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Protein digestion in penaeid shrimp: digestive proteinases, proteinase inhibitors and feed digestibility

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Abstract

Protein is the most abundant ingredient in both natural and prepared diets of penaeid shrimp. The assessment of protein digestion through the developmental stages of penaeids may contribute to the development of more suitable feeding schedules for their cultivation. Among the techniques to study protein digestion, detection and characterization of digestive proteinase inhibitors in proteinaceous feed ingredients can be achieved by substrate-[sodium dodecyl sulphate polyacrylamide gel electrophoresis] (SDS-PAGE). In vitro assays of protein digestibility are also useful tools when testing alternative protein sources in the formulation of shrimp feeds. The present article reviews three methods that have been used to assess protein digestion: (1) detection and characterization of proteinase activity and proteinaceous proteinase inhibitors by substrate-SDS-PAGE, (2) quantification of proteinase activity, and (3) in vitro evaluation of digestibility of dietary protein sources by shrimp proteinases. A compilation of previously reported and unpublished data on some aspects of penaeid protein digestion is presented. Trypsin activity of *Litopenaeus schmitti* varied considerably during larval and postlarval development, showing the highest value at protozoea III. The molecular weight of digestive proteinases from early stages of *Farfantepenaeus paulensis* also differed from the adult pattern, and some activity bands could be characterized as trypsin in adult *F. paulensis*. The digestive proteinase pattern of adult *Farfantepenaeus californiensis*, *F. paulensis*, *L. schmitti* and *Litopenaeus vannamei* in SDS-PAGE showed clear differences among these species, which may be evidence of a species-specific pattern of protein digestion. In vitro evaluation of digestibility of aquafeeds can be achieved by the pH-stat method, which can help in the choice for alternative protein sources. Moreover, the quality

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of postharvest shrimp may also be affected by increased digestive proteinase activity that seems to be stimulated by low nutritional quality feeds.

Keywords: Protein digestion; Digestive enzymes; Proteinases; Digestibility; In vitro; Shrimp

1. Introduction

Feed is a major concern for shrimp farmers, representing up to 60% of the total variable production costs (Akiyama et al., 1992; Sarac et al., 1993). It must fulfill several characteristics including organoleptic properties such as odor, texture, and flavor, and physical properties such as particle size. In addition, the feed should contain all the essential nutrients for the cultured organism, and be readily available at low cost, highly digestible with the nutrients available for assimilation, and devoid of antinutritional factors (Sudaryono et al., 1995). In both natural and prepared diets, protein is the most abundant ingredient for shrimp playing a critical role for growth and development (Smith et al., 1992; Sudaryono et al., 1995). Protein ingredients are the most expensive constituents in aqua feeds. Besides the price, availability of protein ingredients is also a problem for feed producers. The search for alternative protein sources with high nutritional quality at reasonable cost is a current concern among shrimp farmers (Akiyama, 1991; Sarac et al., 1993; Sudaryono et al., 1995, 1999). Locally available feedstuffs such as fish or plant seed meals are normally used for formulation of low cost feeds. Fish meals naturally contain a well-balanced mixture of essential amino acids and other nutrients which are readily digested. However, the raw material can be technically abused causing a loss in its nutritional and functional properties (Garcia-Carreño, 1998) and the world supply of fish meal, mainly derived from capture fisheries, is heavily influenced by climatic events such as the El Niño effect (Cushing, 1975). On the other hand, plant derived ingredients may contain antinutritional factors that impair the efficiency of digestion. The most common are enzyme inhibitors that decrease the ability of digestive enzymes to hydrolyze dietary protein (Olli et al., 1994). A reduced digestion limits the bioavailability of amino acids needed for protein synthesis and growth. Some of these inhibitors are in fact proteins that occur in the protein source used to formulate the diet. As an example, some plant seed meals contain proteinase inhibitors which affect the degree of protein hydrolysis by shrimp digestive enzymes (Garcia-Carreño, 1996), leading to poor growth.

Understanding protein digestion is a relevant issue for the many steps in the shrimp production process. Penaeid life history is marked by changes in morphology and behavior, with a shift from planktonic herbivory to omnivory in late protozoea and the adoption of a benthic existence as postlarvae. These ontogenetic events are accompanied by significant changes in metabolic rates and digestive enzyme activities (Laubier-Bonichon et al., 1977; Lovett and Felder, 1990; Chu and Ovsianico-Koulikowsky, 1994; Lemos et al., 1999). The characterization and quantification of proteolytic enzyme activity at different stages of development may contribute in improving feeding conditions during the entire production process. Moreover, the penaeid digestive gland is known to contain high proteolytic activity (Tsai et al., 1986; Van Wormhoudt et al.,

1992), which may negatively affect the quality of postharvest shrimp by deterioration of muscle protein during storage and processing (Kawamura et al., 1981; Baranoski et al., 1984). Since diet may affect shrimp enzyme activity (Le Moullac et al., 1996; Ezquerra et al., 1997b; Lemos and Rodríguez, 1998), studies on proteolytic enzymes may contribute to improve the postharvest quality of shrimp.

Shrimp farming is, for many countries, a growing economical activity (Lester, 1992). To date, the choice for culturing a given species has mostly depended on known yields. Many farmers have invested in growing species that exhibit a satisfactory performance, regardless of whether the species were native to the area where they would be cultured. On the other hand, culturing autochthonous or native species may be a source of business diversification and high yields can be reached due to their inherent physiological adaptation to the local environmental conditions. Culture techniques for many autochthonous species are still not established, making attempts for cultivation a risky practice subject to low yields. The success of rearing alternative species depends mostly on the knowledge of their biology including nutrition. In this scenario, the availability of high nutritional quality feeds is a crucial factor. Studies on shrimp digestion may be helpful for improving the formulation of feeds in order to promote appropriate growth performances (Jones et al., 1997). The present article describes three different methods that have been used to assess protein digestion in penaeid shrimp: (1) detection and characterization of proteinase activity, and proteinaceous proteinase inhibitors by substrate-[sodium dodecyl sulphate polyacrylamide gel electrophoresis] (SDS-PAGE); (2) quantification of proteinase activity; and (3) in vitro evaluation of digestibility of dietary protein sources by shrimp proteinases. Previously reported data along with unpublished data were used as examples of the utility of such techniques. The generic level taxa used in referring the species follow the recently proposed nomenclature for the members of the family Penaeidae (Pérez-Farfante and Kensley, 1997).

2. Methods to assess shrimp proteinases and protein digestion

This section presents a description of some methods to assess enzymatic protein digestion in penaeids. Enzyme extracts may be obtained from the protein fraction of digestive gland, depending on the size of the organism, or from whole individuals as larvae or postlarvae. To enable the comparison of results, individuals must be sampled at the same stage of the molt cycle (Lovett and Felder, 1990; Fernández et al., 1997). Intermolt seems to be the best physiological stage, comprising from ca. 7.5% to 30% of the total molt period since the last molt (Dall et al., 1990). The homogenization and separation of the enzyme extract must be done at about 5°C. Tissues are homogenized in distilled water and centrifuged for 20 min at $10{,}000 \times g$. After the elimination of tissue debris and lipid, the water soluble extract is the enzyme preparation for activity assays.

