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# Peptidase compensation in the digestive system of whiteleg shrimp *Penaeus vannamei* against dietary Kunitz-type soybean trypsin inhibitor

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## Abstract

The aim of this study was to assess the regulatory process of digestive peptidases of crustaceans in the presence of soybean trypsin inhibitor (SBTI). This naturally occurring inhibitor in soybean meal was used to inhibit the activity of digestive serine peptidases of the whiteleg shrimp *Litopenaeus vannamei*. In vitro, SBTI inhibited the total proteolytic activity and chymotrypsin activity by 65%. Trypsin activity was reduced by SBTI from 40% to 15% from 2 to 4 hr of incubation, which is the average time of residence of feed in the shrimp digestive system. During the bioassays, experimental groups were fed with increasing concentration of supplemental SBTI (1 g $kg^{-1}$ and 2 g $kg^{-1}$) and digestive gland and faeces of individual specimens were collected daily. At the end of the bioassay, peptidase activity of digestive gland and faeces was shown, revealing differential inhibition after feeding for 5 days. Several serine peptidases were observed in zymograms, showing a compensation effect on the digestive gland through the activation of peptidases from different catalytic type. These results provide evidence that the shrimp digestive gland can overcome the effect of SBTI by two adaptive mechanisms: synthesis of additional peptidases of the serine class and other unidentified peptidases.

**KEYWORDS**

decapod crustaceans, digestive peptidases, enzyme compensation

## 1 | INTRODUCTION

Peptidases are protein hydrolytic enzymes involved in all physiological processes. These enzymes are controlled by the synthesis, storage, secretion and activation of zymogens (Muhlia-Almazán, Sánchez-Paz, & García-Carreño, 2008). When peptidases are spontaneously activated, peptidase inhibitors are required as a control mechanism to prevent unwanted hydrolysis (Rai, Aggarwal, Mitra, Das, & Babu, 2010; Roy et al., 2009). Besides peptidase activity regulation, specific inhibitors may be involved in controlling those peptidases from pathogens as a host defence mechanism of invertebrates (Xue et al., 2006). Moreover, the peptidase inhibitors of one species may affect the peptidases of another in a prey–predator interaction (Armstrong, 2006).

During the feeding process, besides nutrients, an animal consumes molecules that may tamper with the digestion process, such as peptidase inhibitors (Bakke et al., 2014; Francis, Makkar, & Becker, 2001). Plant meals made of soybean, pea, cotton seed, corn gluten and wheat gluten have been intended for aquatic animal feeds. Among these plant protein sources, the soybean meal is currently used in fish and shrimp feeds (Sookying & Davis, 2011). However, soybean meal contains trypsin inhibitors and other antinutritional factors that can negatively affect the digestion process. Varieties of soybean have been tested in digestibility trials and all containing trypsin inhibitors (Zhou, Davis, & Buentello, 2014). The inclusion of exogenous enzymes such as carbohydrases, and serine peptidases, has been studied to improve digestibility of feeds containing soybean meal, but trypsin inhibitors remained (Dalsgaard et al., 2012).

  

In invertebrates, there are three known response mechanisms against exogenous peptidase inhibitors: (i) hydrolysis of exogenous peptidase inhibitors by non-target peptidases in the digestive system (Amirhusin et al., 2007), (ii) synthesis of another catalytic type of peptidases that are not target of the inhibitor in feed (Ramalho de Oliveira et al., 2013), and (iii) synthesis of isoenzymes that can be active in the presence of the inhibitor (Schwarzenberger, Zitt, Mueller, & Von Elert, 2010). As any other taxa, decapod crustaceans evolved traits that allow them to strive in different environments, feeding habits, herbivorous, carnivorous or detritivorous, favoured the divergence of digestive enzymes (Bonorino & Anderson, 2009). Enzymes that hydrolyse feed protein in penaeids are mostly serine peptidases (Navarrete del Toro, García-Carreño, & Córdova-Murueta, 2011). In the whiteleg shrimp *Penaeus vannamei* (syn: *Litopenaeus vannamei*), isotrypsins and isochymotrypsins are the most abundant serine peptidases in the digestive gland (Hernández-Cortés, Whitaker, & García-Carreño, 1997; Sainz, García-Carreño, Córdova-Murueta, & Cruz-Hernández, 2005; Sainz, García-Carreño, & Hernández-Cortés, 2004; Sainz, García-Carreño, Sierra-Beltrán, & Hernández-Cortés, 2004); cysteine and metallo peptidase classes are also reported, all of them with a potential role in the digestive process (Stephens, Rojo, Araujo-Bernal, García-Carreño, & Muhlia-Almazán, 2012; Zhao et al., 2007). However, to the best of our knowledge, adaptive mechanisms of the digestive enzymes in shrimp against antinutritional factors has not been described.

Given the presence of trypsin inhibitors in protein ingredients for aqua-feeds, like soybean meal and legumes in general, the aim of this work was to know the physiological response of the whiteleg shrimp *L. vannamei* when fed Kunitz-type soybean trypsin inhibitor (SBTI) to support future biotechnologies in aqua-farming.

## 2 | MATERIALS AND METHODS

### 2.1 | Total alkaline proteolytic activity

Total peptidase activity was measured using 5 g azocasein/L (#A2765; Sigma-Aldrich, St. Louis, MO, USA) as substrate in 50 mM Tris-HCl at pH 8.0 (García-Carreño, Hernández-Cortés, & Haard, 1994). The reaction mixture consists of 10 µl of digestive gland extract, 150 µl of 50 mM Tris-HCl buffer (pH 8.0) and 150 µl of substrate with an incubation time of 10 min. The reaction was stopped with 200 ml trichloroacetic acid/L. Then, the reaction was incubated at −20°C for 5 min and centrifuged for 10 min at 10,000 × *g*. The supernatant was separated from the non-hydrolysed substrate, and absorbance was recorded at 366 nm. One unit of activity was defined as the change in absorbance per min per mg of protein.

