Fluorescence-Based Assessment of Total Protease Activity/Trypsin as a Function of Variable Soybean Content in Fines Fed to the Gray Tilapia (*Oreochromis niloticus*) and the Red Tilapia (*Oreochromis hornorum*)

By

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ABSTRACT

This study evaluated the effect of five diets, differing from one another based upon ratio of raw soybean/processed soybean content, upon both biomass production and intestinal protease activity in gray tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis hornorum*). The tilapia were maintained for 5 weeks in a Lajas' Aquaculture Facility pond, Lajas, Puerto Rico. This pond contained 5 separate cages and each cage contained 16 male tilapia, 8 of which were gray and 8 of which were red. These fishes were provided with one of the following five diets (i.e., 0:4, 1:3, 2:2, 3:1, or 4:0 for raw soybean/processed soybean protein dietary component). At the end of the feeding period, the following were determined: 1) weight, 2) total intestinal protease activity (determined by fluorometric assay), 3) amount of intestinal protease activity attributable to the enzyme trypsin, 4) feasibility of purifying and characterizing trypsin from red or gray tilapia, 5) *in vitro* examination of how the addition of processed soybean homogenate affects intestinal protease activity, and 6) SDS-PAGE zymogram visualization of the effect of increased processed soybean homogenate on total intestinal protease activity. Although an increase in biomass >5% for gray tilapia and >21% for red tilapia was observed, a linear correlation between increased raw soybean feed content and biomass production was not observed. The *in vitro* assays revealed that intestinal proteases of both red and gray tilapia are inhibited by processed soybean homogenate. This effect was greatest for gray tilapia. Trypsin was partially purified from both tilapia species and found to display a pH optimum of 8.0, highest catalytic rate at 50 ^{o}C, and a molecular weight of approximately 23 kDa. In addition, *in vitro* soybean trypsin inhibitor analysis revealed that 82-86% of the total

intestinal protease activity was attributable to trypsin, a finding similar to that from similar analysis of other fish species.

RESUMEN

Este estudio evaluó el efecto de cinco diferentes dietas basadas en el contenido de soya cruda/soya procesada sobre la producción de biomasa y la actividad de proteasas intestinales en tilapia gris (*Oreochromis niloticus*) y tilapia roja (*Oreochromis hornorum*). Las tilapias fueron mantenidas por 5 semanas en un lago de las Instalaciones de Acuacultura de Lajas, Puerto Rico. Este lago contenía 5 jaulas separadas, cada una contenía 16 tilapias machos, 8 de los cuales eran grises y 8 rojas. Estos peces fueron sometidos a una de las cinco diferentes dietas de soya (i.e., 0:4, 1:3, 2:2, 3:1, o 4:0 relación soja cruda/soja procesada como componente de proteína en la dieta). Al final del periodo de alimentación se determinó: 1) peso grupal, 2) actividad total de proteasas (mediante ensayo fluorométrico), 3) cantidad de actividad de proteasas intestinal atribuible a tripsina, 4) purificación y caracterización parcial de tripsina en tilapia roja y gris, 5) examinación *in vitro* de cómo la adición de homogenato de soya procesada afecta la actividad de proteasas intestinales, y 6) zimogramas en SDS-PAGE para visualizar el efecto del incremento de homogenato de soya procesada sobre la actividad total de proteasas. Se observó un aumento en biomasa > 5% para tilapia gris y >21% para tilapia roja; aunque no se observó una correlación lineal entre al incremento en soya cruda y la producción de biomasa. Los ensayos *in vitro* demostraron que las proteasas intestinales de ambas especies son inhibidas por el homogenato de soya procesada. Este efecto fue mayor para tilapia gris. Tripsina fue parcialmente purificada de ambas especies de tilapia y ambas enzimas mostraron un pH óptimo de 8.0, la mayor actividad catalítica a 50 ^{o}C, y un peso molecular de

aproximadamente 23 kDa. Además, el análisis *in vitro* con el SBTI reveló que del 82-86% de la actividad total de proteasas intestinales fue atribuible a tripsina, resultado similar a lo encontrado en los análisis de otras especies de peces.

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INTRODUCTION

The growth of any animal is a function of the efficiency with which it ingests, chemically breaks down, and absorbs nutrients from food. Fishes, among vertebrates, are the most efficient converters of food substances into biomass, but require high levels of dietary protein to promote growth (Spinelli, 1978). Moreover, compared to other vertebrates, rapid growth in fishes is characterized by a rigid requirement for high levels of dietary amino acids. Central to obtaining these amino acids is the breakdown of ingested proteins through the activities of various gut proteases.