Proteinases, also called endopeptidases, is an important group among the digestive enzymes of shrimp, they include trypsin and chymotrypsin which are responsible for more than 60% of total protein digestion in penaeids (Galgani et al., 1984, 1985; Tsai et al., 1986). Proteinases can be characterized after separation of the enzyme extract by SDS-PAGE (Laemmli, 1970), using 12% acrylamide.

In the present study, original data on trypsin activity of larval *Litopenaeus schmitti* and the proteinase pattern in SDS-PAGE of adult *Farfantepenaeus paulensis*, *L. schmitti*, *Farfantepenaeus californiensis*, *Litopenaeus vannamei* are provided. Larval *L. schmitti* were fed with *Chaetoceros calcitrans* (80,000 cells ml^{-1}) and artificial plankton (Nippai Shrimp Feed, Japan; 0.03 mg $larva^{-1}$ day^{-1}, 30 μm particle size) from the stage protozoea I (PZ I). Freshly hatched *Artemia* sp. (15 nauplii $larva^{-1}$ day^{-1}, origin: Great Salt Lake) were added to the diet at protozoea III (PZ III). After metamorphosis to postlarva, the diet was composed of microalgae with increasing amounts of artificial plankton and brine shrimp nauplii. The digestive glands of three to five not sexually mature adult females of *F. paulensis*, *L. schmitti*, *F. californiensis* and *L. vannamei* were extracted, frozen at −20°C and freeze dried. The animals were fasted for 24 h before digestive gland extraction. *F. paulensis* and *L. schmitti* were previously fed with frozen mussel and squid, while *F. californiensis* and *L. vannamei* were fed a prepared feed (Piasa Camarón La Paz, Mexico) containing 35% protein, 8% lipid, and 4% fiber.

2.1. Protein and proteinase composition

Enzyme preparations, 0.02 mg of protein in up to 20 μl of sample buffer were loaded per lane, in twin polyacrylamide gels, in a temperature controlled (4°C) electrophoresis device. Gels of 8×10 cm are fast and have the ability to separate protein extracts. Molecular weight standards (0.0175 mg) were included on each plate. Electrophoresis was done at a constant current, 15 mA per gel. The separation should proceed for 1 to 1.5 h, when the tracking dye will reach a centimeter above the end of the plate. After electrophoresis, one of the two identical SDS-PAGE gels is stained for protein with a solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250. The twin gel is immersed in 3% casein in 50 mM Tris–HCl, pH 7.5, for 30 min at 5°C, to allow the substrate to diffuse into the gel at low enzyme activity. Afterwards, the temperature is raised to 25°C for 90 min, for digestion of the protein substrate by the active fractions. The gel is then washed in water and immediately fixed and stained by immersion in the staining solution. After 2 h staining period, the gel is destained with the same solution without the Coomassie dye, and dried. The gel stained for protein gives the protein pattern of the samples (proteinogram) and the gel stained for activity gives the pattern of enzyme activity (zymogram). Clear zones on a blue background indicate proteinase activities and by comparison with the molecular weight standard bands, the molecular weight of the proteins or proteinases is obtained (Garcia-Carreño et al., 1993). The molecular weight of active bands can be determined by regression between the distance of molecular weight standard bands from the top border of the gel (cm) and the log of their molecular mass (kDa). The zymogram is more sensitive than the proteinogram and usually bands of enzyme activity are observed where no bands of protein are seen. A schematic sequence of these procedures is shown in Fig. 1a.

2.2. Proteinaceous proteinase inhibitors

To evaluate the presence of a proteinaceous proteinase inhibitor for a particular proteinase in complex samples such as legume seed meals, an aqueous protein extract is

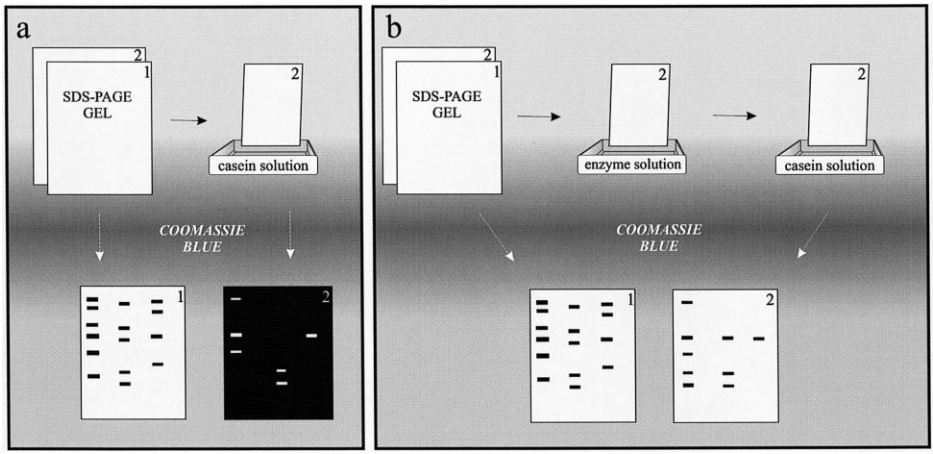

Fig. 1. Schematic sequence of substrate-SDS-PAGE protocols for determining (a) proteinase composition and activity, and (b) proteinaceous proteinase inhibitors (Garcia-Carreño et al., 1993).

separated by SDS-PAGE as above. The twin gel is soaked for 30 min at 5°C in a solution containing the target enzyme, a commercial enzyme preparation or a hepatopancreas extract. Then, it is washed with distilled water and soaked in a solution of the substrate as described for proteinase activity assay (Fig. 1b). The molecular weight of active bands can be determined as described above.

2.3. Characterization of type and class of proteinases by specific inhibitors and SDS-PAGE

Most proteinases from the digestive system belong to the serine class (EC 3.4.21.x), the exception is the stomach proteinase pepsin that belongs to the aspartic class. To determine the class and specificity of the enzyme, the proteinase under study is incubated with specific inhibitors (Table 1) for serine class, trypsin or chymotrypsin (Garcia-Carreño and Haard, 1993). Solutions of 20 mM tosyl-lysine chloromethyl ketone (TLCK) in 1 mM HCl, 200 mM phenylmethylsulphonyl fluoride (PMSF) in 2-propanol, or 0.5 mM carbobenzoxy-phenylalanine chloromethyl ketone (ZPCK) in 50 mM phosphate buffer, pH 7.8, are individually mixed with the enzyme extracts in a ratio of 1:10 (inhibitor/extract) and incubated for 1 h at 25°C. Distilled water is used instead of inhibitors in control. Then the inhibitor–enzyme mixture is diluted with the sample buffer and loaded onto the gel as described above. After electrophoresis, lanes are cut apart for molecular weight standard and total protein lanes, and activity and inhibition lanes. The former lane is immediately stained. Activity and inhibition lanes are immersed in 3% casein following the described procedure for activity. Bands with proteinase inhibitors are compared with control proteinase lanes (no inhibition) to identify the inhibitory effect on active bands. Since TLCK, ZPCK, and PMSF are specific trypsin, chymotrypsin and serine proteinase inhibitors (Table 1), those bands affected by the inhibitors will be smaller or may even disappear. The degree of