### 2.2 | Trypsin and chymotrypsin specific activity

Specific trypsin activity was measured in the digestive gland extracts using 1 mM of the specific substrate BAPNA (#B4875; Sigma-Aldrich) in a 50 mM Tris-HCl, 20 mM $CaCl_2$ buffer at pH 8.0. The amount of *p*-nitroanilide released from BAPNA was quantified by the increase in absorbance at 410 nm for 5 min recorded at intervals of 30 s. One unit of trypsin activity was defined as the amount of protein that hydrolyses 1 µM BAPNA per mg protein.

To determine chymotrypsin activity in the digestive gland extracts, 0.1 mM of the specific substrate SAAPNA (#S7388; Sigma-Aldrich) was used in a 50 mM Tris-HCl, 20 mM $CaCl_2$ buffer pH 8.0. One unit of chymotrypsin activity was defined as the amount of protein that hydrolyses 0.1 µM of SAAPNA per mg of protein (García-Carreño et al., 1994).

### 2.3 | Inhibition of digestive serine peptidases in vitro

A portion of freeze-dried digestive glands from adult shrimp that were not part of the bioassay was solubilized in cold distilled water in a 1:3 (w/v) proportion and centrifuged for 20 min at 10,000 × *g* at 4°C. Soluble protein was quantified by the Bradford method (Bradford, 1976). To assure that the Kunitz-type SBTI (#T9003; Sigma-Aldrich) actively inhibits shrimp digestive peptidases, inhibition assays were performed with 0.2, 0.5, 1, 3, 5, 10, 15 and 20 µg of SBTI and residual activity was measured using azocasein as substrate (as described above).

### 2.4 | Feeding Bioassay

It is known that the average amounts of soybean included in farm-made feeds for shrimps may contain as much as 455 g $kg^{-1}$ soybean meal, such amount of raw soybean meal encloses about 2 g $kg^{-1}$ of SBTI (Clarke & Wiseman, 2005; Suárez et al., 2009). Based in this information, we choose 1 g $kg^{-1}$ and 2 g $kg^{-1}$ to prepare the experimental feeds.

A commercial feed (PIASA, Promotora Industrial de Acuasistemas, La Paz, B.C.S., Mexico) was used as base to prepare three experimental feeds with a supplemental inhibitor (1 g $kg^{-1}$ and 2 g $kg^{-1}$ SBTI), and a control feed without the inhibitor. The commercial feed was soaked in a solution with 1 g $kg^{-1}$ and 2 g $kg^{-1}$ of trypsin inhibitor (#T9003; Sigma-Aldrich) at a ratio of 100 g to 40 ml, then incubated for 12 hr at 4°C and dried in a vacuum oven for 5 hr at 55°C at 67.72 kPa. The feed provided to the control group (without the supplemental inhibitor) was soaked in distilled water and then treated as both experimental feeds. To avoid the inhibitor lixiviation from feed in seawater during the bioassays, all experimental feed were coated with a mixture of carnauba wax (#243213; Sigma-Aldrich) and menhaden oil as an attractant (#F8020; Sigma-Aldrich) in a mixture of 1:6 (w/v), respectively. The feed was kept at 4°C until used.

Enzyme inhibition assays were carried out using feed extracts as the inhibitor source to confirm that the supplemental SBTI was active after the feed preparation and heating. One microgram of each feed extract was incubated individually with 0.5 µg of soluble protein of a digestive gland extract, bovine trypsin (#T8003; Sigma-Aldrich) and bovine chymotrypsin (#C4129; Sigma-Aldrich). Specific activity was measured to ensure that the inhibitor was active before starting the bioassay.

Six specimens of adult whiteleg shrimp (~32.0 g each) per treatment (1 g $kg^{-1}$ and 2 g $kg^{-1}$ SBTI and control) were maintained in individual 19 L aquaria with continuous flow of filtered sea water at 27 ± 1.5°C for 14 days during acclimatization. At this time, all shrimp

were fed to satiation with the control feed. Moulted shrimp before the bioassay were discarded, and only shrimp during intermoult cycle were considered. After acclimatization, shrimp were fed with 0.6 g of each treatment feed divided into two daily rations for 5 days; faeces were collected prior to treatment (D0) and during the following 5 days (D1–D5). Faeces were collected by siphoning and then gently rinsed them with distilled water to eliminate the excess of salt, then were stored at −80°C until processing. In the last day, digestive glands were individually dissected and stored at −80°C until processed. Sampling of faeces allowed us to track possible changes in enzymes released to the lumen from the digestive gland in the same specimen, before and during the bioassay without killing the specimens; same peptidase profile is observed in faeces and digestive gland extracts (Córdova-Murueta, García-Carreño, & Navarrete del Toro, 2003).

