Aquaculture Nutrition

Aquaculture Nutrition 2013 **19**; 731–740 doi: 10.1111/anu.12020

Exogenous proteinases as feed supplement for shrimp: *in vitro* evaluation

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Abstract

To test the efficacy of adding enzyme supplements to feeds, an *in vitro* study was conducted by mixing bovine trypsin or proteinases from the gastric juice of the Cortez swimming crab *Callinectes bellicosus* with an enzyme extract from the digestive gland of the whiteleg shrimp *Penaeus vannamei*. Enzymes alone and mixtures were tested for hydrolysing proteinaceous natural substrates (bovine casein, bovine haemoglobin, and bovine serum albumin). All enzyme preparations hydrolysed casein. Shrimp enzymes hydrolysed haemoglobin but not serum albumin. Bovine trypsin and crab proteinases hydrolysed serum albumin but not haemoglobin. The mixture of shrimp and crab enzymes generated more hydrolytic products of serum albumin than shrimp enzymes alone. Shrimp enzymes mixed with bovine trypsin did not hydrolyse albumin because the bovine trypsin vanished; shrimp enzymes hydrolysed bovine trypsin. Results indicated that it is naive to assume that proteinolytic enzymes from different species will add their catalytic capabilities if mixed; here, we demonstrated that they may act as proteinases and will hydrolyse available protein regardless of its function. Our conclusion is that enzyme supplements should be tested *in vitro* before intending them as exogenous proteinases in feeds. This technique can be used to assess the compatibility and additivity of proteinases when mixed for biotechnological purposes. Besides, the technique can demonstrate who hydrolyses whom.

KEY WORDS: feed supplements, hydrolysis, *Penaeus vannamei*, protein digestibility, proteinases

Received 18 May 2012; accepted 9 November 2012

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Introduction

Protein is the main component in shrimp feeds and also a growth-limiting nutrient, as well as the most expensive ingredient in aqua feeds. Shrimp feeds still rely on fishmeal as the protein source, despite its price, decreasing availability and nutritional quality (Forster *et al.* 2003). The search for alternative protein sources of high nutritional quality focuses on ingredients that are widely available, contain suitable protein, and have no anti-physiological compounds (Tacon & Barg 1998; Davis & Arnold 2000; Lemos *et al.* 2004; Amaya *et al.* 2007). Alternative methods for improving protein digestibility and assimilation are ongoing (Carvalho *et al.* 1997; Córdova-Murueta & García-Carreño 2002). Apparent protein digestibility (APD) is one strategy to evaluate assimilation of protein in whiteleg shrimp *Penaeus* (Syn: *Litopenaeus*) *vannamei* (Lemos *et al.* 2009). Some evidence that *in vitro* hydrolysis (IVH) of feedstuffs by digestive enzymes extracted from the shrimp digestive gland is related to the APD of different types of ingredients, including soybean meal, corn gluten meal, wheat gluten, blood meal, crab meal, fish meal, squid meal, casein and gelatine (De Muylder *et al.* 2008; Lemos *et al.* 2009). Correlations of APD and IVH suggest a close relationship between peptide bond hydrolysis generated by digestive enzymes and apparent nitrogen assimilation in shrimp; IVH has been used as a tool to provide rapid information about the digestibility of food proteins (Lemos *et al.* 2009; Tibbetts *et al.* 2011). Further benefits of the method include selection of supplemental ingredients (García-Carreño *et al.* 1997).

To enhance protein assimilation in shrimp, protein hydrolysates are used to ensure that protein will be digested and assimilated (Carvalho *et al.* 1997; Córdova-Murueta & García-Carreño 2002). Alternatively, some researchers have directed their efforts to assess the benefits of using enzymes as supplements in aquaculture feeds (Dabrowska *et al.* 1979; Maugle *et al.* 1983; Divakaran & Velasco 1999). Supplemental digestive enzymes have been used to counteract deficien-

cies or absence of these catalytic molecules at some developmental stages and even increasing the digestion capacity in juvenile, farmed animals to increase weight more rapidly (Dabrowska *et al*. 1979; Kolkovski *et al*. 1993).

In a preliminary trial, enzymes from the digestive gland of adult *P. vannamei*, yellowleg shrimp *Penaeus californiensis* and bovine chymotrypsin were used as supplements to feed *P. californiensis* postlarvae to increase the final wet weight of treated groups. The highest weight gain occurred in postlarvae fed with feed supplemented with a digestive gland extract of the same species (unpublished data). In other investigations, an overexpressed and microencapsulated proteinase was used to enhance growth and survival of Indian white prawn *Fenneropenaeus indicus* postlarvae, demonstrating that proteinase from the Gram-negative heterotrophic eubacterium *Aeromonas hydrophila* enhanced growth rates (Sirvas-Cornejo *et al*. 2007), which agrees with Kolkovski *et al*. (1993); where the latter group demonstrated the benefit of adding pancreatin to feed to improve growth of gilthead sea bream larvae. They further proposed that feed supplements could replace live food during early stages of development. Digestive enzymes from bovine or porcine pancreas or from microorganisms have been used to supplement aqua farm feeds to improve digestibility (Sirvas-Cornejo *et al*. 2007).