Table 1
Substrates and inhibitors commonly used for shrimp proteolytic enzyme assays

Enzyme	Substrate	Inhibitor
Total proteinases	Azocasein or casein[a]	
Trypsin	TAME (*N*α-*p*-toluenesulphonyl-L-Arg methyl ester)[b]	TLCK (tosyl-lysine chloromethyl ketone)[c]
	BAPNA (*N*α-benzoyl-DL-Arg-*p*-nitroanilide)[d]	
Chymotrypsin	SAPFNA (*N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide)[e]	ZPCK (carbobenzoxy-phenylalanine chloromethyl ketone)[c]
Serine proteinases		PMSF (phenylmethylsulphonyl fluoride)[c]

[a] Garcia-Carreño et al. (1993).
[b] Rick (1984).
[c] Beynon and Salvesen (1989).
[d] Erlanger et al. (1961).
[e] DelMar et al. (1979).

inhibition cannot be measured by visual observation of PAGE zymogram and results are interpreted as presence or absence of casein digestion. Casein is used as substrate because it is readily digested by penaeid proteinases such as trypsin and chymotrypsin (Jiang et al., 1991; Le Moullac et al., 1996). This technique detects only proteinases that form short chain polypeptides. Peptidases (or exopeptidases) are not expected to generate clear zones in the blue background.

2.4. Quantification of proteinase activity

Total proteinases, trypsin and chymotrypsin activities are usually determined by the incubation of enzyme extract with natural or synthetic substrates (Table 1) at controlled temperature and pH. As the substrate is hydrolyzed, changes in absorbancy can be detected at specific wavelengths. Azocasein is a commonly used substrate for the quantification of total proteinase activity (Garcia-Carreño et al., 1993). Ten microliters of enzyme extract are added to 0.5 ml of 1.5% azocasein in 50 mM Tris–HCl buffer, pH 7.5 (Garcia-Carreño, 1992a) at 25°C. The reaction is stopped 10 min later by the addition of 0.5 ml of 20% trichloroacetic acid (TCA), and the mixture is centrifuged for 5 min at $6500 \times g$. The supernatants are separated from the undigested substrate and the absorbance at 366 nm for the released dye recorded. The assay must include appropriate blanks as internal positive and negative controls. Trypsin and chymotrypsin activities are determined by the rate of hydrolysis of synthetic specific substrates. Trypsin activity can be measured by using *N*α-*p*-toluenesulphonyl-L-Arg methyl ester (TAME) or *N*α-benzoyl-DL-Arg-*p*-nitroanilide (BAPNA) substrates. Samples of 300 μl of enzyme extract are mixed with 1.2 ml of 10 mM TAME in 46 mM Tris buffer, pH 8.1, containing 11.5 mM $CaCl_2$ at 25°C, and the change in absorbancy at 247 nm is recorded for 3 min (Rick, 1984). One millimolar BAPNA is dissolved in 1 ml dimethylsulfoxide (DMSO) and then made to 100 ml with Tris–HCl, pH 7.5, containing 20 mM $CaCl_2$.

Ten microliters of the sample are added to 1 ml of the substrate solution at 37°C and the change of absorbancy at 410 nm is recorded for 5 min (Erlanger et al., 1961). Chymotrypsin activity is evaluated using 0.1 mM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (SAPFNA) in 0.1 M Tris–HCl, pH 7.8, containing 0.01 M $CaCl_2$. Samples of 10 μl are mixed with 0.69 ml of substrate solution and the absorbancy at 410 nm is recorded over 3 min at 25°C (DelMar et al., 1979). Each assay must include blanks in which the changes in absorbancy are recorded without the enzyme extract. The relative importance of trypsin and chymotrypsin to the total proteinase activity can be determined by the incubation of enzyme extracts with specific inhibitors (Table 1) prior to the incubation with azocasein as described above (Garcia-Carreño, 1992b; Garcia-Carreño et al., 1994). Total proteinase, trypsin and chymotrypsin activities can be expressed as the change in absorbancy per minute per milligram of protein of the enzyme extract used in the assays or as international units (IU) of μmol of substrate cleaved per minute, based on the substrate extinction coefficient.

2.5. In vitro determination of protein digestibility by pH-stat

For an ingredient of suitable essential amino acid composition, the quality of protein can be evaluated by measuring the digestibility of the ingredient or the feed containing it. The use of a pH controller as the pH-stat (Methohom Ion Analysis, Switzerland), an enzyme extract (Dimes and Haard, 1994; Ezquerra et al., 1997a, 1998), and the concept of degree of hydrolysis (DH) for the in vitro determination of digestibility is based on the fact that the hydrolysis of protein peptide bonds produces a pH drop in the mixture. The DH of a particular feed can be estimated during the digestion process by the automatically recorded titration curve (Dimes and Haard, 1994; Garcia-Carreño et al., 1997). Feeds (substrates) are homogenized in water and the amount of crude protein must be adjusted to 8 mg ml^{-1} (Ezquerra et al., 1997a). Ten grams of the substrate suspension is added to the hydrolysis vessel and the pH adjusted to 8.0 with 0.1 N NaOH. The reaction is started with the addition of 1 ml of shrimp enzyme extract (pH = 8.0). The pH is automatically maintained at 8.0 and the volume of 0.1 N NaOH released is recorded. The assay can be conducted at different temperatures from 23°C to 27°C (Ezquerra et al., 1998). The protein hydrolysis is calculated from the algorithm:

$$\mathrm{DH}\% = \left[\left(B \times N_b \times 1.5/M \times \left[S\%/100\right]\right)/8\right]100$$

where B = volume (ml) of standard alkali (0.1 N NaOH) required to maintain the pH of the reaction mixture at 8.0; N_b = normality of the titrant; M = mass (g) of reaction mixture; S = protein concentration in the reaction mixture (Adler-Nissen, 1986).

The use of the pH-stat method to monitor protein digestibility offers some advantages: (1) the rate of hydrolysis can be estimated quickly during the digestion process by the automatically recorded titration curve; (2) constant pH during the digestion process without the use of a physiological buffer (Pedersen and Eggum, 1983); (3) the method is sensitive to trypsin inhibitors in protein ingredients (Garcia-Carreño et al., 1997); and (4) it significantly correlates with in vivo apparent protein digestibility (APD) (Ezquerra et al., 1998).

