EFFECTS OF FEED DIETS ON DIGESTIVE PROTEASES FROM THE HEPATOPANCREAS OF WHITE SHRIMP (*Penaeus vannamei*)

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ABSTRACT

*Protease activities in the hepatopancreas extract (HP) from white shrimp (*Penaeus vannamei*) fed one of seven test diets for 30 days were evaluated by several methods. The test diet contained 85% of a reference ration for shrimp and 15% of either anchovy meal, tuna waste meal, deboned white fish meal, langostilla meal, soybean meal, and two menhaden meals as a protein replacer. One of the menhaden fish meals tested (B) had the lowest quality as a shrimp feed based on amino acid analysis. SDS-PAGE zymograms of HP from each of the seven diet groups showed similar proteins activity patterns with casein as substrate. The degree of hydrolysis of casein, measured by pH-stat, was also the same for HP from the seven diet groups ($P > 0.05$). However, total protease activity measured by azocasein hydrolysis (units/g HP) was higher for the diet group fed the test ration containing tuna waste as a replacer ($P > 0.05$). Trypsin and chymotrypsin activities measured with synthetic substrates (units/mg protein; units/g HP) from animals reared on the diet with menhaden meal B replacer were*

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greater than in the other diet groups ($P < 0.05$). This study shows that a relatively small amount (15%) of a specific protein replacer in white shrimp rations can influence the protease activity of shrimp HP. Given that digestive proteases such as trypsin can leach into the muscle of postharvest shrimp and thereby cause softening of the meat, the impact of the rearing diet on postharvest shelf-life should be considered along with the standard measures of feed quality that are used by the fish farmer, i.e. animal growth and heatlh.

INTRODUCTION

The white shrimp, *Penaeus vannamei*, is one of the most important penaeid species for commerical production. With the development and expansion of production, processing, and international marketing, the farmed-shrimp quality is becoming increasingly important. Proteases are probably studied more by food scientists and technologists than any other group of enzymes in fish and shell fish (Haard 1992). That these enzymes receive considerable attention is not surprising because protein hydrolysis can contribute to either improvement or loss of seafood quality. Autolysis of muscle protein in crustacean seafood (e.g. shrimp, lobster, crab, krill) occurs rapidly after harvest and has been attributed to digestive proteases (Salem *et al.* 1970). Proteases can be directly responsible for unusual textural defects in seafood, e.g. "belly burst," "gaping," and "mushiness" of bony fish and "tail meat" softening of crustacean species, and causes poor initial quality and fillet yields. The hepatopancreas of penaeids is known to have high proteolytic activities (Tsai *et al.* 1986; Van Wormhoudt *et al.* 1992; García-Carreño *et al.* 1996; Ezquerra *et al.* 1997). In storage and processing of fresh shrimp, the rapid deterioration of muscle protein has been found to be caused by the complexity and high activity of the proteolytic enzymes (Baranoski *et al.* 1984; Kawamura *et al.* 1981).

The growth of the penaeid shrimp is affected by the level and the quality of the protein, among other factors (New 1980). Shrimp fed wih higher quality protein showed higher protein digestibility, better growth, and less susceptibility to disease (Pike and Hardy 1997). Protein synthesis and degradation in muscle tissue of living animals is particularly sensitive to malnutrition and catabolic states caused by disease and stress (Horl *et al.* 1987). Therefore, the physiological state of the animal prior to harvest can be responsible for quality change that occurs during subsequent postharvest storage or processing of the meat (Haard 1992). Dietary protein may influence the capability of an animal to digest protein by regulating the synthesis, secretion, and inactivation of digestive enzymes (Snook and Meyer 1964). An increase in the activity of certain digestive enzymes occurs in *Penaeus japonicus* fed on live clams (Maugle *et al.* 1982). An increase in dietary vegetable

protein decreased the general protease and trypsin activity in *Penaeus vannamei* (Lee *et al.* 1984). Villarreal *et al.* (1990) reported an increase in the protease activity in white shrimp (*P. vannamei*) when dietary fish meal was substituted with langostilla (*Pleuroncodes planipes*) meal.

Because the quality of protein may affect the proteolytic enzyme activity, the aim of this work was to establish the effect of dietary protein quality on total activities of proteases, trypsin, and chymotrypsin in the hepatopancreas of farmed white shrimp (*Penaeus vannamei*). Excessive digestive-enzyme activity can leach into the muscle and decrease the shelf life of shrimp (Nip *et al.* 1985). Shrimp is a major commodity of international trade but little data exist on proteases and their effect on seafood quality. Results of our study will provide a basis of understanding the factors contributing to postharvest quality changes in this economically important commodity.

