The effect of proteinase inhibitors in food protein hydrolysis by digestive proteinases of white shrimp (*Penaeus vannamei*) larvae

Francisco J Alarcón,[1]* Carmen de Oña,[1] Manuel Díaz,[1] Fernando L García-Carreño,[2] Francisco J Moyano[1] and María A Navarrete del Toro[2]

[1]*Dpto. Biología Aplicada, Escuela Politécnica Superior, CITE II-B. Universidad de Almería, La Cañada S. Urbano, 04120-Almería, Spain*
[2]*Centro de Investigaciones Biológicas del Noroeste (CIBNOR), PO Box 128, La Paz, BCS, 23000 México*

Abstract: This study describes the digestive capacities of 10-day-old white shrimp postlarvae (PL10) and how some inhibitors of proteinases affect the digestion of protein in aquafeeds by using *in vitro* hydrolysis techniques. Biochemical data showed eight active proteinases in the PL10 hepatopancreas extract. Enzymes belong to the metallo- and serine-proteinase classes. The effect of inhibitors present in protein ingredients and aquafeeds on PL10 proteinases showed that ovalbumin alone and in commercial microcapsules yielded a significant inhibition in proteolytic activity of PL10 hepatopancreas enzymes. The capacity of PL10 proteinases to hydrolyse the protein fraction within different sources and microcapsules was demonstrated by two *in vitro* approaches, the pH-stat (degree of hydrolysis, DH) and electrophoresis (coefficient of protein degradation, CPD). It was shown that PL10 proteinases hydrolyse, in different extent protein sources and microcapsules. Casein, cuttlefish meal and feeds containing these ingredients are quickly hydrolysed. By contrast, ovalbumin and microcapsules containing ovalbumin are not hydrolysed.

Keywords: digestive enzymes; *Penaeus vannamei*; postlarvae; proteinase inhibitor; pH-stat; SDS-PAGE; white shrimp

INTRODUCTION

The Pacific white shrimp, *Penaeus vannamei*, is the second most important aquafarmed penaeid species. With the development and expansion of production, processing and international marketing, farmed shrimp quality is becoming increasingly important. Aquaculture production of penaeid shrimp larvae is constrained by costly and unreliable supplies of live food, especially *Artemia nauplii*. Despite Japanese and European companies attempts to produce high quality larvae feed commercially, a complete replacement for *Artemia* is not yet possible. For this reason, larvae production constitutes a bottleneck for shrimp production. Microcapsulated feeds have been developed to replace live food. One of the most important questions in the formulation of inert feeds is the quality of the raw materials, mainly the quality of protein.[1] In addition to the amino acid composition, quality involves digestibility, which is dependent on the presence and amount of antinutritional factors such as proteinase inhibitors. So, one limitation in choosing a feedstuff for aquafeeds is the presence of enzyme inhibitors. Inhibitors can affect several proteinase classes, such as serine-, cysteine-, metallo- and acid proteinases.[2] The effect of inhibitors on the shrimp digestive system needs to be studied in order to determine possible adverse side effects of the feedstuff. Likewise, a detection procedure for proteinase inhibitors in raw materials should be included in the quality control of shrimp-feed production.

Since biological experiments are both expensive and time-consuming, and yield results that are only approximate, many authors have sought for appropriate laboratory methods to evaluate protein quality.[3,4] Dimes *et al*.[5] developed an *in vitro* method using enzyme fractions from trout pyloric caeca to estimate the degradation of protein using a pH-stat assay. The use of *in vitro* assays in aquaculture nutrition provides advantages in both economy and animal well-being because fewer animals and facilities are needed.

The crustacean hepatopancreas produces and releases several enzymes into the digestive tract, including proteinases and peptidases.[6,7] Proteinases from shrimp hepatopancreas have been used to evaluate the *in vitro* protein degradation of ingredients for aquafeeds.[4,8]

* Correspondence to: Francisco J Alarcón, Dpto. Biología Aplicada, Escuela Politécnica Superior, CITE II-B, Universidad de Almería, La Cañada S. Urbano, 04120-Almería, Spain
E-mail: falarcon@ual.es
Contract/grant sponsor: Ministerio de Educación y Cultura, Spain
(*Received 21 February 2005; revised version received 8 November 2005; accepted 15 August 2006*)
Published online 11 October 2006; DOI: 10.1002/jsfa.2686

In this study, the overall objective was achieved by following three specific aims: (1) to determine the enzymes involved in the digestion of protein by the PL10, (2) to detect the presence of inhibitors for proteinases in food ingredients and in microcapsules, and (3) to evaluate the kinetics of protein degradation by PL10 digestive enzymes, by using both pH-stat and electrophoresis analysis.

MATERIALS AND METHODS

Reagents

All reagents were supplied by Sigma Chemical Co. (St Louis, MO, USA).