Trypsin, which is specific for the carboxyl side of arginine and lysine residues, is likely the most abundant protease of fish midguts (Ferscht, 1985). Indeed, trypsin activity has been characterized from numerous fish species (Fereidoon and Janak-Kamil, 2001). Additionally, in larval fish higher levels of trypsin activity have been directly correlated to correspondingly higher growth rates and therefore biomass production. Given that feeds provided to commercially reared fish can vary widely in their protein content and that some commonly used fishmeal protein sources (such as that from soybeans) actually contain trypsin and chymotrypsin inhibitors, it is of interest to the fish farming industry to identify and develop feeds that do not impair protease functions and, therefore, biomass production. Clearly, then, a deeper understanding of how general gut protease activity supports digestive physiology and growth in fishes is of interest to aquaculture.

During the past twenty years, commercially raised tilapia has steadily gained popularity as a food fish. Traditional tilapia farming has almost entirely relied upon the fish obtaining nutrients from their cultured environment, in which nutrient input was limited to fertilizers (Alceste, 2000). However, as tilapia have become an international commodity, present and future aquaculturists, seeking to keep tilapia and other cultured fish production costs low, may significantly boost biomass production per unit area through utilizing, in addition to modern equipment and technology, feeds that optimize trypsin activity and hence subsequent amino acid assimilation/biomass production.

While soybeans have provided an important source of protein in prepared fish feeds used in aquaculture, crude soybean contains a natural trypsin inhibitor (Liener, 1989). This inhibitor has the potential to at least partially block trypsin activity and, hence, subsequent protein breakdown/amino acid assimilation (Moyano-Lopéz et al, 1999) in commercially reared fishes. It is thus of interest to aquaculture to determine the effect of crude dietary protein upon gut trypsin levels and fish biomass production.

In an effort to better understand the effect of crude soybean meal upon gut trypsin activity in fishes raised under aquacultural conditions, this study utilized a highly sensitive, fluorescence-based technique to determine relative levels of total protease activity obtained from gray tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis hornorum*) reared in ponds and subjected to one of the five different crude soybean meal content fine

applications. The results of this investigation are considered with respect to biomass production.

Literature Review

Widely cultured in fish farms throughout the world, the tilapia, comprised of both *Oreochromis neloticus* and *Oreochromis hornorum*, is especially important to aquaculture in developing countries. After carps and salmonids, tilapia, with an annual growth rate of about 11.5%, constitute the third largest group of farmed finfish worldwide. It is clear then that tilapia represent an already important commercial fish group that will likely increase in importance as world human populations and food requirements also increase (FAO, 2000; Naylor et al., 2000). Such tilapia production will largely depend on aquacultural practices that promote optimal protein biomass production.

One challenge facing all fish aquaculturists is that of maximizing protein biomass production in a cost effective manner. Indeed, fishmeal protein content alone represents on average 50% of feed costs as protein is the most expensive fish feed component. Moreover, it has been noted that many fish species currently cultured have a high dietary protein requirement, ranging from 30 to 50% (Plascencia et al., 2002). It is therefore clear that an inexpensive, readily available protein source that promotes biomass production is critical for fish farming to prosper.

In an effort to lower fish feed costs, aquaculturists during the past 20 years have made several attempts to partially or totally replace fishmeal protein with less expensive protein

sources, such as that from fishery by-products, terrestrial animal by-products, oilseed plants, aquatic plants, single-cell proteins, grain legumes, plant protein concentrates, and cereal byproducts (Olvera et al, 1988, 1990, 1997, 1998; Begum et al, 1994; Rodriguez-Serna et al., 1996). These more economical protein sources have resulted in fish feeds that promote effective fish production through aquaculture (Foster et al., 2002).

Soybean products relevant to aquaculture can be divided into three categories: 1) protein products; 2) oil; and 3) lecithin. Among these, researchers have paid greatest attention to soy protein/soy concentrate, although soy lecithin is used in commercial aquaculture feeds as both a source of choline and as an emulsifier (Foster et al., 2002). Soybean meal and soy concentrate have been found to be particularly excellent sources of dietary protein for cultured tilapia production (Table 1). This is because its supply is secure, its price is low, and its amino acid composition includes all essential amino acids required by tilapia (Swick, 2002).