MATERIALS AND METHODS

Reagents

The following were obtained from Sigma Chemical Co. (St. Louis, MO). Substrates: succinyl-$(ala)_2$-Pro-Phe-*p*-nitroanilide (SAPNA), benzoyl-Arg-*p*-nitroanilide (BAPNA), azocasein, and casein. Inhibitors: phenylmethanesulfonyl fluoride (PMSF), tosyl-Lys-chloromethyl ketone (TLCK), and tosyl-Phe-chloromethyl ketone (TPCK). Chelator: ethylenediaminetetraacetic acid (EDTA). Buffer: tris-(hydroxymethyl) aminomethane (TRIS) with pH adjusted using HCl. Molecular weight markers (MWM), trichloroacetic acid (TCA), ninhydrin, ethanol, 2-propanol, hydrochloric acid, dimethyl sulfoxide (DMSO). Protein standard: bovine albumin. The Bradford reagent and electrophoresis reagents were from Bio-Rad (Richmond, CA).

Feed and Feeding Trials

Preparation of experimental diets and the feeding trials were done as those reported in detail by Ezquerra *et al.* (1997). The feedstuffs evaluated were provided as indicated: anchovy meal (National Marine Fisheries Service (NMFS), Seattle), tuna waste meal (Productos Pesqueros de La Paz, La Paz, BCS), deboned white fish meal (NMFS-Kodiak), menhaden fish meal (Zapata Protein-USA, Hammond, LA), Atlantic menhaden fish meal (Zapata Protein-USA, Hammond, LA), langostilla meal (Nutrition Laboratory of CIBNOR, La Paz, BCS), and soybean protein (Productos Pesqueros de La Paz, La Paz, BCS). Each diet was prepared with 15% substitution of the fish meal in a reference diet (Table 1). This type of substitution was described by Cho and Slinger (1979) and involves adding a

test ingredient to a reference diet. The resulting diet contained: 84% of the reference diet, 1% chromic oxide as an inert indicator (Tacon and Rodrigues 1984), and 15% of the test ingredient. The protein content in the diets was between 35 and 40%. *Penaeus vannemei* juveniles were fed test diets at the CIBNOR farming facility for 30 days prior to harvest and assay of hepatopancreas digestive proteases. The initial weight of the shrimp was 1.5 to 1.8 g. At the end of the experiment, the average final weights were between 3.0 to 4.0 g for different diet groups.

Amino Acid Analysis

The amino acid composition of the fish meal was determined using an HPLC (Beckman System Gold, UV visible detector 116) with a reverse phase HPLC (column ultra sphere, five μ, 4.6 mm $\times$ 25 cm). The sample (25 μL), after acid hydrolysis using 6 M HCl, was injected, and the running time was 40 min. These analyses were done at the Protein Laboratory, University of California at Davis.

TABLE 1.
COMPOSITION OF THE REFERENCE DIET USED TO DETERMINE GROWTH OF JUVENILE WHITE SHRIMP (% DRY MATTER)[1]

Ingredient	%
Ingredient premix[2]	92.0
Mineral premix[3]	3.5
Vitamin premix[4]	0.50
Fish oil	1.5
Soy lecithin	1.5

[1]Source: Ezquerra *et al.* (1997).
[2]Ingredient premix (percentage in premix): 17.0, fish meal (tuna waste); 10.75, shrimp meal; 19.35, soybean meal; 25.8, wheat meal; 21.5, sorghum meal; 4.7, gelatin.
[3]Mineral premix (percentage in premix): 14.30, KCl; 14.30, $MgSO_4 \cdot 7H_20$; 2.60, $ZnSO_4 \cdot 7H_2O$; 0.70, $MnCl \cdot 4H_2O$; 0.14, $CuCl_2 \cdot 2H_2O$; 0.14, KI; 0.07 $CoCl_2 \cdot 2H_20$; 67.72, $NaHPO_4$.
[4]Vitamin premix (mg or IU/kg diet): 15 000 IU, vitamin A; 7 500 IU, vitamin D_3; 400, vitamin E (tocopherol); 20, vitamin K_3 (menadione sodium bisulphite); 150, thiamin; 10, riboflavin; 50, pyridoxine; 100, pantothenic acid; 300, niacin; 1, biotin; 500, inositol; 0.1, cyanocobalamine; 20, folic acid; 1 000, cellulose; 2 000, ascorbic acid; 400, choline chloride.