### 2.5 | Confirming the presence of the inhibitor in the experimental feed

To guarantee that the inhibitor was active in the feed, 1.0 g of each feed was ground with a mortar and mixed with 5 ml of distilled water and incubated for 2 hr and centrifuged for 20 min at 10,000 × *g* at 4°C. The supernatant was taken apart, and the soluble protein was measured with the Bradford method. To confirm the presence of the SBTI in the feed, a dot blot analysis was carried out using the extracts previously prepared. Each feed extract sample was mixed with sample buffer (0.125 M Tris-HCl, 200 mL glycerol/L, 0.2 g bromophenol blue/L at pH 6.8) in a 1:20 (v/v) ratio, and 1 µl of each feed sample was immobilized in a nitrocellulose membrane. The nitrocellulose membrane was washed twice in 15 ml of TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 10 min and then incubated for 1 hr in a blocking solution (10 mM Tris-HCl, 150 mM NaCl, 0.5 ml Tween-20/L, 50 g skim milk/L). The nitrocellulose membrane was washed twice in 15 ml of TNT buffer (10 mM Tris-HCl, 150 mM NaCl, 0.5 mL Tween-20/L, pH 8.0). The membrane was incubated for 2 hr at room temperature with a polyclonal-specific antibody for the SBTI inhibitor (#PA1-85939, Thermo Fisher) diluted 1:2000 (v/v) with a TNT buffer. The nitrocellulose membrane was washed twice in 15 ml of TNT buffer. Then, the membrane was incubated for 2 hr at room temperature with an anti-rabbit IgG peroxidase antibody (#A6154; Sigma-Aldrich) diluted 1:2000 (v/v) with a TNT buffer. The nitrocellulose membrane was washed 4 times in 15 ml of TNT buffer and then incubated in 2 mM 3,3′-diaminobenzidine dissolved in TBS buffer until colour development.

### 2.6 | Native PAGE and zymogram for alkaline peptidases

Each digestive gland collected in the bioassay was homogenized in a 1:2 ratio (w/v) with cold distilled water. Homogenates were centrifuged for 30 min at 4°C at 10,000 × *g*, and the supernatant was separated from lipids and sediments. A second centrifugation was performed for 10 min at 4°C at 10,000 × *g*.

The faeces previously collected every day from individual shrimp were separately homogenized in 200 µl cold distilled water and centrifuged for 10 min at 4°C at 10,000 × *g*, and the supernatant was separated (Córdova-Murueta et al., 2003). The total soluble protein content of digestive gland and faeces extracts was quantified by the Bradford method (Bradford, 1976); then, 5 µg of soluble protein of each sample was loaded in wells of 0.75 mm gel (native PAGE) and 12% acrylamide and separated at 4°C and 15 mA. Before loading in the gel, each sample was mixed with sample buffer (0.125 M Tris-HCl, 200 ml glycerol/L, 0.2 g bromophenol blue/L at pH 6.8) in a 1:1 (v/v) ratio. After electrophoresis, gels were stained with 1 g Coomassie brilliant blue R-250/L in an aqueous solution of 50 mL methanol/L and 75 mL acetic acid/L and kept overnight at room temperature. Gels were distained with a 400 mL methanol/L and 100 mL acetic acid/L solution.

To assess the composition of peptidases in each sample, 0.005 U of total alkaline proteolytic activity was loaded into a gel. After electrophoresis, the gel was incubated in a solution with 3 g casein/L and 50 mM Tris-HCl at pH 8.0 at 4°C for 30 min. The gel was then incubated in the same solution at room temperature for 90 min, and subsequently washed with distilled water, and transferred to a clean tray with staining solution for 2 hr and then distained until peptidase activity appeared as white bands on a blue background (García-Carreño, Dimes, & Haard, 1993). The same procedure was used for the enzymatic extracts from faeces.

### 2.7 | In vitro inhibition assay of faeces extracts

The identity of the serine peptidases was determined using the specific inhibitor Pefabloc (#76307; Sigma-Aldrich). Enzymatic extracts from faeces collected before the trial and after 2 days of feeding with the supplemental inhibitor (2 g $kg^{-1}$ SBTI) were assayed with Pefabloc to observe changes in time in the same individuals, because this group showed the majority of unidentified peptidases. Then, 5 µg of soluble protein from the faeces extract was incubated with 2 mM Pefabloc for 1 hr at room temperature. Then, samples were mixed with the sample buffer (0.125 M Tris-HCl, 200 mL glycerol/L, 0.2 g bromophenol blue/L at pH 6.8) in a 1:1 (v/v) ratio and separated by native electrophoresis. Afterwards, casein zymograms at pH 8.0 were prepared to reveal peptidase activity, as described earlier (García-Carreño et al., 1993).

### 2.8 | Statistical analysis

Total proteolytic activity, trypsin and chymotrypsin activities were statistically analysed, using one-way ANOVA followed by a Tukey HSD test for six individual specimens per treatment. A significant difference between treatments was set at $p < .05$. Statistical analyses were processed using Statistica 8.0 (StatSoft, Tulsa, OK, USA).

## 3 | RESULTS

### 3.1 | In vitro inhibition of serine peptidases

Increasing concentrations of SBTI were mixed in vitro with the peptidases of the digestive gland of the whiteleg shrimp. Inhibition of the total proteolytic activity reached 80% when 1 µg of inhibitor

was added to the mixture of reaction with 20 μg of digestive gland extract; 2 μg of SBTI produced an inhibition of 90% (Fig. 1a). The total proteolytic, trypsin and chymotrypsin activity were measured at time 0 and then every hour after incubated 4 hr with 1 μg of inhibitor. It was noticeable that total proteolytic activity and chymotrypsin activity showed 35% of residual activity (65% inhibition); while residual trypsin activity was ~60% (~40% inhibition), at 2 hr of incubation, the inhibitory effect begun to decline reaching 90% of residual activity at 4 hr of incubation (Fig. 1b). This phenomenon was also observed and confirmed in zymogram after 2-hr incubation, when trypsin activity was evident (Fig. 1d). The SBTI with a molecular mass of 20.1 kDa; isotrypsins C: 23 kDa, B: 22 kDa and A: 21 kDa; and isochymotrypsins 24.1 kDa and 35.6 kDa were identified, based on previous reports (Hernández-Cortés et al., 1997; Sainz, García-Carreño, & Hernández-Cortés, 2004; Sainz, García-Carreño, Sierra-Beltrán, et al., 2004), by electrophoresis and zymography (Fig. 1c,d).

