Aquaculture Nutrition

Aquaculture Nutrition 2016 doi: 10.1111/anu.12408

Advances in the study of activity additivity of supplemented proteases to improve digestion of feed protein by *Penaeus vannamei*

J.H. CÓRDOVA-MURUETA, M.A. NAVARRETE-DEL-TORO & F.L. GARCÍA-CARREÑO

Biochemistry, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico

Abstract

In vitro assays used porcine or bovine trypsin as models of exogenous enzymes to determine functioning in the presence of enzymatic extracts from the digestive gland of whiteleg shrimp *Penaeus vannamei*. Using electrophoresis and zymograms, when enzymes from the shrimp were mixed in the absence of protein substrate, they hydrolysed the trypsin from bovine or porcine origin. Porcine or bovine trypsin, when mixed with shrimp enzymes in pH-stat assays in the presence of shrimp commercial feed, fish meal, or casein, there was added activity to hydrolyse the protein substrate. Hydrolysis of protein substrate was two-fold to threefold stronger if exogenous enzymes were added. Results suggest that porcine or bovine trypsin could be used as feed supplements for whiteleg shrimp *P. vannamei* to enhance hydrolysis of proteins in feeds, because the commercial enzymes contributed to the hydrolysis of the protein in the three substrates in the presence of shrimp enzymes.

KEY WORDS: crustaceans, digestion, feed, hydrolysis, *in vitro*, proteases

Received 2 June 2015; accepted 28 October 2015

Correspondence: J.H. Córdova-Murueta, Biochemistry, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Calle IPN 195, La Paz, Baja California Sur 23096, Mexico. E-mail: jcordova@cibnor.mx

Introduction

The feed industry needs novel strategies to produce improved feeds to increase digestibility of food proteins. Exogenous (commercial) enzymes in feeds that promote high assimilation rates of the feed components are an alternative that is under consideration. This alternative must be studied, bearing in mind factors that may affect the functionality of the exogenous enzymes in the digestive tract, and mainly, exogenous enzymes must not be inactivated by endogenous ones or by the conditions in the gastrointestinal tract, such as pH (Marquardt *et al.* 1996). Proteases actively hydrolyse proteins, so added proteases in feeds should hydrolyse food protein but not the digestive proteases or tissues in the intended beneficiary. Some studies dealing with the use of proteases as feed supplements for aquatic animals have only assessed variables, such as growth or protein efficiency ratio and apparent digestibility, but the mechanisms for success or failure were not discussed (Divakaran & Velasco 1999; Farhangi & Carter 2007; Carneiro *et al.* 2008). Inclusion of proteases in aquaculture feeds is a relatively new approach to improve the digestibility of protein ingredients. However, some questions related to the functioning of mixtures of exogenous proteases and endogenous ones in aquatic animals are currently unanswered. In a previous investigation, proteases from the whiteleg shrimp, *Penaeus vannamei* (synonym *Litopenaeus vannamei*), or the warrior swimming crab, *Callinectes bellicosus*, were mixed with bovine trypsin *in vitro*, where the crustacean proteases hydrolysed the mammalian trypsin (González-Zamorano *et al.* 2013). This observation supposed that, when crustacean and mammalian proteases are mixed in the digestive tract of the shrimp, the bovine trypsin would be hydrolysed.

Enzyme technology is used in industrial chemical transformations (Christensen 1989), and their applications are influenced by the availability and feasibility of the catalyst (Schmid *et al.* 2001). A question is the role of proteases from different sources in mixtures that may be used as feed supplements. Enzymes used in food processing are removed or inactivated from the food products after performing their activity. Ingested active enzymes may cause damage to other enzymes and tissues inside the gastrointestinal

tract (Pariza & Cook 2010). It is known that specific proteases are essential in several physiological processes, but others may affect proteins and tissues causing unwanted effects if they are present in inappropriate quantities or places in the digestive tract (Harrison & Bonning 2010). *In vitro* studies are essential to help predict ways that enzyme cocktails act under varied conditions: pH, concentration of enzymes and the presence of a protein substrate affects the activity of proteases (Qiao *et al*. 2005).

Proteases have been used to supplement aquafeeds to enhance protein digestibility to advance assimilation of amino acids. Contradictory results have been found. For example, Davis *et al*. (1998) report that 0.4% feed-grade protease supplementation to a commercial shrimp feed yielded an increase from 65% to 74% apparent protein digestibility and a decrease in growth and feed utilization. Divakaran & Velasco (1999) report that, although *in vitro* digestibility indicated the presence of active enzymes in the feed, the feeding trial revealed that shrimp growth was not enhanced by the inclusion of proteases in the feed. Basic research must be carried out to understand the mechanisms of aquafeed enzymology.

