4.9^{f}	4.9^{ef}
Tyr	–	3.2^{abc}	3.0^{a}	3.0^{ab}	4.1^{d}	3.4^{bc}	4.1^{e}	4.2^{e}	3.0^{a}	3.3^{bc}	2.9^{a}	3.5^{c}	4.7^{e}	4.4^{ef}
Val	4.0	5.5^{cd}	4.7^{a}	7.2^{i}	6.2^{f}	6.1^{f}	6.4^{g}	5.7^{de}	5.7^{e}	6.6^{gh}	5.4^{bc}	5.3^{b}	6.6^{h}	5.6^{de}

† R = Recommended for shrimp feeds (Akiyama et al., 1991).
* Different letters in the same row mean significant differences ($P < 0.05$).
** Names explained in Table 2.

When evaluating survival., there were no significant differences among groups (Table 5, $P > 0.05$). The evaluation of the FCR indicates that, except for F15, SQ9, and SQ15, all the experimental groups performed better than the control group (Table 5, $P < 0.001$). The two-way ANOVA showed significant effects of protein-source supplement ($P < 0.05$) and quantity in feeds ($P < 0.001$) on shrimp growth. Also a high correlation was found between shrimp weight gained and FCR ($r^2 = 80.9$, $P < 0.01$), The regression equation was $y = -0.5x + 6.98$, where y is the FCR and x is final weight, indicating a negative slope. This means that larger shrimp required less feed per gram of weight gained than smaller ones.

Table 4
Composition of shrimp muscle at the end of the experiment in dry basis expressed in %

Group	Protein *	Lipid	Ash
C32	79.5^{a}	9.6	6.7
F3	80.6^{c}	10.1	7.1
F9	80.5^{c}	9.7	7.9
F15	82.2^{f}	9.6	7.2
K3	81.0^{d}	10.0	6.9
K9	82.4^{f}	10.1	7.1
K15	82.2^{f}	9.4	7.0
SQ3	81.7^{e}	9.1	7.0
SQ9	81.4^{d}	10.2	6.9
SQ15	79.9^{b}	11.0	6.6

C32 = Control feed; K3 = 3% krill hydrolysate; K9 = 9% krill hydrolysate; K15 = 15% krill hydrolysate, F3 = 3% Fish hydrolysate; F9 = 9% fish hydrolysate; F15 = 15% fish hydrolysate; SQ3 = 3% squid; SQ9 = 9% squid, SQ15 = 15% squid.
* Different letters mean significant differences ($P < 0.01$).

Table 5
Weight gain, survival, feed conversion ratios, apparent protein digestibility, trypsin, chymotrypsin and total activities

Group *	Final weight (g) of shrimp	Survival ($P>0.05$) (%)	Feed conversion ratios	Units ** of total proteolytic activity	Units*** of trypsin activity	Units*** chymotrypsin activity
C32	8.0^{ab}	83	3.2^{cd}	14.5^{c}	5.4^{bc}	23.9^{cde}
F3	8.7^{cd}	79	2.6^{ab}	14.4^{c}	5.8^{cd}	20.7^{bcd}
F9	8.8^{d}	67	2.6^{ab}	14.5^{c}	7.1^{e}	22.5^{cd}
F15	8.2^{bc}	67	2.9^{bcd}	11.4^{b}	4.2^{b}	16.2^{b}
K3	8.7^{cd}	75	2.7^{ab}	15.9^{c}	5.2^{bc}	24.5^{de}
K9	9.1^{d}	81	2.6^{ab}	15.2^{c}	5.0^{bc}	28.4^{e}
K15	8.7^{cd}	67	2.4^{ab}	10.8^{ab}	4.9^{bc}	24.1^{cde}
SQ3	9.3^{d}	69	2.4^{ab}	15.4^{c}	4.9^{bc}	22.7^{cd}
SQ9	8.2^{bc}	79	2.8^{abc}	10.6^{ab}	4.9^{bc}	20.1^{bc}
SQ15	7.6^{a}	75	3.2^{d}	8.4^{a}	2.2^{a}	11.2^{a}

* As explained in Table 2. Different letters in the same column mean significant differences ($P<0.05$).

** One unit of activity = abs_{366} min^{-1} mg^{-1} protein.

*** One unit of activity for synthetic substrate = the amount of enzyme needed to hydrolyze 1 μmol of substrate in 1 min.