When feed was supplemented with bovine trypsin to feed post-larval kuruma shrimp *Marsupenaeus japonicus* in combination with a carbohydrase, the experimental group had a weight gain of 7.7%, compared with the control group; the cause was the activation of the endogenous proteinase zymogens of the shrimp by bovine trypsin, and this resulted in increased growth and total proteinase activity, although the authors made no effort to demonstrate this (Maugle *et al*. 1983). In another study, the effect of supplementing feed with bovine trypsin to feed common carp fry, rainbow trout, grass carp and whitefish has been assessed (Dabrowski & Glogowski (1977). The authors suggested that the exogenous enzymes participate in hydrolysis of dietary proteins in the fish, work that constitutes a starting point for studies on supplementing fish feed with exogenous enzymes. However, the cause and effect relationship, if any, of the supplemental enzymes has never been assessed. In spite of a general belief that digestive enzyme-supplements work, in fact, the mechanism by which supplemental digestive enzymes in feeds exert their effects and if digestive enzyme preparations work to help the digestive system of the animal has never been disclosed in peer-reviewed literature. Our group proposes a new approach for the study of using enzymes as supplements to enhance digestibility of feed protein. To arrive at a mechanism of action, this study intended to ascertain what happens when enzymes from different species are mixed *in vitro*. We prepared two digestive enzymes systems: one was an enzyme extract from the digestive gland of *P. vannamei*, and the other was either bovine trypsin or gastric juice from *Callinectes bellicosus* as the supplement. Supplements are from one species related to the one to be supplemented, crabs, and the other phylogenetically distant, from a mammal.

Materials and methods

Exogenous proteinase feed supplements

Bovine trypsin (10 mg; EC 3.4.21.4; #T4665; Sigma-Aldrich St. Louis, MO, USA) was dissolved in 1 mL distilled water; the solution is called bovine trypsin (TRY). Gastric juice from *C. bellicosus* (size 13.2 ± 1.8 cm, spine to spine) was extracted by inserting a flexible probe attached to a 3 mL syringe into the esophagus to reach the cardiac chamber. The gastric juice is composed by the whole set of digestive enzymes secreted by the digestive gland (Díaz-Tenorio *et al*. 2006). The gastric juice was centrifuged at 10 000 ***g*** at 4 °C for 10 min. The supernatant was recovered and maintained at −20 °C for further assays; the enzyme extract is called CB.

Digestive gland proteinases of shrimp

Juvenile *P. vannamei* (weight: 15.0 ± 1.0 g) were obtained from CIBNOR hatchery facilities. Specimens were kept under controlled conditions for acclimatization (28 °C under continuous flow of filtered seawater and aeration). The digestive gland of 200 specimens were excised, pooled and stored in a beaker in an ice bath, weighed and homogenized twice with cold dH_2O (1 : 3 w/v) for 20 s each time, using a kitchen blender operating at low speed. The homogenate was centrifuged at 10 000 ***g*** for 30 min at 4 °C to eliminate lipids and tissue debris. The aqueous supernatant is the enzyme extract, which was freeze-dried and stored at 4 °C for later analysis.

Enzymatic activity

Freeze-dried enzyme extract (100 mg) was solubilized in 0.5 mL dH_2O; this solution is called PV. Soluble protein in PV, CB and TRY was assayed (Bradford, 1976) by adapting the method for microassays, using BSA as the standard. Absorbance was measured at 595 nm in an microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA).

Total proteinolytic activity of PV, CB and TRY was assayed (García-Carreño & Haard 1993). In a 1.5 mL

microtube, 5 μL PV was diluted 1 : 50 with dH_2O; 10 μL CB was diluted 1 : 100 with dH_2O; or 10 μL TRY was diluted 1 : 10 with dH_2O. Each sample was mixed with 100 μL TRIS–HCl buffer at pH 8.0 and 100 μL 0.5% (w/v) azocasein (#A2765; Sigma-Aldrich) in 50 mM TRIS–HCl buffer at pH 8.0 at 25 °C. After incubation (10 min), the reaction was stopped by adding 100 μL 20% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 10 000 ***g*** for 10 min. The supernatant was recovered; absorbance was measured spectrophotemetrically at 366 nm in the microplate reader. Control assays (blanks) received TCA solution before the azocasein was added. All assays were conducted in triplicate to obtain an accurate value. Total protease units of activity were expressed as change in absorbance per minute per mL of enzyme extract ($U = Abs_{280}\ min^{-1}\ mL^{-1}$).