Larvae culture and sampling

Postlarvae of *Penaeus vannamei* (stage PL10) were obtained from a commercial hatchery (NAUPLIO SA de CV, La Paz, BCS, México). From PL1 to PL10, only live *Artemia nauplii* were supplied to the culture. PL10 were collected from the experimental tanks and anaesthetised in an ice-cold bath. A sample of 250 hepatopancreas of postlarvae was obtained by dissection under a binocular microscope. Postlarvae bodies were separated into cephalothorax and abdomen segments, and the abdominal portions discarded. Whole cephalothorax was considered representative of hepatopancreas tissue since at this developmental stage the hepatopancreas approximately represents 80% of the cephalothorax weight and the reproductive organs are not developed yet. Samples of hepatopancreas were washed in distilled water and then freeze-dried for the determination of dry weight and stored at −20 °C until use.

Sample preparation

Extracts of PL10 hepatopancreas were obtained by homogenising organs in 0.05 mol L^{-1} Tris.HCl buffer, 0.01 mol L^{-1} $CaCl_2$, pH 7.0. Homogenates were centrifuged for 15 min at 12 000 × *g* and 4 °C using a refrigerated EBA 12R Hettich centrifuge (Tuttlingen, Germany) with a rotor for Eppendorf tubes (radius = 92 mm). The supernatant was kept at −20 °C until use. Total protein of the crude enzyme extract was determined by the Bradford technique.[9]

Proteins and microcapsules

Table 1 shows the protein sources and microcapsules used in the study.

Table 1. Protein content in raw sources and microcapsules (mean ± SD)

Protein		Crude protein (%)
Casein[a]		90.5 ± 1.9
Hydrolysed krill meal[b]		49.7 ± 2.4
Cuttlefish meal[c]		73.7 ± 1.3
Ovalbumin[d]		89.3 ± 0.7
Microcapsules (MC)		**Composition**
MC 1[e]	Hydrolysed marine protein	52.1 ± 1.7
MC 2[f]	Ovalbumin, mainly	51.8 ± 0.6
MC 3[g]	Casein and fish meal	61.4 ± 0.9
MC 4[h]	Casein, cuttlefish meal, and fish meal	60.7 ± 1.3

[a] Hammerstein casein purchased from ICN Biomedical (Aurora, Ohio).
[b] From Biozyme Systems Inc., Vancouver, Canada.
[c] Provided by Dr J.P. Cañavate, CICEM ''El Toruño'', Cádiz, Spain.
[d] Sigma Chemical (St Louis, MO).
[e] Artificial diet for shrimp from Inve Aquaculture, Dendermonde, Belgium.
[f] Artificial diet for shrimp from Nippon Formula Feed Manufacturing Co Ltd, Kanagawa, Japan.
[g,h] Laboratory-made artificial diet for shrimp provided by Dr M.Yúfera, ICMA, Cádiz, Spain.

Proteinolytic activity in PL10 extracts

Total proteinase activity of extracts was assayed in test tubes using a modification of the procedure described by García-Carreño and Haard.[6] The main modification of the original method was to omit the step of making the stopped reaction mixture alkaline with NaOH 0.1 mol L^{-1} because it simplifies the assay and makes it possible to use light at 366 nm instead of 410 nm to measure the azo-peptides liberated during enzymatic reaction. In brief, 20 μL of the enzyme preparation was mixed with 0.5 mL of 0.1 mol L^{-1} Tris.HCl, pH 7.0 at 25 °C. Reaction was initiated by the addition of 0.5 mL of 20 g L^{-1} azocasein and stopped 30 min later by adding 0.5 mL of 200 g L^{-1} trichloroacetic acid. After 10 min at 4 °C, the reaction mixture was centrifuged at 16 500 × ***g*** for 5 min and the absorbance at 366 nm recorded. For the control, trichloroacetic acid was added before the substrate. Units of proteinase activity (UA) were calculated by using the equation: $UA = \Delta ABS_{366\,nm} \times time^{-1} \times$ volume of $enzyme^{-1}$, where the time is in minutes and the volume of enzyme is in millilitres.

The proteinase classes in shrimp extracts were established by using specific inhibitors following the methods described by Dunn.[10] The enzyme extract (20 μL) was mixed with 0.5 mL of 0.1 mol L^{-1} Tris.HCl, 0.01 mol L^{-1} $CaCl_2$ buffer, pH 7.0 and 10 μL of the inhibitor and incubated for 60 min at 25 °C. The mixture was assayed for proteinase activity as above. The assay included internal controls for inhibition of solvents of inhibitors and enzyme. The percentage of inhibition was established taking the activity without inhibitor as 100%.

Zymograms of proteinase activity were carried out by dissociating discontinuous PAGE following the procedure described by García-Carreño *et al.*[11] Proteinases were visualised as clear bands on a blue background after staining the gel with Coomassie brilliant blue and could be compared to molecular mass standard bands.