We already know that the destiny of commercial enzymes (bovine and porcine trypsins) in the presence of shrimp enzymes, lose their activity (González-Zamorano *et al*. 2013). Here, we compare the stability and hydrolytic capabilities of commercial porcine and bovine trypsins, alone or mixed with shrimp enzymatic extract in the presence or absence of proteinaceous substrates.

Materials and methods

Enzymes

Penaeus vannamei specimens (15.0 ± 1.0 g) from CIBNOR hatchery facilities were fed *ad libitum* twice daily, using commercial feed (PIASA; Productora Industrial Acuasistemas, La Paz, BCS, Mexico). They were kept under controlled conditions (28 °C under continuous flow of filtered seawater and aeration); 24 h prior to sampling, feeding was suspended. The digestive gland of 200 shrimp was 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 pelletize lipids and tissue debris. The aqueous supernatant is the enzyme extract, which was freeze-dried and stored at 4 °C for later analysis. Enzyme extract (named PV) was mainly composed of five serine proteases: two isochymotrypsins H and L (Sellos & Van Wormhoudt 1992; Hernández-Cortés *et al*. 1997) and three isotrypsins as follows: A, B and C (Sainz *et al*. 2004), and it also contained other proteases in negligible amounts. Bovine trypsin type IX and porcine trypsin type I (T4265 and T0134; Sigma-Aldrich, St. Louis, MO, USA) were prepared in concentration of 10 mg mL^{-1} in distilled water and used immediately. They were named as BT and PT, respectively.

Total proteolytic activity

Soluble protein in the enzyme preparations was quantified by the Bradford method (Bradford 1976). Bovine serum albumin (BSA and B4267; Sigma-Aldrich) was used as the standard. Each enzyme preparation was diluted with distilled water and analysed for total proteolytic activity (Garcia-Carreño & Haard 1993), using 0.5% (w/v) azocasein (A2765; Sigma-Aldrich) as the substrate; all assays were conducted in triplicate. Total protease activity units were expressed as change in absorbance per minute per mL of enzyme extract ($U = Abs_{366}\ min^{-1}\ mL^{-1}$).

Stability of enzyme mixtures without the substrate

Penaeus vannamei enzyme extract (PV), PT and PV + PT were analysed using electrophoresis methods to compare protein composition and migration performance and observe proteolytic activity of each enzyme preparation when enzymes are isolated or mixed. The mixture PV + BT was previously analysed in González-Zamorano *et al*. (2013). Protein composition of enzyme samples was analysed by SDS-PAGE (Laemmli 1970), as follows. Samples containing 12 μg protein for PV or 2 μg for PT, previously mixed with 2× sample buffer (0.125 M Tris-HCl at pH 6.8, 4% SDS, 20% v/v glycerol, 0.02% w/v bromophenol blue), were loaded into a 12% acrylamide gel. A 4 μL sample of low molecular weight standard mixture (17-0446-01; GE Healthcare, Little Chalfont, UK) was loaded. Proteins were separated at 15 mA, using a mini-vertical electrophoresis device (SE260; GE Healthcare). Once electrophoresis was completed, the gel was stained with an 0.05% (w/v) aqueous solution Coomassie brilliant blue R-250 (B0149; Sigma-Aldrich), 40% (v/v) methanol and 7% (v/v) acetic acid and then distained with 40% (v/v) aqueous solution of methanol and 7% (v/v) acetic acid. The gels were digitalized using an image analyser (EZ-Doc; Bio-Rad Laboratories, Hercules, CA, USA).

Proteolytic activity was analysed, as follows. The equivalent volume containing 125 mU activity of PT was

sequentially mixed with several amounts of PV containing 125, 62.5, 31.2, 15.6, 7.8, 3.9 or 1.9 mU activity, and each one made up to a final volume of 100 μL with 50 mM Tris-HCl at pH 8. The reaction mixtures were incubated for 5 min at 28 °C. Subsamples containing 5 mU activity from each tube were mixed with 2× sample buffer and immediately loaded into 12% acrylamide gels; 4 μL of a low molecular weight standard mixture was loaded in one lane. Electrophoresis conditions were performed as above. Zymograms for alkaline protease activity were prepared by substrate SDS-PAGE (García-Carreño *et al*. 1993). After electrophoresis, the gel was transferred to a tray and washed with distilled water to eliminate SDS. Then, 60 mL cold [3% (w/v)] casein in 50 mM Tris-HCl at pH 8.0 was poured and incubated on an ice bath under orbital shaking. After 30 min, the temperature was raised to 25 °C and incubated for 90 min. Then, the gel was washed thoroughly with distilled water to remove remaining substrate and immediately stained, distained and digitalized, as described earlier. Proteases were identified as clear bands against a blue background.