Electrophoresis

Proteins in the enzyme preparations and their mixtures were separated by electrophoresis (8 cm × 10 cm; 0.75 mm thick gel size, 12% acrylamide), described by Laemmli (1970). The samples, containing 12 μg protein, were mixed with the same volume of 2× sample buffer (0.125 M TRIS –HCl, 4% SDS, 20% v/v glycerol, 0.02% w/v bromophenol blue at pH 6.8). Each sample was loaded into the gel. A low molecular weight protein standard mixture (4 μL; #17-0446-01; Amersham Biosciences, GE Healthcare, Little Chalfont, UK) was loaded. Electrophoresis was performed at 15 mA per gel and 4 °C in a circulating bath in a mini-vertical gel electrophoresis unit (SE260; Amersham Biosciences). Once the tracking dye reached the bottom of the gel, electrophoresis was stopped. The gels were stained with 0.05% Coomassie brilliant blue R-250 in an aqueous solution of 40% methanol and 7% acetic acid for at least 4 h and then destained with the same solution without dye.

S-SDS-PAGE

Proteins with proteinase activity were evaluated by the method of García-Carreño *et al.* (1993). A volume of each enzyme preparation or mixture containing 5 mU enzyme activity was combined with the same volume of 2× sample buffer (0.125 M TRIS–HCl, 4% SDS, 20% v/v glycerol, 0.02% w/v bromophenol blue at pH 6.8). Without boiling, each sample was loaded into the gel. The same low molecular weight protein standard was loaded. Electrophoresis was performed, as described earlier. After electrophoresis, the gel was soaked in 3% (w/v) casein in 50 mM TRIS–HCl at pH 8.0 for 30 min in an ice bath under circular agitation for the substrate to penetrate into the gel. The temperature was raised to 25 °C for 90 min. Gels were washed in dH_2O and immediately fixed and stained with Coomassie brilliant blue R-250 in the same aqueous methanol-acetic acid solution for at least 4 h and then destained with the same solution without dye.

Hydrolysis of proteinaceous substrates

To determine the contribution to protein hydrolysis by enzyme preparations, bovine casein (CAS), bovine haemoglobin (Hb) and bovine serum albumin (BSA); (#C7078; #H2625; #B4287; Sigma-Aldrich) were used as proteinaceous substrates. The hydrolysis products generated in each substrate by enzyme preparations and their mixtures were incubated and assayed by SDS-PAGE at 5, 30, 60 and 120 min. Each substrate (500 μL), containing 4 mg mL^{-1} of protein in 50 mM TRIS–HCl at pH 8.0, was mixed with a volume of enzyme preparation containing 125 mU of activity or a mixture of two enzyme preparations containing 125 mU of each preparation. The control treatments of all the substrates were incubated with dH_2O instead of enzyme preparations. Each mixture was incubated at 28 °C and stirred at 500 rpm on a thermomixer (Comfort model; Eppendorf, Hamburg, Germany). Subsamples of 20 μL were taken at 5, 30, 60 and 120 min, and each subsample was treated with one volume of 2× sample buffer and immediately boiled to inactivate the enzymes and stop the reaction until the last sampling was completed. Once incubation was completed, 12 μg of proteinaceous substrates of each subsample and low molecular weight standard were loaded in a 12% acrylamide gel. Electrophoresis conditions were described earlier.

Fractionation of PV by ion exchange and size-exclusion chromatography

Enzyme extract from digestive gland of *P. vannamei* is a crude extract, mainly composed of five serine proteinases (SPs): two isochymotrypsins, including a high molecular weight (MW) of 33.2 kDa (Chy H; Hernández-Cortés *et al.* 1997) and a low MW of 26 kDa (Chy L; Sellos & Wormhoudt 1992), and three isotrypsins (A, B and C; Sainz *et al.* 2004) and other minor proteinases. To determine which SPs from PV could hydrolyse TRY, trypsins and chymotrypsins from PV were partly separated by size-exclusion chromatography and ion exchange. SP fraction containing five SPs was obtained by size-exclusion chromatography, using the modified method of Rivera-Pérez *et al.* (2011). For ion