Figure 2a shows a dot blot analysis to demonstrate the presence of the SBTI in the feed used to fed the experimental groups, while Fig. 2b shows the inhibition of bovine trypsin by an extract from the experimental feeds, both figures prove that the inhibitor was present and active in the feeds. In test tubes, the activity of bovine trypsin decreased 50% when was incubated with the extract of 1 g $kg^{-1}$ SBTI

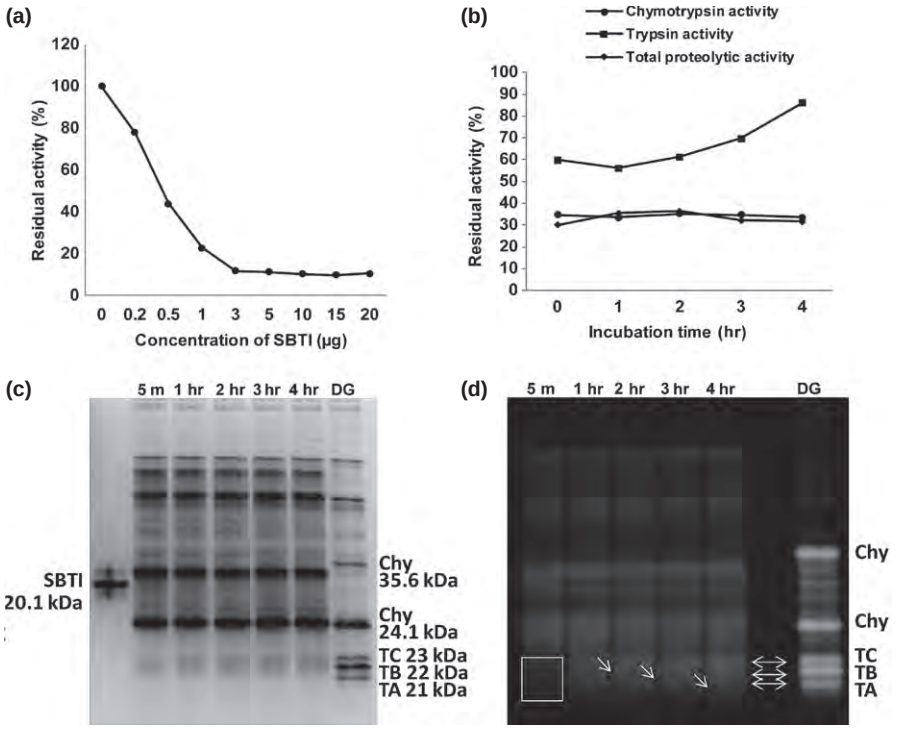


**FIGURE 1** *In vitro* interaction of digestive peptidases and SBTI. (a) Inhibition assay with increasing concentrations of SBTI, each dot represents the average of triplicate samples. (b) Total alkaline proteolytic activity and specific chymotrypsin and trypsin activities of triplicate samples. (c) Native PAGE 12% with digestive gland extract mixed with the trypsin inhibitor at different intervals of incubation. (d) Zymogram to reveal alkaline peptidase activity, showing inhibition of serine peptidases. Dotted white box indicates the inhibition of isotrypsins; dotted white arrows show how inhibition of isotrypsins is decreasing. SBTI, Kunitz-type soybean trypsin inhibitor; DG, digestive gland enzyme extract; Chy, chymotrypsins; TC, isotrypsin C; TB, isotrypsin B; TA, Isotrypsin A