Performance of protease mixtures with substrate

The pH-stat method (Ezquerra-Brauer *et al*. 1997) was used to compare *in vitro* performance of digestive enzymes extract from *P. vannamei*, porcine and bovine trypsin, alone or combined. Casein-Hammerstein (US2840; USB Corporation, Cleveland, OH, USA), fishmeal (F6381; Sigma-Aldrich) and a commercial shrimp feed (PIASA) were used as protein substrates. The proximate composition of fishmeal and the commercial feed is provided in Table 1. The appropriate amount of each substrate was weighed to yield 0.08 g protein. The substrates were stirred in distilled water, adding water to complete 10 g of substrate mixture (substrate + water + enzyme = 10 g). The pH of this mixture (without enzyme) was adjusted to 7.9, with 1 N NaOH solution and stirred for 1 h to facilitate protein solubilization, using a titrator (718 Stat Titrino; Metrohom, Herisau, Switzerland). Prior to adding the enzyme to start the hydrolysis reaction, the temperature was set to 28 °C in a jacketed reaction vessel, and the pH of the substrate was raised to 8.0 by the pH-stat device by adding 0.1 N NaOH. Then, the selected enzyme or enzyme mix, previously adjusted to pH 8.0, was added to the reaction vessel. The hydrolysis reaction was followed for 1 h, recording the pH and the volume of consumed 0.1 N NaOH to keep the pH at 8.0 during the reaction time. The appropriate volume of enzyme solution containing 2.0 U activity was used for each enzyme when assayed alone. When a mix of enzymes was used, 2.0 U of each was added to observe additive effects on hydrolysis of the substrates. Degree of hydrolysis (DH%) was calculated (Lemos *et al*. 2004): $DH\% = 100\ [(B \times N_B)\ (1/\alpha)]\ [(1/Mp)\ (1/h_{tot})]$, where: $N_B = 1.0$, $1/\alpha = 1.13$, $h_{tot} = 8.6$.

SDS-PAGE of hydrolysis products

Hydrolysis products from the commercial feed and casein were followed in 12% SDS-PAGE. Additional samples were run in the pH-stat, as described earlier, except that during the reaction time, subsamples were taken at 0, 5, 30 and 60 min. Soluble protein was measured using the Bradford method (Bradford 1976). Subsamples were immediately placed in an ice bath to stop the reaction. The samples were boiled and mixed with sample buffer-containing DTT (0.5 M). The appropriate volume, containing 30 μg protein, was loaded into each well of the gel. After electrophoresis, the gels were stained, distained and digitalized as described earlier.

Statistical analysis

All assays were carried out in triplicate. ANOVA was carried out to compare mean DH% values. Tukey's range test was used to find the differences, setting significance at $P < 0.05$.

Results and discussion

Enzymes interaction in the absence of the substrate

González-Zamorano *et al*. (2013) demonstrated that crustacean enzymes hydrolyse bovine trypsin when mixed *in vitro*. We used porcine trypsin as a model to determine the potential for using exogenous enzymes as a feed

Table 1 Proximate composition of commercial feed and fishmeal (g kg^{-1})

Source	Protein	Lipids	Ash	Fibre	NFE[1]	Moisture
Commercial feed	386.3 ± 1.8	68.7 ± 0.4	110.1 ± 0.2	18.8 ± 0.9	372.1	43.9
Fishmeal	669.7 ± 3.0	108.2 ± 2.0	190.8 ± 1.7	0.3 ± 0.1	11.3	19.7 ± 0.7

[1] NFE calculated by difference.

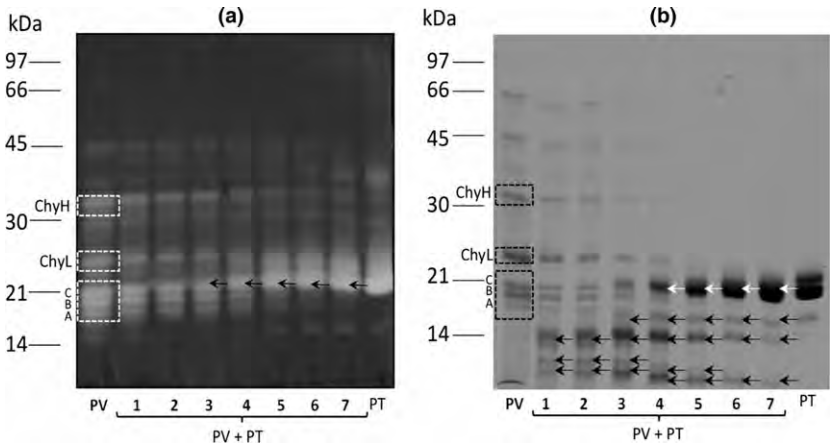

Figure 1 Substrate SDS-PAGE and SDS-PAGE of enzyme mixtures with constant amount of porcine trypsin with decreasing amounts of digestive proteases from *Penaeus vannamei*. (a) Zymogram of extract from *P. vannamei* (PV), porcine trypsin (PT) and mixture of them in Lanes 1–7, with decreasing amounts of PV. The black arrows indicate hydrolysis products from PT (Lane PV as the control; 125 mU), PT (Lane PT; 125 mU) and mixture; Lanes 1–7 show decreasing activities of PV: 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 1.9 mU). The boxes in the Lane PV indicate chymotrypsins (ChyH and ChyL) and trypsins (A, B and C). (b) Proteins in the PV, PT and the mixture in the Lanes 1–7 show decreasing amounts of PV. Samples for substrate SDS-PAGE and SDS-PAGE analysis were taken after incubation for 5 min at 28 °C. Black arrows indicate hydrolysis products from PT; white arrows indicate the decline of the main protein in PT.