exchange chromatography, 100 mg PV freeze-dried enzyme extract was dissolved in 2 mL 50 mM TRIS–HCl buffer at pH 7.5; 0.1 N NaOH was used to readjust the pH to 7.5. Then, 50 μL of this solution was loaded into a Q-Sepharose (#71-7128-00; Amersham Biosciences, GE Healthcare, Little Chalfont, UK) column; previously, the column was equilibrated with 50 mM TRIS–HCl at pH 7.5. The proteins were eluted in seven steps (0.1, 0.2, 0.3 0.4, 0.5, 0.7 and 1.0 M NaCl). Fractions of 250 μL were collected and analysed for chymotrypsin or trypsin activity. Fractions with Chy H were eluted with 0.4 M NaCl (Fraction B-I); Chy L and a part of the three isotrypsins (A, B and C) were eluted with 0.7 M NaCl (Fraction B-II). Finally, the remaining three isotrypsins A, B and C were eluted with 1.0 M NaCl (Fraction ABC). The fractions were desalted and concentrated at 4000 ***g***, using a centrifugal filter unit (Amicon Ultra-15; EMD Millipore, Billerica, MA, USA). S-SDS-PAGE was used to assess the composition of proteinases. Fractions B-I, B-II and ABC were incubated with TRY and asses their activity by S-SDS-PAGE.

Kinetics of concentration of PV

Mixtures of PV with TRY were assessed individually in 0.5 mL microtubes. To visualize bands of activity, eight microtubes containing TRY (125 mU) were added in decreasing concentrations of PV (125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9 and 0.97 mU) and taken to 100 μL volume with 50 mM TRIS–HCl buffer at pH 8.0 at 28 °C. Five mU per sample were loaded into S-SDS-PAGE. To visualize bands of protein, eight microtubes containing TRY (125 mU) were added in decreasing concentrations of PV (125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9 and 0.97 mU) and taken to 25 μL with 50 mM TRIS–HCl buffer at pH 8.0 at 28 °C. Each sample (12 μg) was loaded into SDS-PAGE. Separately, PV and TRY were used as controls.

Statistics

Enzyme activity of each enzyme preparation was done in triplicates, and significant differences were determined by ANOVA at $P \leq 0.05$. Results are presented as means ± SD.

Results

Enzymatic activity

Proteinolytic activities of enzyme preparation were CB 85.5 ± 1.1 U mL^{-1}, PV 27.1 ± 0.7 U mL^{-1} and TRY 15.2 ± 0.4 U mL^{-1}. Each enzyme preparation was diluted to obtain the activity needed for assays.

Hydrolysis of proteinaceous substrates generated by exogenous enzymes, TRY and CB, digestive enzymes, PV and their mixtures

To investigate the contribution of TRY or gastric juice of *C. bellicosus* (CB) in hydrolysing protein when mixed with digestive proteinases of *P. vannamei* (PV), three different proteinaceous substrates (CAS, BSA and Hb) were tested. Each proteinaceous substrate was incubated individually with different enzyme preparations and their mixtures according to Table 1. Proteinaceous substrate without enzymes was used as control. Paramount for this investigation was to assess whether PV, TRY and CB, individually, hydrolysed CAS in 30 min (Table 2). Only PV was able to hydrolyse Hb (Table 2), but not BSA (Fig. 1a). TRY or CB hydrolysed BSA (Fig. 1) and Hb in a lesser grade (Table 2). Experiments were run to assess whether the mixture of PV with TRY or PV with CB using BSA as the substrate can improve the PV limited capability in hydrolysing BSA. Contrary to expectations, PV with TRY did not hydrolyse BSA (Fig. 1a and Table 2) and PV with CB generated more products of hydrolysis from BSA compared to PV and CB alone (Fig. 1b and Table 2).

These results suggested that TRY, but not CB, loses activity in the presence of PV. To elucidate this, S-SDS-PAGE was used to evaluate activity in the reaction mixtures of PV with either TRY or CB. Figure 2 shows the outcome. PV, TRY and CB enzyme preparations and their

Table 1 Reaction mixtures to determine hydrolysis of BSA by enzyme preparations

	Enzyme preparation plus proteinaceous substrate
Control	500 μL BSA
PV	125 mU PV + 500 μL BSA
PV + TRY	125 mU PV + 125 mU TRY + 500 μL BSA
TRY	125 mU TRY + 500 μL BSA
PV + CB	125 mU PV + 125 mU CB + 500 μL BSA
CB	125 mU CB + 500 μL BSA

Proteinaceous substrates (4 mg mL^{-1}), BSA, Hb and CAS were mixed with PV or TRY or CB or mixtures of PV + TRY and PV + CB. Here, we exemplify for BSA, and it was the same for Hb and CAS. The reaction was kept at 28 °C and stirred at 500 rpm. Samples for analysis by SDS-PAGE were taken at 5, 30, 60 and 120 min.

Abbreviations: BSA, bovine serum albumin; CAS, bovine casein; CB, gastric juice from *Callinectes bellicosus*; Hb, bovine haemoglobin; PV, enzyme extract from digestive gland of *Penaeus vannamei*; and TRY, bovine trypsin.