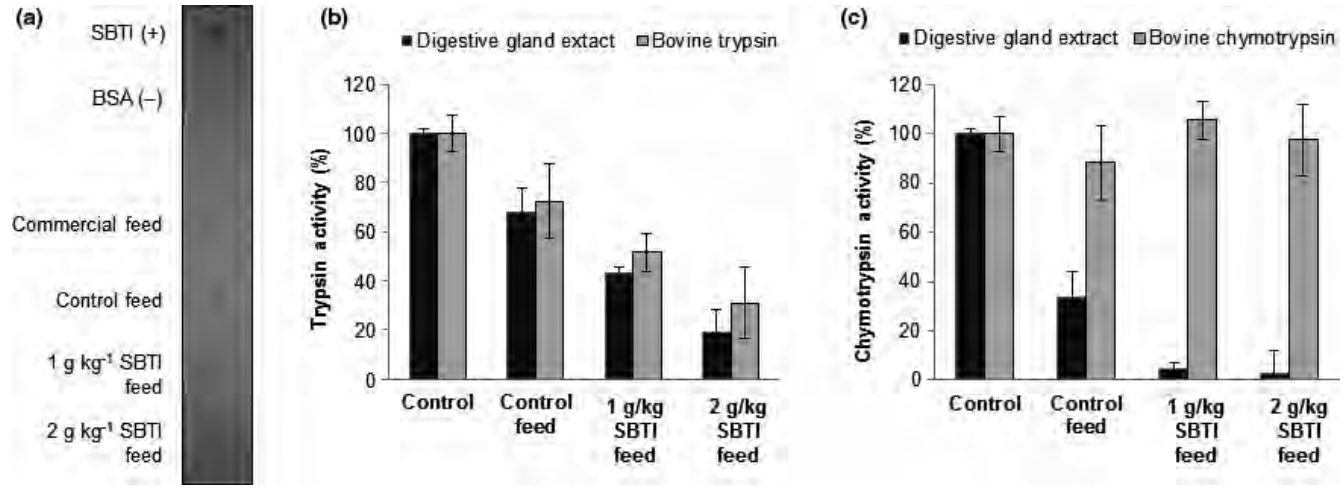


**FIGURE 2** Presence and activity of the SBTI in the feeds. (a) Dot blot analysis of the feeds before the bioassay. (b) Trypsin activity of shrimp digestive gland extract and commercial bovine trypsin incubated with feed extracts. (c) Chymotrypsin activity of shrimp digestive gland extract and commercial bovine chymotrypsin incubated with feed extracts. SBTI, Kunitz-type soybean trypsin inhibitor; BSA, bovine serum albumin

feed and 70% with the extract of 2 g $kg^{-1}$ SBTI feed. Trypsin activity of the shrimp digestive gland extract decreased 60% when incubated with the extract of 1 g $kg^{-1}$ SBTI feed and 80% with the extract of 2 g $kg^{-1}$ SBTI feed (Fig. 2b). Bovine chymotrypsin was not affected by the feed extract; however, chymotrypsin activity of the shrimp digestive gland extract decreased 90% (Fig. 2c).

## 3.2 | Effect of SBTI on the activity of peptidases

The effect of supplemental SBTI on the digestive peptidases from experimental group fed feed containing 1 g $kg^{-1}$ or 2 g $kg^{-1}$ SBTI was assessed and compared with the control group fed feed with no inhibitor. Results are that either 1 g $kg^{-1}$ and 2 g $kg^{-1}$ SBTI in feeds significantly reduced total proteolytic activity in the digestive gland by 20% and 40%, respectively ($p < .05$; Fig. 3a). Trypsin activity was significantly reduced by 60% and 70%, at 1 g $kg^{-1}$ and 2 g $kg^{-1}$ SBTI, respectively ($p < .05$; Fig. 3b). Chymotrypsin activity from the digestive gland was not affected in the group fed feed containing 1 g $kg^{-1}$ SBTI and reduced by 30% in the digestive gland of organisms consuming 2 g $kg^{-1}$ SBTI ($p < .05$; Fig. 3c).

Peptidases composition of the digestive glands extracts in control and treated groups were observed in zymograms (Fig. 4). Isotrypsins intensity of band (TC, TB and TA) was reduced indicating partial inhibition in treatment group fed 2 g $kg^{-1}$ SBTI. Isochymotrypsins were slightly inhibited as expected. Unidentified bands of peptidases were observed in all groups and marked U1–U7. The seven unidentified peptidase bands had different band intensities in the digestive gland extracts from control and treated groups. Bands U3, U5 and U6 were observed in control group. In treated group fed 1 g $kg^{-1}$ SBTI, additional bands were observed: U4 and U7. In treated group fed 2 g $kg^{-1}$ SBTI, bands not present in control and treated group 1 g $kg^{-1}$ fed SBTI were observed: U1, U2; band U3, which also occurred in the control group and in treated group feed 1 g $kg^{-1}$ fed SBTI increased its intensity. Band U4, which was not present in the control group, faintly present in treated group 1 g $kg^{-1}$ fed SBTI increased intensity in treated

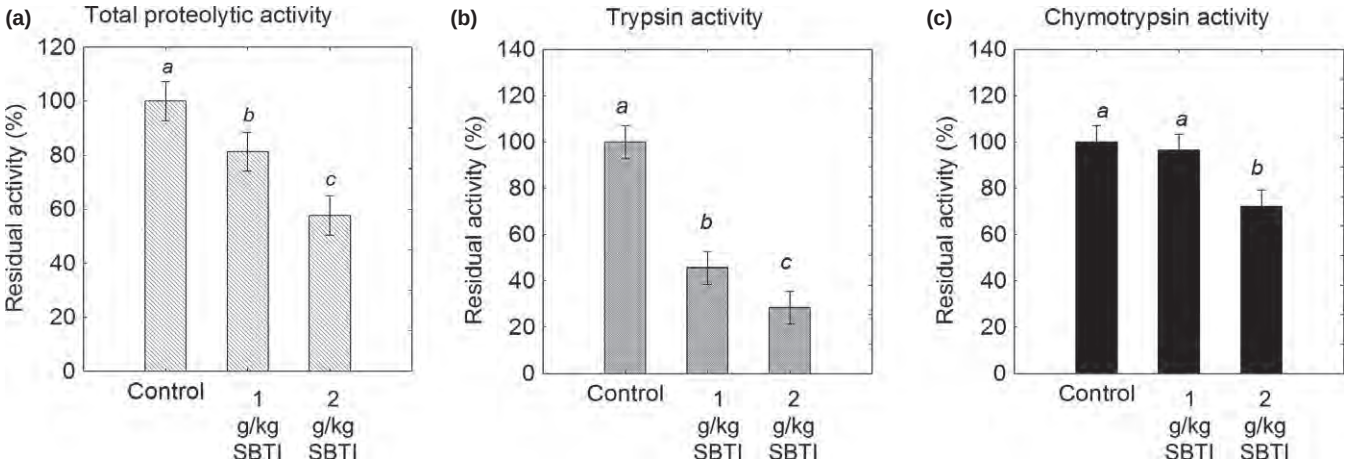


**FIGURE 3** Effect of soybean trypsin inhibitor (SBTI) on in vivo digestive peptidases. (a) Total alkaline proteolytic activity; (b) specific activity of trypsins; (c) specific activity of chymotrypsins. Data correspond to triplicates samples from digestive glands of shrimp fed with SBTI. Different letters on bars mean significant differences ($p < .05$)

