FLUORESCENT BASED ASSESSMENT OF TRYPSIN ACTIVITY VS TOTAL PROTEASES IN THE QUEEN TRIGGERFISH, *Balistes vetula*

by

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ABSTRACT

Amino acids that comprise proteins constitute essential nutrients for any animal organism. Indeed they are directly related to its growth and maintenance. Protein assimilation depends on the catalytic properties of proteases in the alimentary canal. Such protein-degredative enzymes include trypsin, chymotrypsin and elastase, with trypsin being the most important and abundant. The main objective of this research project was to use a highly sensitive fluorometric technique to assess both total protease and trypsin activities from intestines of the queen triggerfish, *Balistes vetula*, and determine if these were affected by fish size. To this end, 32 west coast and 4 east coast intestinal samples were obtained. Each sample contained from 2 – 10 total intestines. The weight (wet weight) was obtained and recorded for each sample. Samples were then individually homogenized in distilled water to produce a supernatant. Filtered supernatant was assayed for the following three properties: 1) protein content; 2) total protease activity; 3) trypsin activity. The remaining supernatant was pooled and subjected to size exclusion chromatographic procedure in order to obtain a partial purification of proteins conferring trypsin activity. The kinetic analysis of this trypsin activity was assessed as per the following four variables: 1) protein concentration; 2) pH; 3) temperature; and 4) presence of soybean trypsin inhibitor. The data are considered with respect to both the utility of the fluorometric technique in this analysis and the significance of trypsin with respect to the biochemistry/physiology of the queen triggerfish.

RESUMEN

Los aminoácidos contenidos en las proteínas son nutrientes esenciales para todo organismo animal y su asimilación depende de proteasas presentes en el tracto digestivo, tales como tripsina, quimotripsina y elastasas, siendo tripsina la más abundantes e importante. El objetivo principal de esta investigación, fue determinar mediante un sensible método fluorométrico, la actividad total de proteasas y la actividad de tripsina en el tracto intestinal del queen triggerfish, *Balistes vetula* y determinar si dichas actividades son afectadas por la edad de los peces. Con propósito se obtuvieron 32 muestras intestinales de la costa oeste y 4 muestras de la costa este de Puerto Rico; cada muestra conteniendo de 2 a 10 intestinos. El peso húmedo de cada muestra fue determinado y registrado. Las muestras fueron después individualmente homogenizadas en agua destilada para producir sobrenadantes (homogenatos crudos). A los sobrenadantes filtrados se les determinó 1) Contenido de proteína, 2) actividad total de proteasas y 3) actividad de tripsina. El homogenato crudo fue además sometido a cromatografía de exclusión por peso molecular, con el objetivo de obtener la purificación parcial de tripsina. La tripsina parcialmente purificada fue caracterizada cinéticamente de acuerdo a los siguientes 4 parámetros 1) actividad de tripsina, 2) pH, 3) temperatura y 4) inhibición usando el inhibidor de soya de Kunitz. Los resultados de este estudio son útiles para evaluar la utilidad del método fluorométrico usado y la importancia de tripsina con respecto a la fisiología digestiva del queen triggerfish.

To my Mother

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INTRODUCTION

The growth of any animal is directly related to the breakdown of ingested proteins into their constituent amino acids and the subsequent assimilation of these breakdown products into the body. The enzymes involved in this protein-digestive process include trypsin, chymotrypsin and elastase (Solomon et al, 1996). All of these are endopeptidases that occur in the alimentary canal. Trypsin is specific for peptides and esters of the amino acids lysine and arginine; chymotrypsin is specific for the hydrophobic amino acid side chains of phenylalanine, tyrosine and tryptophan; elastase is specific for small hydrophobic side chains such as alanine. Collectively, these three endopeptidases play essential roles in converting ingested proteins into individual amino acids, which are in turn absorbed across the gut epithelium (Rivera, 2003).

Various studies have shown that trypsin, trypsin precursors, and/or equivalent protease activities have been used as indicators of the nutritional condition of fish (García–Carreño et al., 2002; Hjelmeland et al. 1988; Ueberschar, 1988). The basic premise is that a diet rich in proteins, and therefore also amino acids, will promote fish protease secretion and its activity within the midgut. On this point, investigators have observed that trypsin ranks as the more important midgut (i.e., intestine, anterior midgut caeca, and posterior midgut caeca) protease in fishes (Martínez et al., 1988; Hideki and Hayasi, 2002; Castillo–Yañez, 2005). Thus, most fish digestive protease studies have focused on this enzyme.

Various methodologies have been used in assessing levels of fish trypsin activity and abundance. One such method is that of the radioimmunoassay (RIA), in which radioactive antibodies specific for trypsin hybridize with and thereby identify the enzyme in midgut tissues (Ueberschar et al., 1992). Another approach utilizes a trypsin specific substrate (N-alpha-pTosyl-L-Arginine-Methyl Ester, or TAME), the breakdown of which is evaluated over time via a spectrophotometer (Hummel, 1959). One drawback with each of these trypsin assays is that one must perform a labor-intensive and time-consuming process of first partially or completely purifying the enzyme, and may also require relatively large amounts of tissue.

Ueberschar (1988) introduced a highly sensitive fluorometric assay that determines total protease activity from crude tissue homogenates. Though lacking the specificity for its direct determination, it is here hypothesized that this methodology can be made specific, in an indirect way, for trypsin activity by running the assay once in the presence of a trypsin inhibitor and then once again in the absence of that inhibitor. Trypsin activity would then be measured as the increased level of activity obtained in the latter. All other protease activity is that obtained in the presence of trypsin inhibitor.

The queen triggerfish, *Balistes vetula*, a moderately important food fish inhabiting shallow Puerto Rican marine waters, feeds exclusively upon crustaceans (notably crabs), mollusks (major predation upon oysters), echinoderms (especially sea stars and sea urchins), sponges and other marine invertebrates, as well as some small fishes. Given the

fish's feeding habits, the queen triggerfish is potentially an excellent species to assess the proportion of the trypsin activity in relation to the total intestinal proteases, to better understand the contribution that trypsin has on fish digestion.

In an effort to more precisely assess the protease activity within queen triggerfish intestines, a methodology was developed whereby trypsin and total protease activities could be individually, yet simultaneously, determined. This method makes use of soybean trypsin inhibitor (SBTI) added to certain assays. The resulting protease activity in these assays is lower because the trypsin activity has been wholly, or nearly so, blocked by the inhibitor. In this way, one can determine the contribution of trypsin to the total protease activity. In addition, the direct assay of trypsin activity via the Hummel (1959) assay was utilized in efforts to chromatographically obtain a partial purification of the trypsin enzyme.

The data obtained in this investigation help develop a better understanding of the queen triggerfish's chemical digestion functions. In addition, analysis of the possible influence of fish age on trypsin and total intestinal protease activities helped to meet this goal as well.

LITERATURE REVIEW

Fish Proteases

As with others animals, fish growth depends on a diet that provides sufficient levels of protein. Indeed, amino acids are needed for maintenance, growth, and regeneration of tissues. Protein is therefore a critical component of the fish diet.

Among the 23 naturally-occurring amino acids, 10 of these must be obtained through diet to support growth and maintenance as fish, especially non-adults, lack the capacity to synthesize them in sufficient quantities for rapid growth (Bureau and Young, 2000). It is thus clear that an analysis of the enzymes which allow midgut breakdown of ingested proteins into their constituent amino acids is central to understanding the biochemistry that supports fish growth and development.

It has been established that fishes and higher vertebrates utilize the same enzymes and hormones in the breakdown of proteins into amino acids (Smith, 1989). In fish, the specific levels of digestive enzyme activities are dependent on age, diet, season, and/or ambient temperature (Munilla-Morán and Saborido-Ray, 1996). Moreover, some researchers have demonstrated that the production of the three fish midgut proteolytic enzymes, including trypsin, chymotrypsin, and elastase, is regulated at the transcriptional level as a function of diet (Peres et al., 1998; Muhlia-Almazán et al., 2003).

In the past three decades several studies of chymotrypsin, elastase and/or trypsin from digestive tissue of numerous fish species have been made and several generalizations have emerged. First, these enzymes have an approximate molecular weight of 25 to 27 kilodaltons (kDa). In addition, the enzymes are not stable at low pH but retain their activity at a neutral pH in presence of Ca^{2+} (Cohen et al., 1981; Arnt and Walter, 1989). Because carbonate salts are secreted into the frontal portion of the intestine to neutralize the acidity of the bolus, fish midgut digestion is carried out under neutral to alkaline conditions. Both trypsin and chymotrypsin characteristically display higher activities than elastase (Von Elert at al., 2004).

Trypsin

Given its higher catalytic rate and therefore greater overall contribution to the breakdown of ingested proteins for animals in general, it is perhaps not surprising that trypsin has been at the center of attention for researchers wishing to better understand the digestive biochemistry/physiology of many different fish species. For example, in 1960, Bradford was among the first to characterize a fish trypsin-like enzyme activity, when he identified proteolytic hydrolysis to be present in homogenates of pyloric caeca from the Chinook Salmon, *Onchorhynchus tshawytscha*. The enzyme was found to have similar properties to both mammalian trypsin and chymotrypsin. This trypsin-like enzyme exhibited an optimal pH of 9.0 and maximum activity at a temperature of 49^0C.

Prahl and Neurath (1966) described an anionic form of pancreatic trypsinogen, as well as other digestive enzymes, in the spiny dogfish, *Squalus acanthias*. The dogfish enzyme was found to have a molecular weight of 24 to 25 kDa and exhibited an optimum pH of 7.9 to 8.0. Reeck et al. (1970) identified three forms of trypsinogen in the African lungfish, *Protopterus annectens*. One form was later purified and characterized (Reeck et al., 1970). It displayed a molecular weight of 24 kDa and optimum activity at pH 8.0. The lungfish trypsin amino acid composition was found to be similar to that of other animal trypsins especially those obtained from bovine and dogfish sources. Its properties including, pH and temperature optimum, and molecular weight, were found to lie between those of invertebrate and mammalian trypsins. It resembled invertebrate enzymes in being anionic and stable at neutral pH in the absence of calcium. However, like cationic mammalian trypsins, the lungfish form was found to be stable at pH 3.0.

Yasunaga (1972) identified protease activity from digestive organs of three flatfish species, the marbled flounder, *Pseudopleuronectes yokohamae*, the stone flounder, *Platichtys bicoloratus*, and the olive flounder, *Paralichtys olivaceus*. He found an optimum pH of 8.0 and maximal activity at a temperature of 40 ^{o}C. Overnell (1973) made a comparative study of the digestive enzymes from pyloric caeca and its associated mesentery in the cod, *Gadus morhua*. Activities of the following were identified: trypsin, chymotrypsin, carboxipeptidase A and B, leucine aminopeptidase, ribonuclease, amylase, acid phosphatase, and alkaline phosphatase. The trypsin-like enzyme showed a molecular weight of 18 to 22 kDa. Its pH optimum ranged from 8.0 to 9.0. It is notable that the

principal pancreatic enzymes were found in the mesentery of the caeca and not in the caeca themselves.