Enzyme Analysis

At the completion of feeding trials (30 days), shrimp were fasted for 24 h and then sacrificed. Hepatopancreas of 3.0 to 4.0 g white shrimp from triplicate tanks (each tank with ten animals) for each diet group were separately collected, frozen, freeze-dried, and assayed for total protease and trypsin activities (García-Carreño and Haard 1993), and chymotrypsin activity (García-Carreño *et al.* 1994). The hepatopancreas tissues (n = 10) were removed from decapitated animals and kept frozen prior to extraction of enzymes. The thawed tissue was homogenized at 10C, the homogenate was centrifuged at 11,300 × g for 20 min, the aqueous supernatant was freeze-dried and stored at −20C following the procedure of Garcıa-Carreña and Haard (1993). The total protein content of the extract was determined using the method of Bradford (1976).

Substrate-SDS-Polyacrylamide Gel Electrophoresis (S-SDS-PAGE)

Electrophoresis separation of the protein in the enzyme preparations was done according to Laemmli (1970) using 12.5% acrylamide. Enzyme preparations were diluted 1:4 in sample buffer and 5 μL was loaded into a vertical electrophoresis device (Hoffer, San Francisco, CA). Zymograms of endopeptidase activity fractions separated by electrophoresis were made according to García-Carreño *et al.* (1993). Molecular weight standards were included on each plate.

Enzyme classification was done by SDS-PAGE. Enzyme preparations were mixed with 10 mM TLCK, 5 mM TPCK, or 20 mM PMSF and incubated for 60 min at 25C. Controls incubated without the inhibitor were prepared with TRIS buffer, pH 7.8. They were diluted 1:4 in sample buffer and 5 μL was loaded on to SDS-PAGE plates.

Azocasein Hydrolysis

Proteolytic activity was assayed in test tubes using 2% azocasein in 50 mM TRIS, pH 7.5 (García-Carreño and Haard 1993). In brief, 10 mL of the enzyme preparation was mixed with 0.5 mL of 0.5 M TRIS·HCl, pH 7.5, at 25C. Reaction was initiated by the addition of 0.5 mL of 2% azocasein and stopped 10 min later by adding 0.5 mL of 20% TCA. After incubation at 4C for 10 min, the reaction mixture was centrifuged at 16,500 × g for 5 min and the $Abs_{440\ nm}$ recorded. For the controls, TCA was added before the substrate.

Trypsin Activity

Trypsin activity was assayed using benzoyl-Arg-*p*-nitroanilide as substrate according to García-Carreño and Haard (1993). BAPNA was dissolved in 1 mL

of DMSO to make it 1 mM, and then made to 100 mL with 50 mM TRIS buffer, pH 7.5, containing 20 mM $CaCl_2$. The substrate solution and the reaction mixture were maintained at 37C during the enzyme assay. To 1.25 mL of fresh substrate solution was added 10 mL of the enzyme preparation. After 10 min, 0.25 mL of 30% acetic acid was added and the Abs_{410nm} was recorded against a water blank. BAPNA units were evaluated according to Dimes *et al.* (1994). BAPNA activity was obtained from the following formula:

$$[(Abs_{410nm}/min \times 1000 \times mL \text{ of reaction})/(8800 \times mg \text{ protein})]$$

where 8800 is the extinction coefficient of *p*-nitroaniline.

Chymotrypsin Activity

Chymotrypsin activity was assayed using succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide as substrate, recording the increase in Abs_{410nm}. Ten mL of enzyme preparation was added to a mix containing 100 mL of substrate (0.02 mM SAPNA) and 590 mL of 0.1 M TRIS, pH 7.8, containing 0.01 M $CaCl_2$. The absorbance was recorded for 3 min. One unit of chymotrypsin activity was defined as the absorbance Δ Abs_{410nm}/min, according to García-Carreño *et al.* (1994).

Degree of Hydrolysis of Casein

The degree of hydrolysis of casein was determined in triplicate for each hepatopancreas extract preparation by pH-stat titration at 25C, following the method described by García-Carreño *et al.* (1996). The concentration of the enzymes was adjusted to the same proteolytic activity with 2% azocasein. The time for complete hydrolysis was normally about 60 min/sample.

Statistics

All experiments were done three times, each with three replicates. Results were analyzed by ANOVA. Differences between the means were analyzed by the HSD Tukey test. The data were analyzed using the program STATISTICS for PCS (Microsoft Co., Tulsa, OK).