supplement for shrimp by following its stability in the presence of substrate when exposed to enzymes from *P. vannamei* digestive gland. The zymogram (Fig. 1a) shows PV activity bands of trypsins A, B and C, and the two chymotrypsins L and H (see boxes in lane PV). Enzyme activity can be observed even at the lowest concentration (Lanes 1 to 7; 125 to 1.9 mU activity, respectively). The band of porcine trypsin activity is fully shown in lane PT. When PT is mixed with PV, the main band of activity decreased until it is not detected at 125 or 62.5 mU of PT (Fig. 1a; Lanes 1 and 2). There is an inverse correlation between the activity of PV and the intensity of the main band of PT (Fig. 1a; Lanes 3 to 7), where PT protease activity is observed in the zymogram when the PV concentration is four times lower, than PT or more (Fig. 1a; Lanes 3 to 7, black arrows). In Fig. 1b, the electropherogram shows that the main band of PT is hydrolysed by PV, generating low molecular weight protein products (Fig. 1b; Lanes 1 to 7; white arrows). This means that the extent of PT hydrolysis was related to the amount of shrimp extract in the reaction. González-Zamorano *et al.* (2013) and our results show that PV proteases hydrolysed bovine and porcine trypsin.

Enzymes interaction in the presence of the substrate

Additionally, we studied the activity of proteases and their mixtures on proteinaceous substrates by *in vitro* digestion by pH-stat. The DH% of the evaluated substrates showed that PV hydrolysed, to a lesser extent, the protein in the commercial feed and fishmeal (Fig. 2a,b) than PT and BT, these enzymes doubled or tripled hydrolysis of each substrate. When casein was used as the substrate, the enzymes PV, PT and BT produced similar amounts of DH%, with PT producing the lowest ($P < 0.05$; Fig. 2c). When PV was mixed with PT or BT, the amount of hydrolysis in mixes of enzymes was higher than the amount of hydrolysis from a single source of enzymes (Fig. 2a–c). This indicates that adding an exogenous enzyme (BT or PT) to PV in the presence of a substrate, the enzymes act on the protein substrate, not on other enzymes. The degree of hydrolysis of the substrate increases with the additional enzyme activity. A paramount result is that PV added activity when mixed with either BT or PT, regardless of the substrate, commercial feed or fishmeal. This contradicts what occurred in González-Zamorano *et al.* (2013). These results suggest that mammalian proteases may be used to supplement shrimp feeds.

Molecular interaction

When enzymes were mixed and incubated with or without substrate, the results were contradictory. Trypsins have specificity for Lys and Arg in the carboxylic side of the scissile peptide bond in the protein substrate (Olsen *et al.* 2004), and trypsin primary structure contains Lys and Arg that can be hydrolysed for trypsin molecules (Vestling *et al.*