3.3. Enzyme activities

Total proteolytic activity of the hepatopancreas extracts showed significant differences between the control and some of the concentrations of protein substitutions ($P<0.001$, Table 5). The higher the ingredient substitution, the lower the values for total activity for the three experimental ingredients. Significant differences were also found for trypsin activity in some groups ($P<0.001$, Table 5). F9 group had the highest activity and SQ15 group the lowest, when compared to the control group. Chymotrypsin activity in groups F15 and SQ15 was lowest, when compared to the control group ($P<0.001$, Table 5).

3.4. Protein digestibility

When analyzing variables of digestion of protein (Table 6), the ADC of protein was dramatically enhanced in all the experimental groups compared with the control. ADC was increased between 15 and 25% in the F groups, between 30 and 44% in the K groups, and between 10 and 30% in the SQ groups. The DH obtained in the in vitro protein hydrolysis, which is an evaluation of the ability of the digestive enzymes from the hepatopancreas to digest feed protein (except for K15 and SQ3), was considerably enhanced in all the experimental groups. There was no correlation of DH vs. ADC of feeds using shrimp enzymes, but when data from groups fed hydrolyzed protein were analyzed (F3, F9, F15, K3, K9, and K15), a negative correlation ($P<0.1$) was observed (the higher the DH, the lower the ADC). The opposite tendency was apparently observed in the remaining data (C32, SQ3, SQ9, and SQ15), but there was no significant correlation ($P>0.1$).

When using commercial enzymes instead of the shrimp enzymes the DH was higher in all groups, including the control. There was no correlation of DH vs. ADC when

Table 6
Apparent digestibility coefficients of protein (ADC) and degree of hydrolysis (DH) of feed using shrimp enzymes and commercial enzymes, and DH of casein with shrimp enzymes, all values presented as %

Group *	ADC of protein	Feed DH with shrimp enzymes	Feed DH with commercial enzymes	Casein DH with shrimp enzymes
C32	67.8^{a}	3.1^{a}	4.0^{a}	12.2^{cd}
F3	82.9^{d}	4.1^{cd}	6.0^{d}	11.0^{a}
F9	78.3^{c}	5.4^{e}	6.7^{e}	12.3^{d}
F15	85.2^{d}	5.5^{e}	6.1^{d}	12.1^{cd}
K3	88.3^{e}	3.8^{bc}	4.5^{ab}	11.6^{bc}
K9	87.8^{e}	4.4^{d}	5.2^{c}	11.9^{bcd}
K15	91.8^{f}	3.4^{ab}	4.8^{bc}	12.3^{d}
SQ3	78.3^{c}	3.4^{ab}	4.6^{b}	11.5^{ab}
SQ9	88.6^{e}	4.1^{cd}	4.9^{bc}	11.7^{bc}
SQ15	75.1^{b}	3.7^{bc}	6.0^{d}	12.4^{d}

* As explained in Table 2. Different letters mean significant differences ($P < 0.001$).

commercial enzymes were used. Also, there was a negative correlation with groups fed hydrolyzed protein ($P < 0.05$). Shrimp enzymes digested casein, a reference protein, to a higher degree than feed protein. Except for the F3 and SQ3 groups, there were no significant differences among groups when comparing the DH of casein by the shrimp digestive enzymes. The weight gain of shrimp in the different groups also was analyzed for correlation against ADC, but there was no significant relation between the variables ($P > 0.1$).

3.5. Proteolytic activity in protein ingredients

For proteolytic activity of squid meal and krill hydrolysate, the squid meal did not show any proteolytic activity. Krill hydrolysate had 0.33 U/ml of total proteolytic activity of extract. The SDS PAGE on hydrolysates revealed that FH had peptides mainly under 14.4 kDa, and no larger than 20 kDa. The KH presented smaller bands, under 14.4 kDa, and presence of peptides larger than 66 kDa (picture not shown). Related to SQ, the SDS PAGE revealed important bands of peptide fractions under 14.4 kDa and larger bands between 29 and 45 kDa. The proteolytic activity in the krill hydrolysate did not show changes in the activity bands after 6 h of incubation in the S-SDS PAGE.

4. Discussion

We used the term feed to describe the food substance(s) supplied to feed an animal, in contrast to diet, that should be used to describe the food substance(s), including the amount and how frequently it is provided (McGraw-Hill Dictionary of science and technical terms).