RESULTS AND DISCUSSION

Chemical Quality of the Meals

The proximate composition of protein sources included in shrimp diets is summarized in Table 2 (from Ezquerra *et al.* 1997). The protein content, expressed

TABLE 2.
CHEMICAL COMPOSITION OF THE PROTEIN SOURCES USED IN SHRIMP FEED[1]

Test ingredients	Moisture (%)	Crude Protein (% dry matter)	Crude Fat (% dry matter)	Ash (%)
Anchovy meal[2]	10.4±0.3^{d}	60.2±0.1^{c}	12.5±0.6^{c}	14.8±0.1^{b}
Tuna waste meal[3]	4.9±0.4^{a}	61.3±0.4^{c}	6.4±0.4^{b}	21.5±.1^{c}
Deboned white fish[4]	5.4±0.3^{a}	75.7±0.9^{d}	7.2±0.1^{b}	9.3±0.05^{a}
Menhaden fish meal A[5]	7.8 ±0.5^{b}	63.8±1.0^{c}	11.9 ±0.6^{c}	18.1±0.5^{c}
Menhaden fish meal B[5]	8.3±0.2^{b,c}	60.9±1.0^{c}	14.5±0.2^{d}	15.9±0.4^{b}
Langostilla meal[6]	5.9±1.0^{a}	39.4±1.0$^{a,(7)}$	2.9±0.6^{a}	42.6±0.1^{d}
Soybean Protein [3]	9.3±0.5^{c}	49.5±1.0$^{b,(8)}$	5.3±0.7^{b}	21.5±0.3^{c}

[1]Source: Ezquerra *et al.* (1997).
[2]National Marine Fisheries Service
[3]Productos Pesqueros de La Paz (PROPEPAZ)
[4]NMFS-Kokiak
[5]Zapata Protein-USA
[6]Nutrition Laboratory of CIBNOR, La Paz, BCS.
[7]10% the N is from chitin
[8]N x 5.71
Triplicate samples of the meals, as received, were analyzed following the standard methods of AOAC (1990).
Values with different letter for each column, are statistically different (P<0.05).

on a moisture-free basis, ranged from 39.4 to 75.5%. The tuna waste meal had 21.5% ash, the highest among the five fish meal samples tested. The ash content of langostilla meal was 42.6%, the highest among all meals tested. The highest lipid content was in Menhaden B meal. The relationship between the chemical composition of the protein sources in shrimp feed and the protease activity of the hepatopancreas extract of shrimp is discussed later.

The amino acid composition of meals tested are shown in Table 3. All meals had an amino acid content similar to those recommended for shrimp. An indication of biological value of protein is the lysine and arginine dietary relationship known as the lysine-arginine antagonism. This antagonism occurs with excessive levels of either amino acid and results in depressed growth. The lysine:arginine ratio should be maintained at 1:1 to 1:1.1 (Akiyama *et al.* 1992). Somewhat lower lysine:arginine ratios (Table 3) were observed for anchovy (1:0.7), tuna waste (1:0.7), and menhaden meals (1:0.7), i.e., arginine contents were relatively low compared to lysine contents. As with chemical composition, the amino acid content of feed proteins was related to the enzyme activity recovered after feeding and is discussed later.

SDS-Gel Electrophoresis (SDS-PAGE)

Several activity bands were observed in the extracts from shrimp hepatopancreas. Regardless of the diet, all organisms displayed similar active zones having caseinolytic activity (Fig. 1). The molecular mass range of the active zones was 14 to 64 kDa (Fig. 2, lane B). When PMSF was included in the zymogram assay buffer, some active zones were partially inhibited. The inhibited active zones in extracts obtained from animals fed with control diet are shown in Fig. 2 (lane D). The reduction in proteolytic activity in the presence of PMFS is an indication of the presence of serine proteases in the shrimp extract. Active zones in the molecular mass range of 24 to 30 kDa were similar to langostilla and crayfish extracts inhibited by the serine protease inhibitor PMSF (García-Carreño and Haard 1993; García-Carreño *et al.* 1993). The activity of shrimp hepatopancreas extracts was sensitive to the trypsin inhibitor TLCK (Fig. 2, lane E). The shrimp hepatopancreas extract zymograms showed at least four fractions bearing protease activity sensitive to trypsin inhibitor. A 22 kDa protease, corresponding in molecular mass to porcine trypsin (García-Carreño 1992), was detected as a minor zone in all shrimp hepatopancreas extracts. Similar results were reported by García-Carreño *et al.* (1994) working with crayfish proteases. Shrimp extracts were insensitive to TPCK (Fig. 2, Lane F), which is an inhibitor for mammal chymotrypsin. Because most of the studies looking for chymotrypsin activity were done using this inhibitor as a reporter, conflicting reports and the presence or absence of chymotrypsin in decapod digestive systems have been reported (García-Carreño *et al.* 1994).