Table 1: Essential amino acids composition of SPC (Soy Protein Concentrate) and requirement of fish (% of diet)

	SPC1 (cp 65%) % as is	SPC % at 30% protein level	Requirement2 Channel catfish	Rainbow trout	Common carp	Tilapia
Arginine	4.94	2.3	1.2	1.5	1.31	1.18
Histidine	1.82	0.8	0.42	0.7	0.64	0.48
Isoleucine	3.19	1.5	0.73	0.9	0.76	0.87
Leucine	5.2	2.4	0.98	1.4	1	0.95
Lysine	4.23	2.0	1.43	1.8	1.74	1.43
Met + Cys	1.89	0.9	0.64	1.0	0.94	0.9
Phe + tyr	3.45*	1.6*	1.4	1.8	1.98	1.55
Threonine	2.73	1.3	0.56	0.8	1.19	1.05
Tryptophan	0.78	0.4	0.14	0.2	0.24	0.28
Valine	3.38	1.6	0.84	1.2	1.10	0.78

1, Soycomil FG, ADM Europoort bv, Rotterdam, The Netherlands; 2, NRC, 1993 *, Only phenylalanine

Despite its positive attributes, the use of soybeans as a fish meal protein component is not without some drawbacks. First, soybeans/soymeal contain/s naturally-occurring inhibitors of the digestive proteases chymotrypsin and/or trypsin, this latter enzyme being the most important and abundant protease present in fish intestines and pyloric caeca (Fereidoon and Janak-Kamil, 2001). Thus, the function of trypsin in breaking down ingested protein into individual amino acids that may be absorbed across the fish gut epithelium and subsequently used in protein biomass production will be lessened. Another soybean/soy meal component that may curtail effective nutrient absorption is lecithin, which has been found to combine with carbohydrate receptors in cell membranes and thereby interfere with nutrient absorption and transportation (Tacon, 1995). Oligosaccharide, specifically alcohol-soluble carbohydrate, removed from soybean meal significantly increased nutrient utilization in trout and salmon (Murai et al., 1989, cited by

Krogdahl, 1989). Further still, certain soy proteins/peptides may function as antigens which damage portions of the gut wall and therefore impair its capacity to effectively absorb nutrients (Rumsey et al., 1994). Finally, soy saponins may cause soy-based fish feeds to have a bad taste. Bureau et al. (1998) reported observing this in trout and salmon and suggested that this accounted for an observed decrease in the growth of cultured examples of these species.

Among these soybean/soy extract components that may negatively impact fish growth and development, it is the soybean protease/soybean trypsin inhibitors that likely have the greatest such effect (Liener, 1994). Soybean protease/trypsin inhibitors are protein molecules which have the ability to inhibit the activity of proteolytic enzymes within the alimentary tract. In raw soybeans these account for about 6% of the protein content. Their main constituents are the relatively heat-labile and acid-sensitive Kunitz inhibitors as well as the heat stable Bowman-Birk inhibitor. The former molecule is a larger protein (20 – 25 kDa) that at a concentration of one mole will abolish the activity of one mole of trypsin or chymotrypsin. The latter is a smaller molecule (6 – 10 kDa), one mole of which will simultaneously inhibit, for example, one mole each of trypsin and chymotrypsin (Liener, 1989).

The evolution of plant protease inhibitors is likely a response to deter both herbivorous insects and vertebrates (Moyano-López, 2006). It is notable that the presence of these inhibitors in plants in general was first detected by fish aquaculturists (Moyano-López, 2006) who had utilized plants as fish feed protein sources.

In the past ten years, some studies have been undertaken on the effect of protease inhibitors upon different fish species. For example, Moyano-López et al. (1999) evaluated the inhibitory effect of three different vegetable meals (soybean, gluten and wheat) on protease activity of tilapia, seabream, and sole. This study found that tilapia (*Oreochromis niloticus*) proteases exhibited the highest inhibitor sensitivity to inhibitors from all three feed preparations. By contrast, sole (S*olea senegalensis*) proteases were found to be least sensitive. Clearly, then, protease inhibitors within fish meal preparations are of concern to aquaculture because these molecules can have an effect upon protein breakdown and subsequent assimilation/biomass production.

It is of interest to develop a sensitive methodology that quickly determines the effect of crude soy protein inhibitors upon fish intestinal protease activities. On this point, Moyano-López et al. (1999) utilized the casein method of Kunitz (1947) to address this issue in gray tilapia. However, our study used a more highly sensitive fluorescence-based protease assay to assess the effect crude soybean homogenate upon protease activity from grey and red tilapia intestinal homogenate. Indeed, this fluorescence-based procedure is at least ten times more sensitive to protease activity than spectrophotometric protease assays (Puerta-Martínez, 2007), such as those used by Moyano-López et al. (1999).