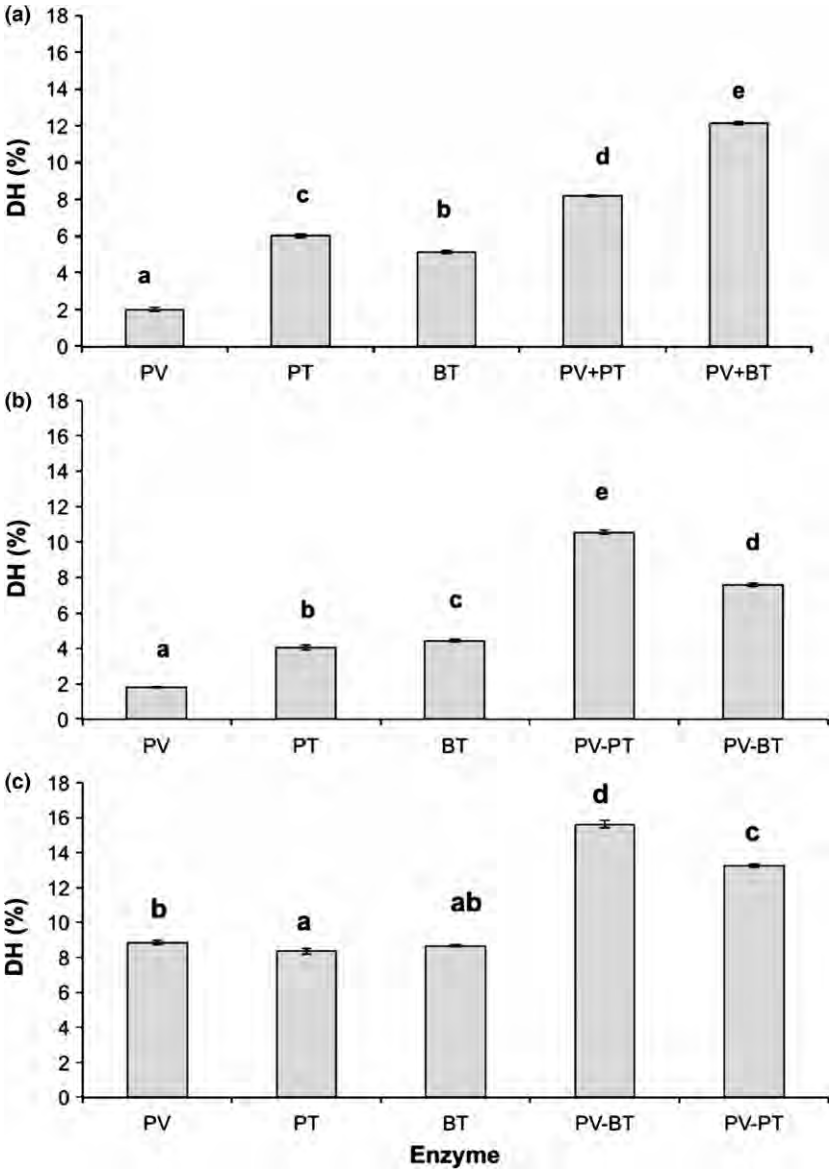

Figure 2 Degree of hydrolysis of commercial shrimp feed (a), fishmeal (b) and casein (c). Different letters in columns in the same graph mean significant differences ($P < 0.05$). *Penaeus vannamei* enzymatic extract from the digestive gland (PV); PT, porcine trypsin; BT, bovine trypsin, also showing PV + BT and PV + PT.

1990). Qiao *et al*. (2005) found full enzyme activity, when a proteinaceous substrate was present in enzyme mixtures and suggests substrate protection. With an *in vitro* study, Wang & Hsu (2006) report that trypsin and chymotrypsin were significantly more resistant to acid and pepsin hydrolysis in the presence of casein or soybean protein as substrate, amounting to eight times more residual activity than without the substrate. In the absence of a protein substrate, trypsins in PV hydrolysed PT as occurred with BT in previous work (González-Zamorano *et al*. 2013). Shrimp isotrypsins A, B and C are glycoproteins (Sainz *et al*. 2004) that have carbohydrate residues that function as stabilizers of the three-dimensional structure of the protein. This helps avoid hydrolysis by other enzymes or auto hydrolysis because the unfolded protein molecules are more susceptible to proteolysis than folded forms (Villalonga *et al*. 2000).

As indicated by the pH-stat tests, when a large number of substrate molecules, such as commercial shrimp feed, casein or fishmeal were present in the reaction mixture, the probability of one molecule of PV to collide at the right angle and with enough energy to react with one molecule of porcine or bovine trypsin was significantly lower than when only PV and PT or PB were in the mixture. Substrate molecules occupying the active site of the enzyme add in preventing action on other enzymes (Wang & Hsu 2006). The DH% of the substrates assayed shows that when PV is mixed with BT or PT, the extent of the hydrolysis was significantly increased (Fig. 2a–c), implying that BT or PT