Inhibition of proteinases by feedstuffs

Aqueous extracts of protein raw material and microcapsules (100 mg mL^{-1}) were prepared by

shaking for 120 min at room temperature, and for 22 h at 4 °C. Then they were centrifuged for 20 min at 12 000 × ***g*** and 4 °C. The supernatant was stored at 4 °C until use. The effect of extracts on digestive proteinase activity of PL10 was tested according to Alarcón *et al*.[12] Values of inhibition are expressed as a percentage of residual activity, considering the activity of the control as 100%. Controls were made by replacing test substance with distilled water. Dose–response curves were obtained from assays varying the ratio inhibitor/enzyme according to Alarcón *et al*.[12] Such plots were constructed by using two commercial microcapsules, MC 1 and MC 2.

Kinetics of protein hydrolysis

The degree of hydrolysis (DH) of the protein ingredients and protein in aquafeeds was evaluated by pH-stat titration using PL10 enzymes.[13] Five millilitres of an ingredient or feed suspension, containing 40 mg crude protein, was adjusted to pH 8.0 with 0.1 mol L^{-1} NaOH and equilibrated at 37 °C for 10 min in a 10-mL jacketed reaction vessel at 37 °C. Reaction was started by addition of 0.5 mL of the enzyme solution, containing 1.55 units of activity (UA). The evaluation of DH was done in a pH-stat 718 Stat Titrino (Methrom Ion Analysis, Herisau, Switzerland). The DH after 90 min reaction was used as a measure of *in vitro* protein digestibility.[8,14,15] Evaluation of DH values without enzymatic extracts was also made to evaluate potential autohydrolysis in all samples. Each evaluation was made in triplicate.

Analysis of protein hydrolysis by electrophoresis

Along with the DH evaluation, sampling of the reaction mixture was done at intervals (0–5000 s) to assess the progress of hydrolysis of proteins in the following raw materials and microcapsules: casein, cuttlefish meal, ovalbumin, MC 2 (composed mainly of ovalbumin) MC 3 (composed mainly of casein, mainly) and MC 4 (composed mainly of casein and cuttlefish meal). At each time interval samples of the mixture were diluted (1:1) in sample buffer (0.125 mol L^{-1} Tris.HCl, pH 6.8, 100 g L^{-1} β-mercaptoethanol, 100 g L^{-1} glycerol, 2 g L^{-1} bromophenol blue, and 15 g L^{-1} SDS) and quickly boiled for 5 min in order to stop the enzymatic reaction. The samples were stored at −20 °C until analysis by SDS-PAGE according to Laemmli.[16] An amount of 35–40 µg of protein per well was loaded. The progression of the hydrolysis of proteins was evaluated in the electrophoresis gels by lane densitometry using software for image analysis (1-D Manager™, T.D.I., Madrid, Spain). The numerical coefficient of protein degradation (CPD) was obtained according to Alarcón *et al*.[12]

Statistical methods

All experiments were repeated three times with replicas. Data were expressed as mean ± SD. Comparison of means was obtained using ANOVA followed by a Tukey's multicomparison test (CSS-Statistica®. Statsoft Co.; Tulsa, OK, USA). The data were analysed and plotted using the program Microsoft Excel (Microsoft Co., Seattle WA, USA).

RESULTS AND DISCUSSION

Proteinolytic activity in PL10 extracts

The use of artificial feed for larvae has been assessed by a number of researchers.[17,18] To improve aquafeeds recent investigations have focused on larvae digestive enzymes.[19,20] Proteinase activity in enzyme extracts of PL10 is shown in Table 2. The effect of specific inhibitors on the proteinase activity from the hepatopancreas extracts is shown in Table 3. According to the results, the main proteinolytic activities in PL10 extracts belong to the metallo- and serine-proteinase classes. Among serine-proteinases, trypsin was the most abundant enzyme (23% of inhibition), followed by chymotrypsin (7% with TPCK and 14% with ZPCK, respectively). The cysteine protease inhibitor E-64 produces a slight inhibition, which suggests that cysteine protease-like enzymes did not contribute to total proteinase activity to a great extent. The inhibition caused by metallo-proteinase inactivators ranged from 69 to 34%. Results indicated that the proteinase activity inhibited by these compounds may be attributed to metallo-proteinase like enzymes. By contrast, several authors have reported that most of the digestive proteinases of decapod crustaceans belong to the serine class.[6,7] Taking into account this fact, there are two possibilities to explain the results obtained: (1) enzymes that belong to the metallo-proteinase class are present in the hepatopancreas extracts and/or 2) chelators may affect also some serine-proteinases or others classes of proteinases that require Ca^{2+}, Mg^{2+} or Zn^{2+} to be active. The composition of proteinases in the enzyme extracts is shown in Fig. 1. By the substrate-SDS-PAGE analysis, eight active fractions (range, 16–50 kDa) each representing a putative proteinase found in the enzyme extracts. These results agree with