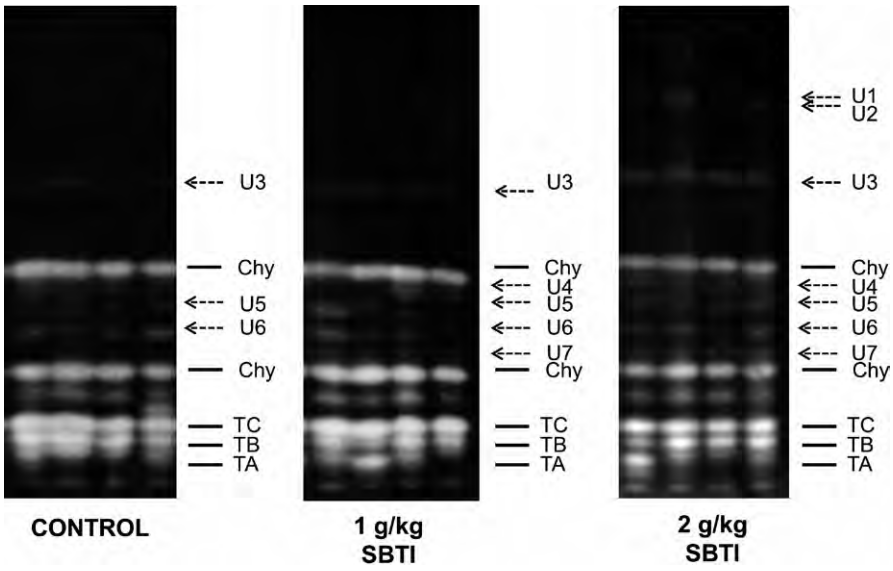


**FIGURE 4** Zymograms of enzyme extracts from digestive glands of shrimp. Each gel contains enzymatic extracts from four different individuals. Dotted black arrows indicate unidentified peptidases (U) in extracts of the digestive gland of shrimp fed soybean trypsin inhibitor. Chy, chymotrypsins; TC, isotrypsin C; TB, isotrypsin B; TA, isotrypsin A

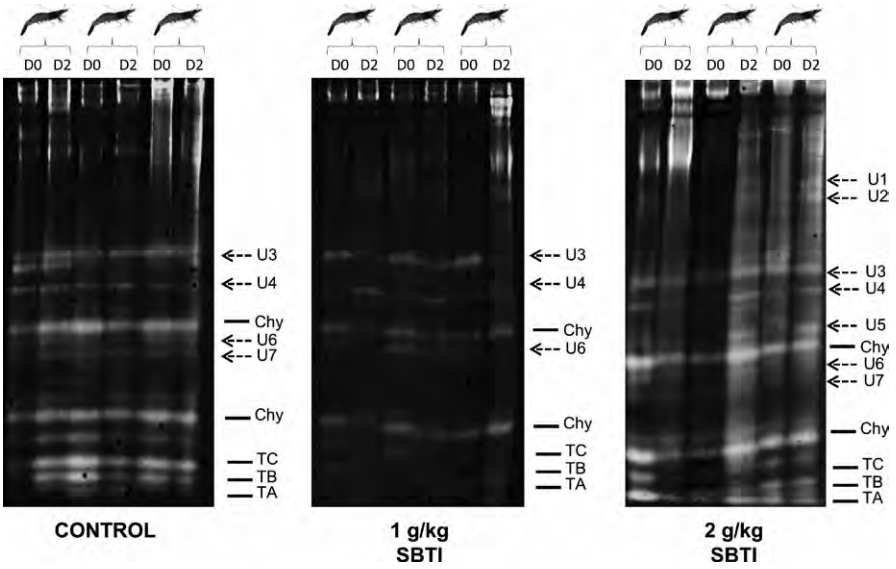


**FIGURE 5** Zymogram of extract from faeces of three shrimp sampled from each group. Serine peptidases are marked as: Chy, chymotrypsins; TC, isotrypsin C; TB, isotrypsin B, TA, isotrypsin A. Dotted black arrows indicate unidentified peptidases. D0 = Enzyme extract of faeces before the bioassay, D2 = Enzyme extract of faeces in the second day of the bioassay

group fed 2 g $kg^{-1}$ SBTI. Bands U5 and U6 occurred in all treatments, but with greater intensity in the 2 g $kg^{-1}$ SBTI group.

Peptidases from shrimps sampled at day 2 of the bioassay were compared in zymograms with peptidases observed from faeces sampled at day 0 (Fig. 5). Isotrypsins and chymotrypsins were identified in extracts from faeces of day 0 and unidentified bands from faeces of day 2 marked in Fig. 4 (U1–U7). Greater inhibition was observed in faeces extracts from group fed 1 g $kg^{-1}$ SBTI. Bands U1 and U2 were seen in the group fed 2 g $kg^{-1}$ SBTI. Bands U4, U5 and U6 were not observed in the 1 g $kg^{-1}$ SBTI group and hardly noticeable in the control and 2 g $kg^{-1}$ SBTI groups, but several other peptidases were found. Unidentified peptidase bands were observed in the group fed 2 g $kg^{-1}$ SBTI.