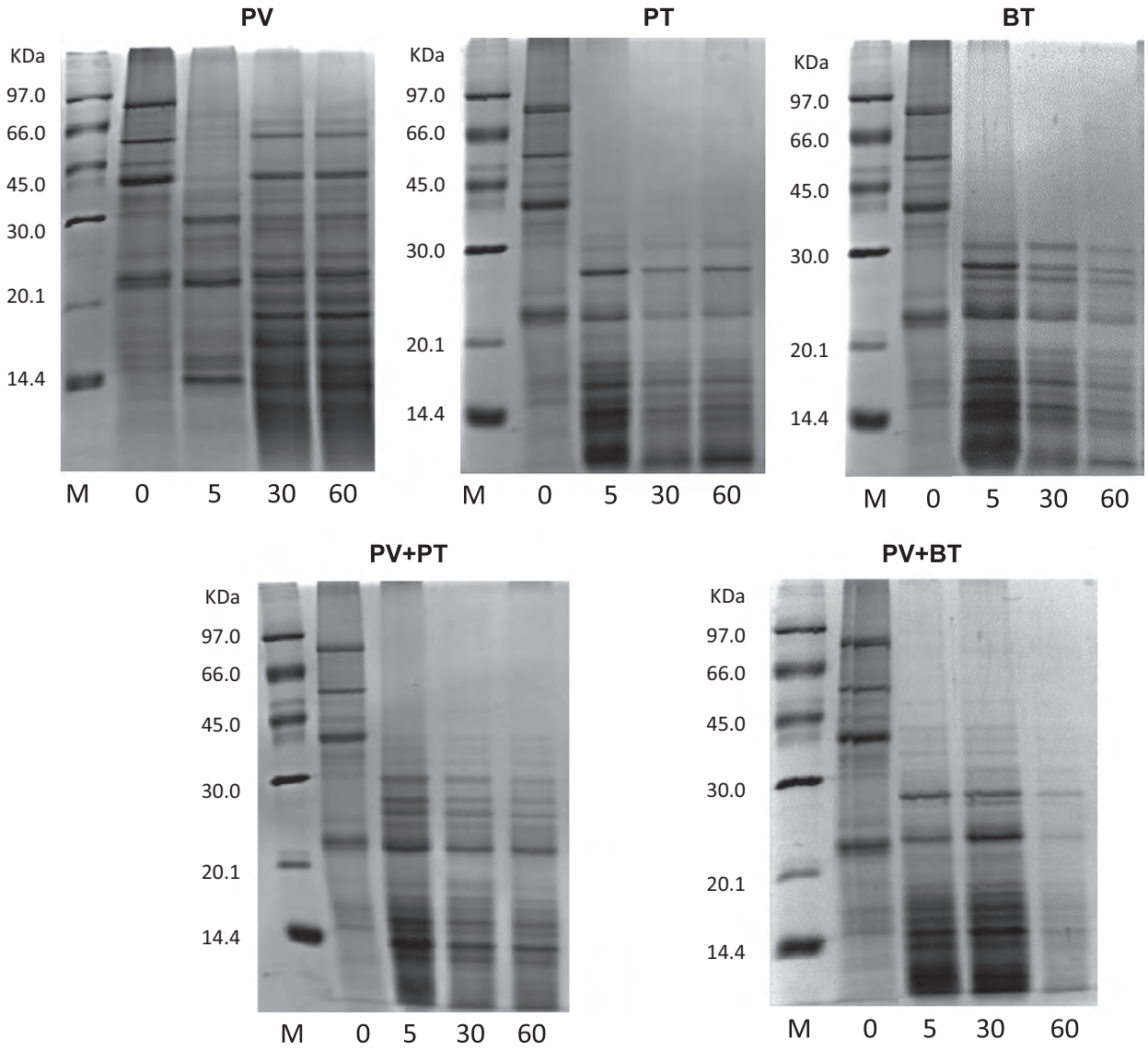

Figure 3 SDS-PAGE of hydrolysis of commercial shrimp feed by *Penaeus vannamei* enzyme extract (PV); porcine trypsin (PT); bovine trypsin (BT); and mixtures PV + PT and PV + BT. Samples taken at 0, 5, 30 and 60 min. Lane M shows molecular weight markers.

mixed with PV was active during the reaction time and was not suppressed in the presence of shrimp enzymes. This finding was confirmed by the electropherogram analysis of the hydrolysis products of commercial feed and casein (Figs 3 & 4). The pattern and size of the peptides generated during substrate hydrolysis over time have lower molecular weights than the control (Lane 0), demonstrating the advantage of protein hydrolysis with mixtures (PV and PT or PV and BT).

Shrimp enzymes yielded the lowest DH% of commercial feed and fishmeal. Fishmeal is the main protein ingredient of shrimp feeds, but if the drying process is performed at high temperature, bioavailability of the protein is negatively affected (Córdova-Murueta *et al.* 2007). Shrimp feeds are usually supplemented with different sources of protein, including plants that may contain protease inhibitors. This reduces catalytic activity of the endogenous enzymes (Lemos *et al.* 2004).

Protease supplemented feeds should improve the digestibility of feed protein (Carneiro *et al.* 2008; Cowieson & Ravindran 2008), which occurred in the pH-stat assays. In all treatments, regardless of the substrate, the mix of PV with BT or PT yielded at least a twofold increase in hydrolysis over the three enzymes applied separately. In