TABLE 3.
AMINO ACID COMPOSITION OF THE MEALS TESTED

Amino acid	Recommended[1]	Anchovy	Tuna	Deboned	Menhaden A	Menhaden B	Langostilla	Soybean
	g of amino acid/100 g of total protein							
Alanine	-	9.2	9.5	8.7	8.8	10.9	7.9	6.5
Arginine*	5.5[2]	4.7	4.7	7.0	5.4	5.0	4.9	4.9
Aspartic acid	-	9.0	9.3	8.3	9.5	6.3	10.4	11.3
Cysteic acid	-	1.4	0.8	1.0	1.1	0.7	1.2	1.7
Glutamic acid	-	12.2	11.5	12.8	12.3	9.2	12.2	16.7
Glycine	-	11.5	13.0	5.2	10.2	5.5	10.7	7.5
Histidine*	2.1	3.1	2.3	1.8	2.2	1.7	2.4	2.1
Isoleucine*	3.5	4.3	4.4	5.0	4.7	3.6	4.5	4.2
Leucine*	5.4	7.3	7.5	7.5	7.8	6.2	6.9	6.9
Lysine*	5.3	7.0	6.6	7.4	7.6	5.2	5.5	4.8
Methionine*	2.4	3.1	1.6	2.1	2.0	1.5	1.0	0.6
Phenyalanine*	4.0	3.2	3.3	2.4	3.4	1.7	4.2	3.6
Proline	-	5.2	5.6	5.0	4.9	6.6	5.4	5.9
Serine	-	4.6	4.7	4.3	4.6	4.8	5.1	5.7
Threonine*	3.6	4.6	4.7	4.7	4.8	3.6	4.9	3.7
Thryptophan*	0.8	1.0	0.4	1.2	1.4	0.7	0.7	N.D.
Tyrosine	-	2.0	2.2	2.5	2.5	2.3	4.9	2.3
Valine*	4.0	5.8	6.0	5.1	5.9	3.6	6.0	5.0
Lysine:Arginine	1:1	1:0.7	1:0.7	1:0.9	1:0.7	1:1.0	1:0.9	1:1.0

*Essential amino acid of protein sources in shrimp.

[1]Suggested pattern of requirement from Akiyama *et al.* (1992).

[2]Value suggested by Chen *et al.* (1992).

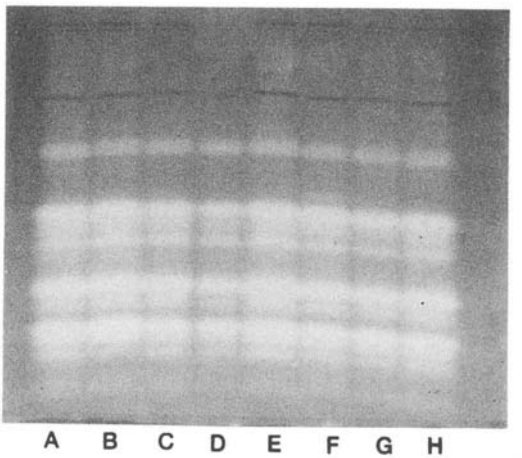

FIG. 1. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF HEPATOPANCREAS ENZYMES FROM SHRIMP (SHE) REARED WITH DIFFERENT DIETS

Gels were prepared as described under Materials and Methods. The protein applied to gels was the same for all extracts, 12 μg. Lane A, control (Hepatopancreas enzymes from shrimp before growth assay); lane B, anchovy diet; lane C, tuna waste diet; lane D, langostilla diet; lane E, menhaden fish diet; lane F, deboned white fish diet; lane G, soybean protein diet, and lane H, Atlantic menhaden fish diet.