Alternate protein ingredients were used to supplement a commercial feed containing fish meal as the main protein source. Such protein ingredients were produced with gentle processes intended to preserve the nutritional, functional, and organoleptic characteristics

of the product. Protein hydrolysates are the product of solubilizing protein enzymatically in the raw material by a limited protein hydrolysis. The hydrolyzed protein is obtained by physical means and may be a liquid or dry product. Drying is also achieved by gentle processes, such as freeze-drying. The production process is so benign that, in the krill hydrolysate, the digestive enzymes of krill remain active. The enzyme activity in the KH was resistant to heat for several h (data not shown), which suggests that, though the activity was not found when feed extracts were assayed for total proteolytic activity in test tubes, they remained active in the feeds.

All the experimental feeds rendered improved performances compared with the control group. The protein content in the muscle of shrimp fed supplemental protein was higher than the control group (Table 4). It is unusual that a 3–15% protein component in feeds can affect the protein content in muscle. This could be related to a higher protein synthesis in shrimp provided with better dietary protein sources. Except for groups F15, SQ9, and SQ15, all the experimental groups had higher final weight than the control group, which suggests that it is better to use low protein supplement in feeds of these protein sources (Table 5).

It was established that the digestive system of shrimp is modulated by the components of the supplemented feeds, yielding a 10–44% improved ADC of feeds over the control. This was confirmed by in vitro protein digestibility. In the experimental feeds, the DH of the proteins by shrimp enzymes fed these feeds were, in general, higher that the DH of the proteins in the control feed by shrimp enzymes. Commercial enzymes are more potent than shrimp enzymes, which is the reason the DH of feeds was higher in all cases when commercial enzymes were used to hydrolyze feeds and casein.

The results also showed some limits for quantity in feed for the protein sources tested. Protein hydrolysates and squid meal have been recognized as good supplements for feeds, and for squid (*D. gigas*) this study and a previous one (Córdova-Murueta and García-Carreño, 2001) demonstrated growth enhancement properties for *P. vannamei*, but only at low rates of feed supplementation.

Regarding the fish hydrolysate, the highest supplementation in feeds (F15 feed) yielded less growth than shrimp fed F3 and F9 feeds. Also trypsin, chymotrypsin, and total proteolytic activity were also lower for the shrimp fed the F15 feed. Hence, high quantities of protein hydrolysate are not necessary for shrimp feed production. Kolkovski and Tandler (2000) suggested no more than 50% of protein hydrolysate in fish larval feed. In their experiment, Zambonino-Infante et al. (1997) found better growth performance with fish (*Dicentrarchus labrax*) larvae fed feeds supplemented with the lowest quantity of fish hydrolysates. They also observed that chymotrypsin activity of the larvae was enhanced with low quantity of hydrolysates in feed. The properties of protein hydrolysates also depend on their chemical characteristics, which are a function of the degree of hydrolysis (Kristinsson and Rasco, 2000). The results of the present study on shrimp fed protein hydrolysate appear to be related to the DH of the protein of the commercial products. The SDS PAGE revealed that the fish hydrolysate had peptides of lower size than the krill hydrolysate, which included considerable amount of peptides of high (>66 kDa) and low molecular mass (<20 kDa). The KH supplements did not affect growth negatively when higher or lower quantities of KH were supplied to shrimp, as all KH fed shrimp gained more weight than the control group. The lower degree of hydrolysis and presence of larger

peptide chains may explain these results. The existence of low enzymatic activity in KH could help to enhance the digestion of feeds containing this ingredient. The KH is a freeze-dried product and conserves much of its natural properties. Related to KH, the present results demonstrated that there is a limit in shrimp growth response derived from protein supplementation, since all the K groups grew equally at the three levels of supplementation. There was no negative effect with the highest supplementation, as observed with the other protein ingredients tested.

The presence of large amount of peptides in fish hydrolysate of low molecular mass (<14 kDa) and almost no fractions above 20 kDa, could explain the lower growth observed in the F15 group (more concentration of small peptides and probably free amino acids in feeds). The most important feature of a protein hydrolysate for good nutritional quality is the degree of hydrolysis of the product. In fish, high levels of dietary free amino acids can change the rate of absorption in the gastrointestinal tract, inducing premature absorption of certain free, essential amino acids in relation to the absorption of the amino acids in the polypeptide chains (Hardy, 1991). As a result, an imbalance of amino acid absorption can occur. Also, it has been shown that free amino acid supplementation does not support growth of shrimp as well as peptide or protein amino acids (Akiyama et al., 1991; Fox et al., 1994; Shiau, 1998). Also supplementation of polyamino acids (prepared by thermal condensation from crystalline amino acids) of feed demonstrated no advantage against crystalline amino acids for shrimp growth and FCR (Divakaran, 1994).