Titani et al. (1975) purified pancreatic trypsins from the spiny dogfish through chromatographic and related procedures. This purified enzyme revealed two bands, one of 11 kDa and the other of 23 kDa. This finding suggests the possibility of a mixture of single chain and double chain trypsins. In addition, the amino acid sequence of these trypsins was determined, indicating that there was one less amino acid residue in the spiny dogfish than had been identified in bovine and porcine trypsin. In 1976, Klaus-Dieter found a trypsin-like activity in the intestine and hepatopancreas of the bonefish, *Carassius auratus gibelio*. This fish is the wild type, eastern european form of the familiar goldfish. He determined its optimum pH to be 9.0 and that optimal activity occurred within a temperature range of 30^{o}C to 50^{o}C. This study also found that trypsinogen was synthesized by the hepatopancreas. Subsequently, this zymogen became activated through enterokinase activity upon being secreted into the intestine.

Cohen et al. (1981) purified pancreatic proteolytic enzymes including trypsin from carp, *Cyprinus carpio*. The enzyme showed an approximate molecular weight of 25 kDa. The carp trypsin was found to be anionic protein that is unstable at low pH. Hjelmeland and Raa (1982) purified two trypsin-like enzymes from the gut of the artic capelin, *Mallotus villosus*. Both enzymes had a molecular weight of about 28 kDa. The enzymes were inhibited by standard trypsin inhibitors and displayed a pH optimum of 8.0 to 9.0.

In 1984, Simpson and Haard purified and characterized trypsin from the pyloric caeca of Greenland cod, *Gadus ogac*. Trypsin was isolated by ammonium sulfate fractionation followed by acetone precipitation and affinity chromatography techniques. Electrophoretic analysis of the enzyme revealed a single band with an estimated molecular weight of 23.5 kDa. Characterization of the enzyme included identifying its catalytic specificity for amide or ester bonds involving the carboxyl group or arginine, capacity to hydrolyze the trypsin-specific synthetic substrate TAME, its sensitivity to serine protease inhibitors, and lowering or cessation of enzyme activity when in the presence of SBTI.

Yoshinaka et al. (1984) isolated an anionic trypsin from the pancreas of the amur catfish, *Silurus asotus*. This enzyme had a molecular weight of 26 kDa. Its pH optimum was 8.3. The amino acid composition of this trypsin was similar to that of cationic bovine trypsin. Clark et al. (1985) examined protease activities in the intestine of the sole, *Solea solea*. Experiments using synthetic substrates suggested the presence of a trypsin-like enzyme. This activity revealed a pH optimum of 8.0. Uys and Hecht (1987) characterized pancreatic enzymes, including trypsin, from the sharptooth catfish, *Clarias gariepinus*. Trypsin displayed optimal activity at pH 8.2 and at temperatures ranging from 30^{o}C to 40^{o}C.

Purification and characterization of two trypsin-like enzymes from the digestive tract of the anchovy, *Engraulis encrasicholus*, was realized through a combination of affinity

and ion exchange chromatographic procedures (Martínez et al., 1988). These enzymes displayed molecular weights in the range of 27 and 28 kDa. Their isoelectric points were between 4.6 and 4.9. The enzyme displayed optimal activity in a pH range of 8 to 9. They resembled other fish trypsins in their molecular weights, kinetic properties, and instability at low pH.

Guizani et al. (1991) purified and characterized a trypsin from the pyloric caeca of mullet, *Mugil cephalus*. The enzyme exhibited optimal activity at a pH of 8.0 and at a temperature of 55^{o}C. It was stable within a pH range of 7.5 to 9.0. This stability is similar to that reported for most marine organism trypsins, which are highly unstable under acidic conditions but very stable at neutral to slightly alkaline conditions. Electrophoretic analysis determined the molecular weight of the enzyme to be 24 kDa. Trypsin from the pyloric caeca of rainbow trout, *Oncorhynchus mykiss*, was purified and characterized by Kristjansson (1991). The isolated enzyme had an estimated molecular weight of 25 kDa. The enzyme was stable at temperatures in the range of 40 to 50^{o}C and at a pH range of 5.4 to 8.0. However, this thermal stability was shown to be calcium-dependent. Hydrolisis of substrate was maximal at approximately 60^{o}C.

Sabapathy and Teo (1993) conducted a comparative study of the rabbitfish, *Siganus canaliculatus*, and sea bass, *Lates calcarifer*, digestive tracts and digestive enzymes distributions and activities. Proteases including trypsin, were found in both species. Trypsin activity was higher in the rabbitfish, in which the enzyme was detected in all

regions of the digestive tract. By contrast, sea bass trypsin was confined to the intestine and pyloric caeca. Later, Sabapathy and Teo (1995) identified some properties of the rabbitfish's intestinal enzymes. Comparison of trypsin and chymotrypsin from the viscera of anchovy, *Engraulis japonicus*, was undertaken by Heu et al. (1995). The molecular weight of the trypsin was estimated to be 25.6 kDa. Maximal activity was found at pH 9.0 and 45^{o}C for casein, and at pH 8.0 and 45^{o}C for TAME.

Four differently charged trypsins were purified from pyloric caeca of Atlantic salmon, *Salmo salar* (Outzen et al. 1996). The four isoforms of trypsin were differentiated as anionic trypsin I, II and III, and cationic trypsin. Al were found to have a molecular weight of approximately 25 kDa. Cationic salmon trypsin displayed optimal activity at a pH range of 8.5 to 10.5. By contrast, the anionic salmon trypsin was optimally active at pH 10.5. This study was the first to identify, isolate, and characterize a cationic trypsin from marine species.

The proteolytic activities in the gut of three carnivorous fish species, the deepwater redfish, *Sebastes mentella*, the turbot, *Scophthalmus maximus* and the gilthead bream, *Sparus aurata*, were compared by Munilla-Morán and Saborido-Rey (1969). Optimum stomach trypsin activity for all three was detected at pH 2.0, while such activity in the intestinal forms of the enzyme showed activity at a pH range of 9.5 to 10.0. The temperature range at which both enzymes displayed maximal activity was 35 to 40^{o}C.

Díaz (1999) obtained a 50-fold level of purification for trypsin from pyloric caeca of the red hind grouper, *Epinephilis guttatus*. This study revealed that the red hind trypsin displayed optimal activity at a pH range of 6.0 to 8.0. Activity was also found to be temperature-dependent, with highest catalysis occurring at 40^{o}C. The enzyme's molecular weight was somewhere within the range of 17 to 44 kDa. This trypsin was inhibited by the SBTI.

Quiñones (2000) obtained a 20-fold level of purification for trypsin from pyloric caeca and intestinal tissues of the queen snapper, *Etelis occulatus* (Valenciennes, 1928). This study showed that the queen snapper trypsin displayed optimal activity in a pH range of 8.0 to 9.0. The temperature-dependent activity was highest at 50^{o}C for pyloric caeca trypsin and 60^{o}C for the intestinal tissues. Like the red hind grouper trypsin, that from the queen snapper had a molecular weight in the range of 17 to 44 kDa and was inhibited by SBTI.

Sekizaki et al. (2000) purified an anionic trypsin from the pyloric caeca of chum salmon, *Onchorhynchus keta*. The molecular weight was around 24 kDa as determined by SDS-PAGE. The enzyme displayed moderate activity toward artificial substrate TAME and tosyl –L-lysine methyl esther. The main band of the anionic enzyme showed an isoelectric point (pIs) of 5.10. The effect of temperature on the hydrolysis of TAME suggested that the enzyme is an efficient catalyst for peptide synthesis at low temperature.

Purification and characterization of trypsin-like enzyme from the pyloric caeca of cod (Beirao et al., 2001) was obtained through affinity chromatography on CHOM sepharose 4B. Characterization of the enzyme was established through both its observed catalytic activity upon TAME and its inhibition in the presence of serine protease inhibitors. The enzyme showed pIs of 5.30 and 5.89 and was found to have similar amino acid composition to that of bovine trypsin.

We can glean from the previous studies that trypsin is a standard protease of fishes and that it may readily be characterized from digestive fish tissues. These trypsins are optimally active at pH ranges of 7.5 to 9.0. In addition, all of the trypsins displayed greatest catalytic rate at temperatures of 45 to 60^{o}C.

While trypsin is clearly the most significant enzyme with regard to fish digestion of ingested proteins, it is nonetheless of great interest to evaluate the proteolytic contribution of both chymotrypsin and elastase in fishes. Indeed, trypsin cannot by itself supply the enzyme activity to breakdown food proteins into individual amino acids. Thus an assessment of total protease activity, including that of trypsin, chymotrypsin, and elastase is essential in the quest to better understand fish nutrition.

It has been demonstrated that different sources of dietary protein can affect gut protease activity levels in at least some, and perhaps most, fish species (García-Carreño et al., 2002; Bureau and Young, 2000). On this point, Jobling (1995) and Kapoor et al.

(1975) have established that in freshwater fishes that an increase in dietary protein levels results in a concomitant increase in proteolytic activities. It is reasonable to assume that this generalization may well apply to certain marine species as well.

The Queen Triggerfish *Balistes vetula*

The queen triggerfish, *Balistes vetula*, is an important predator of Caribbean coral reef and other tropical marine ecosystems (Gonziles, 2001). This fish feeds upon a variety of crustaceans, echinoderms, mollusks, and small fishes. The queen triggerfish is therefore an excellent candidate for use in a comparative study aimed at examining the total protease activity as well trypsin activity from intestinal tissue.

Fluorescence

Dyes and stains have long been used to detect and visualize structures and processes in biological samples. Today the most used dyes and stains have a fluorescent component, because fluorescent molecules can be detected with extraordinary sensitivity and selectivity (Sisken, 1989). There are a wide variety of fluorescent compounds such as ethidium bromide, alexa fluor dyes, cy dyes and fluorescein; all of them are able to emit different color of light at different wave lengths. These molecules are capable of being excited, via absorption of light energy, to a higher energy state, also called an excited state. The energy of the excited state which cannot be sustained for long

"decays" or decreases, resulting in the emission of light energy; this process is called fluorescence.

Fluorescence based techniques have proven useful in quantifying total proteases activity from different biological samples such as intestinal tissue from fish larvae of the herring, *Clupea harengus* (Ueberschar, 1988; Ueberschar et al., 1992). This technique may also be applied in determining total midgut protease/trypsin activity from queen triggerfish as presented in the previous paragraph.

From the above literature review, the following conclusions can be drawn: 1) like other animal organisms, fishes have a need for enzymatically processing ingested proteins into their constituent amino acids; 2) the major midgut proteases of fishes are trypsin, chymotrypsin and elastase; 3) trypsin is the most extensively studied of these three proteases and this enzyme or its equivalent activity has been described from numerous fish species; 4) the contribution of chymotrypsin and elastase is essential in fish protein digestion and the study of these enzymes is critical in better understanding fish protein nutrition; 5) a direct correlation has been established between levels of dietary protein and levels of proteolytic activities in fresh water fishes; 6) the queen triggerfish is an important predator occurring in Caribbean tropical marine habitats and its midgut proteolytic activity may be affected by factors such as diet; and 7) a highly sensitive fluorometric technique can be utilized in evaluating total queen triggerfish midgut protease activity.

OBJECTIVES

- To develop a strategy to determine the trypsin activity verses total protease activity in queen triggerfish intestinal tissue homogenates

- To determine the influence of fish age upon trypsin and total protease activities contained within queen triggerfish tissue homogenates

- To purified and characterize kinetically the queen triggerfish trypsin regarding pH, temperature and inhibition using Trypsin Soybean Inhibitor.