The serine peptidase inhibitor Pefabloc was used to assess the class of peptidases in three specimen faeces extracts seen in zymograms of the 2 g $kg^{-1}$ SBTI group (Fig. 6). Isotrypsins and chymotrypsins were completely inhibited with Pefabloc (dotted boxes), confirming their identity. Band U2 and U4 are serine peptidases because they were inhibited with Pefabloc. The Pefabloc failed to inhibit U1 and U3 bands, indicating they are not serine peptidases. Bands U5 and U6 were faint, so, its identity remains unclear. Other peptidases of high molecular mass were well-defined in faeces in the 2 g $kg^{-1}$ SBTI group, but not in the digestive gland and were not inhibited by Pefabloc, suggesting that most of these peptidases are in higher concentration in faeces extracts and they are not from the serine class.

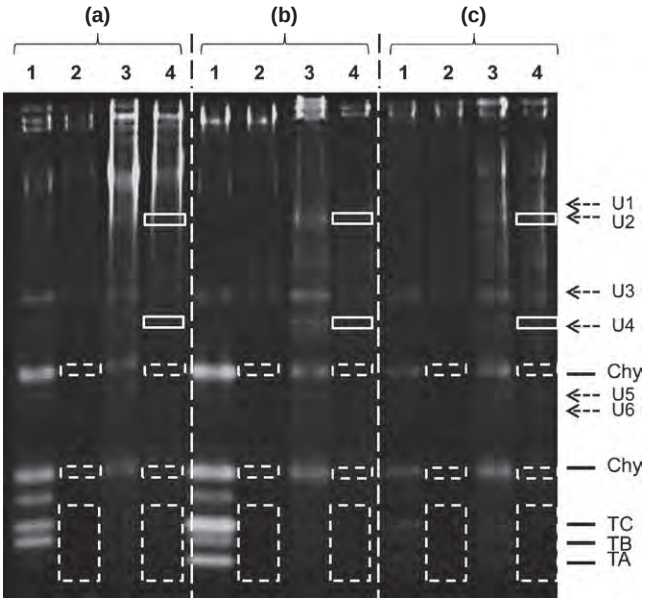


**FIGURE 6** Zymograms from faeces extracts of three specimens sampled from the 2 g $kg^{-1}$ soybean trypsin inhibitor group. White boxes indicate unidentified peptidases inhibited with Pefabloc. White dotted boxes indicate inhibition by Pefabloc of already known serine peptidases. Chy, chymotrypsins; TC, isotrypsin C; TB, isotrypsin B; TA, isotrypsin A; U, unidentified peptidases. Lane 1 = faeces extract sampled before the bioassay, Lane 2 = faeces extract sampled before the bioassay and incubated with 2 mM Pefabloc, Lane 3 = faeces extract sampled the second day of the bioassay, Lane 4 = faeces extract sampled the second day of the bioassay and incubated with 2 mM Pefabloc. Letters a, b and c below the gels represent different specimens

## 4 | DISCUSSION

The inclusion of plant meals in aquaculture feeds is not a trend; these meals are a current ingredient in fish and shrimp feeds. The aim of many producers is a complete substitution of fishmeal, however the more plant meals in feds, the more antinutritional molecules; with an effect been specie specific. The proportion of soybean meal in aquaculture feeds can vary from 75 to 300 g/kg (Collins et al., 2012). Shrimp farming demand feed containing up to 300-400 g protein/kg diet (Córdova-Murueta & García-Carreño, 2002), paradoxically, suitable protein ingredients used in fabricating feed may also contain antiphysiological (aka antinutritional) compounds as was evidenced by Lemos, Navarrete del Toro, Córdova-Murueta, and Garcia-Carreño

(2004), and their effect will depend on the final concentration of these molecules in the feed. Besides been a fitting source of essential amino acids, soybean meal also contains digestive peptidase inhibitors, which interfere with digestion and assimilation of food proteins (Francis et al., 2001; Yu, Gong, Yuan, & Lin, 2013).

Based on nutrient profile, cost and availability, soybean meal is a good protein source to fulfil the 400 g/kg of protein needed to fabricate shrimp feed (Fuertes, Celada, Carral, Sáez-Royuela, & González-Rodríguez, 2012). On the other hand, it contains antinutritional compound like trypsin inhibitors, the so-called SBTI. SBTI are antinutritional factors because they tamper the most important digestive peptidases, trypsins and, to a lesser extent, chymotrypsins (De Vonis Bidlingmeyer, Leary, & Laskowski, 1972). In an attempt to learn if shrimp physiology evolved adaptations to defeat the presence of peptidase inhibitors, our group launched a research programme. Previously, we reported that the content of digestive peptidases in crustaceans is identical in gastric juice (Celis-Guerrero, García-Carreño, & Navarrete del Toro, 2004) and in faeces (Córdova-Murueta et al., 2003), making possible to expand the capacity of analysis of physiological processes by sampling biological material to analyse along a treatment within the specimens. In the current study, immediate and strong inhibition of serine peptidases occurred in the shrimp digestive gland and faeces extracts after consuming the supplemental inhibitor (2 g $kg^{-1}$ SBTI). This effect has been widely observed in insects, which are highly exposed to natural inhibitors such as the SBTI; for example, chymotrypsin from the digestive gland of the red flour beetle (*Tenebrio molitor*) and trypsin from the Indian mealmoth (*Plodia interpunctella*) were strongly inhibited by 10 g $kg^{-1}$ and 2 g $kg^{-1}$ SBTI, respectively (Amorim et al., 2008; Elpidina et al., 2005).