Table 2. Morphometric variables and values of proteinase activity in PL10 extracts

Variables	mean ± SD
Corporal length (mm)[a]	13.6 ± 0.04
Body dry weight (mg)	0.20 ± 0.02
Proteinase activity (U mL^{-1})[b]	3.11 ± 0.19
Specific proteinase activity (U mg $protein^{-1}$)[c]	0.96 ± 0.05
Proteinase activity $PL10^{-1}$[d]	0.24 ± 0.02
Soluble protein concentration of extract (mg mL^{-1})	3.80 ± 0.20

[a] Measured from the top of the rostrum to the end of the telson.
[b] Proteinase activity per mL of crude enzyme extract.
[c] Proteinase activity per mg of soluble protein in the enzyme extract.
[d] Proteinase activity per postlarvae (the expression of proteinase activity based in the body weight of postlarvae was obtained dividing the proteinase activity expressed in U per mL between the number of postlarvae homogenised in one mL of crude enzyme extract).

Table 3. Effect of several commercial inhibitors on proteinase activity of PL10 extracts (mean ± SD). Enzymatic inhibition due to solvents never exceeded 5%

Inhibitor	Percentage of inhibition
PMSF	30.6 ± 1.0
SBTI	29.4 ± 4.0
TLCK	23.3 ± 5.5
TPCK	7.4 ± 1.5
ZPCK	14.4 ± 5.3
E-64	3.5 ± 0.5
1,10-Phenanthroline	69.4 ± 0.4
EGTA	58.6 ± 4.3
EDTA	34.7 ± 0.3

PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; ZPCK, *N*-CBZ-L-phenylalanine chloromethyl ketone; E-64, *trans*-epoxy-succinyl-L-leucylamido-(4-guanidino)butane; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid and EDTA, ethylenediaminetetraacetic acid.

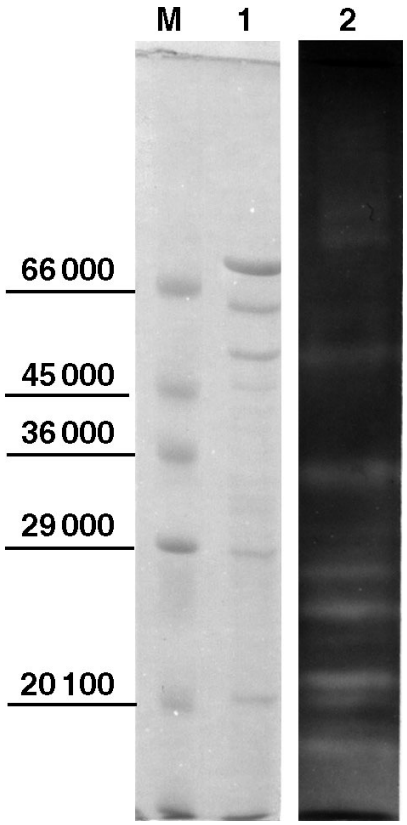

Figure 1. SDS PAGE of PL10 enzyme extracts. M = molecular weight markers, lane 1 = protein profile of PL10 extracts and lane 2 = caseinogram of PL10 extracts. MWM = albumin, bovine (66 000), albumin, egg (45 000), glyceraldehyde-3P-dehydrogenase (36 000), carbonic anhydrase, bovine (29 000) and trypsin inhibitor, soybean (20 100).

previous reports in other crustacea, including shrimp, crab, krill and crayfish.[7,21,22]

Inhibition of proteinases by feedstuffs

The inhibition of PL10 digestive proteinases by protein ingredients and microcapsules is summarised in Table 4. Some protein ingredients reduced the proteinolytic activity in PL10 hepatopancreas extracts. Ovalbumin inhibited about 80% and krill meal 33%. The MC 2 reduced the enzyme activity in 66%. A negligible inhibition by other protein ingredients and food was obtained. Proteinase inhibitors may affect digestive enzyme reducing the digestibility of protein in food and hence the availability of amino acids. The inhibition of digestive proteinases in aquacultured species by antinutritional compounds is well-documented by Krogdahl *et al*.[23] and Tacon.[2] An inhibitor for digestive proteinases can have a two-fold effect: by reducing the assimilation of food protein it disturbs nutrition and thus further reduces the growth of organisms. Usually, to alleviate the problem, farmers increase the amount of food supplied or increase the concentration of protein in the food. Both strategies cause an increase in the waste from the culture. Besides nutrition issues, the nondigested protein will be discarded, with the faeces increasing the organic material in the farming ponds and severely affecting the conditions of the culture. Both effects influence the economics of the project and the sustainability of the biological system. In this study, ovalbumin, a protein used in the MC 2, inhibited the PL10 proteinase activity by 80% (Table 4). Ali[24] reported that juvenile *Penaeus indicus*, fed on ovalbumin feeds, discarded higher amounts of faecal nitrogen. Similar results were reported in larvae of the marine fish, *Sparus aurata*, fed with ovalbumin-based feed that inhibited the digestive proteinases,[25] and yielded lower growth and survival of the larvae.[26]