METHODS

Queen Triggerfish Collection

Queen triggerfish samples were obtained from commercial fishermen either as complete digestive tract, including stomach, pyloric caeca, and intestines (Fig. 1) and as a whole fish. Samples were collected from August 2005 through August 2006, from both the east and wet coasts of Puerto Rico (Fig. 2). All samples were sealed in plastic bags, transported on ice, and stored at -20 ^{0}C prior to processing. Whole fish were measured for standard length and their intestines dissected out. A total of 36 digestive tracts samples (each sample consisting of a pool of digestive tracts obtained in a day of fishing) were analyzed for total protease activity, and a total of 30 digestive tracts samples were used for the trypsin activity determination study, while a number of 18 whole fish were used for the correlation length/trypsin activity study.

Intestinal Tissue Crude Homogenate Preparation

Each frozen digestive tract sample was separately thawed at room temperature, weighed (wet weight), and homogenized in 100 mL of deionized, distilled water at 4 ^{0}C with an Osterizer blender set at medium high speed for 1 to 2 minutes. The resulting homogenate was filtered through gauze to remove large tissue/cellular debris. The solution was distributed into 50-ML Oak Ridge polycarbonate centrifuge tubes (Nalgene Corp, Rochester, NY) and subsequently centrifuged in a J2-21 Beckman centrifuge equipped with a Ji-20 rotor and set at 15,000 rpm for 30 min at 4 ^{0}C. The resulting

supernatants were filtered through 0.45 µm (Millipore Corp.) and then stored at 4 ^{0}C until analysis for protease activities.

Protein Concentration Determination

The protein concentration for each of the crude homogenate samples was separately determined using a BCA protein assay (Pierce, Rockford, IL) as per the manufacturer's directions (Smith et al., 1985) in conjunction with a pharmacia Ultrospec 4000 computer-assisted spectrophotometer (Pharmacia, Piscataway, NY).

Fluorometric Assay Standardization

Employing the methodology described by Ueberschaer et al. (1992), a Turner BioSystems TD-700 Laboratory Fluorometer was used in conjunction with a Molecular Probe's EnzChek^{TM} Protease Assay Kit to provide direct fluorescence-based assay for detecting protease activity from intestinal tissue crude homogenate of queen triggerfish. Prior to the protease activity determination, different assays were conducted to test the effectiveness and sensitivity of the fluorometric technique. These included 1) a sensitivity comparison test between the spectrophotometric technique described by Hummel (1959) and the fluorometric technique, 2) a temperature stability test of the fluorogenic substrate (consisting of regularly increasing 10 ^{o}C intervals of temperatures ranging from 0 to 60 ^{0}C); and 3) a pH stability test (consisting of protease activities at all integer pH values ranging from and including 3 to 11). The standard microassay (Fig. 3)

was performed at room temperature in a mini borosilicate glass cuvette (Turner Biosystems) containing 198 µL of digestion buffer, 100 µL of fluorogenic substrate solution, and 2 µL of enzyme solution (crude homogenate adjusted to1 µg/µL of protein concentration) read at 485 +/- 12.5 and 530 +/- 15 nanometers (nm).

To prepare a 1.0 mg/mL stock solution for green fluorescence assay, 0.2 mL of phosphate buffer saline (0.1 M, pH 7.2) were directly added to a plastic vial containing lyophilized fluorogenic substrate (BODIPY FL casein). Both the vial and the BODIPY FL casein were provided with the kit. The vial was capped, inverted several times, and allowed to set at room temperature for 10 minutes to ensure substrate solubility. In preparing a 1X digestion buffer solution, 2.5 mL of 20X digestion buffer were brought to 50 mL total volume with distilled water. In addition, a 10 µg/mL working solution of BODIPY FL casein was prepared by adding 0.2 mL of stock solution (prepared in step #1) to 19.8 mL of the 1X digestion buffer (prepared in step # 2).

Total Intestinal Proteases activity determination in *Balistes vetula*

Digestion buffer was used to bring 2 µL of intestinal crude homogenate solution to 100 µL total volume in a mini borosilicate cuvette. A 100 µL volume of BODIPY casein working solution was added to the buffer + crude homogenate solution to obtain a 200 µL assay volume. The assay was immediately homogenized and read in a Turner Biosystems TBS-380 fluorometer using excitation and emission filters of 485 +/- 12.5 nm and 530 +/-

15 nm. The values were recorded and plotted and the enzymatic activity was expressed in µmol/minute using the following equation (Beer-Lambert law):

$$v = \frac{m.V_R.10^3}{\varepsilon.V_E} = \frac{\mu mol}{min.mL_{Enz}}$$

Where: v = Velocity of enzymatic reaction

m = Slope

V_R = Reaction volume (volume of substrate + volume of enzyme)

ε = Molar extinction coefficient

V_E = Volume of enzyme

The Specific enzymatic activity (SA) was also calculated, using the following equation (a derivative of the Beer-Lambert equation):

$$\text{Specific Activity (SA)} = \frac{U/mL\ Enz.}{V_E.\ C_E.10^{-3}\ mg/mL\ Enz}$$

Where: U = Units of Enzymatic Activity/mL

C_E = Enzyme Concentration

Specific Trypsin inhibition assays

To totally and specifically inhibit trypsin activity contained in crude homogenate, 10 µg of SBTI (Sigma Aldrich) were added to the standard assay. This was accomplished by producing a 200 µL solution made up of 96 µL digestion buffer, 100 µL BODIPY casein working solution, and 2 µL (10 µg) trypsin inhibitor. Assays were conducted by adding 2 µL of crude homogenate preparation to this solution. The resulting protease activity was determined with the fluorometer as per the manufacturer's directions. Observed values were recorded, plotted, and compared with those values obtained in absence of the SBTI.

Correlation Between Fish Size and Trypsin Activity levels

Fish were classified into three different size groups (each group consisting of 3 individuals with similar length and weight) and their intestines processed separately for each group. Total proteases activity was determined for each size group, and thereafter the trypsin activity from the crude homogenates were totally and specifically inhibited, using trypsin soybean inhibitor, as was mentioned above. The results for each group were analyzed and compared to each other. This experiment was performed twice under identical conditions.

Partial Purification of Queen Triggerfish Trypsin

Queen triggerfish trypsin was partially purified using the ammonium sulfate precipitation procedure as described by Deutscher (1990) in conjunction with size exclusion chromatography. Supernatant produced through crude homogenate centrifugation was brought to 25% saturation with ammonium sulfate and subsequently centrifuged at 20,000 rpm for 30 min at 0 ^{o}C. The resulting supernatants were pooled and stored at 4 ^{o}C. Each pellet was resuspended in 2 ml of deionized water. Resuspended pellets were then pooled to form a pellet solution. The 25% ammonium sulfate supernatant was brought to 50% saturation with ammonium sulfate and centrifuged in a manner identical to that just described for the 25% ammonium sulfate precipitation procedure. A 75% saturated ammonium sulfate solution was obtained as per the methodology just described for the 25% and 50% forms. The total volume and protein concentration of each supernatant and pellet fraction were quantified and recorded. Trypsin activity was individually determined for each of the three fractions using the Hummel (1959) assay, which is specific for this enzyme. Determinations of trypsin activity were obtained with a Pharmacia (Piscataway, NY) Ultrospec 4000 spectrophotometer set at 247 nm maximum absorption. The 50% fraction displayed highest trypsin activity was therefore selected for further purification analysis.

Size exclusion chromatographic procedures were performed using a BioRad Econosystem chromatographic system equipped with a glass column (1.5 cm X 100 cm, 250 ml total volume) loaded with P-60 Polyacrylamide gel prepared as per the

manufacturer's instructions (BioRad, Hercules, CA). Running solution was Buffer Tris-HCL, pH 7.0. Flow rate was 0.18 ml/min. Total run volume was 240 ml. All fractions were collected using a BioRad Model 2128 fraction collector (Hercules, CA). A trypsin activity screening was performed and the positive chromatographic fractions were pooled and preserved under refrigeration at 4^{o}C for further analysis.

Kinetic Characterization of the partially purified Queen Triggerfish trypsin

Temperature effect upon the trypsin activity was examined by establishing the temperature of the trypsin assay solution (Hummel, 1959) within each, individual cuvette at either 10, 20, 30, 40, 50, 60, 70 or 80 ^{0}C. As temperature was the only parameter being changed in this line of analysis, all other aspects of the assay are identical to those already described for the standard trypsin assay. Cuvette reaction temperatures were obtained through use of either a hot bath or immersing the cuvette in ice water.

Analysis of pH effect upon hydrolysis of artificial substrate TAME was determined at pH values ranging from 3.0 to 11.0. All other aspects of the trypsin assay were as described for our standard assay conditions.

The effect of soybean trypsin inhibitor (SBTI) upon queen triggerfish trypsin activity was examined at each of the four different inhibitor concentrations (i.e., 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 1.1 µg) in the otherwise standard fluorometric assay buffer

(Hummel, 1959). All other aspects were identical as described for our standard assay conditions.

Molecular weight determination of queen triggerfish trypsin was performed through SDS PAGE gel electrophoretic procedures. SDS PAGE electrophoresis was performed under non-denaturing conditions (using native sample buffer) in a 12% acrylamide gel. BioRad Blue Precision Plus protein standards (BioRad, Hercules, CA) were used for electrophoretic determination of molecular weight. The SDS gel was run at 200 volts for one hour. Proteins, including molecular weight standards, purified porcine pancreatic trypsin (Sigma, St. Louis, MO), as well as partially purified queen triggerfish trypsin, fractions were identified in gel lanes through visualization with coomassie blue staining procedure (BioRad, Hercules, CA). Molecular weights of unknown proteins were calculated using the protein's RF values.

RESULTS

Fluorometric Assay Standardization

The fluorometric technique used in this study, was found to be at least 10 times more sensitive than the spectrophotometric technique (Fig. 4) as the former could detect the proteolytic activity produced by even less than 10 ng of purified commercial porcine trypsin (Sigma – Aldrich). By contrast, the spectrophotometric technique (i.e., Hummel assay) required a minimum of 100 ng purified porcine trypsin to obtain detectable readings. However, it must be noted that while the fluorometric technique is more sensitive, its direct application is limited to analyzing total protease activities while the spectrophotometric version is specific for detecting trypsin activity.

The fluorogenic substrate (BODIPY FL casein, consisting of 100 µL of substrate solution mixed with 100 µL of digestion buffer) was found to be stable at 0 to 40 ^{0}C (Fig. 5). However, temperatures of 50 to 60 ^{0}C resulted in false positive protease readings. In assays conducted above 60 ^{0}C there was no detection of fluorescence emission.

When the fluorogenic substrate was exposed to a pH range of 3 to 11 it displayed stability (Fig. 6), meaning an absence of significant fluorescence emission in the absence of proteases. Above a pH of 11 the substrate displayed a slightly increase of fluorescence.

Total Intestinal Protease Activity Determinations in *Balistes vetula*

A total of 36 intestinal tract samples of queen triggerfish (32 samples from the west coast and 4 samples from the east coast of Puerto Rico) were processed and their protease activity was fluorometrically determined (Table 1). Average total protease activity and specific enzymatic activity for west coast samples were 0.26 µmol/mL/min and 64.79 U/mg (Fig.7), respectively. The average values for samples from east coast were, respectively, 0.28 µmol/mL/min and 70.02 U/mg.