Total Protease Activity

The total protease activity assayed with azocasein substrate as units of activity/g hepatopancreas (Table 4) was not significantly affected ($P > 0.05$) except for tuna waste meal. Maugle *et al.* (1982) examined the enzyme activities in hepatopancreas of *P. japonicus* fed live and freeze-dried clam diets. They also found the protease activity in the hepatopancreas was influenced by the type of protein in the diet. The extracts from animals fed with tuna waste meal had the highest protease activity (102 units of activity/g of hepatopancreas) followed by those obtained by langostilla meal (81 units of activity/g of hepatopancreas). This could be caused by the high ash content of the meals. It is known minerals induce digestive protease activity (Lan and Pan 1993). The use of soybean protein did not increase the total protease activity as was reported by Lee *et al.* (1984). They evaluated the influence of the level of plant protein in the feed. In our study, only 15% of the test protein was used as a replacer in the basal diet.

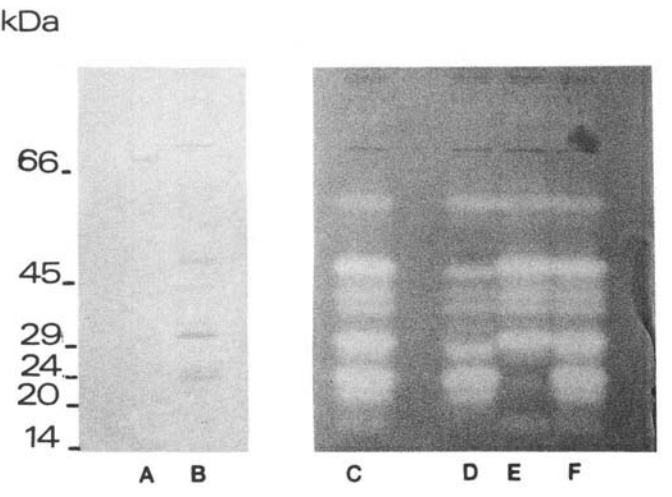

FIG. 2. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF HEPATOPANCREAS ENZYME FROM SHRIMP FED WITH CONTROL DIET (SHE)

Gels were prepared as described under Materials and Methods. Lane A, molecular weight markers used were: Bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactoalbumin (14,400); lane B, 12 μg SHE; lane C, 12 μg SHE without inhibitor; lane D, 12 μg SHE with protease inhibitor (PMSF); lane E, 12 μg SHE with trypsin inhibitor (TLCK); lane F, 12 μg SHE with chymotrypsin inhibitor (TPCK).

Trypsin and Chymotrypsin Activity

Extracts from the shrimp (*P. vannamei*) hepatopancreas also hydrolyzed trypsin- and chymotrypsin-specific substrates (Table 5 and 6). Proteases have been described in a variety of decapods, including shrimp, crab, krill, and crayfish. A common characteristic of digestive enzymes from decapods is the presence of enzymes with trypsin activity (Gibson and Barker 1979; Zwilling *et al.* 1981; Osnes 1985; Kim 1991; García-Carreño and Haard 1993; García-Carreño *et al.* 1994) and chymotrypsin activity (García-Carreño *et al.* 1994). The trypsin-specific activity seems to be a function of the protein source (Van Wormhoudt *et al.* 1980; Lee *et al.* 1984).

The highest trypsin-specific activity was shown by animals fed on the menhaden fish B meal (Table 5, $P < 0.05$). It appears the enzyme activity is independent

TABLE 4.
WEIGHT OF HEPATOPANCREAS SOLUBLE PROTEIN AND GENERAL PROTEASE ACTIVITIES IN SHRIMP HEPATOPANCREAS EXTRACTS FROM SHRIMP FED WITH DIFFERENT PROTEIN SOURCES

Meals tested	Hepatopancreas (g)	Protein (mg/mL)	Total protein (mg per g hepatopancreas)	Protease activity(1) (units/mg protein)	Protease activity(2) (units/g hepatopancreas)	Total Activity(3) (units)	Casein DH(4) (%)
Anchovy	0.15±0.01[a]	8.8±0.2[a]	1.3±0.1[a]	1.1±0.2[a]	63.4±11.6[a]	1.4±0.4[a]	19.3+0.1[a]
Tuna waste	0.14±0.02[a]	11.9±0.8[b]	1.6±0.1[a]	1.1±0.4[a]	102.3±11.9[b]	1.7±0.5[a]	20.4+1.0[a]
Deboned white	0.13±0.01[a]	11.2±0.5[b]	1.4±0.1[a]	0.8±0.1[a]	68.4±10.0[a]	1.1±0.1[a]	22.4+1.4[a]
Menhaden A	0.14±0.01[a]	12.3±1.1[b]	1.7±0.1[a]	0.7±0.1[a]	62.7±4.5[a]	1.3±0.7[a]	21.1+1.5[a]
Menhaden B	0.14±0.01[a]	11.6±1.7[b]	1.6±0.2[a]	0.7±0.3[a]	60.7±13.9[a]	1.2±0.2[a]	22.0+0.8[a]
Langostilla	0.13±0.01[a]	10.7±1.8[b]	1.4±0.3[a]	1.0±0.3[a]	81.1±11.0[a,b]	1.4±0.5[a]	21.2+0.6[a]
Soybean protein	0.14±0.02[a]	11.1±0.2[b]	1.6±0.2[a]	0.8±0.1[a]	64.9±12.6[a]	1.3±0.3[a]	21.7+1.1[a]