Table 4. Effect of protein sources and microcapsules on proteinase activity of postlarval-shrimp enzyme extracts. Values with the same superscript are not significantly different ($P > 0.05$)

Extract	Inhibition (%) ± SD
Casein	2.1 ± 0.4^{f}
Hydrolysed krill meal	33.8 ± 0.5^{c}
Cuttlefish meal	11.1 ± 1.0^{d}
Ovalbumin	79.9 ± 1.5^{a}
MC 1	15.8 ± 1.8^{d}
MC 2	65.5 ± 0.7^{b}
MC 3	7.8 ± 1.0^{e}
MC 4	3.9 ± 2.6^{e}

The kinetics of inhibition of shrimp proteinases using MC 1 and MC 2 was obtained and results shown in Fig. 2. Curves and equations are calculated by measuring the inhibition of digestive proteinases of PL10 at increased amount of the evaluated ingredient or microcapsule extract, from zero to amounts beyond those ingested in a day by the larvae. Obvious differences in the kinetics of inhibition of shrimp proteinase activity were found, yielding curves which fitted logarithmic (MC 2) or polynomial (MC 1) functions. The ingested amount of feed by a single larva per day (208 mg per postlarvae) was calculated by Lemos.[27] A hypothetical intake of 208 mg of MC 2 reduced 40% of proteinase activity, whereas MC 1 only reduced 5%. Such curves could be used

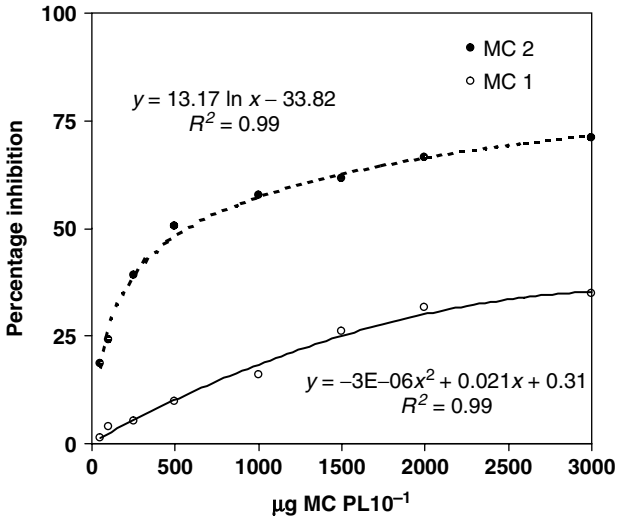

Figure 2. Inhibition curves of MC 1 and MC 2 obtained using increased relative concentrations of protein ingredient (µg MC) vs the same units of proteolytic activity (proteinase activity measured in a PL10).

to evaluate the effect of the amount of ingested feeds having enzyme inhibitors can affect digestive proteinases in a given organism.[12]

Kinetics of protein hydrolysis

Because we know from previous experience[28] that, in spite of the presence of proteinase inhibitors in some feeds for aquaculture, there is some digestion of the protein evaluated as digestibility, we conducted some assays to evaluate the *in vitro* digestibility of protein in the ingredients and microcapsules. The highest DH values were obtained for MC 4, MC 3, casein and cuttlefish meal (Table 5). In contrast, ovalbumin was digested poorly, yielding a lower DH. A correlation between ingredient quality and DH was found by Ezquerra *et al*.[4] With the information gained in this study we can assume that the lower digestibility of ovalbumin is because it is not a pure product and has potent inhibitors for proteinases derived from the egg white, such as ovomucoid and ovoinhibitor. The DH of MC 2 was half the value of MC 1. Microcapsules based on casein and cuttlefish meal (MC 3 and 4) were better hydrolysed by the shrimp larvae enzymes. These data agree with results reported previously.[18,29] The results obtained indicate that the use of ovalbumin as the protein ingredient for the formulation of microcapsules can negatively affect digestive proteinases of the shrimp larvae. Ovalbumin may be used if the product lacks inhibitors or they are eliminated by denaturation using some food technique such as heating.[30]

Table 5. Degree of hydrolysis (%) of protein sources and microcapsules obtained with postlarval shrimp enzyme extracts

Protein	DH (%) ± SD	Microcapsules (MC)	DH (%) ± SD
Casein	6.83 ± 0.53^{d}	MC 1	4.60 ± 0.22^{c}
Hydrolysed krill	1.70 ± 1.29^{b}	MC 2	2.50 ± 0.17^{b}
Cuttlefish meal	5.15 ± 0.34^{c}	MC 3	7.21 ± 0.49^{d}
Ovalbumin	0.16 ± 0.11^{a}	MC 4	8.96 ± 0.34^{e}

Values with the same superscript are not significantly different ($P > 0.05$).
In all the samples, the spontaneous DH (auto-hydrolysis) was negligible.