Figure 8 shows a typical, fluorometrically monitored protease assay read for 2 minutes at intervals of 10 seconds with a fluorometer using filters corresponding to the wavelengths within the following range: 485 +/- 12.5 nm to 530 +/- 15 nm.

Specific Trysin Inhibition Assays

The optimal concentration of SBTI required to completely inhibit the trypsin activity contained in 2 µL of any intestinal crude homogenate tested was experimentally determined to be 10 µg (Fig. 9).

A total of 30 samples of queen triggerfish tissue (26 samples from the west coast and 4 samples from the east coast) were each fluorometrically assayed for total protease activity. Subsequently the trypsin activity of each one was totally and specifically inhibited using 10 µg of SBTI (Table 2). The average uninhibited (meaning no addition

of SBTI) protease activity and specific activity of the samples from west coast were, respectively, 0.27 µmol/mL/min and 68.79 U/mL (Fig.10); these average values for east coast samples were, respectively, 0.28 µmol/mL/min and 70.02 U/mL. These same average values for west coast protease activities in the presence of inhibitor were, respectively, 0.069 µmol/mL/min and 17.15 U/mL. For the east coast samples, they were, respectively, 0.059 µmol/mL/min and 14.8 U/mL.

In the presence of SBTI, there was an average loss of 74.93% of total protease activity for west coast samples and a total loss of 78.9 % for east coast samples. Thus, these data indicate that approximately 75 to 80% of total protease activity was attributable to trypsin.

Correlation Between Fish size and Trypsin Activity levels

A number of three fish with similar length were assigned to each of the following three size groups: 10-15 cm (group 1), 20-25 cms (group 2), and 30-35 cm (group 3). Their intestines per each group were separately homogenized and analyzed for total protease activity and indirectly for trypsin activity through the trypsin inhibition assay (table 3). Each experiment was repeated twice. The average protease activity for groups 1, 2 , and 3 were respectively, 0.28, 0.28 and 0.29 µmol/mL/min (Fig.11). After the trypsin inhibition assay, the average protease activity for groups 1, 2, and 3, were respectively 0.066, 0.070 and 0.060 µmol/mL/min. The indirectly estimated trypsin activity for the groups 1, 2, and 3 were respectively, 76.43, 75.00 and 79.31 % (Fig. 12).

Partial Purification of Queen Triggerfish Trypsin

Table 4 summarizes the levels of purification obtained from crude homogenate subjected to ammonium sulfate precipitation fractionation and subsequent size exclusion chromatographic procedures. The highest trypsin activity level was detected in the 50 % ammonium sulfate fraction (0.79µmol/mL/min) and this was therefore utilized for further trypsin purification. Figure 13 shows the trypsin activity determined through the Hummel (1959) assay for all the three ammonium sulfate fractions: 25, 50, and 75%. The 50% fraction, which displayed highest activity, was subjected to size exclusion chromatographic purification procedure and yielded eighty 3-mL fractions, of the which seven fractions (18 – 24) displayed trypsin activity (Fig. 14). The highest activity (0.42 µmol/mL/min) was observed in the fraction number 20; this fraction was used for pH, temperature, and inhibition kinetic assays.

Trypsin Molecular Weight Determination via SDS-PAGE

Figure 15 shows SDS-PAGE analysis results for each of the following: purified pancreactic porcine trypsin (lane 1), size exclusion chromatographic fractions 18 – 24 (lanes 2 -8), and protein size standards (lane 9). Lanes 2 – 8 reveal a band of ~24 kDa, which presumably is the queen triggerfish trypsin. The molecular weight of this band was determined as per the mobility factor (Rf) analysis methodology. The molecular weight of the porcine trypsin band was experimentally determined to be ~23.8 kDa.

Kinetic Analysis Results

All partially purified trypisn activities were determined via the Hummel (1959) assay. In the determination of pH effect upon trypsin activity, the pH was adjusted to all whole number values ranging from 3 to 11. Optimal trypsin activity was obtained at pH 8.0 (fig. 16). The enzyme displayed no activity at pH 3.0. However, it retained 60% of its highest activity at pH 11.

Queen triggerfish trypsin activity was determined by taking measurements at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ^{o}C. Highest activity was obtained at 50 ^{o}C (Fig. 17). The enzyme activity remained stable up to 80 ^{o}C, above which activity was lost (probably as a result of denaturation).

The effect of SBTI on queen triggerfish trypsin activity was examined at inhibitor concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, and 1.1 µg. The partially purified queen triggerfish trypsin was totally inhibited by the SBTI at a concentration of 1100 ng (Fig. 18). Below this concentration level, a progressive increase in activity was noted for all progressively lower concentration levels.

DISCUSSION

The results obtained in this study for examination of total queen triggerfish intestinal protease activity are in agreement with similar such findings from other investigators (Krogdahl and Sundbay, 1999; German et al., 2004; Castillo – Yañez, 2005, Gawlicka and Horn, 2006), all of whom concluded that trypsin is the more important abundant and significant intestinal protease of fish. Most (65 to 83 %, statistical mean 75.5%) of the total queen triggerfish protease activity was attributable to the enzyme trypsin (Table 2). In fact, this level of trypsin activity was maintained regardless of how high or how low the determined total protease activity for a sample.

It was also found that the factors of size and/or age did not affect the total protease activity from queen triggerfish intestines (Fig. 11). German et al. (2004), Sunde et al. (2004), and Gawlicka and Horn, (2006) examined proteases from a number of different fish species and reached an identical conclusion. Taken together, these findings all indicate that size and ontogeny have no bearing upon total protease activity.

The approximately 24 kDa partially purified trypsin is similar in molecular weight with those trypsins purified from other fish species. For example, trypsin from spiny dogfish was found to have a moleculr weight of 23 kDa (Titani et al., 1975) while those from greenland cod, *Gadus ogac*, spiny dog fish, *Squalus acanthias*, and African lungfish, *Protopterus annectens*, were 23.5 kDa (Simpson and Haard, 1984), 24 kDa (Prahl and Neurath, 1966), and 24 kDa (Reeck et al., 1970), respectively.

The pH optimum of 8.0 obtained for queen triggerfish trypsin (Fig. 16) is the same as that determined for many other fish trypsins. Trypsins from spiny dogfish (Prahl and Neurath, 1966), African lungfish (Reeck et al., 1970), and three flatfish species, *Pseudopleuronectes yokohamae, Platichtys bicoloratus*, and *Plactichtys olivaceus* (Yasunaga, 1972), were all determined to display optimal activity at pH 8.0. As with other fish species studied, trypsin activity was largely lost or absent altogether at or near the pH extremes of 3.0 and 11.0. The loss of activity at the lowest pH is similar to the other fish trypsins already mentioned above.

Optimal queen triggerfish trypsin activity was obtained at a temperature of 50 ^{o}C (fig.17). This is similar to that of other fish trypsins, which were found to be optimally active at temperatures between 30 and 60 ^{o}C. For example, Croston (1965) reported that optimal trypsin activity for salmon was 49 ^{o}C. In addition, the artic capelin, *Mallotus villosus*, was found to display optimal trypsin activity at 42 ^{o}C (Hjelmaland and Raa, 1982).

Partially purified queen triggerfish trypsin was inhibited in a concentration-dependent manner by SBTI (Fig. 18). It was found that 1.1 µg inhibitor/3.2 ml total assay solution totally inhibited trypsin activity when the source of trypsin was those proteins contained in the size exclusion chromatographic fraction tube displaying the highest activity. Lower concentrations of inhibitor resulted in less inhibition, with concentration and inhibition level being related in a linear manner. These inhibition data indicate that

the observed trypsin activity from partially purified queen triggerfish proteins was obtained from an actual trypsin and not from the collective activities of several other proteases. We thus conclude that the observed hydrolysis is actual trypsin activity.

This study documents the presence of trypsin in the intestinal tract of the queen triggerfish. Given its high rate of activity compared to that of the other gut proteases, trypsin clearly accounts for the majority of queen triggerfish gut protease activity. This finding supports the assertion that trypsin is the most abundant of fish gut proteases and therefore contributes most significantly to catabolizing ingested proteins into their constituent amino acids.

It would be of interest to examine the factors that govern queen triggerfish trypsin transcription, translation, and perhaps storage in zymogen form (i.e., trypsionogen) to gain a better understanding of how the 3:1 ratio of this enzyme to other gut proteases is maintained. This could lead to a better understanding of those mechanisms controlling gut protease production and excretion. Such a determination may lead to identifying general protease production/regulation for fishes in general.

The specific inhibition of trypsin, SBTI proved to be an effective strategy to indirectly quantify trypsin activity. The results obtained through the inhibition assay were very reproducible and therefore reliable (Table 2). Figure 9 shows the effect of the inhibitor on both purified porcine trypsin (1µg/µL) and on trypsin contained in 2 µL of

crude homogenate (1µg/µL total protein). Some 10 µgof SBTI was sufficient to wholly inhibit total proteolytic activity of the purified porcine trypsin in the standard fluorometric assay. However, when applying this same assay to the queen triggerfish crude homogenate, approximately 25% of the protease activity remained. This result held true even when the amount of inhibitor was doubled (data not shown). The remaining protease activity is logically the result of intestinal proteases other than trypsin, such as chymotrypsin and elastase (Castillo-Yáñez, 2005) in the crude homogenate. Indeed, these enzymes are unaffected by the SBTI, which is specific for trypsin.

Various techniques have been used to quantify trypsin in different animal tissues. Ueberschar (1992) used both a radioimmunoassay and a fluorescence technique to quantify trypsin activity in herring larvae. In addition, Muhlia–Almazán et al. (2003), German et al. (2004), and Gawlicka and Horn (2006) used a hybridization mRNA technique to quantify mRNA concentration in shrimp and fishes. These techniques are expensive and time-consuming. By contrast, the fluorometric technique used in this study is a very simple and cost-effective strategy that proved to be useful in quantifying intestinal trypsin activity in the queen triggerfish. In all likelihood, it would therefore also be useful as applied towards assaying trypsin and other protease activities of fishes and animal species in general.

CONCLUSIONS

- Trypsin is responsible for most of the protease activity in the intestinal tract of queen triggerfish, yielding an average activity of around 75%.

- According to the results obtained from the trypsin analysis conducted on three different queen triggerfish age groups, size (and in consequence age) does not have a significant influence on trypsin activity levels.

- The proportion trypsin activity versus total proteases activity is relatively constant, and is independent on whether the total proteases activity levels are either high or low in a queen triggerfish intestinal tract.

- The strategy used in this study consisting in to specifically inhibit trypsin activity from the crude homogenate, to determine the proportion of the trypsin verses total proteases activity, proved to be a very efficient and cost-effective methodology compared with those methods of other researchers.

- Queen triggerfish trypsin was found to be very similar as regards its kinetic characteristics and molecular weight to many trypsins from other fishes species studied.

RECOMMENDATIONS

To better understand the role that trypsin plays in fish digestion, further studies focused in the following trypsin aspects are recommended:

A complete study of all the possible variables that could affect intestinal trypsin activity levels, including fish intrinsic factors: age, health, physiological state, and sex; random paternal and maternal effects; and random environmental effects: temperature, season, depth, geographical distribution, diet, and feeding regime.

A comparative trypsin study among carnivorous and herbivore fishes, would be very valuable to better understand fish physiology.