Table 2 Hydrolysis of proteinaceous substrates produced by the enzyme preparations and their combinations

Proteinaceous substrates	PV	TRY	PV + TRY	CB	PV + CB
CAS	++++	++++	++++	++++	++++
Hb	++++	+	++++	++	++++
BSA	+	++++	+	+++	++++

The samples were loaded into a 12% acrylamide gel. Signs indicate whether the main band of proteinaceous substrate was fully hydrolysed, ++++ (0% of the band remained) or partially hydrolysed, +++ (25% of the band remained), ++ (50% of the band remains), and +, (75% of the band remained) at 120 min of reaction. Abbreviations: PV, enzyme extract from digestive gland of *Penaeus vannamei*; CB, gastric juice from *Callinectes bellicosus*; TRY, solution of bovine trypsin; CAS, bovine casein; Hb, bovine haemoglobin, and BSA, bovine serum albumin.

mixtures (PV plus TRY or CB) were incubated for 2 h, and subsamples of 20 μL were taken periodically (5, 30, 60 and 120 min); each subsample was treated with one volume of 2× sample buffer and immediately placed in an ice bath to stop the reaction. Once incubation was completed, 5 mU of activity of each subsample and low molecular weight standard were loaded in a 12% acrylamide gel. Electrophoresis conditions were described earlier. Because the bands of activity of PV and TRY did not overlap, characteristic bands of activity of each sample were displayed (Fig. 2). In the mixture of PV with TRY, TRY band of activity was never observed (Fig. 2a, centre). In the mixture of PV with CB, characteristic bands of activity of both PV and CB were displayed (Fig. 2b). This information gave us some clues about the reason why the mixture of PV with TRY did not hydrolyse BSA; because PV is hydrolysing TRY. As PV is a complex system of proteinases, so far, at this research, it was not known whether all proteinases are involved in the hydrolysis of TRY.

Hydrolysis of TRY by digestive proteinases from shrimp

To assess whether the loss of activity of TRY in the presence of PV was from hydrolysis, S-SDS-PAGE was used to

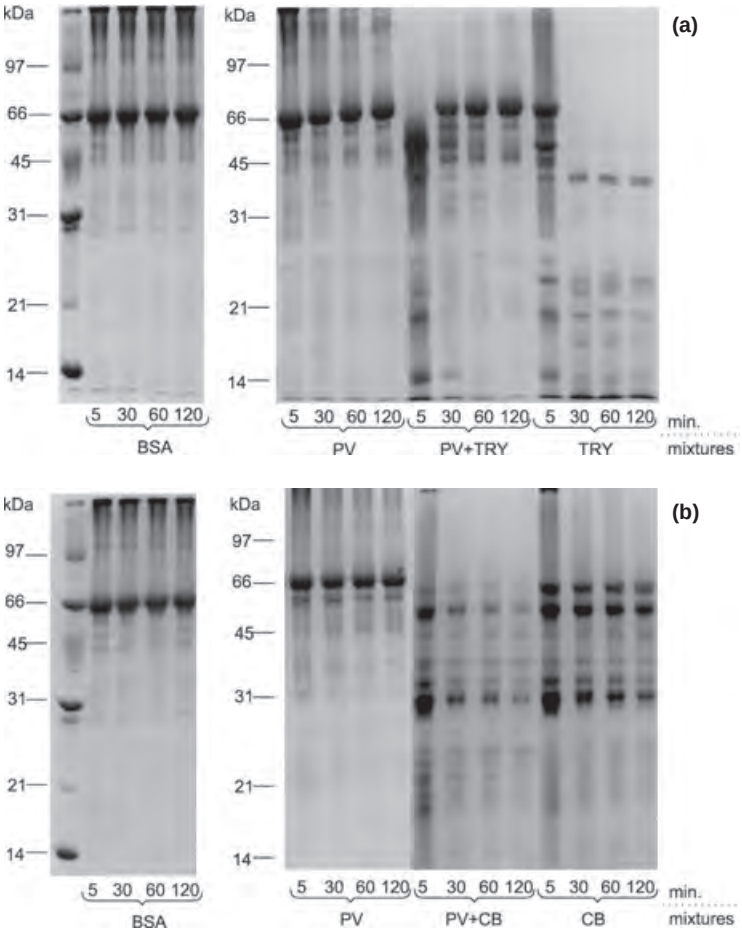

Figure 1 Hydrolysis of BSA by digestive enzymes. BSA and enzymes preparation were mixed and sampled at 5, 30, 60 and 120 min. Hydrolysis products were separated by SDS-PAGE and stained with Coomassie blue. (a) PV and TRY and mixture of PV + TRY. BSA without enzymes was used as controls (far left). (b) CB and the mixture with PV (PV + CB). Abbreviations: BSA, bovine serum albumin; CB, gastric juice from *Callinectes bellicosus*; PV, enzyme extract from digestive gland of *Penaeus vannamei*; and TRY, solution of bovine trypsin.