(1)Specific activity with azocasein, $Abs_{366\ nm}$/min/mg protein.
(2)Specific activity with azocasein, $Abs_{366\ nm}$/min/g of hepatopancreas.
(3)Total activity of proteases per total protein in the shrimp hepatopancreas preparation.
(4)The degree of hydrolysis (DH) was determined by pH stat using the hepatopancreas extract normalized for total protease activity.
The activities are reported as the population means±standard deviation (Triplicate determinations for each of three groups of shrimp for each diet group). Activities with different letter for each column, are statistically different ($P<0.05$).

TABLE 5.
TRYPSIN ACTIVITIES IN SHRIMP HEPATOPANCREAS
FROM SHRIMP FED WITH DIFFERENT PROTEIN SOURCES

Meals tested	Trypsin activity[1] (units/mg protein)	Trypsin activity[2] (units/g hepatopancreas)	Total trypsin activity[3] (units per mg protein)
Anchovy	0.1±0.01[a]	65.6±9.6[a]	0.1±0.02[a]
Tuna waste	0.1±0.02[a]	77.8±11.0[a]	0.1±0.04[a]
Deboned white	0.1±0.02[a]	65.7±10.3[a]	0.1±0.02[a]
Menhaden A	0.1±0.01[a]	72.3±10.6[a]	0.1±0.01[a]
Menhaden B	0.1±0.03[b]	120.8±3.2[b]	0.2±0.04[b]
Langostilla	0.1±0.00[a]	58.6±3.9[a]	0.1±0.02[a]
Soybean protein	0.1±0.01[a]	76.0±11.1[a]	0.1±0.02[a]

[1]Specific activity with BAPNA (Abs_{410nm}), μMol of *p*-nitroanilide released/min/mg protein.
[2]Specific activity with BAPNA (Abs_{410nm}), μMol of *p*-nitroanilide released/min/g of hepatopancreas.
[3]Total activity (BAPNA units) of trypsin per total protein in the shrimp hepatopancreas preparation.
The activities are reported as the population means±standard deviation (Triplicate determinations for each of three groups of shrimp for each diet group). Activities with different letter for each column, are statistically different ($P<0.05$).

TABLE 6.
CHYMOTRYPSIN ACTIVITIES IN SHRIMP HEPATOPANCREAS FROM SHRIMP FED WITH DIFFERENT PROTEIN SOURCES

Meals tested	Chymotrypsin activity[1] (units/mg protein)	Chymotrypsin activity[2] (units/g hepatopancreas)	Total chymotrypsin activity[3] (units per mg protein)
Anchovy	0.5 ± 0.10^{c}	$32.1\pm6.0^{a,b}$	$0.7\pm0.16^{a,b}$
Tuna waste	$0.4\pm0.02^{a,b}$	$32.4\pm5.6^{a,b}$	$0.6\pm0.07^{a,b}$
Deboned white	0.3 ± 0.04^{a}	28.0 ± 6.9^{a}	0.5 ± 0.04^{a}
Menhaden A	0.3 ± 0.01^{a}	28.8 ± 4.7^{a}	$0.6\pm0.05^{a,b}$
Menhaden B	$0.5\pm0.09^{b,c}$	41.3 ± 6.1^{b}	0.8 ± 0.09^{b}
Langostilla	$0.4\pm0.08^{a,b}$	$29.7\pm6.7^{a,b}$	$0.5\pm0.19^{a,b}$
Soybean protein	$0.4\pm0.0^{b,c}$	$32.9\pm3.9^{a,b}$	$0.7\pm0.16^{a,b}$