Finally it would be interesting to study the upregulation mechanisms involved in the trypsin genes expression.

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TABLES

Table 1: Pooled samples of queen triggerfish intestinal tissue, analyzed for total protease activity (TA) and specific enzymatic activity (SA). Total protease activity and specific enzymatic activity averages for samples from west coast (WC) were respectively 0.26 µmol/mL/min. and 64.79 U/mg. The average values for samples from east coast (EC), were respectively 0.28 µmol/mL/min and 70.02 U/mg.

Sample #	Origin	Wet Weight (gr)	Protein Concentration (mg/mL)	Total Protease Activity (µmol/mL/min)	Specific Enzymatic Activity (U/mg)
1	WC	29	1.70	0.30	75.51
2	WC	39	1.40	0.23	58.38
3	WC	23	1.60	0.17	41.40
4	WC	17	1.75	0.06	14.71
5	WC	21	1.80	0.05	11.40
6	WC	60.73	2.58	0.04	11.21
7	WC	126.9	2.41	0.18	43.79
8	WC	25	1.86	0.22	54.04
9	WC	24	1.94	0.31	76.51
10	WC	17	1.57	0.29	73.27
11	WC	45	2.21	0.26	64.89
12	WC	28.3	2.15	0.29	74.04
13	WC	70.2	2.35	0.26	66.03
14	WC	35.6	1.80	0.29	73.16
15	WC	26	2.35	0.29	71.58
16	WC	45	2.10	0.28	71.10
17	WC	60.5	1.80	0.32	80.70
18	WC	53.7	2.20	0.28	70.84
19	WC	61	2.50	0.28	70.37
20	WC	23.4	2.05	0.29	72.32
22	WC	15.4	2.05	0.28	70.99
23	WC	71	2.11	0.28	69.85
24	WC	60	2.54	0.29	72.98
25	WC	62	2.34	0.28	69.12
26	WC	51.2	2.07	0.29	71.51
27	WC	24	1.74	0.30	74.23
28	WC	43	1.70	0.31	77.57
29	WC	28	2.01	0.31	77.17
30	WC	31	2.50	0.27	68.31
31	WC	44	1.80	0.35	88.53
32	WC	23.5	1.45	0.37	92.87
1	EC	23.5	1.40	0.27	67.28
2	EC	28	1.63	0.30	75.66
3	EC	35.4	1.55	0.22	55.96
4	EC	39.1	1.83	0.32	81.18

Table 2: Total protease activity in samples from west coast (WC) and east coast (EC) before and after the specific trypsin inhibition assay.

Sample #	Wet Weight (gr)	Protease Activity (µmol/mL/min) / Specific Enzymatic Activity(U/mg) Before Trypsin Inhibition	Protease Activity (µmol/mL/min) / Specific Enzymatic Activity (U/mg) after Trypsin Inhibition	Percent of Loss Activity
WC				
1	60.2	0.25 / 62.25	0.069 / 17.32	72.40
2	55	0.21 / 53.56	0.049 / 12.17	76.67.
3	48	0.33 / 83.46	0.079 / 19.71	76.06
4	45	0.23 / 58.68	0.049 / 12.13	78.70
5	28.3	0.29 / 73.27	0.092 / 22.94	68.28
6	70.2	0.26 / 64.89	0.089 / 22.43	65.76
7	35.6	0.29 / 74.04	0.085 / 21.32	70.69
8	26	0.26 / 66.03	0.088 / 22.06	66.15
9	45	0.29 / 73.16	0.079 / 19.93	72.76
10	19.5	0.22/ 55.15	0.049 / 12.32	77.72
11	53.7	0.28 / 71.10	0.067 / 16.76	76.07
12	61	0.32 / 80.70	0.104 / 25.88	67.50
13	23.4	0.28 / 70.84	0.068 / 16.91	75.71
14	15.6	0.22 / 55.66	0.050 / 12.50	77.27
15	71	0.29 / 72.32	0.066 / 16.40	77.24
16	60	0.28 / 70.99	0.062 / 15.51	77.86
17	62	0.28 / 69.85	0.082 / 20.58	70.71
18	51.2	0.29 / 71.51	0.082 / 20.59	71.72
19	24	0.30 / 74.23	0.049 / 12.21	83.67
20	43	0.31 / 77.57	0.052 / 12.94	83.23
22	28	0.31 / 77.17	0.075 / 18.82	75.81
23	31	0.28 / 70.29	0.068 / 16.95	75.71
25	44	0.24 / 59.67	0.048 / 11.86	80.00
26	23.5	0.26 / 64.67	0.046 / 11.43	82.31
EC				
1	23.5	0.27 / 67.28	0.062 / 15.55	78.13
2	28	0.30 / 75.66	0.067 / 16.73	77.67
3	35.4	0.22 / 55.96	0.038 / 9.52	77.04
4	39.1	0.32 / 81.18	0.07 / 17.39	82.73

Table 3: trypsin activity comparison among queen triggerfish individuals of different sizes

Size Group	Average Protease Activity (µmol/mL/min) / (U/mg)	Average Protease Activity after Trypsin Inhibition (µmol/mL/min) / (U/mg)	Estimated Trypsin Activity
G1 (10-15 cm)	0.28 / 73.02	0.066 / 16.32	76.43 %
G2 (20-25 cm)	0.28 / 68.37	0.070 / 17.68	75.00 %
G3 (30-35 cm)	0.29 / 74.01	0.060 / 14.67	79.31 %

Table 4: summary of the queen triggerfish purification process using ammonium sulfate precipitation and size exclusion chromatography.

Protein Source	Protein concentration (mg/mL)	Trypsin Activity (µmol/mL/min)
Crude Homogenate (CH)	2.5	0.68
25% Resuspended Pellet	1.58	0.59
50% Resuspended Pellet	1.51	0.79
75% Resuspende Pellet	2.05	0.73
Size Exclusion Chromathography Of 50% Resuspended Pellet		
(3 mL Fractions)		
18	0.67	0.3
19	0.7	0.33
20	0.69	0.42
21	0.6	0.38
22	0.51	0.3
23	0.55	0.17
24	0.48	0.1

FIGURES

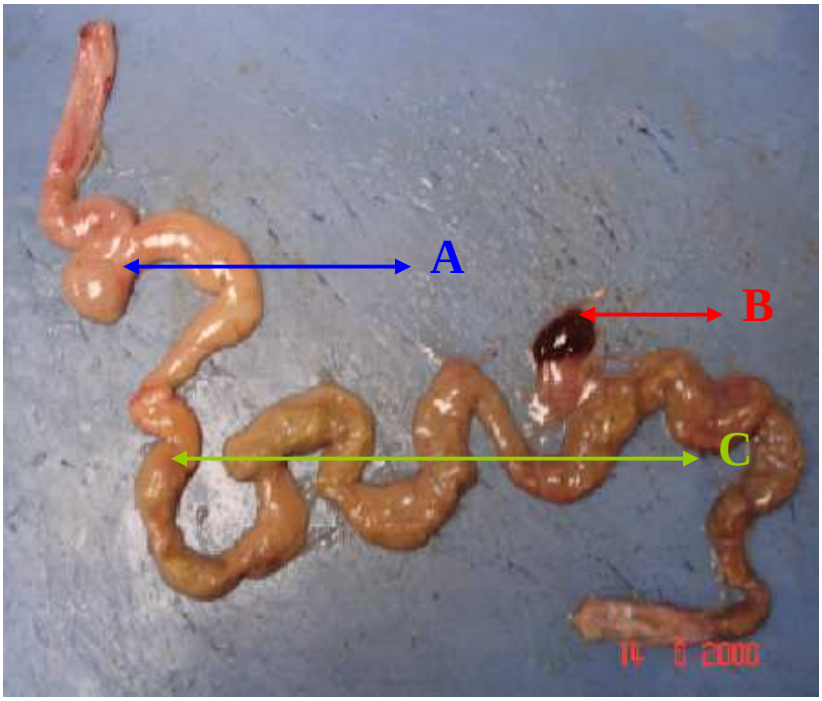


Figure 1: Digestive tract of queen triggerfish, *Balistes vetula*: stomach (A), liver (B), and intestinal tract extending from the stomach (C).

Figure 2: queen triggerfish individuals and/or intestinal tissue were collected in both the west coast (A) at Cabo Rojo, Mayagüez and Rincon, and in the east coast (B) at Naguabo, Playa Ucares.

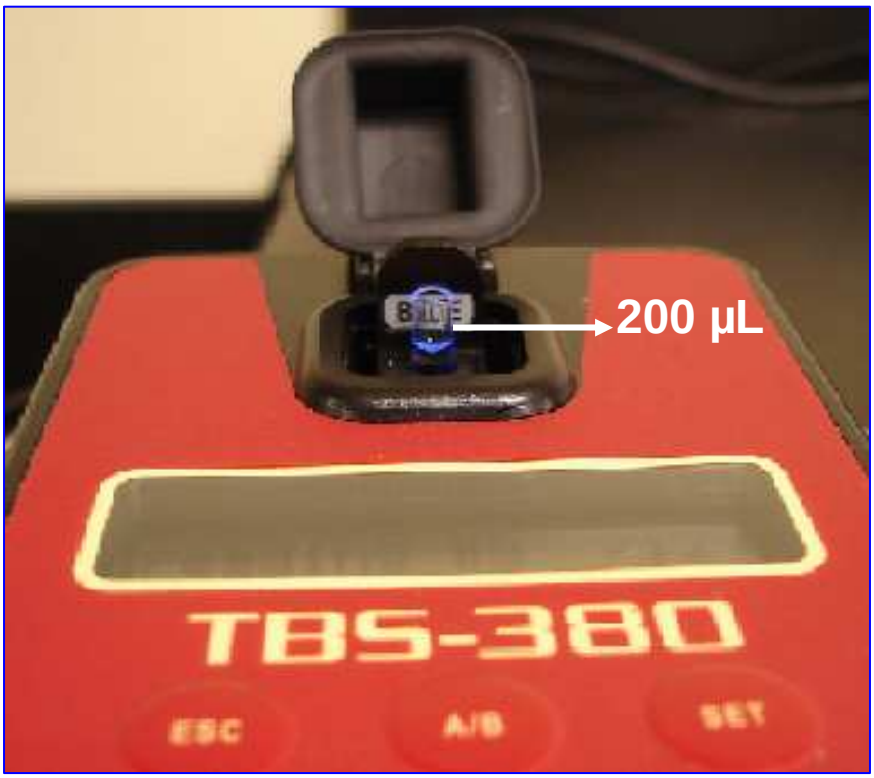
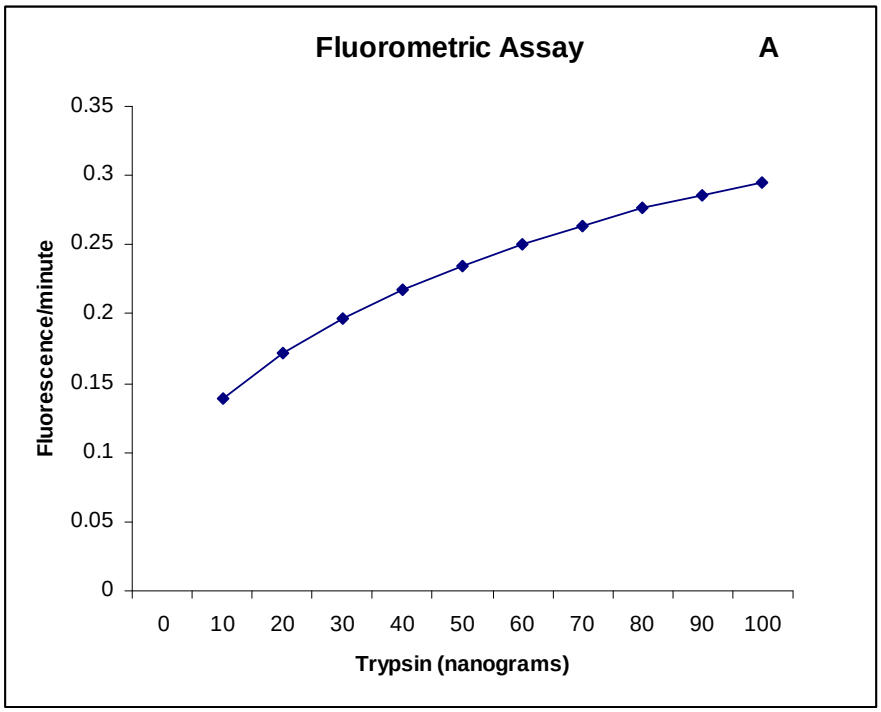


Figure 3: Picture showing the fluorometer and the mini borosilicate cell used in the fluorometric assay.