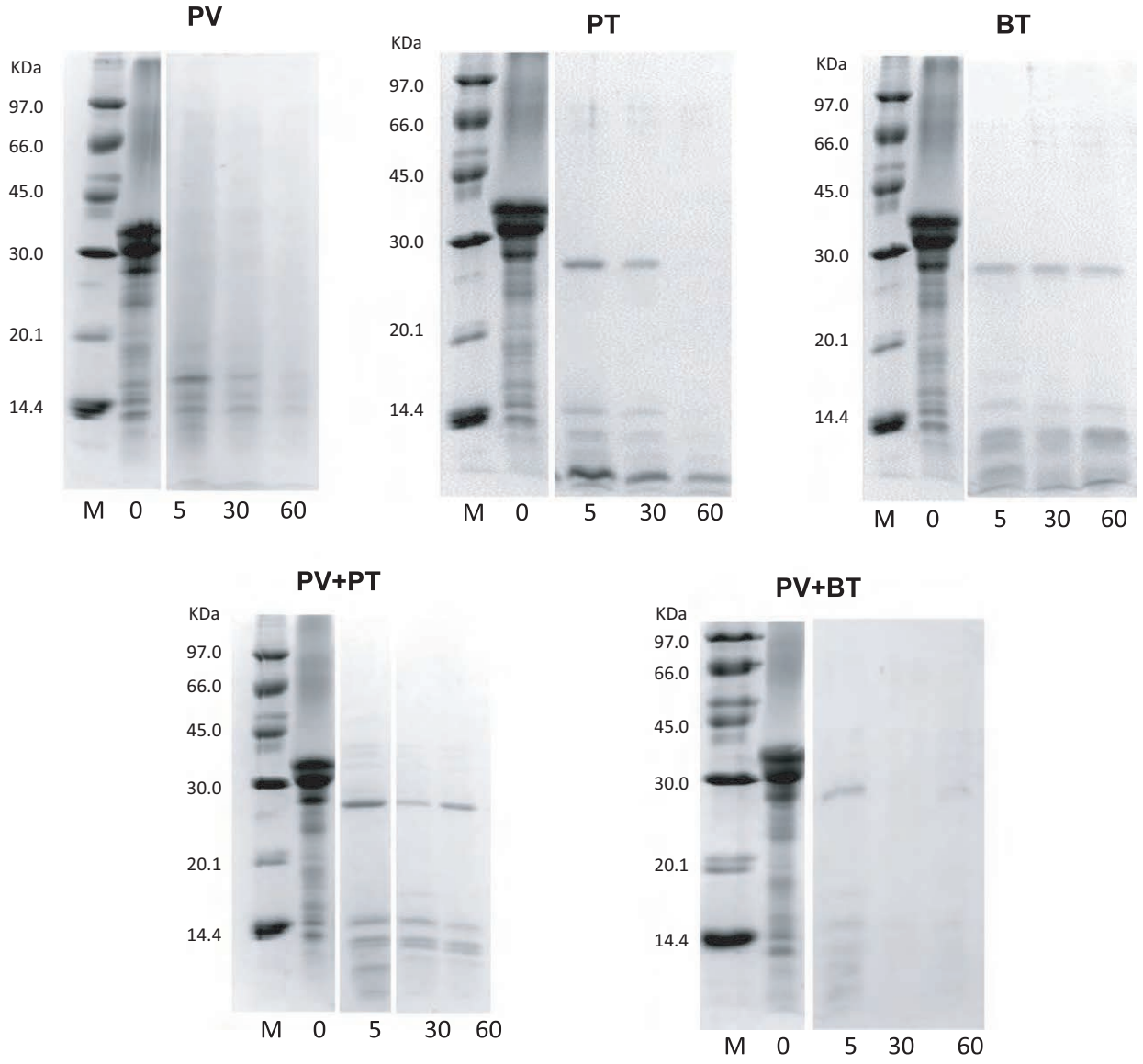

Figure 4 SDS-PAGE of hydrolysis of casein by *Penaeus vannamei* enzyme extract (PV); porcine trypsin (PT); bovine trypsin (BT); and mixtures PV + PT and PV + BT. Samples taken at 0, 5, 30 and 60 min. Lane M contains molecular weight markers.

several investigations where enzymes were added to feed, there was no noticeable effect on the targeted animals (Divakaran & Velasco 1999; Miller *et al*. 2008), probably because the enzymes were degraded during feed processing or one or more of the factors discussed earlier. Another consideration is that exogenous proteases may hydrolyse amylases, lipases and other digestive enzymes. An obvious essential criterion for enzyme supplements is that they must remain active in the digestive system and not obstruct the digestion process. *In vitro* assessments of enzymes help to find appropriate supplements for the targeted species, although *in vivo* studies are still necessary, considering the physiology of the digestive system.

In summary, we demonstrated that assessing *in vitro* proteases as feed supplements requires understanding compatibility of exogenous proteases with digestive enzymes of the intended species before live trials. Our results suggest that porcine or bovine trypsin can be tested as feed supplements for whiteleg shrimp to enhance hydrolysis of proteins in feeds, because the exogenous enzymes contributed to the hydrolysis of protein in three substrates in the presence of PV enzymes.

It is an obvious outcome of the study that *in vivo* assays are to challenge the hypothesis stated in the previous paragraph. Other species, in aquaculture production, mostly crustaceans, can be tested with the same strategy. Fish, having active stomachs, may need a different approach.

Acknowledgements

J. González and M. González of CIBNOR provided technical assistance. I. Fogel of CIBNOR provided editorial services. Funding was provided by Consejo Nacional de Ciencia y Tecnología (CONACYT grant CB-2010-01 0155119).