Table 6. Coefficient of protein degradation, CPD (%) from some protein and microcapsules obtained for *P. vannamei* postlarvae

Protein sources and MC	CPD (%) ± SD
Casein	99.0 ± 1.90^{a}
Cuttlefish meal	55.0 ± 1.53^{d}
Ovalbumin	2.0 ± 0.03^{f}
MC 2	12.0 ± 0.25^{e}
MC 3	87.1 ± 1.82^{b}
MC 4	67.0 ± 1.29^{c}

Values with the same superscript are not significantly different ($P < 0.05$).

Analysis of protein hydrolysis by electrophoresis

Samples of casein, cuttlefish meal, MC 2 and MC 4 were analysed by SDS-PAGE (Fig. 3), to assess the course of protein hydrolysis when either protein ingredient or microcapsules were incubated in the presence of PL10 enzyme extracts. In Fig. 3(A), the hydrolysis of casein is shown. In fewer than 100 s of hydrolysis, the main proteins present almost disappeared when testing this protein. When assaying cuttlefish meal (Fig. 3(B)), proteinograms showed a progressive and less marked tendency (see protein with 45 kDa) of proteolysis. In MC 4 (Fig. 3(D)) these two effect can be seen at the same time since this microcapsules contain both protein sources: casein and cuttlefish meal. In contrast, the main protein present in MC 2 is ovoalbumin (45 kDa) and remained unaffected throughout the enzymatic hydrolysis (Fig. 3(C)). Digital images of SDS-PAGE gels were processed to obtain densitometry values of main protein bands. The coefficient of protein degradation (CPD) was calculated for each protein ingredient and microcapsule (Table 6). CPD and DH values showed a direct linear correlation ($R^2 = 0.760$). The reduction in the concentration of bands of protein was used as to evaluate the protein degradability. By using this criterion, it was possible to show the limited hydrolysis of ovalbumin as reagent and in MC 2 (Fig. 3(C)), where the main protein band remained unaltered during the assay period. Casein and casein-based microcapsules were quickly hydrolysed (Fig. 3(A and D)). Analysis of lane densitometry made quantitative the information from SDS-PAGE. Results corroborate data obtained by pH-stat.

CONCLUSION

From the results it can be concluded that some compounds present in food ingredients and feed may reduce the digestion capability shrimp postlarvae. This