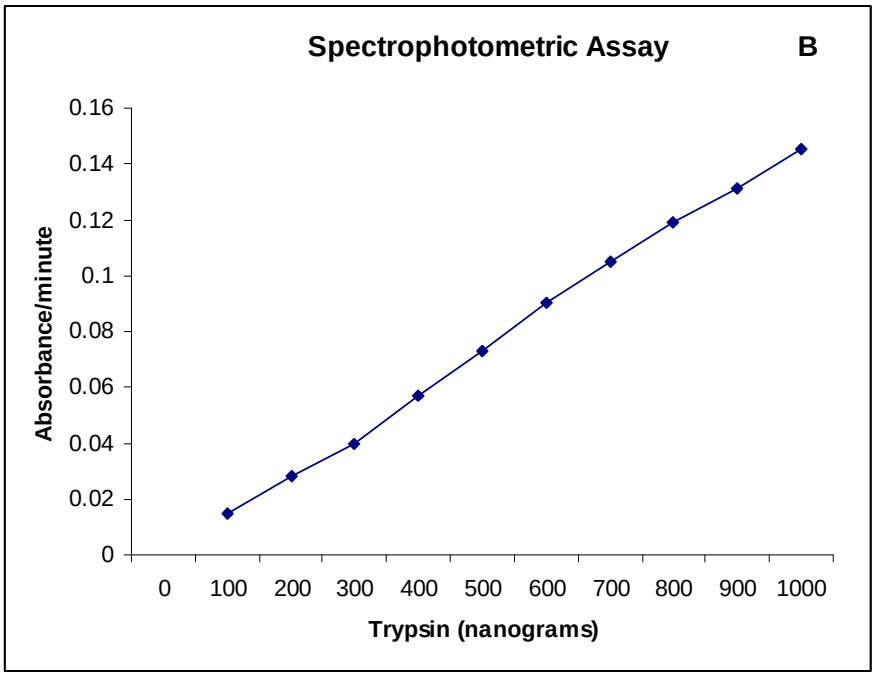


Figure 4: The fluorometric assay (A), detects enzymatic activity in concentration of 10 ng or higher of purified porcine trypsin. The spectrophotometric assay (B), using the methodology described by Hummel, detects 100 ng or higher

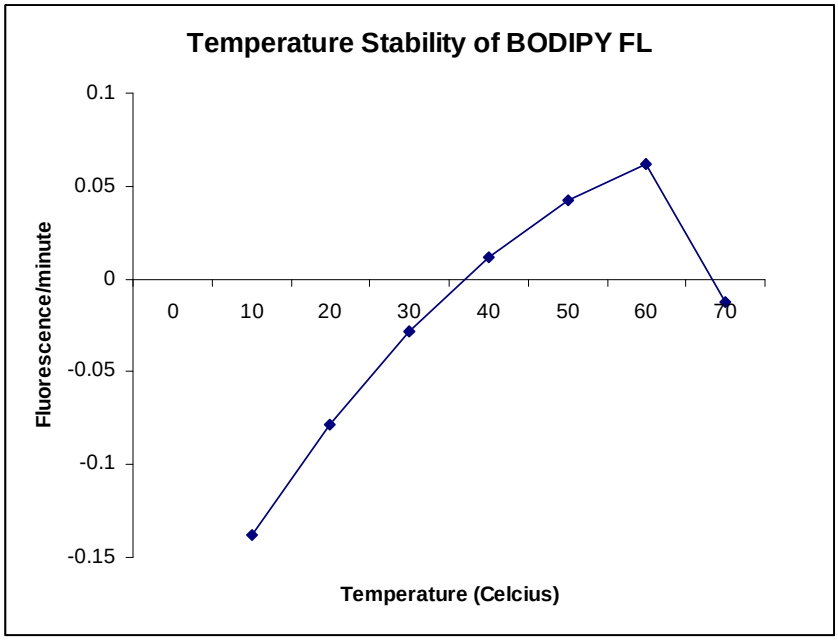


Figure 5: BODIPY FL substrate, was found stable in a range of temperature of 0 – 40 ^{0}C. From 40 to 60^{0}C, fluorescence emission (background) increase. Above 60^{0}C there is no background .

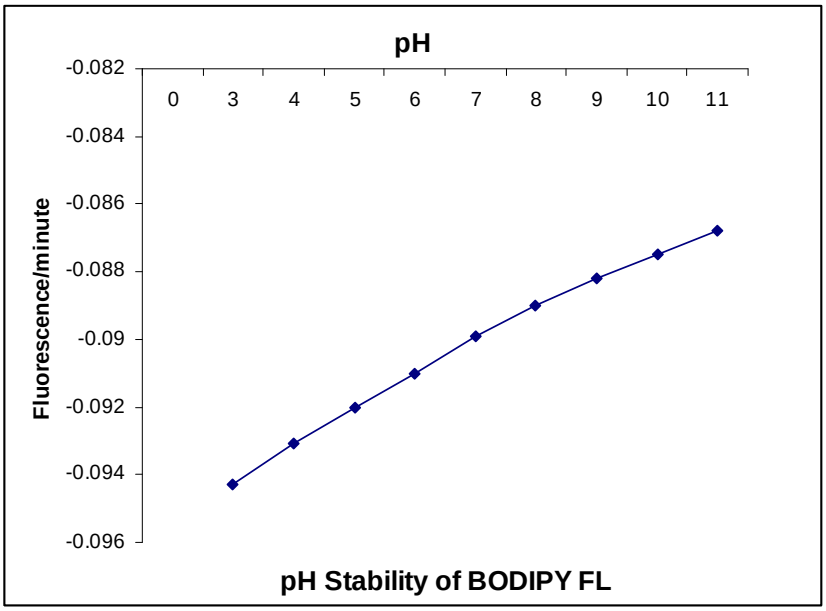


Figure 6: BODIPY FL casein substrate was found stable (there was no fluorescence emission) in a pH range of 3 to11.

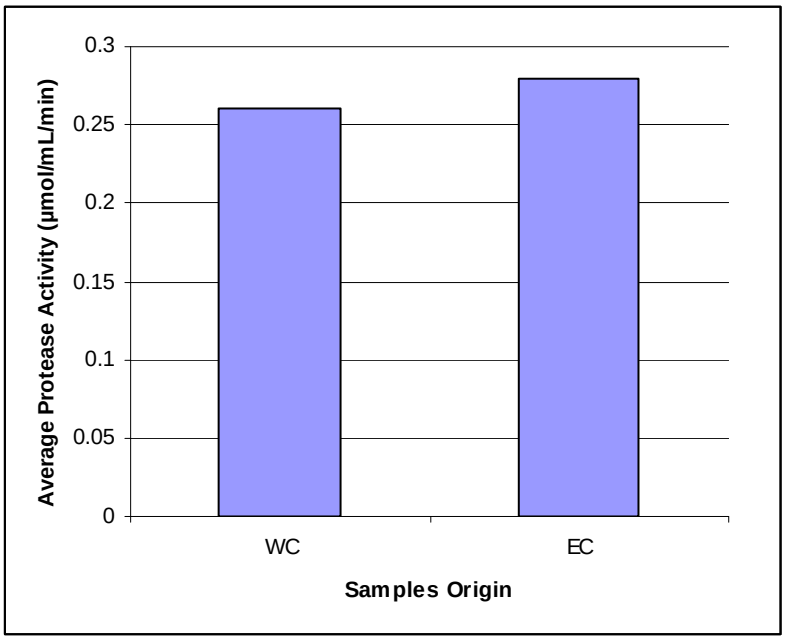


Figure 7: Average protease activity for samples from west coast (WC) and east coast (EC), were respectively 0,26 and 0,28 µmol/mL/min.

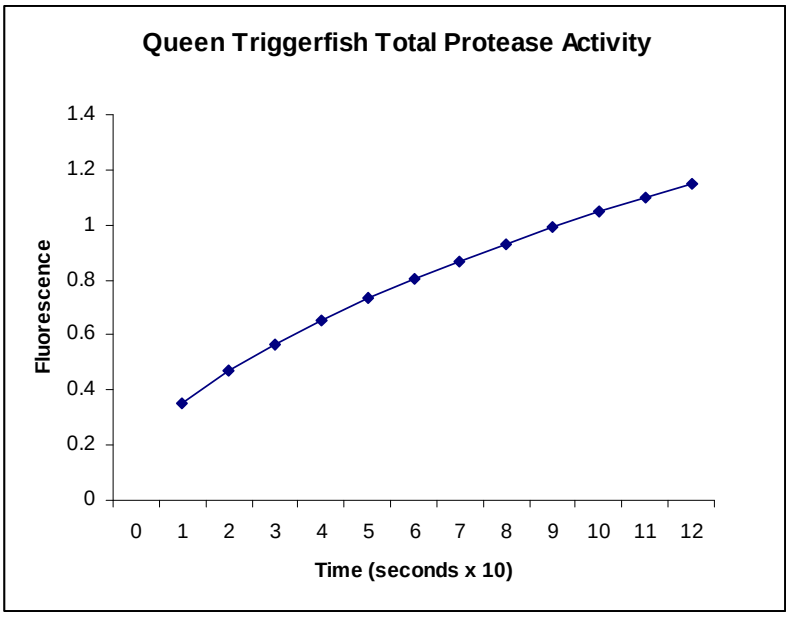
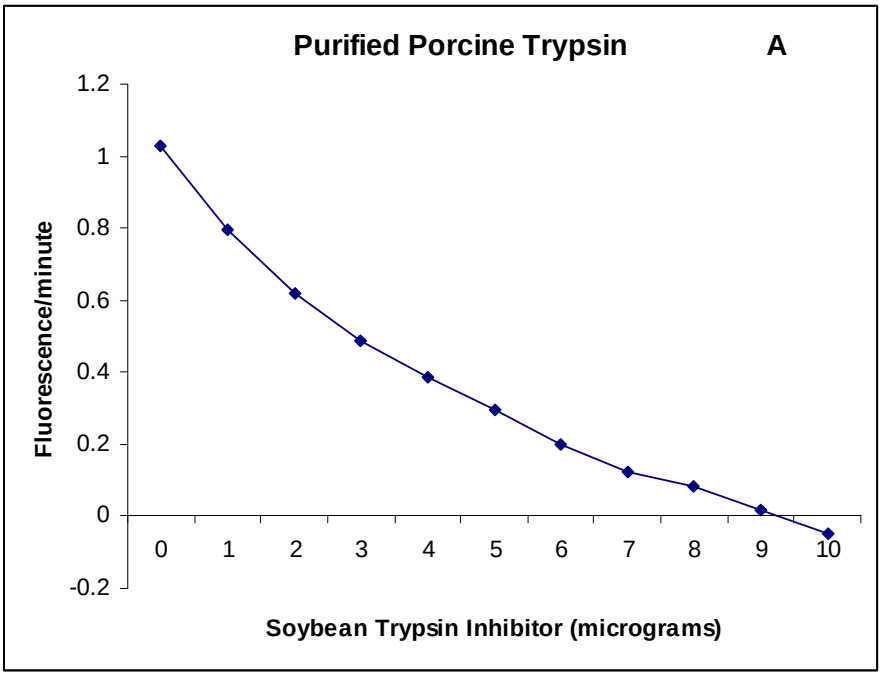


Figure 8. Total protease assay fluorometrically monitored during 2 minutes.