3. Results and discussion

Good growth performance of shrimp in ponds is associated with survival during larval stages (Castille et al., 1993). Looking for the production of good quality postlarvae, research on larval nutrition has traditionally focused on the establishment of suitable feeding protocols for the different developmental stages (Loya-Javellana, 1989; Gallardo et al., 1995). Digestive enzyme activities were used as an index of the trophic state in order to allow for suitable diet formulation (Van Wormhoudt, 1973; Laubier-Bonichon et al., 1977; Lee et al., 1984; Galgani et al., 1985). It may also indicate differences in the digestive potential among the life stages of cultured shrimp. Trypsin specific activity of *L. schmitti* was low in egg and nauplius IV–V, increased in PZ I and reached a peak in PZ III (Fig. 2). The activity decreased from mysis I, maintaining low values during the initial postlarval stages. This activity pattern was found to be similar in several penaeid species such as *Marsupenaeus japonicus*, *Litopenaeus setiferus*, *Penaeus monodon*, *L. vannamei* and *F. paulensis* (Laubier-Bonichon et al., 1977; Lovett and Felder, 1990; Fang and Lee, 1992; Le Moullac et al., 1992; Lemos et al., 1999). The mechanism of enzyme regulation remains unclear, but the variations seem to be closely related to developmental events rather than changes in diet (Lovett and Felder, 1989, 1990). The increased enzyme activity occurs at stages with intense energy turnover such as protozoea, when swimming, ingestion and metabolic rates are high (Emmerson, 1980; Loya-Javellana, 1989; Dall et al., 1990; Chu and Ovsianico-Koulikowsky, 1994). The

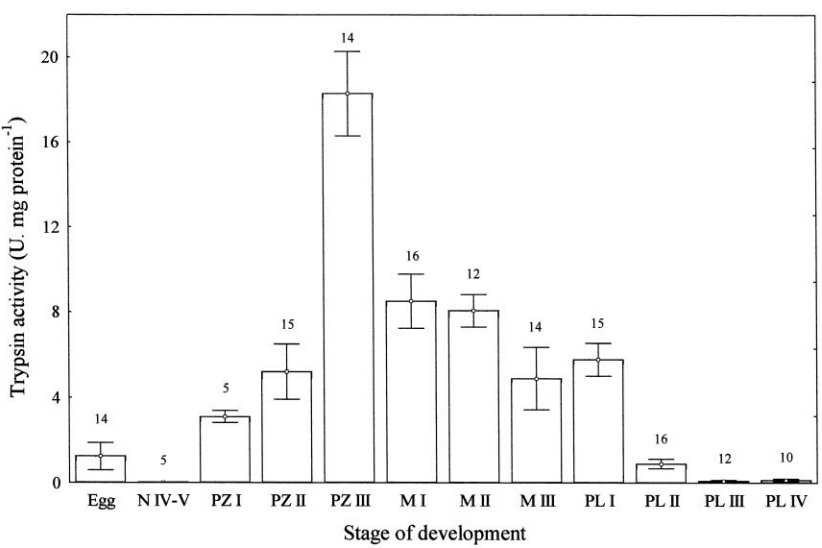

Fig. 2. Trypsin activity using TAME as substrate, during larval and postlarval development of *L. schmitti*. E = egg, N = nauplius, PZ = protozoea, M = mysis and PL = postlarva. Error bars = standard deviation. The number of activity assays for each stage is denoted above the error bars. Eggs at the early nauplius embrionary stage according to Primavera and Posadas (1981). Activity was expressed as U mg protein^{-1} based on an extinction coefficient of 0.54 cm^2 μmol^{-1} for the substrate TAME (Rick, 1984).

digestive potential through larval development may be a clue to assess optimal nutritional conditions for cultured penaeid species (Jones et al., 1997).

As observed for enzyme activity, the characteristics of digestive proteinases may also vary during the early stages of penaeid species (Fang and Lee, 1992; Lemos et al., 1999). The number of enzymes, isoenzymes and their molecular weight, as demonstrated by substrate-SDS-PAGE, varies significantly through ontogenetic development of *F. paulensis* (Lemos et al., 1999) (Fig. 3). Intense activity zones of 16.4 to 19.5 kDa were observed in protozoea II and mysis I. The number of active bands (14.6, 16.4, 17.5, 19.5, 22.5, 23.9, 25.8, 28.9, 32.0, 34.4, 37.7 and 42.2 kDa) was higher in adults compared to early stages and may be due to an increase in cellular and physiological complexity of the digestive gland during development (Morgan et al., 1978; Biesiot and Capuzzo, 1990; Dall et al., 1990). The characterization of digestive enzymes involves determining their specificity and kinetic properties. The importance of a given enzyme to shrimp digestion can be quantitatively assessed after its identification and characterization. For aquaculture purposes, the variation in the proteinase pattern between postlarva and adult (Fig. 3) shows these stages may have different nutritional requirements, and possibly a need for specific feeds.

Trypsin and chymotrypsin fractions can be identified in SDS-PAGE by using synthetic proteinase inhibitors (Fig. 4). However, the proteinases of *F. paulensis* were not inhibited by tosyl-phenylalanine chloromethyl ketone, lane 3 (TPCK), an inhibitor of mammalian chymotrypsin (Fig. 4). TLCK is a trypsin inhibitor and PMSF is a specific serine proteinase inhibitor. Since trypsin and chymotrypsin are serine proteinases, active bands inhibited by both TLCK and PMSF were considered as trypsin while the inhibition by PMSF only may be an evidence of chymotrypsin. Four trypsins from 14.6

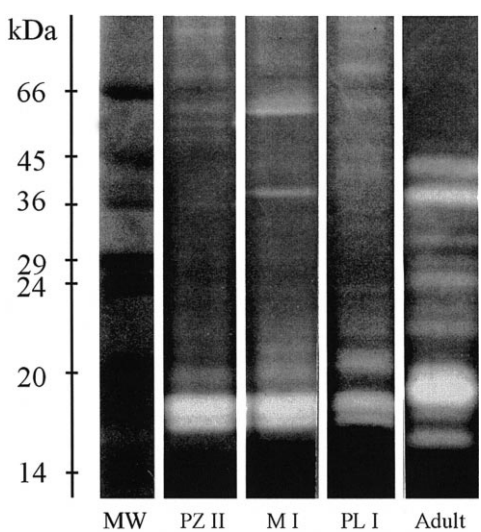

Fig. 3. Digestive proteinases of different ontogenetic stages of *Farfantepenaeus paulensis* in substrate-SDS-PAGE (Lemos et al., 1999). MW = molecular weight markers, Abbreviations as in Fig. 2.

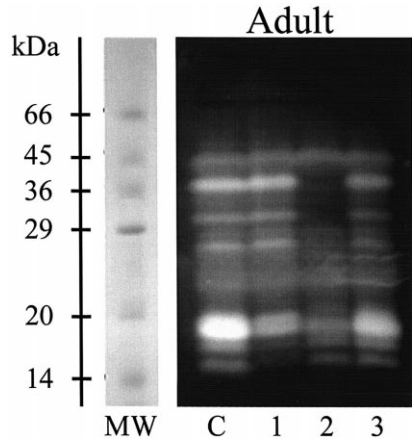

Fig. 4. Digestive proteinases from *F. paulensis* adult hepatopancreas in substrate-SDS-PAGE (Lemos et al., 1999). MW = molecular weight markers; P = protein pattern. C, 1, 2, and 3 lanes denote the activity pattern of the samples incubated with different inhibitors as follows: C, control (without inhibitor); 1, TLCK; 2, PMSF; 3, TPCK. Other abbreviations are as in Fig. 2.

to 21.7 kDa (lane 1) and three possible chymotrypsins of 28.9, 32 and 37.7 kDa (lane 2) were found in adult *F. paulensis* (Fig. 4). Their molecular weight is similar to that reported for *P. monodon* trypsin (Jiang et al., 1991) and *L. vannamei* chymotrypsin (Hernández-Cortés et al., 1997).