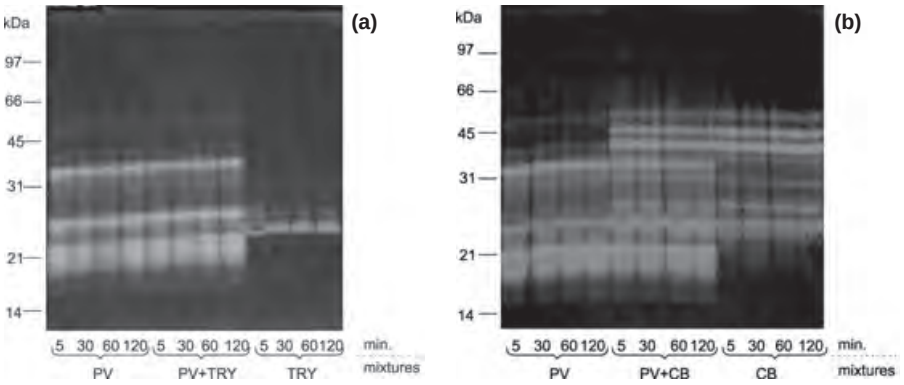

Figure 2 S-SDS-PAGE for enzyme preparations and their mixtures. (a) PV and TRY and the mixture of both (PV + TRY). (b) CB and the mixture with PV (PV + CB). Samples for analysis were taken at 5, 30, 60 and 120 min. Abbreviations: CB, gastric juice from *Callinectes bellicosus*; PV, enzyme extract from digestive gland of *Penaeus vannamei*; and TRY, solution of bovine trypsin.

evaluate the activity in enzyme preparations, TRY and PV, and their mixture. A kinetic of concentration of PV was run, keeping the concentration of TRY constant (125 mU). We expected that when PV was sufficiently diluted, no effect on TRY activity would happen. When the total proteinolytic activity from PV was diluted enough in the reaction mixture, a residual activity of TRY was start to display (Fig. 3b, lanes 3–8, note the band indicated by the arrow). SDS-PAGE was used to evaluate the protein present in TRY, PV and their mixture. The Fig. 3a (lane 1 and 2) shows that the protein of 23 kDa of TRY disappears in the presence of PV; however, if PV concentration decreases, then TRY remains almost unmodified (Fig. 3a, lane 5–8). The cause for the loss activity of TRY is the result of hydrolysis.

Hydrolysis of TRY by individual fractions from enzyme extract of shrimp

As PV is a crude extract, mainly composed of SPs; see Fig. 3. We hypothesized that one or all SPs could hydrolyse TRY. In order to challenge the hypothesis, the fractions BI, BII and ABC were mixed with TRY and their activity tested by S-SDS-PAGE (Fig. 4). The three fractions derived from PV partially or totally hydrolysed TRY in a few minutes. Isochymotrypsins fractions B-I and B-II

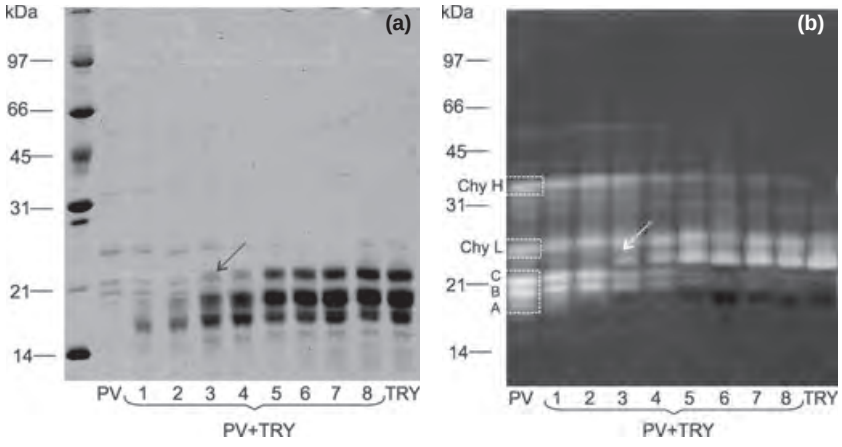

Figure 3 Hydrolysis of trypsin caused by PV. (a) Proteins present in PV, TRY and the mixture of both (PV + TRY). (b) Bands of activity in PV, TRY and the mixture of both. The reaction mixture PV + TRY, lanes 1–8, containing 125 mU TRY were mixed with decreasing amounts of PV, as follows: 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9 and 0.97 mU. Samples for SDS-PAGE and S-SDS-PAGE analysis were taken after 5-min incubation at 28 °C. The arrows indicate the presence of TRY in the first mixture in which protein or residual activity was detected. The boxes indicate the main proteinases in PV; two isochymotrypsin (Chy H and Chy L); and three isotrypsins (A, B and C). Abbreviations: PV, enzyme extract from digestive gland of *Penaeus vannamei* and TRY, solution of bovine trypsin.