[1]Specific activity with SAPNA (ΔAbs_{410nm}), mol of *p*-nitroanilide released/min/mg protein.
[2]Specific activity with SAPNA (ΔAbs_{410nm}), mol of *p*-nitroanilide released/min/g of hepatopancreas.
[3]Total activity (SAPNA units) of chymotrypsin per total protein in the shrimp hepatopancreas preparation.
The activities are reported as the population means±standard deviation (Triplicate determinations for each of three groups of shrimp for each diet group). Activities with different letter for each column, are statistically different ($P<0.05$).

of the fat and ash content as long as contents of protein and basic amino acids are kept at the same level (Lan and Pan 1993). Trypsin cleaves protein at the peptide bonds adjacent to the basic amino acids. The sum of lysine and arginine, obtained from amino acid analysis (Table 3), showed the lowest values were in soybean protein (9.7), menhaden fish B meal (10.2), and langostilla meal (10.4). The trypsin activity was not affected by the lysine and arginine values, but it was by the lipid content and the drying process of the fish meal. Menhaden fish B showed high lipid content (Ezquerra *et al.* 1997) and had a dark color, which is indicative of overheating. The overheating could cause reactions of lysine and other amino acids, diminishing the quality of the protein (Anderson *et al.* 1993), which can increase the trypsin activity (Rodriguez *et al.* 1994) and reduce the storage life of the shrimp (Nip *et al.* 1985).

There were significant differences ($P < 0.05$) in chymotrypsin activity in extracts from shrimp reared with different diets (Table 6). The highest chymotrypsin activity was in organisms reared on menhaden fish B meal. Chymotrypsin cleaves proteins at the aromatic amino acids. The total content of tyrosine and phenylalanine in test ingredient (Table 3) was associated with the chymotrypsin activity, and also with the general quality of the meals. The lowest value of tyrosine and phenylalanine was in menhaden fish B, and this also had the highest lipid content and a dark color. These observations suggest this meal had a low protein quality, which is known to stimulate the trypsin and chymotrypsin activities (Rodriguez *et al.* 1994). The alkaline proteases like trypsin have been implicated in texture deterioration of crustacean seafood (Salem *et al.* 1970), so the high trypsin and chymotrypsin response from shrimp fed with menhaden B may affect shelf-life of the postharvest shrimp.

Degree of Hydrolysis of Casein

The total protease activity of hepatopancreas extracts was also measured by the degree of casein hydrolysis (DH). The DHs of casein from shrimp reared on diets containing different sources of protein were not significantly different ($P > 0.05$) (Table 4). Similar results were reported by Dimes and Haard for rainbow trout and coho salmon fed different protein sources (1994). Earlier we showed that *in vitro* digestibility by shrimp hepatopancreas extracts using a pH-stat method can predict the protein digestibility of alternate sources of protein for white shrimp diets. These results demonstrated the evaluation of *in vitro* protein quality using pH-stat approach is not affected by extracts obtained from shrimp fed with different diets, but showed this method was not a useful indicator of effect of the protein quality on the protease activity in this study.

CONCLUSIONS

Because culture conditions can influence the amount and type of enzymes that can affect postharvest quality, knowledge of the composition of the tissue proteases in animals reared on different diets was needed. We found all shrimp hepatopancreas extracts showed the same protease zymogram pattern, and when specific inhibitors were used (TLCK for trypsin and PMSF for alkaline proteases), the inactivation of specific zones was detected.

The results of this work have shown that total protease, trypsin, and chymotrypsin activities of hepatopancreas enzyme extracts from organisms fed on the same basal diet with only 15% replacer of different protein sources showed some significant differences. The menhaden fish B meal, which had the lowest protein quality according to the chemical composition and tyrosine and phenylalanine content, was shown to stimulate trypsin and chymotrypsin activity in hepatopancreas tissue.

The degree of hydrolysis of casein by hepatopancreas extracts normalized for total protease activity was not affected by the diet used to feed shrimp, so this method was not a useful indicator of the effect of the protein quality on the protease activity in this study.

We conclude, under the conditions of these studies, protease activity of trypsin and chymotrypsin are affected by the protein source used to feed the juvenile *P. vannamei*, but not the protease composition and the overall proeinolysis ability of the hepatopancreas extracts. This knowledge will aid the aquaculturist to manipulate dietary protein sources, to optimize postharvest shelf life and other production considerations.

Further work on the relationship between digestive enzyme activity, sources of protein, and shelf-life of shrimp is necessary to prove that the increase of digestive enzyme activity affects the quality of postmortem shrimp.

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