The nutritional requirements of penaeid species are quite variable (Tacon, 1987; Akiyama et al., 1992). Nevertheless, the bulk of studies on penaeid nutrition has been focused on the most widely cultured species such as *M. japonicus*, *P. monodon* and *L. vannamei* (García, 1996). The success in culturing these species is mostly based on the availability of suitable formulated feeds that yield satisfactory growth. When growing alternative penaeid species, farmers depend on the use of standard diets that have not been developed according to the species nutritional needs (Akiyama et al., 1992). The different characteristics of digestive proteinases from *F. californiensis*, *L. vannamei*, *F. paulensis*, and *L. schmitti* (Fig. 5) are evidences of a species-specific pattern of protein digestion. A common feature among these species is the occurrence of enzymes with molecular mass equal to or lower than 20 kDa. *F. californiensis* (lane 2) exhibited a higher number of low molecular weight enzymes than the other species, while in *L. vannamei* (lane 3), intense bands are observed between 20 and 29 kDa. Few activity zones heavier than 29 kDa were detected. Because of the diverse proteinase pattern among these species, different properties related to the digestion of feed protein fraction may be expected.

The lack of nutritional information has limited the number of potential species for shrimp culture. As an example, several Brazilian shrimp farmers have chosen the exotic *L. vannamei* instead of autochthonous species because of its better growth performance. The availability of a suitable commercial feed for *L. vannamei* has contributed to the adoption of this species. On the other hand, the cultivation of the native *F. paulensis* in

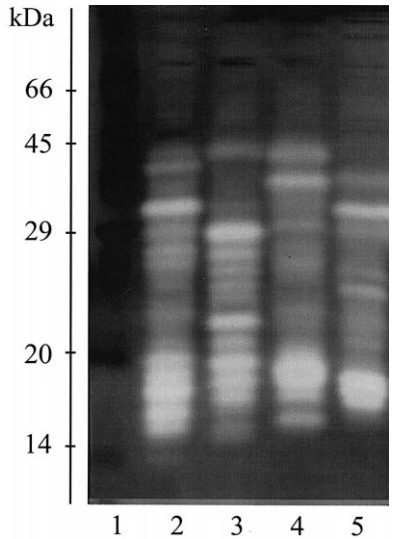

Fig. 5. Digestive proteinases from the hepatopancreas of some penaeid species in substrate-SDS-PAGE. 1 = Molecular weight markers, 2 = *F. californiensis*, 3 = *L. vannamei*, 4 = *F. paulensis*, and 5 = *L. schmitti*.

southern Brazil has decreased drastically because of the lack of adequate feeds to sustain profitable production (Beltrame, personal communication).

Nutritional background is an important issue for the choice of shrimp feeds (Akiyama et al., 1992; Lee and Lawrence, 1997). Some regionally available feed ingredients as plant seed and fish meals may be an inexpensive source of protein for diet formulation. However, these ingredients may contain antinutritional factors that inhibit digestive enzyme activity, decreasing growth rates (Garcia-Carreño, 1996), or may be of poor quality because of the handling and processing of the raw material (Garcia-Carreño, 1998). The proteinaceous proteinase inhibitor from soybean (soybean trypsin inhibitor [SBTI]) can be detected and characterized by substrate-SDS-PAGE (Fig. 6). The inhibition of trypsin activity seems to be proportional to the amount of SBTI assayed. This inhibitor can reduce trypsin activity and weight gain in the Atlantic salmon in a dose-dependent manner (Olli et al., 1994). Meals made from some legume seeds also seem to inhibit the degree of protein hydrolysis by the digestive gland extract of *F. californiensis* and *L. vannamei* (Garcia-Carreño et al., 1997). Measuring the DH by the digestive enzymes helps to assess the potential digestibility of a particular feedstuff (Dimes et al., 1994). The in vitro results of DH should positively correlate with in vivo measurements of APD in order to validate the method (Lee and Lawrence, 1997; Ezquerra et al., 1998). The DH by the pH-stat method of some feed ingredients by *L. vannamei* digestive gland extract may vary from 23.2% (tuna waste meal) to 33.99% (deboned white fish meal) (Table 2). The low digestibility of some fish meals has been attributed to excessive heat treatment which reduces the nutritional value of dietary protein by causing cross-linking reactions or amino acid racemization (Garcia-Carreño, 1996, 1998; Ezquerra et al., 1997b). In vitro measurements of the DH of some prepared

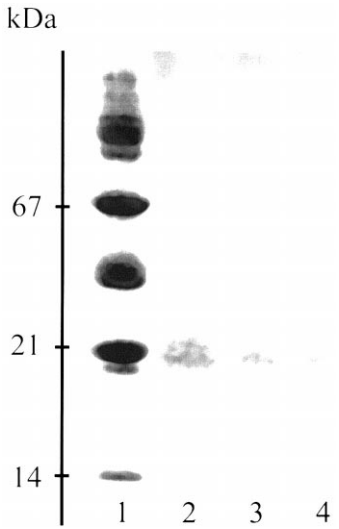

Fig. 6. Substrate-SDS-PAGE for the proteinaceous proteinase inhibitor SBTI (Garcia-Carreño et al., 1993). MW = molecular weight markers, 1 = 12 μg SBTI, 2 = 120 ng SBTI, 3 = 12 ng SBTI. After electrophoresis, the gel was incubated in 50 mM Tris buffer, pH 7.5, containing 0.1 mg l^{-1} porcine trypsin for 30 min at 5°C. Then, the gel was washed, assayed for casein hydrolysis and stained as described in Section 2.

feeds by *L. vannamei* correlated ($P < 0.05$) with APD of shrimp fed these diets (Fig. 7). These results indicate that in vitro DH determination may be a useful tool in estimating the biological value of alternative protein sources for shrimp diets (Ezquerra et al., 1998).

The sustainability of the shrimp farming industry greatly depends on the market price reached by the product (Shang, 1992). The physiological state of farmed animals prior to harvest may affect their quality during storage or processing (Haard, 1992). Digestive and muscle enzymes may contribute to the loss of seafood quality. Autolysis of muscle

Table 2
Degree of hydrolysis of some feed ingredients by *L. vannamei* hepatopancreas extract, and trypsin and chymotrypsin activities of *L. vannamei* fed different protein sources (from Ezquerra et al., 1997b, 1998). Results expressed as mean ± s.d.