References

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Carneiro, M.S.C., Lordelo, M.M., Cunha, L.F. & Freire, J.P.B. (2008) Effects of dietary fibre source and enzyme supplementation on faecal apparent digestibility, short chain fatty acid production and activity of bacterial enzyme. *Anim. Feed Sci. Technol.*, **146**, 124–136.

Christensen, F.M. (1989) Enzyme technology versus engineering technology in the food industry. *Biotechnol. Appl. Biochem.*, **11**, 249–265.

Córdova-Murueta, J.H., Navarrete-del-Toro, M.A. & García-Carreño, F.L. (2007) Concentrates of fish protein from bycatch species produced by various drying processes. *Food Chem.*, **100**, 705–711.

Cowieson, A. & Ravindran, V. (2008) Effect of exogenous enzymes in maize-based diets varying in nutrient density for young broilers: growth performance and digestibility of energy, minerals and amino acids. *Br. Poult. Sci.*, **49**, 37–44.

Davis, D.A., Johnston, W.L. & Arnold, C.R. (1998) The use of enzyme supplements in shrimp diets. *In IV International Symposium on Aquatic Nutrition*, pp. 1–20. 18 November (1998) La Paz. B.C.S, Mexico.

Divakaran, S. & Velasco, M. (1999) Effect of proteolytic enzyme addition to a practical feed on growth of the Pacific white shrimp, *Litopenaeus vannamei* (Boone). *Aquacult. Res.*, **30**, 335–339.

Ezquerra-Brauer, J.M., Garcia-Carreño, L.F., Civera, R. & Haard, N.F. (1997) pH-stat method to predict protein digestibility in white shrimp (*Penaeus vannamei*). *Aquaculture*, **157**, 251–262.

Farhangi, M. & Carter, C.G. (2007) Effect of enzyme supplementation to dehulled lupin-based diets on growth, feed efficiency, nutrient digestibility and carcass composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquacult. Res.*, **38**, 1274–1282.

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

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

González-Zamorano, M., Navarrete del Toro, M.A. & García-Carreño, F.L. (2013) Exogenous proteinases as feed supplement for shrimp: *in vitro* evaluation. *Aquacult. Nutr.*, **19**, 731–740.

Harrison, R.L. & Bonning, B.C. (2010) Proteases as insecticidal agents. *Toxins (Basel).*, **2**, 935–953.

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

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

Lemos, D., Navarrete-del-Toro, M.A., Córdova-Murueta, J.H. & Garcia-Carreño, F. (2004) Testing feeds and feed ingredients for juvenile pink shrimp *Farfantepenaeus paulensis*: *in vitro* determination of protein digestibility and proteinase inhibition. *Aquaculture*, **239**, 1–4.

Marquardt, R.R., Brenes, A., Zhang, Z. & Boros, D. (1996) Use of enzymes to improve nutrient availability in poultry feedstuffs. *Anim. Feed Sci. Technol.*, **60**, 321–330.

Miller, D.R., Granzin, B.C., Elliott, R. & Norton, B.W. (2008) Effects of an exogenous enzyme, Roxazyme® G2 Liquid, on milk production in pasture fed dairy cows. *Anim. Feed Sci. Technol.*, **145**, 194–208.

Olsen, J.V., Ong, S.-E. & Mann, M. (2004) Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Mol. Cell Proteomics*, **3**, 608–614.

Pariza, M.W. & Cook, M. (2010) Determining the safety of enzymes used in animal feed. *Regul. Toxicol. Pharmacol.*, **56**, 332–342.

Qiao, Y., Gumpertz, M. & Van Kempen, T. (2005) Stability of a pancreatic enzyme cocktail during *in vitro* protein digestibility assays. *J. Food Biochem.*, **29**, 205–220.

Sainz, J.C., Garcia-Carreño, L.F. & Hernandez-Cortés, P. (2004) *Penaeus vannamei* isotrypsins: purification and characterization. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **138**, 155–162.

Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M. & Witholt, B. (2001) Industrial biocatalysis today and tomorrow. *Nature*, **409**, 258–268.

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.

Vestling, M.M., Murphy, C.M. & Fenselau, C. (1990) Recognition of trypsin autolysis products by high-performance liquid chromatography and mass spectrometry. *Anal. Chem.*, **62**, 2391–2394.

Villalonga, R., Villalonga, M. & Gómez, L. (2000) Preparation and functional properties of trypsin modified by carboxymethylcellulose. *J. Mol. Catal. B Enzym.*, **10**, 483–490.

Wang, H.T. & Hsu, J.T. (2006) Usage of enzyme substrate to protect the activities of cellulase, protease and α-amylase in simulations of monogastric animal and avian sequential total tract digestion. *Asian-Aust. J. Anim. Sci.*, **19**, 1164–1173.