DOI: 10.1002/jsfa

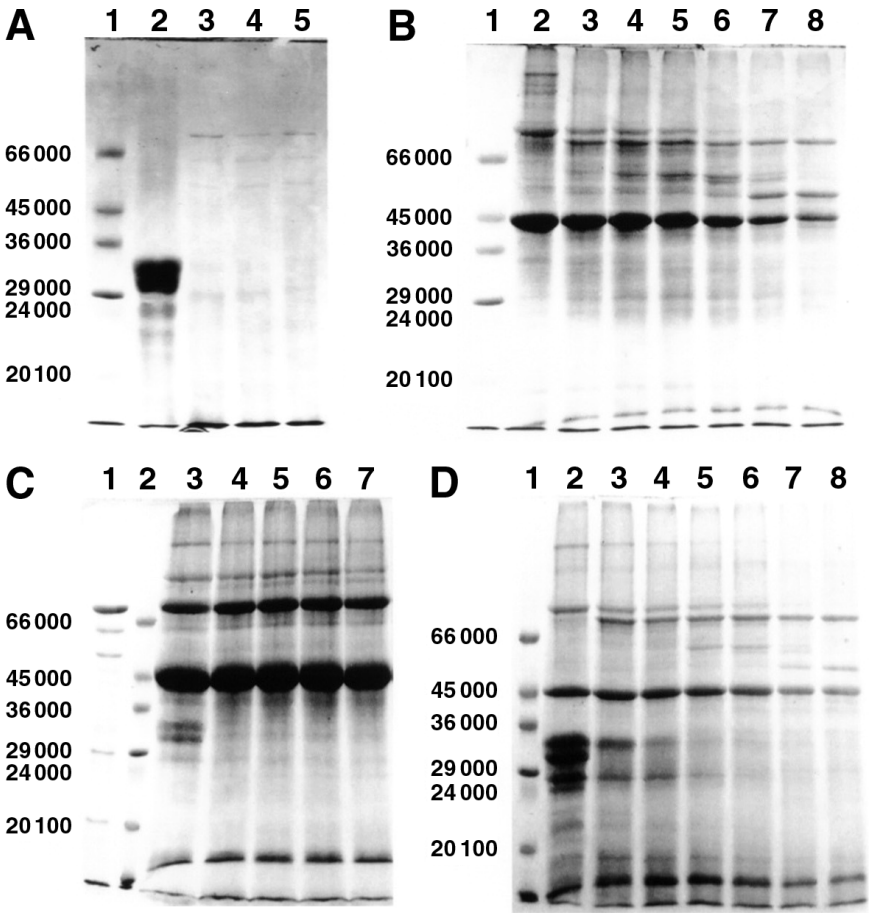

Figure 3. SDS-PAGE profile of proteins obtained at different time intervals during pH-stat enzymatic digestion by PL10 enzymes. (A) Casein. Lane 1 = molecular weight markers (MWM), lanes 2 to 5 = protein profile of reaction mixture after 0, 100, 250 and 500 s of enzymatic hydrolysis. (B) Cuttlefish meal. Lane 1 = MWM, lanes 2 to 8 = protein profile of reaction mixture after 0, 100, 250, 500, 1000, 2000 and 3000 s of enzymatic hydrolysis. (C) MC 2. Lane 1 = PL10 hepatopancreas extract, lane 2 = molecular weight markers (MWM), lanes 3 to 7 = protein profile of reaction mixture after 0, 100, 500, 1000 and 3000 s of enzymatic hydrolysis. (D) MC 4. Lane 1 = MWM, lanes 2 to 8 = protein profile of mixture after 0, 100, 250, 500, 1000, 2000 and 3000 s of enzymatic hydrolysis.

study provides a series of techniques to: (1) evaluate the ability of any organism intended for aquafarming to use enzymes from its digestive system to digest protein; (2) determine the presence of inhibitors in the feedstuff; and (3) calculate the maximum amount of the feedstuff containing inhibitors to be included in a feed in order that the digestive function is not affected.

Further studies are needed to determine whether, as in other organisms, shrimp postlarvae and juveniles show a physiological response to foods containing proteinase inhibitors. Such a phenomenon is called compensation and has being found in fish and mammals.[15,31] The techniques provided herein can help in such studies by identifying the amount of inhibitors and the ability of the organism to hydrolyse protein in food containing them.

In a world that needs increased amounts of aquafarmed organisms, the lack of suitable protein ingredients to produce the feeds to support the demand could limit the impact of aquaculture. Alternative protein ingredients have to be assayed and laboratory techniques that save time and money will help in the rapid evaluation of such ingredients.

ACKNOWLEDGEMENTS

The authors are indebted to B. Guillermo Portillo and Ing. Mayra Vargas for supplying white shrimp larvae and assistance during shrimp culture. F.J. Alarcón was a recipient of a postdoctoral fellowship from the Ministerio de Educación y Cultura, Spain.

REFERENCES

1 Utne F, Standard methods and terminology in finfish nutrition, in, *Proceedings of a World Symposium on Nutrition and Finfish Technology*, Hamburg, 20–23 June 1978, ed by Halver JE and Tiews K. Heenemann, Berlin, Vol. 11, pp. 437–444 (1979).

2 Tacon AGJ, Fishmeal replacers: Review of antinutrients within oilseeds and pulses – A limiting factor for the aquafeed Green Revolution? *Cah Opt Méditerr* **22**:153–182 (1997).

3 Pedersen B and Eggum BO, Prediction of protein digestibility by an *in vitro* enzymatic pH-stat procedure. *J Anim Physiol Anim Nutr* **49**:265–277 (1983).

4 Ezquerra JM, García-Carreño FL and Carrillo O, *In vitro* digestibility of dietary protein sources for white shrimp (*Penaeus vannamei*). *Aquaculture* **163**:123–136 (1998).

5 Dimes LE, Haard NF, Dong FM, Rasco BA, Forster IP, Fairgrieve WT, *et al.*, Estimation of protein digestibility. II. *In vitro* assay of protein in salmonid feeds. *Comp Biochem Physiol* **108A**:363–370 (1994).