Test ingredients	DH% at 25°C	Trypsin activity (U mg protein^{-1})	Chymotrypsin activity (U mg protein^{-1})
Deboned white fish meal	33.99 ± 0.45	0.1 ± 0.02	0.5 ± 0.04
Mexican tuna waste meal	23.20 ± 0.21	0.1 ± 0.04	0.6 ± 0.07
Chilean anchovy meal	31.22 ± 0.27	0.1 ± 0.02	0.7 ± 0.16
Langostilla meal	28.22 ± 0.43	0.1 ± 0.02	0.5 ± 0.19
Menhaden fish meal A	25.32 ± 0.23	0.1 ± 0.01	0.6 ± 0.05
Menhaden fish meal B	28.60 ± 0.10	0.2 ± 0.04	0.8 ± 0.09
Soybean protein	31.13 + 0.10	0.1 ± 0.02	0.7 ± 0.16

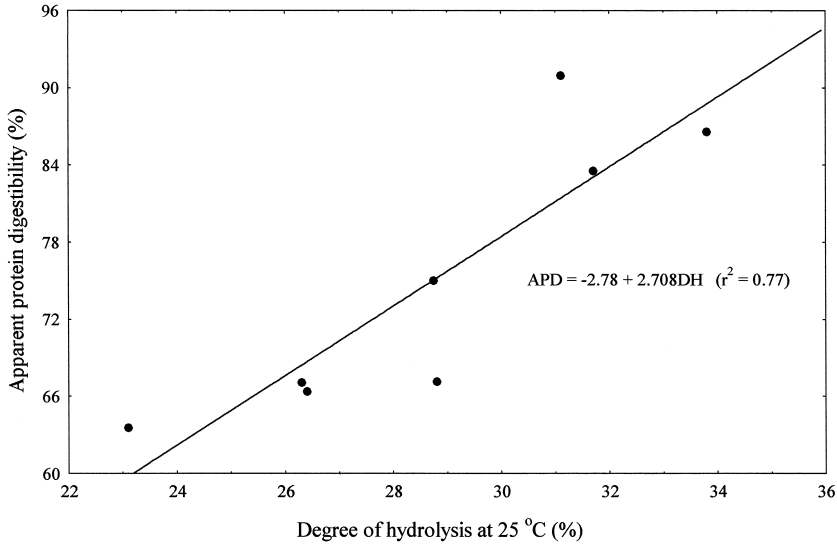

Fig. 7. Relationship between APD and DH of some meal samples (Table 2) in juveniles *L. vannamei* (Ezquerra et al., 1998). DH measured by the pH-stat method at 25°C. APD was determined by the chromic oxide method (Cho and Slinger, 1979; Tacon and Rodrigues, 1984). Significant correlation at $P < 0.05$.

protein in crustacean seafood occurs rapidly after harvest and has been attributed to digestive proteases (Salem et al., 1970). As observed in other cultured animals, the activity of shrimp digestive proteinases seems to be stimulated by low quality feeds (Lee and Lawrence, 1986; Rodríguez et al., 1994; Le Moullac et al., 1996; Lemos and Rodríguez, 1998). Thus, the supply of a low nutritional quality feed may reduce the shelf life of shrimp (Nip et al., 1985), decreasing its trade value. Both trypsin and chymotrypsin of *L. vannamei* had higher activities when fed with a low quality protein meal (menhaden B) when compared to other feeds (Table 2). The low nutritional value of feeds containing menhaden B meal may be due to the heat-treatment during drying which decreased the amount of some essential amino acids available in the feed (Ezquerra et al., 1997b). In shrimp farming, the use of low quality feeds may result in lower growth rates but also in the reduction of the product storage life.

Penaeid nutritional background is scarce and few research projects are devoted to understanding the biochemical capabilities of shrimp during digestion. So far, most of the proteinases in the shrimp digestive system have been described and some have been characterized (Maugle et al., 1982; Van Wormhoudt et al., 1992; Hernández-Cortés et al., 1997). There is evidence that some of these proteinases are translated as zymogens (Sellos and Van Wormhoudt, 1992). However, the zymogen has never been isolated. Recently, an inhibitor for trypsin was found in *L. vannamei* digestive gland (Garcia-Carreño et al., 1999). There is a lack of information about the transcription and translation processes as well as the mechanisms of regulation of both processes. It is unclear if the enzymes are constitutive or inducible, how many mRNAs are coded and their half-life, and how many proteins are translated by any mRNA. Also, posttrans-

lational processes such as zymogen activation and enzyme activity regulation remain unclear. The development of techniques devoted to evaluating the proteinase activity and composition in digestive systems, the presence and effect of antinutritional factors in feedstuff, and the evaluation of protein digestibility allows a closer evaluation of the feeds and the ability of shrimp to digest feed protein. The knowledge on the tools used by the organisms to digest protein can lead to the adequate formulation of feeds. Now, it is a simple task to predict the digestibility of a proteinaceous ingredient or a feed before being used in aquafeeds. With the development of the so-called second generation protein ingredients (Garcia-Carreño, 1998), the evaluation in the laboratory of such ingredients, and the feeds made using them, a lot of evaluations are expected. By using the in vitro technique to evaluate digestibility, a lot of labor may be saved.

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References

Adler-Nissen, J., 1986. Enzymic Hydrolysis of Food Protein. Elsevier, London, 427 pp.

Akiyama, D.M., 1991. Soybean meal utilization by marine shrimp. In: Akiyama, D.M., Tan, R.K.H. (Eds.), Proceedings of the Aquaculture Feed Processing and Nutrition Workshop, Thailand and Indonesia, September 19–25. American Soybean Assoc., Singapore, pp. 207–225.

Akiyama, D.M., Dominy, W.G., Lawrence, A.L., 1992. Penaeid shrimp nutrition. In: Fast, A.W., Lester, L.J. (Eds.), Marine Shrimp Culture: Principles and Practices. Elsevier, Amsterdam, pp. 535–568.

Baranoski, E.S., Nip, W.K., Moy, J.H., 1984. Partial characterization of a crude enzyme extract from the freshwater prawn, *Macrobrachium rosembergii*. J. Food Sci. 49, 1494–1501.

Beynon, R.J., Salvesen, G., 1989. Commercially available protease inhibitors. In: Beynon, R.J., Bond, J.S. (Eds.), Proteolytic Enzymes: A Practical Approach. IRL Press, Oxford, pp. 241–249.

Biesiot, P.M., Capuzzo, J.M., 1990. Changes in digestive enzyme activities during early development of the American lobster *Homarus americanus* Milne Edwards. J. Exp. Mar. Biol. Ecol. 136, 107–122.

Castille, F.L., Samocha, T.M., Lawrence, A.L., He, H., Frelier, P., Jaenike, F., 1993. Variability in growth and survival of postlarval shrimp (*Penaeus vannamei* Boone 1931). Aquaculture 113, 65–81.

Cho, C.Y., Slinger, S.J., 1979. Apparent digestibility measurement in feedstuffs for rainbow trout. In: Halver, J.E., Trews, K. (Eds.), Finfish Nutr. Fishfeed Technol. Vol. 2 Heinemann, Berlin, pp. 241–245.

Chu, K.H., Ovsianico-Koulikowsky, N.N., 1994. Ontogenetic changes in metabolic activity and biochemical composition in the shrimp, *Metapenaeus ensis*. J. Exp. Mar. Biol. Ecol. 183, 11–26.

Cushing, B.H., 1975. Marine Ecology and Fisheries. Cambridge Univ. Press, 252 pp.

Dall, W., Hill, B.J., Rothlisberg, P.C., Staples, D.J., 1990. The biology of Penaeidae. Adv. Mar. Biol. 27, 1–489.

DelMar, E.G., Largman, C., Brodrick, J.W., Geokas, M.C., 1979. A sensitive new substrate for chymotrypsin. Anal. Biochem. 99, 316–320.