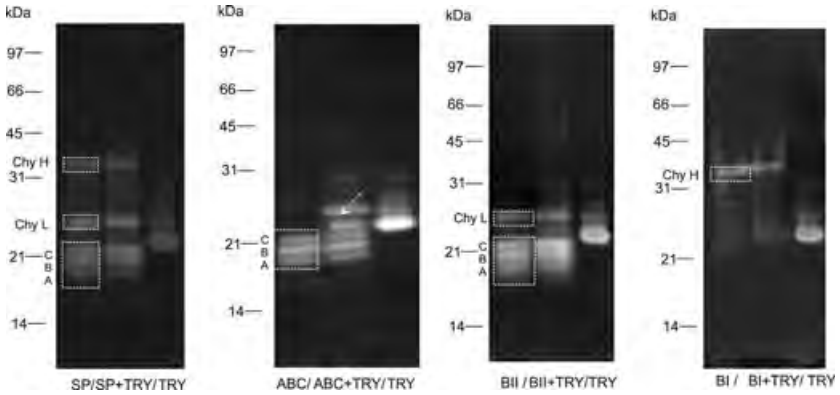

Figure 4 Loss of TRY activity from hydrolysis produced by SPs from shrimp. Fractions SP, ABC, B-II and B-I, alone or mixed with TRY were incubated for 5 min. Arrow indicates the residual activity of TRY in the enzyme mixture. Abbreviations: ABC, fraction chromatography with trypsin activity; B-II, fraction chromatography with Chy L and trypsins A, B and C; B-I, fraction chromatography with Chy H; SP, chromatography fraction with activity of serine proteinases; and TRY, solution of bovine trypsin.

hydrolysed TRY. Isotrypsins only partially decreased activity of TRY (Fig. 4).

Discussion

Proteinases are enzymes that specifically hydrolyse internal peptide bonds on proteins. Digestive proteinases hydrolyse food proteins. A crucial step in food protein digestion is catalysis by SPs synthesized and secreted by the digestive system of decapods. Given the participation of proteinases in digestion process, it is believed that to improve the nutritional value of feed, proteinases have been used as feed supplements. Although feed supplementation with proteinases or mixed enzymes might have positive effects on functioning of animals (Maugle *et al*. 1983; Kolkovski *et al*. 1993), in many cases, contradictory results in feed trials are reported because the enzymes are inactivated by some components in the digestive system of the fed animals (Campbell & Bedford 1992; Divakaran & Velasco 1999; García-Ruiz *et al*. 2006; Miller *et al*. 2007). In practice, when enzymes are used as feed supplements, it is expected that they should be unaffected by the physiological conditions of the digestive system and remain active to exert a positive effect. While there is a great deal of knowledge about the general properties of enzymes used as supplements, scarce information is known about the effect of gastric or pancreatic fluids of the host on the supplements. Studies are needed to fill the gap of knowledge about the use of exogenous enzymes when interacting with proteinases present in digestive fluid of the host. This study is the first attempt to investigate, *in vitro*, the acting of exogenous enzyme within the host digestive system. It shows that SPs from *P. vannamei* are able to hydrolyse bovine trypsin. The same results were obtained with other decapods proteinases *Panulirus interruptus*, *C. bellicosus* and *P. californiensis* (unpublished data). It is remarkable that digestive proteinases from decapods (*C. bellicosus* or *P. interruptus*) remain active when mixed with digestive enzymes from digestive gland of *P. vannamei in vitro*. Gastric fluids of marine decapods mainly contain SPs (Navarrete del Toro *et al*. 2006), as is the case with *P. vannamei* (Celis-Guerrero *et al*. 2004) and *C. bellicosus* (Díaz-Tenorio *et al*. 2006). Decapod's gastric juices hydrolyse TRY (bovine trypsin) and other enzymes, including bovine chymotrypsin and bromelain from pineapple stems (unpublished data). The significance of this finding remains to be investigated. The results suggest that chymotrypsins from *P. vannamei* play an important role in hydrolysing TRY, while isotrypsins from *P. vannamei*, only partly hydrolyse TRY. This is probably related to molecular characteristics, kinetic parameters and specificity of the bovine enzyme and the shrimp enzymes. In the cell, proteolysis is the most common mechanism for inactivating proteins and regulating physiology; proteinases are paramount in modulating enzyme activity and protein–protein interactions within multicomponent signalling pathways, thereby acting as master regulators (Ehrmann & Clausen 2004), but proteinases do not attack protein substrates at random. Rather, they display a high degree of specificity in binding and processing the substrates, and even in the extent of the hydrolysis; most regulation mechanisms are achieved by limited proteolysis. Substrate specificity is often defined by the structural properties of the active site, the specificity pocket and surface accessibility for potential cleavage sites (Gottesman 2003). The biological function of a protein is closely related to its three-dimensional structure, which is held by different types of

covalent and non-covalent bond interactions, among which are formation of hydrogen bonds, hydrophobic interactions, Van der Waals force and disulfide bonds (Pace *et al*. 1996). Other structural features that confer protein stability are post-translational modifications, such as glycosylation. Glycosylated enzymes are more resistant to hydrolysis than non-glycosylated ones (Marshall 1978; Wang *et al*. 1996). Some glycoproteins may increase their resistance to the action of proteinases because of steric hindrance or masking of recognition sites to proteinases by presence of carbohydrate moieties near the sites of proteinolytic action (Davis 2002).