Supplemental SBTI in the extracts from feed used to feed the experimental group reduced the total proteolytic activity from the shrimp digestive gland extracts. After 3 hr exposure to SBTI, trypsin activity was observed both in test tubes and zymogram, suggesting that the SBTI was hydrolysed by unidentified enzymes in the digestive gland extract or trypsin itself, allowing trypsin to display activity. The reaction between trypsin and SBTI is a reversible one forming a 1:1 stoichiometric complex inhibiting trypsin mole-for-mole and to a lesser extent chymotrypsin, which means that the complex trypsin-SBTI associates and dissociates in proportion to the dissociation constant. When dissociated, trypsin or other peptidases can hydrolyse it. In the bean beetle larvae, *Callosobruchus maculatus*, SBTI was able to inhibit serine peptidases effectively; at 8-hr exposure, the effect was overcome by an aspartic peptidase, demonstrating that the inhibitor was hydrolysed (Amirhusin et al., 2007). Aspartic peptidases have not been described in any stage of whiteleg shrimp (Wei, Zhang, Yu, Li, & Xiang, 2014); however, cysteine and metallo peptidases have been reported in the shrimp (Stephens et al., 2012; Zhao et al., 2007), and it is likely that any or all of these enzymes be responsible for hydrolysis of SBTI.

The hydrolysis of peptidase inhibitors is a mechanism to fight back antinutritional factors in food and feed by the digestive gland that still requires the identification of the peptidases that are taking over the inhibited ones. In this study, inhibition of chymotrypsins was only 25% inhibition at 2 g $kg^{-1}$ SBTI, while total proteolytic and trypsin activity were inhibited 60%–70%. This distinctive difference has been attributed to the affinity to serine peptidases of the Kunitz-type trypsin inhibitors: trypsin, Ki = 1.87 pM and chymotrypsin Ki = 2.8 pM (Patthy, Molnár, Porrogi, Naudé, & Gráf, 2015).

Invertebrates have shown adaptation to tamper natural inhibitors. Three types of adaptation have been demonstrated: (i) hydrolysis of the inhibitor by endogenous enzymes in the cowpea bruchid *C. maculatus* (Amirhusin et al., 2007), (ii) compensation by synthesis of a different catalytic type of peptidases in the red flour beetle *Tribolium castaneum* (Oppert, Morgan, Hartzer, & Kramer, 2005), and (iii) compensation by synthesis of the same class of enzymes in the water flea *Daphnia magna* (Schwarzenberger et al., 2010). The results from this study suggest that the shrimp digestive system shift their battery of peptidases (Figs 3 & 4), which implies a composition of peptidases with alternative synthesis of serine peptidases as observed when using Pefabloc inhibition. The findings of this study suggest that enzyme adaptation depends not only on the physiological condition of the shrimp (Muhlia-Almazán & García-Carreño, 2002) but also on the length of exposure and the concentration of the antinutritional factors, such as trypsin inhibitors. Yu et al. (2013) reported that replacing fish meal protein with soybean meal by >40% significantly reduces the activity of digestive peptidases leading to physiological disorders in the Chinese sucker *Myxocyprinus asiaticus*, also in the Atlantic salmon *Salmo salar* L., trypsin activity decreases in the pyloric, mid and distal intestine when fed with soy protein concentrate (Penn, Bendiksen, Campbell, & Krogdahl, 2011) leading to enteropathy (Baeverfjord & Krogdahl, 1996). In the giant tiger prawn *Penaeus monodon*, there are injuries in the cells of the digestive gland when feeds are prepared with raw, soaked and germinated cow pea and mug bean. These feeds have concentrations from 12 to 44 mg/g of trypsin inhibitor, leading to sever vacuolization of B cells of tubules of the digestive gland, infiltration of inflammatory haemocytes in to the gut lumen and degeneration of the gut wall (Kumaraguru-Vasagam, Balasubramanian, & Venkatesan, 2007). A cladoceran crustacean, the water flea *D. magna*, is tolerant to peptidase inhibitors. It compensates when 20% of feed is substituted with a cyanobacteria strain rich in chymotrypsin inhibitor (Schwarzenberger et al., 2010).

In the present study, the fact that 1 g $kg^{-1}$ SBTI did not reduce the activity of chymotrypsin and the total proteolytic activity was reduced only by 20% suggests a threshold for the SBTI in commercial feeds. Adding 2 g $kg^{-1}$ SBTI reduced the total proteolytic activity more than 40%, and trigger a compensation mechanism based on synthetizing yet unidentified peptidases. More work is needed to understand the mechanisms of compensation against the inhibitors in feeds for shrimp, as the growth may be affected when rebasing the compensation capacity.

## 5 | CONCLUSIONS

The current study demonstrates that the whiteleg shrimp *P. vannamei* has the capacity to counteract the Kunitz-type SBTI present in soybean meal. The results obtained, provided evidence that the SBTI present

in the soybean meal is resistant to heat denaturation as previously demonstrated (Lemos et al., 2004) and in the processed experimental feeds in this study, the shrimp digestive system synthesized other peptidases when SBTI was present in feed; some of those enzymes were identified as peptidases from the serine class and others from a different class that remains to be identified. This study provides compelling evidence of the adaptive mechanism of shrimp digestive gland to overcome the presence of peptidase inhibitors.

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