The negative correlation observed on DH vs. ADC, using shrimp enzymes and commercial enzymes in pH-stat, may be explained by the presence of partially hydrolyzed protein in feeds, and that there are less peptide bonds to hydrolyze. The in vivo method (ADC) accounts for all nitrogen present in the feeds and in feces estimated by the Kjeldahl method (no difference, if it proceeds from a dipeptide or tripeptide or even if it is from free amino acids).

When ADC is compared with the DH, the latter tends to be lower when there is more hydrolyzed protein in feeds, thus explaining the negative correlation observed. Also, as the evaluation of the ADC depends on shrimp feces collection, it is likely the leaching of small peptides and free amino acids from the feces producing an underestimation of protein in feces, and as a result, yields a higher ADC value. Dimes et al. (1994) concluded that the pH-stat method is not suitable for feed samples that have been partially hydrolyzed, as observed in this investigation, especially for prediction of ADC based on DH values of feeds containing hydrolyzed protein. This is so because the correlation can be affected by the quantity of the hydrolysate included in feeds and the degree of hydrolysis of the product. Previous workers (Dimes et al., 1994, Ezquerra et al., 1998) have found a positive correlation for DH vs. ADC on non-hydrolyzed protein.

We observed that the growth of the SQ15 group (15% squid protein) was statistically equal to the control. We expected that giant squid (*D. gigas*) meal contained a growth factor, but it was not clear in these results because more squid in the feed led to decreased growth, while the FCR increased. When feed was supplemented with a lower squid concentration, the growth was greater. If the growth factor (Cruz-rique et al., 1987) exists in the Mexican giant squid, it may be unavailable for shrimp at high rates of feed supplementation. This negative response may be related to the presence of a high content of small peptides and free amino acids in the squid mantle, as revealed by SDS PAGE.

Also the enzymatic activity was observed to be lower for total, trypsin, and chymotrypsin activities.

The presence of free amino acids or small polypeptides in feeds can reduce the substrate for enzymes (mainly endopeptidases), especially if the specific sites of the enzyme attack along the protein chain are previously hydrolyzed. This may explain the lower activity observed in the hepatopancreas from shrimp fed with the highest quantities of these supplements.

P. vannamei could modulate the enzymatic secretions according to the type of protein eaten, such as hydrolysate protein concentrate and low-heat-dried giant-squid meal. We observed diverse growth responses, enzymatic activity, and digestibility in vivo and in vitro analyses. The two commercial hydrolysates tested (FH and KH) have different degree of hydrolysis and processes that affect the protein structure and digestive-enzymes recognition sites. Further investigation is needed to find an appropriate DH and processes for protein hydrolysates used for shrimp feeds, because shrimp have specific protein requirements.

For squid protein supplementation, small quantity of low-heat dried giant squid protein in feeds for shrimp is enough to enhance the performance of the cultivated shrimp *P. vannamei* (3% protein fraction in this study). Le Moullac et al. (1994) found that squid meal improved the larval growth in *P. vannamei*. Le Moullac et al. (1996) also reported an enhancement of trypsin activity in adult shrimp fed protein from squid, but growth performance was not evaluated. In our study, trypsin and chymotripsin were lower compared to the control when shrimp were fed 15% SQ as a protein supplement, and growth was not enhanced. For growth, Cruz-Rique et al. (1987) reported an improvement of growth in *P. vannamei* fed squid protein at various concentration.

Protein hydrolysates can benefit shrimp at low concentration and a controlled degree of hyrolysis is needed to keep low quantities of free amino acids. For the SQ meal, we observed by SDS-PAGE high quantities of small peptides. This might be the result of protein hydrolysis by endogenous cathepsin and cysteine-proteinases enzymes in the squid mantle during the postmortem period (Ponce-A. et al., 1999) occurred between the catch and muscle drying time (24–48 h). There is evidence that myosin is the main protein hydrolyzed during storage (Dublán-G. et al., 2000). The presence of hydrolyzed protein in squid muscle may explain the growth improvement in shrimp fed SQ protein, similar to that observed with the FH supplemented in feeds.

The amino acid content of feed did not suggest any relationship to the present results. It is evident that the feed can contain all the nutritive requirements for shrimp. However, if protein in feeds is not in the appropriate form for the physiological requirements of shrimp, it cannot be completely assimilated and it will be released to the pond water.

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