Bovine trypsin is a pancreatic SP with substrate specificity based upon positively charged Lys and Arg side chains. It is derived from a 34 kDa inactive precursor. Enzymatic removal of the N-terminal results in the 23.8 kDa trypsin molecule (http://www.uniprot.org/uniprot/P00760). The optimum pH is 8.0. Bovine trypsin, found in most vertebrates and has six disulfide bridges; in *P. vannamei*, there are eight Cys residues and four disulfide bonds (Klein *et al*. 1996). According to Sainz *et al*. (2004), shrimp isotrypsins are glycosylated; bovine trypsin, in contrast, lacks glycosylation. Other important difference between decapod trypsin and bovine trypsin is autoproteinolytic stability of trypsins in decapods (Hehemann *et al*. 2008). Chymotrypsins in *P. vannamei* preferentially hydrolyse peptide bonds involving Tyr, Phe and Trp. In general; proteinases of decapods have maximum activity from neutral to alkaline pH, similar to mammalian enzymes, and they are thermostable from 25 to 37 °C (Hernández-Cortés *et al*. 1997; Celis-Guerrero *et al*. 2004; Sainz *et al*. 2004; Díaz-Tenorio *et al*. 2006). With the bioinformatics tools, NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/), which predict N- and O-glycosylation sites, we found $Thr^{43,150,162,163,165,167}$ prone to glycosylation in shrimp chymotrypsin B-I and UniProt ID: O18487_LITVA, $Thr^{156,157,159,161,170}$, and Asp^{141} in chymotrypsin B-II, UniProt ID: O18488_LITVA. The same sequences were uploaded to the predictive program SWISS-MODEL (http://www.swissmodel.expasy.org/workspace). The three-dimensional structures allowed us to determine that some of the Thr are located near positively charged amino acids (Arg and Lys) sites susceptible to hydrolysis by bovine trypsin; this suggests that the carbohydrates at the sites of proteinolytic action may be protecting, by steric hindrance or masking, of recognition sites for proteases. These structural characteristics of chymotrypsins and isotrypsins in *P. vannamei* probably give them protection against hydrolysis by other proteases. Complementary studies are necessary to explain how the bovine trypsin is hydrolysed by SPs in *P. vannamei*. The sites of proteinolytic action in bovine trypsin are exposed and accessible to shrimp proteinases.

Commercial proteinases from the bovine pancreas (Dabrowski & Glogowski 1977; Maugle *et al*. 1983), the porcine pancreas (Kolkovski *et al*. 1993), bacterial enzymes (Sirvas-Cornejo *et al*. 2007), enzymes in living food (Lauff & Hofer 1984; Kuz'mina & Golovanova 2004) or commercial enzymes mixtures (Divakaran & Velasco 1999) have been used as feed supplements, even supplementing foods for humans. Thus far, our results suggest that enzymes from related species could remain active inside the digestive tract of the shrimp and exert a positive effect. As an alternative method for evaluating the effectiveness of enzymes as supplements, because the enzymes maybe inactivated or hydrolysed by some proteinases in the digestive tract of the host, it is important to use *in vitro* studies to determine the fate of enzymes before supplementing feeds.

Conclusions

Our results show that digestive enzymes from the host could hydrolyse exogenous enzymes. It is necessary that exogenous enzymes remain active to contribute to hydrolysis of food proteins. In particular, it seems that serial trials will be needed to find the right proteinase preparation as supplements in the feed industry. The digestive enzymes from crustaceans are more resistant to hydrolysis than mammalian enzymes; this characteristic could be a potential source for supplements in the feed industry.

Acknowledgements

We thank Balam Martínez-Pérez of CIBNOR for donating of SP (chromatography fraction with activity of serine proteinases). Ira Fogel of CIBNOR provided valuable editorial services. Funding was provided by the Consejo Nacional de Ciencia y Tecnología (CONACYT grant CB-2010-01 and 000000000155119). M.G.Z. is a recipient of a CONACYT doctoral fellowship (214851). We also thank the two anonymous reviewers of the manuscript as their comments strengthen and improve the quality of the paper.

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