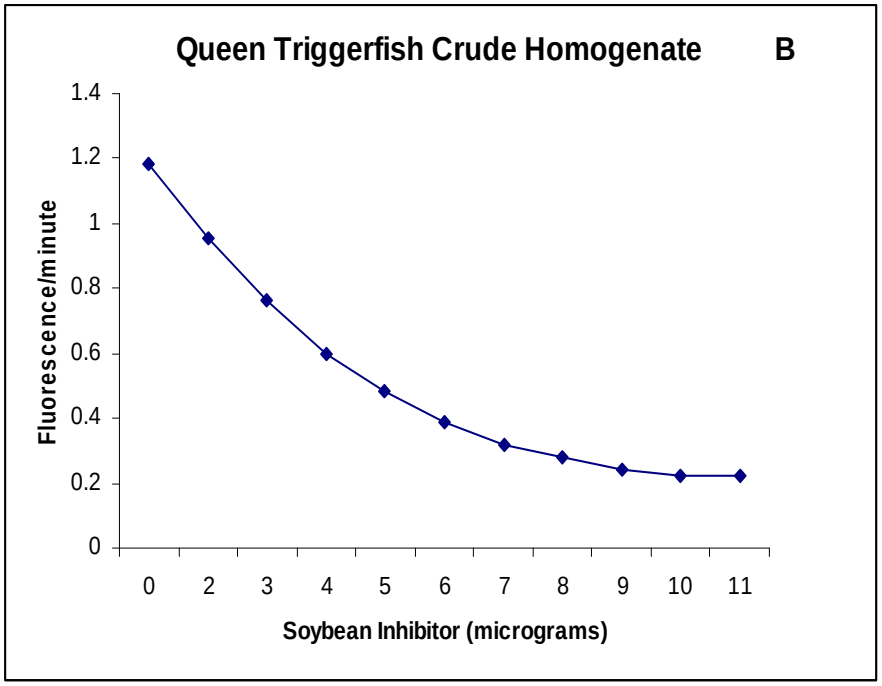
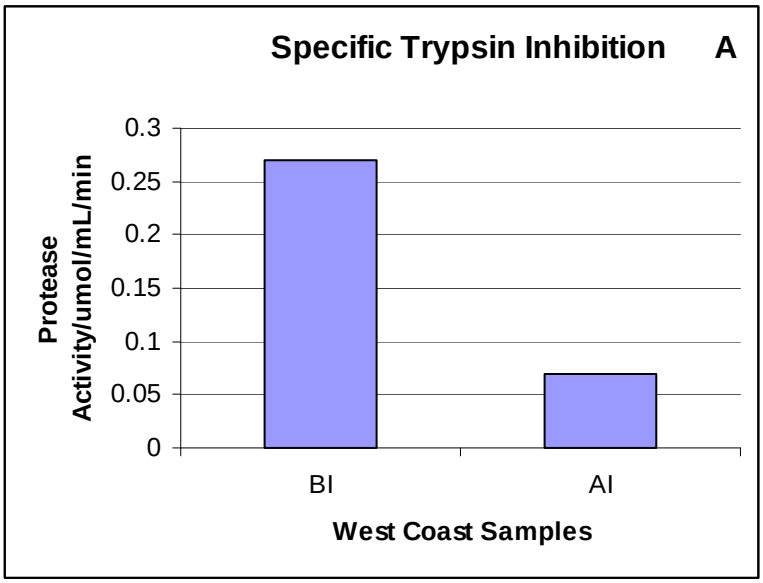


Figure 9: 10 µg of soybean trypsin inhibitor totally inhibit the activity of 1 µg/µL of purified porcine trypsin (A) and most of the activity in the crude homogenate (B).



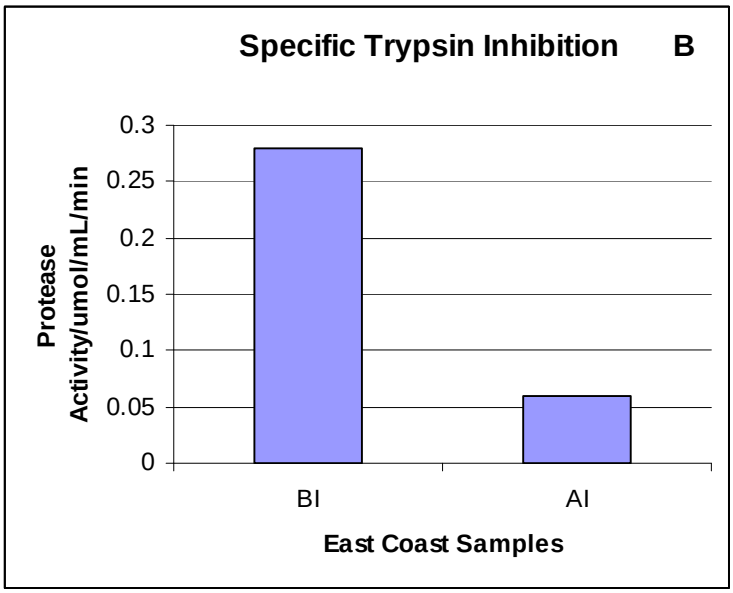


Figure 10: total protease activity fluorometrically assessed before (BI) and after (AI) the specific trypsin inhibition, in samples from west (A) and east (B) coasts.

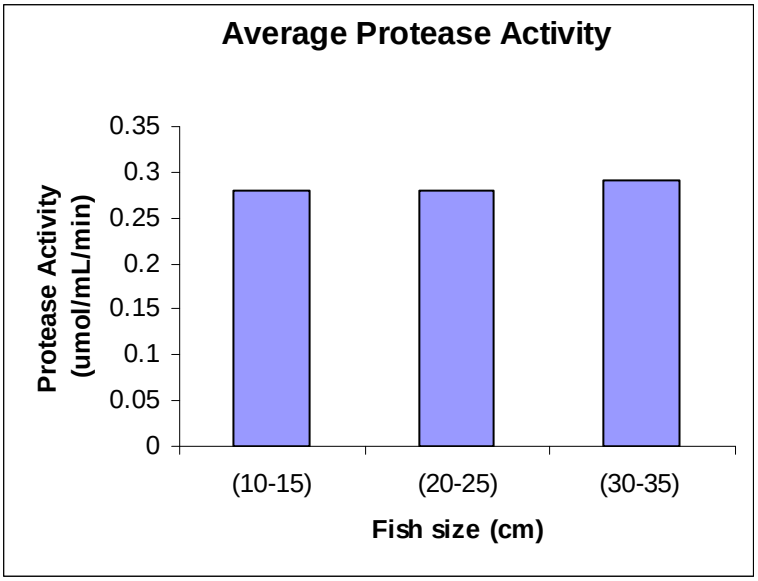


Figure 11: total protease activity comparison among queen triggerfish individuals of different sizes.

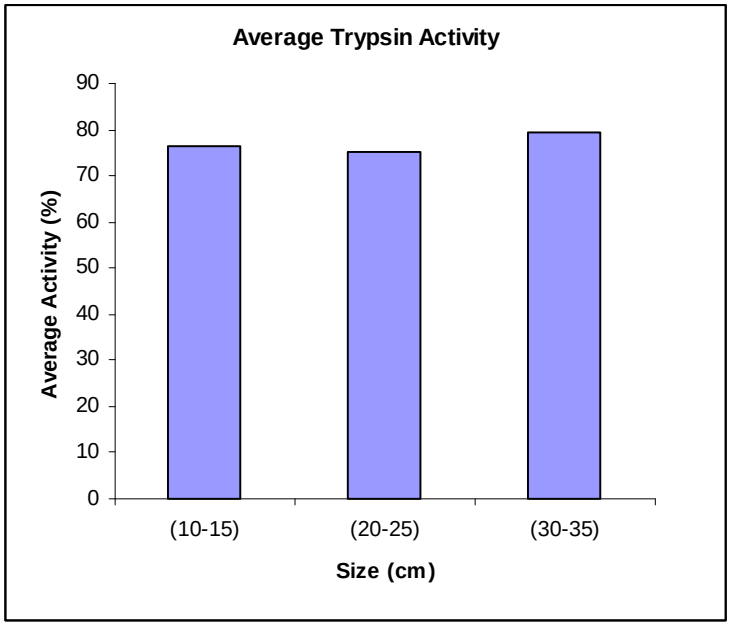


Figure 12: trypsin activity comparison among queen triggerfish individuals of different sizes.

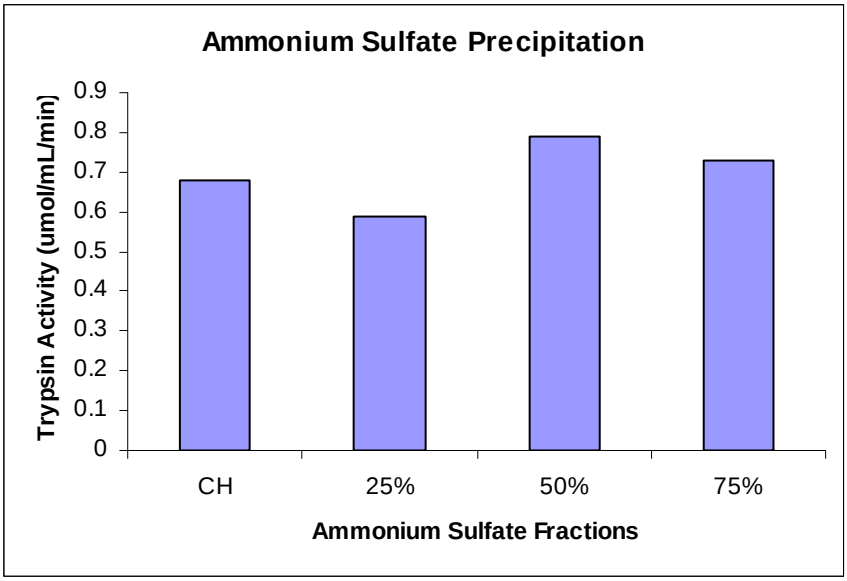


Figure 13: queen triggerfish trypsin activity determined through the Hummel assay for all the three ammonium sulfate fractions: 25, 50, and 75% and for the crude homogenate (CH).