Dimes, L.E., Haard, N.F., 1994. Estimation of protein digestibility: I. Development of an in vitro method for estimating protein digestibility in salmonids (*Salmo gairdneri*). Comp. Biochem. Physiol. 108A, 349–362.

Dimes, L.E., Haard, N.F., Dong, F.M., Rasco, B.A., Forster, F.T., Fairgrieve, W.T., Arndt, R., Hardy, R.W., Barrows, F.T., Higgs, D.A., 1994. Estimation of protein digestibility: II. In vitro assay of protein in salmonid feeds. Comp. Biochem. Physiol. 108A, 363–370.

Emmerson, W.D., 1980. Ingestion, growth and development of *Penaeus indicus* larvae as a function of *Thalassiosira weissflogii* cell concentration. Mar. Biol. 58, 65–73.

Erlanger, B., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.

Ezquerra, J.M., Garcia-Carreño, F.L., Carrillo, O., 1998. In vitro digestibility of protein sources for white shrimp (*Penaeus vannamei*). Aquaculture 163, 123–136.

Ezquerra, J.M., Garcia-Carreño, F.L., Civera, R., Haard, N.F., 1997a. pH-stat method to predict digestibility in white shrimp (*Penaeus vannamei*). Aquaculture 157, 249–260.

Ezquerra, J.M., Garcia-Carreño, F.L., Haard, N.F., 1997b. Effects of feed diets on digestive proteases from the hepatopancreas of white shrimp (*Penaeus vannamei*). J. Food Biochem. 21, 401–419.

Fang, L.-S., Lee, B.-N., 1992. Ontogenetic change of digestive enzymes in *Penaeus monodon*. Comp. Biochem. Physiol. 103B, 1033–1037.

Fernández, I., Oliva, M., Carrillo, O., Van Wormhoudt, A., 1997. Digestive enzyme activities of *Penaeus notialis* during reproduction and moult cycle. Comp. Biochem. Physiol. 118A, 1267–1271.

Galgani, M.L., Benyamin, Y., Ceccaldi, H.J., 1984. Identification of digestive proteinases of *Penaeus kerathurus* (Forskal): a comparison with *Penaeus japonicus*. Comp. Biochem. Physiol. 78B, 355–361.

Galgani, M.L., Benyamin, Y., Van Wormhoudt, A., 1985. Purification, properties and immunoassays of trypsin from the shrimp *Penaeus japonicus*. Comp. Biochem. Physiol. 81B, 447–452.

Gallardo, P.P., Alfonso, E., Gaxiola, G., Soto, L.A., 1995. Feeding schedule for *Penaeus setiferus* larvae based on diatoms (*Chaetoceros ceratosporum*), flagellates (*Tetraselmis chuii*) and *Artemia* nauplii. Aquaculture 131, 239–252.

García, T.G., 1996. Estado del arte de la investigación científica en nutrición de peneidos. In: Calderón, J., Magallon, F., Andreatta, E., Sanchez, R. (Eds.), Memorias del taller ''La Investigación Científica en Camarones Peneidos de Iberoamerica''. CENAIM-CYTED, Guayaquil, Ecuador, pp. 45–57.

Garcia-Carreño, F.L., 1992a. The digestive proteases of langostilla (*Pleuroncodes planipes*, decapoda): their partial characterization, and the effect of feed on their composition. Comp. Biochem. Physiol. 103B, 575–578.

Garcia-Carreño, F.L., 1992b. Protease inhibition in theory and practice. Biotechnol. Educ. 3, 145–150.

Garcia-Carreño, F.L., 1996. Proteinase inhibitors. Trends Food Sci. Technol. 7, 197–204.

Garcia-Carreño, F.L., 1998. Prediction of protein digestibility in shrimp and use of second generation protein ingredients in aquaculture feeds. In: IV International Symposium Nutrition in Aquaculture. La Paz, BCS, México, November 15–18. CIBNOR, La Paz, BCS, Mexico, Conference.

Garcia-Carreño, F.L., Carrillo, O., Navarrete del Toro, M.A., 1999. Control of digestive functions in shrimp: I. An inhibitor of trypsin activity in the hepatopancreas. In: Proceedings of the Fourth International Crustacean Congress, Amsterdam, The Netherlands, July 20–24. Schram, F.R., von Vaupel Klein, J.C. (Eds.), Crustaceans and the Biodiversity Crisis Vol. I Brill, Leiden.

Garcia-Carreño, F.L., Dimes, L.E., Haard, N.F., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. Anal. Biochem. 214, 65–69.

Garcia-Carreño, F.L., Haard, N.F., 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J. Food Biochem. 17, 97–113.

Garcia-Carreño, F.L., Hernández-Cortés, M.P., Haard, N.F., 1994. Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and a marine decapod. J. Agric. Food Chem. 42, 1456–1461.

Garcia-Carreño, F.L., Navarrete del Toro, A., Ezquerra, J.M., 1997. Digestive shrimp proteases for evaluation of protein digestibility in vitro: I. Effect of protease inhibitors in protein ingredients. J. Mar. Biotechnol. 5, 36–40.

Haard, N.F., 1992. Protein hydrolysis in seafoods. In: Shahidi, F., Botta, J.R. (Eds.), Seafoods: Chemistry, Processing Technology and Quality. Chapman & Hall, London, pp. 10–33.

Hernández-Cortés, M.P., Whitaker, J., Garcia-Carreño, F.L., 1997. Purification and characterization of chymotrypsin from *Penaeus vannamei* (Crustacea: Decapoda). J. Food Biochem. 21, 497–514.

Jiang, S.T., Moody, M.W., Chen, H.C., 1991. Purification and characterization of proteases from digestive tract of grass shrimp (*Penaeus monodon*). J. Food Sci. 56, 322–326.

Jones, D.A., Kumlu, M., Le Vay, L., Fletcher, D.J., 1997. The digestive physiology of herbivorous, omnivorous and carnivorous crustacean larvae: a review. Aquaculture 155, 285–295.

Kawamura, Y., Nishimura, K., Igrashi, S., Doi, E., Yonezawa, D., 1981. Characteristics of autolysis of Antarctic krill. Agric. Biol. Chem. 45, 93–100.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227, 680–685.

Laubier-Bonichon, A., Van Wormhoudt, A., Sellos, D., 1977. Croissance larvaire contrôlée de *Penaeus japonicus* Bate: enzymes digestives et changements de regimes alimentaires. Publs. Cent. Natn. Exploit Océans (CNEXO) Sér. Act. Colloques 4, 131–145.

Lee, P.G., Lawrence, A.L., 1986. Effects of diet and size on growth, feed digestibility and digestive enzyme activities of the marine shrimp, *Penaeus setiferus* Linnaeus. J. World Maric. Soc. 16, 275–287.

Lee, P.G., Lawrence, A.L., 1997. Digestibility. In: D'Abramo, L.R., Conklin, D.E., Akiyama, D.M. (Eds.), Crustacean Nutrition, Advances in World Aquaculture Vol. 6 World Aquaculture Society, Baton Rouge, LA, pp. 194–260.

Lee, P.G., Smith, L.L., Lawrence, A.L., 1984. Digestive proteases of *Penaeus vannamei* Boone: relationship between enzyme activity, size and diet. Aquaculture 42, 225–239.