6 García-Carreño FL and Haard N, Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish

(*Pacifastacus astacus*) extracts. *J Food Biochem* **17**:97–113 (1993).
7 García-Carreño FL, Hernández-Cortés MP and Haard NF, Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and marine decapod. *J Agric Food Chem* **42**:1456–1461 (1994).
8 García-Carreño FL, Navarrete del Toro A and Ezquerra JM, Digestive shrimp proteases for evaluation of protein digestibility *in vitro*. I: Effect of protease inhibitors in protein ingredients. *J Mar Biotechnol* **5**:36–40 (1997).
9 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:48–254 (1976).
10 Dunn BM, Determination of proteinase mechanism, in, *Proteolytic Enzymes: A Practical Approach*, ed. by Beynon RJ and Bond JS. IRL Press, Oxford, pp. 57–81 (1989).
11 García-Carreño FL, Dimes LE and Haard NF, Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal Biochem* **214**:65–69 (1993).
12 Alarcón FJ, Moyano FJ, Díaz M, Fernández-Díaz C and Yúfera M, Optimization of the protein fraction of microcapsules used in feeding of marine fish larvae using *in vitro* digestibility techniques. *Aquaculture Nutr* **5**:107–114 (1999).
13 Adler-Nissen J, *Enzymic Hydrolysis of Food Proteins*. Elsevier Applied Science Publishers, London, p. 427 (1986).
14 Dimes LE and Haard NF, Estimation of protein digestibility: I. Development of an *in vitro* method for estimating protein digestibility in salmonids. *Comp Biochem Physiol* **108A**:349–362 (1994).
15 Haard NF, Dimes. E, Arndt RE and Dong FM, Estimation of protein digesibility. IV. Digestive proteinases from the pyloric caeca of coho salmon (*Oncorhynchus kistch*) fed diets containing soybean meal. *Comp Biochem Physiol* **110B**:533–540 (1996).
16 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685 (1970).
17 Le Moullac GA, Van Wormhoudt A and Aquacop, Adaptation of digestive enzymes to dietary protein, carbohydrate and fibre levels and influence of protein and carbohydrate quality in *Penaeus vannamei* larvae (crustacea, decapoda). *Aquat Living Resour* **7**:203–210 (1994).
18 Kumlu M and Jones DA, The effect of live and artificial diets on growth, survival, and trypsin activity in larvae of *Penaeus indicus*. *J Eur World Aqua Soc* **26**:406–414 (1995).
19 González R, Fraga V and Carrillo O, Cambios ontogenéticos en la actividad de las principales enzimas digestivas de *Penaeus schmitti*. *Rev Invest Mar* **15**:262–268 (1994).
20 Rodríguez A, Le Vay L, Mourente G and Jones DA, Biochemical composition and digestive enzyme activity in larvae and postlarvae of *Penaeus japonicus* during herbivorous and carnivorous feeding. *Mar Biol* **118**:45–51 (1994).
21 Jiang S, Moody MW and Chen H, Purification and characterization of proteases from digestive tract of grass shrimp (*Penaeus monodon*). *J Food Sci* **56**:322–326 (1991).
22 Fang L and Lee B, Ontogenic change of digestive enzymes in *Penaeus monodon*. *Comp Biochem Physiol* **103B**:1033–1037 (1992).
23 Krogdhal A, Lee TB and Olli JJ, Soybean protease inhibitors affect intestinal trypsin activities and amino acids digestibilities in rainbow trout (*Oncorhynchus mykiss*). *Comp Biochem Physiol* **107A**:215–219 (1994).
24 Ali SA, Comparative evaluation of four purified dietary proteins for the juvenile *Penaeus indicus*. *J Aqua Trop* **9**:95–108 (1994).
25 Yúfera M, Sarasquete MC and Fernández-Díaz C, Testing protein-walled microcapsules for the rearing of first-feeding gilthead sea bream (*Sparus aurata* L.) larvae. *Mar Freshwater Res* **47**:211–216 (1996).
26 Yúfera M, Fernández-Díaz C, Pascual P, Sarasquete MC, Moyano FJ, Díaz M, *et al.*, Towards an inert diet for first-feeding gilthead seabream *Sparus aurata* L. larvae. *Aquaculture Nutr* **6**:143–152 (2000).
27 Lemos D, Avaliação do estado nutricional do camarão *Penaeus japonicus* Bate, no início da fase pós-larval, em cultivo. MSc dissertation, Instituto Oceanográfico, Universidade de São Paulo, Brazil, p. 150 (1996).
28 Alarcón FJ, Moyano FJ and Díaz M, Evaluation of different protein sources for aquafeeds by an optimised pH-stat system. *J Sci Food Agri* **82**:1–8 (2002).
29 Koshio S, Kanazawa S and Teshima S, Search for effective protein combination with crab protein for the larval kuruma prawn *Penaeus japonicus*. *Nippon Suisan Gakkaishi* **58**:1083–1089 (1992).
30 Alarcón FJ, García-Carreño FL and Navarrete del Toro MA, Effect of plant protease inhibitors on digestive proteases in two fish species, *Lutjanus argentiventris* and *L. novemfasciatus*. *Fish Physiol Biochem* **24**:179–189 (2001).
31 Pusztai A, Grant G, Bardocz S, Baintner K, Gelencser E and Ewen SW, Both free and complexed trypsin inhibitors stimulate pancreatic secretion and change duodenal enzyme levels. *Am J Physiol* **272**:340–350 (1997).