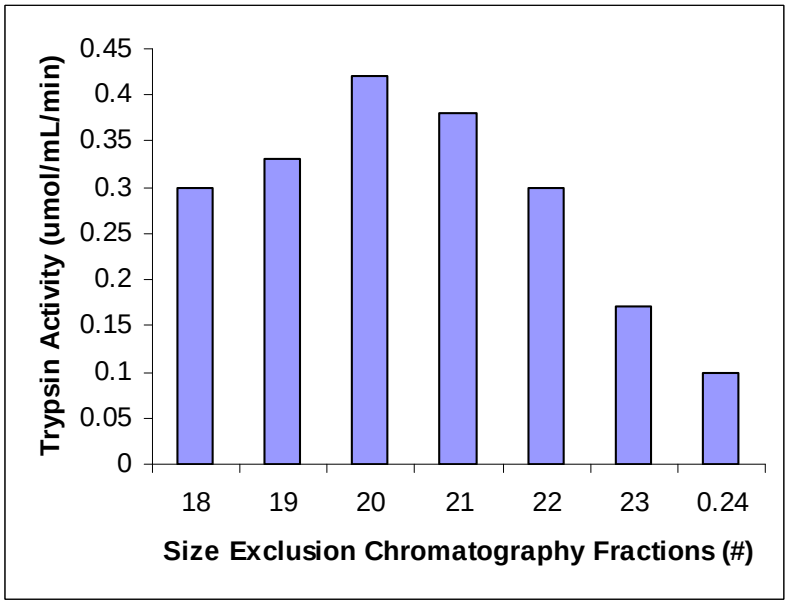


Figure 14: queen triggerfish trypsin activity determined through the Hummel assay displayed for seven size exclusion chromatography fractions.

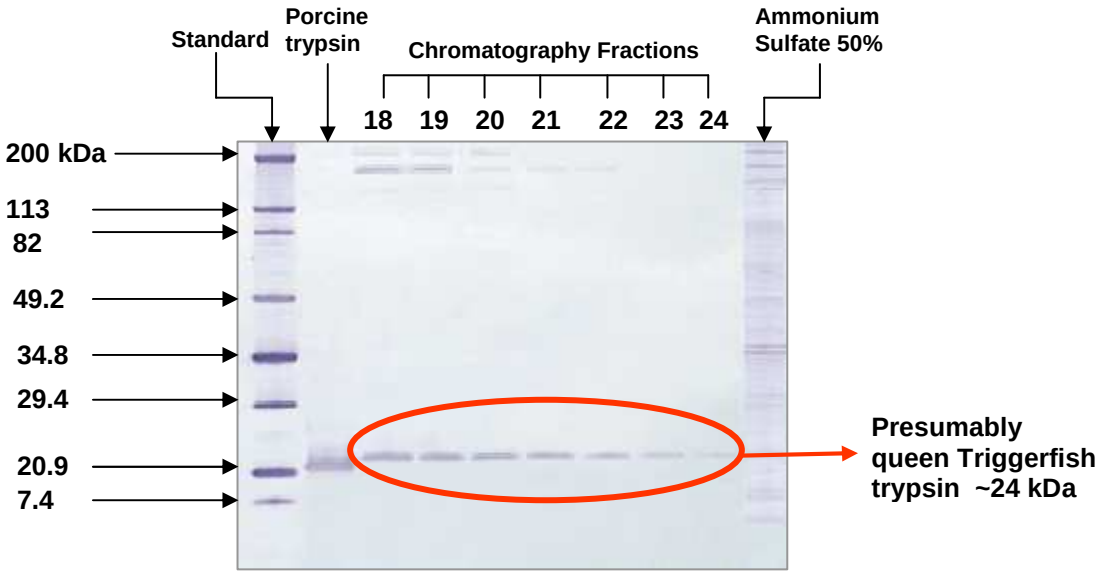


Figure 15: SDS-PAGE analysis results for each of the following: protein size standards (lane 1), purified pancreactic porcine trypsin (lane 2), size exclusion chromatographic fractions 18 – 24 (lanes 3 -9), and 50% ammonium sulfate fraction (lane 10)

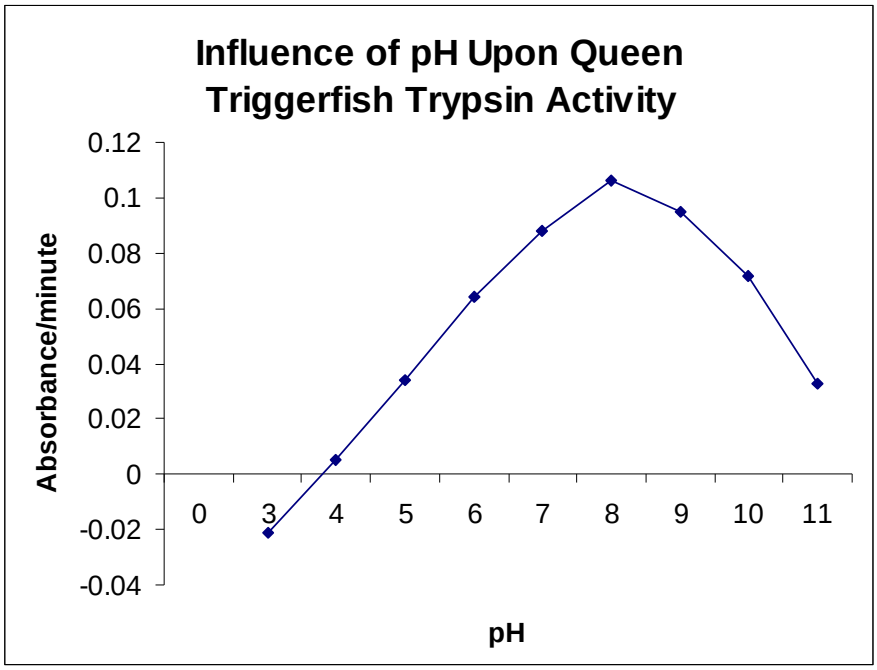


Figure 16: queen triggerfish trypsin showed optimal activity at pH 8.0, while at pH 3.0 showed no activity and retained ~60% of its activity at pH 11.

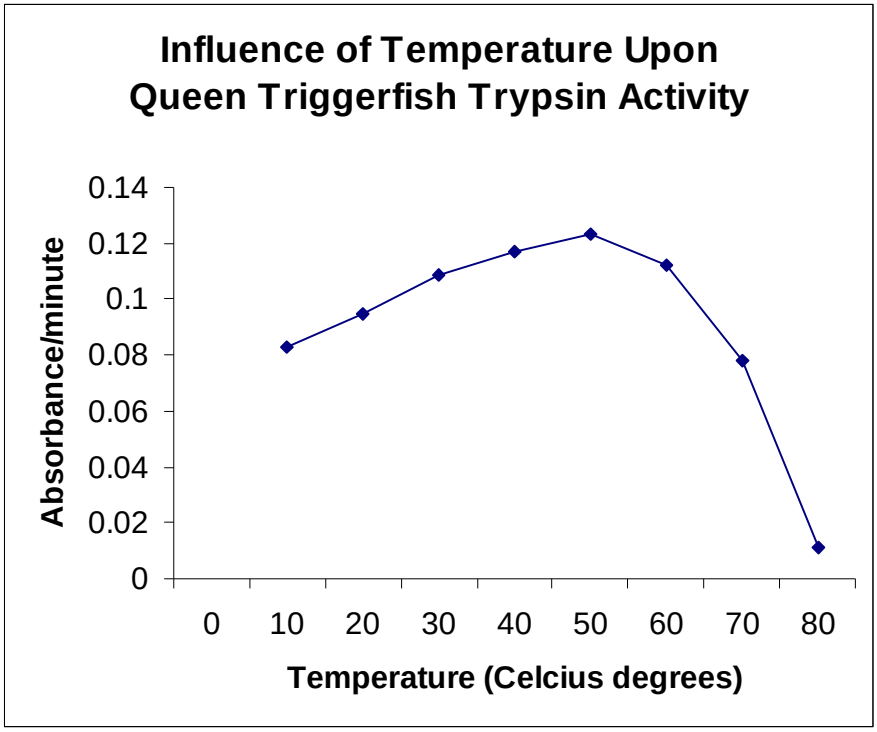


Figure 17: queen triggerfish trypsin displayed the highest activity at 50^{o}C, and its activity remained stable up to 80^{o}C.

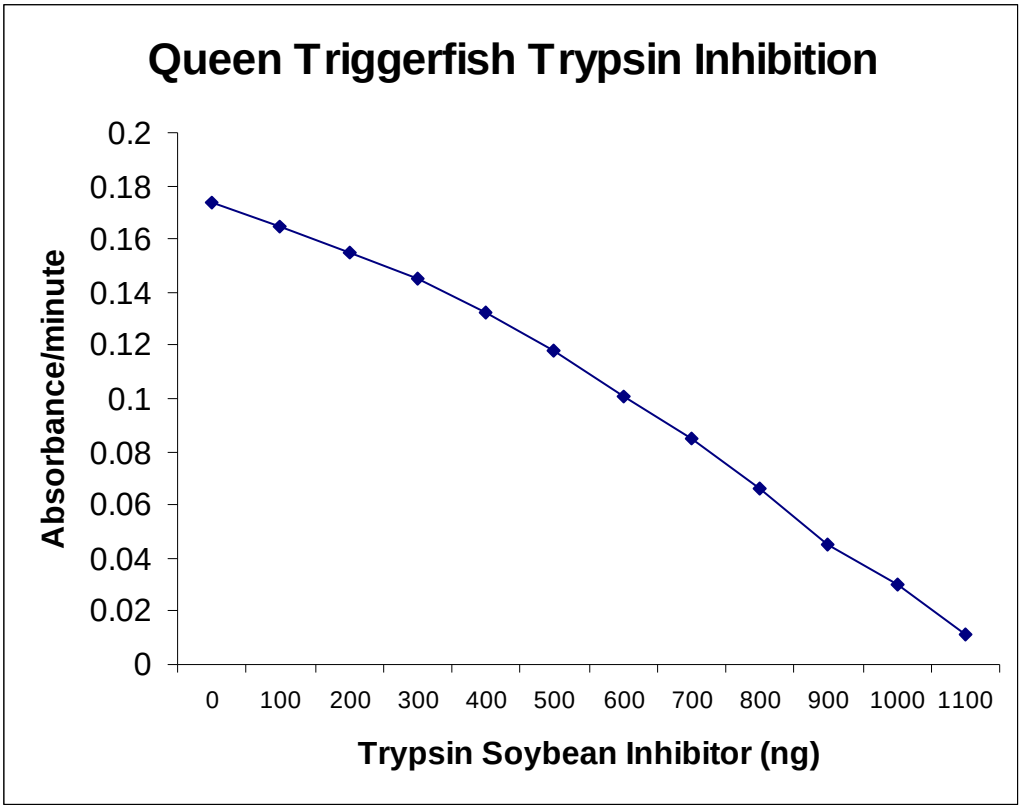


Figure 18: The partially purified queen triggerfish trypsin was totally inhibited by the soybean trypsin inhibitor at a concentration of 110ng