Lemos, D., Hernández-Cortés, M.P., Navarrete, A., Garcia-Carreño, F.L., Phan, V.N., 1999. Ontogenetic variation in digestive proteinase activity of larval and postlarval shrimp *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeidae). Mar. Biol. 135, 653–662.

Lemos, D., Rodríguez, A., 1998. Nutritional effects on body composition, energy content and trypsin activity of *Penaeus japonicus* during early postlarval development. Aquaculture 160, 103–116.

Le Moullac, G., Klein, B., Sellos, D., Van Wormhoudt, A., 1996. Adaptation of trypsin, chymotripsin and α-amylase to casein level and protein source in *Penaeus vannamei* (Crustacea Decapoda). J. Exp. Mar. Biol. Ecol. 208, 107–125.

Le Moullac, G., Roy, P., Van Wormhoudt, A., 1992. Effects of trophic prophylactic factors on some digestive enzymatic activities of *Penaeus vannamei* larvae. In: Calderón, J., Sandoval, V. (Eds.), Memorias del Primer Congreso Ecuatoriano de Acuicultura. CENAIM, San Pedro de Manglaralto, Ecuador, pp. 81–86.

Lester, L.J., 1992. Overview of shrimp farming in the western hemisphere. In: Fast, A.W., Lester, L.J. (Eds.), Marine Shrimp Culture: Principles and Practices. Elsevier, Amsterdam, pp. 771–782.

Lovett, D.L., Felder, D.L., 1989. Ontogeny of gut morphology in the white shrimp *Penaeus setiferus* (Decapoda Penaeidae). J. Morphol. 201, 253–272.

Lovett, D.L., Felder, D.L., 1990. Ontogenetic change in digestive enzyme activity of larval and postlarval white shrimp *Penaeus setiferus* (Crustacea Decapoda, Penaeidae). Biol. Bull. 178, 144–159.

Loya-Javellana, G., 1989. Ingestion saturation and growth responses of *Penaeus monodon* larvae to food density. Aquaculture 81, 329–336.

Maugle, P.D., Deshimaru, O., Katayama, T., Simpson, K.L., 1982. Characteristics of amylase and protease of the shrimp *Penaeus japonicus*. Bull. Jpn. Soc. Sci. Fish. 48, 1753–1757.

Morgan, R.P., Kramarsky, E., Sulkin, S.D., 1978. Biochemical changes during larval development of the xanthid crab, *Rithropanopeus harrisii*: III. Isozyme changes during ontogeny. Mar. Biol. 48, 223–226.

Nip, W.K., Lan, C.Y., Moy, J.H., 1985. Partial characterization of a collagenolytic enzyme fraction from the hepatopancreas of the freshwater prawn, *Macrobrachium rosembergii*. J. Food Sci. 50, 1178–1187.

Olli, J., Hjelmeland, K., Krodahl, 1994. Soybean trypsin inhibitor in diets for atlantic salmon (*Salmo salar*, L): effects on nutrients digestibilities and trypsin in pyloric caeca homogenate and intestinal content. Comp. Biochem. Physiol. 109A, 923–928.

Pedersen, B., Eggum, B.O., 1983. Prediction of protein digestibility — an in vitro enzymatic pH-stat procedure. Tierphysiol. Tieternahrg u Futtermittelkde 49, 277–286.

Pérez-Farfante, I., Kensley, B.F., 1997. Penaeoid and sergestoid shrimps and prawns of the world. Keys and diagnoses for the families and genera. Mém. Mus. Natn. Hist. Nat. Paris 175, 1–233.

Primavera, J.H., Posadas, R.A., 1981. Studies on the egg quality of *Penaeus monodon* Fabricius, based on morphology and hatching rates. Aquaculture 22, 269–277.

Rick, W., 1984. Trypsin: measurement with *N*α-toluenesulfonyl-L-arginine methyl ester as substrate. In: 2nd edn. Bergmeyer, H.U. (Ed.), Methods Enzym. Anal. Vol. 2 Academic Press, New York, pp. 1021–1024.

Rodríguez, A., Le Vay, L., Mourente, G., Jones, D.A., 1994. Biochemical composition and digestive enzyme activity in larvae and postlarvae of *Penaeus japonicus* during herbivorous and carnivorous feeding. Mar. Biol. 118, 45–51.

Salem, H., Youssef, A.M., El-Nakkadi, A.M.N., Bekeit, M., 1970. Proteolytic decomposition of shellfish muscle proteins under different conditions. Alex J. Agric. Res. 8, 61–66.

Sarac, Z., Thaggard, H., Saunders, J., Gravel, M., Neill, A., Cowan, R.T., 1993. Observations on the chemical composition of some commercial prawn feeds and associated growth responses in *Penaeus monodon*. Aquaculture 115, 97–110.

Sellos, D., Van Wormhoudt, A., 1992. Molecular cloning of a cDNA that encodes a serine protease with chymotryptic and collagenolytic activities in the hepatopancreas of the shrimp *Penaeus vannamei* (Crustacea, Decapoda). FEBS Lett. 309, 219–224.

Shang, Y.C., 1992. Penaeid markets and economics. In: Fast, A.W., Lester, L.J. (Eds.), Marine Shrimp Culture: Principles and Practices. Elsevier, Amsterdam, pp. 589–604.

Smith, D.M., Dall, W., Moore, L.E., 1992. The natural food of some Australian penaeids. In: Allan, G.L., Dall, W. (Eds.), Proc. Aquaculture Nutrition Workshop. NSW Fisheries, Salamander Bay, Australia, pp. 95–96.

Sudaryono, A., Hoxey, M.J., Kailis, S.G., Evans, L.H., 1995. Investigation of alternative protein sources in practical diets for juvenile shrimp, *Penaeus monodon*. Aquaculture 134, 313–323.

Sudaryono, A., Tsvetnenko, E., Hutabarat, J., Evans, L.H., 1999. Lupin ingredients in shrimp (*Penaeus monodon*) diets: influence of lupin species and types of meals. Aquaculture 177, 121–133.

Tacon, A., 1987. The nutrition and feeding of farmed fish and shrimp — a training manual: 1.The essential nutrients. FAO, United Nations, Rome, Italy.

Tacon, A., Rodrigues, A.M.P., 1984. Comparison of chromic oxide, crude fibre, polyethylene and acid-insoluble ash as dietary markers for the estimation of apparent digestibility coefficients in rainbow trout. Aquaculture 43, 391–399.

Tsai, I.H., Chuang, K.L., Chuang, J.L., 1986. Chymotrypsins in digestive tracts of crustacean decapods (shrimps). Comp. Biochem. Physiol. 85, 235–240.

Van Wormhoudt, A., 1973. Variations des protéases, des amylases et des protéines soluble au curs du développement larvaire chez *Palaemon serratus*. Mar. Biol. 19, 245–248.

Van Wormhoudt, A., Le Chevalier, P., Sellos, D., 1992. Purification, biochemical characterization and *n*-terminal sequence of a serine-protease with chymotrypsic and collagenolytic activities in a tropical shrimp, *Penaeus vannamei* (Crustacea Decapoda). Comp. Biochem. Physiol. 103B, 675–680.