OBJECTIVES

The aims of this study, then, consisted of the following two objectives:

- Obtain a deeper understanding of how protease/trypsin activity and fish feed content relate to biomass production in fish species of aquacultural importance, such as Gray Tilapia (*Oreochromis niloticus*) and the Red Tilapia (*Oreochromis hornorum*)

- Through use of a fluorometric technique, assess the effect of increasing levels of dietary soybean protein upon gut protease/trypsin activity in tilapia; this will simultaneously evaluate the effectiveness of the fluorometric technique towards this application

MATERIAL AND METHODS

Tilapia Tissue

Oreochromis niloticus (gray tilapia) and *Oreochromis hornorum* (red tilapia) utilized in this study were reared in the Lajas' Aquaculture Facilities, Lajas, Puerto Rico. Dr. John Kubaryk was oversaw this aspect of the study. The tilapia were maintained for 5 weeks in one pond (Fig. 1). This pond contained 5 separate cages. Each cage contained 16 male tilapia, 8 of which were gray and 8 of which were red. Thus, each cage contained two study groups, one having 8 gray and the other having 8 red tilapia. On a daily basis, each cage was consistently fed one of the following fine preparations, each such preparation being characterized by one of the following five raw soybean to processed soybean ratios: 1) 0:4; 2) 1:3; 3) 2:2; 4) 3:1; 5) 4:0 (Table 2). Total amount of daily soybean received by each cage was 391.8 g. Each of the 10 fish groups was weighed at the beginning and the end of the study.

Figure 1: Pond and cages where tilapia were maintained for 5 weeks

Table 2: Formulation of the five diets, all of which differed from one another based solely upon the ratio of raw soy protein to processed soy protein

Component (gr)	Levels of Raw soybeans (%)				
	100	**75**	**50**	**25**	**0**
Raw soybean	391.8	293.8	195.9	98	0
Processed soybean	0	98	195.9	293.8	391.8
Fish flour	144	144	144	144	144
Maize	322.2	322.2	322.2	322.2	322.2
Wheat	169.2	169.2	169.2	169.2	169.2
Alphalpha	99.6	99.6	99.6	99.6	99.6
Premixes	38.4	38.4	38.4	38.4	38.4
Oil (Maize/fish)	34.8	34.8	34.8	34.8	34.8

At the end of the rearing component of the study, tilapia representing the individuals from one of the ten study groups were collectively weighed, placed in a labeled plastic bag, tied shut and placed on ice for transport from the University of Puerto Rico Aquacultural Experiment Station, Lajas, Puerto Rico to the Fish Physiology/Biochemistry Research Laboratory at the University of Puerto Rico-Mayaguez (Department of Biology) (Fig. 2). Each of the ten tilapia groups was separately treated in this manner. Upon arrival to the University, the bags were placed in a freezer set at -20ºC until the dissection procedure.

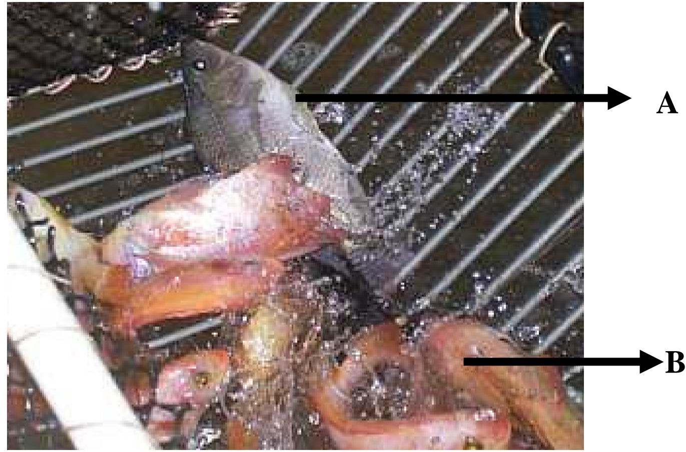

Figure 2: ***Oreochromis niloticus*** **(gray tilapia; A) and** ***Oreochromis hornorum*** **(red tilapia; B)**

Crude Homogenate Preparation

Each group of tilapia was thawed at room temperature and fish were dissected to obtain the intestinal tracts. Group intestinal tracts were pooled, weighed (wet weight), and homogenized in an Osterizer® blender set at medium high speed for 2 min with and containing deionized, distilled water in a quantity of mL that was 1.5 X the wet weight of the tissue in grams. The resulting homogenate was filtered through gauze to remove large cellular debris. This filtered solution was then distributed into 50-mL Oak Ridge® polycarbonate centrifuge tubes (Nalgene Corp, Rochester, NY). The tubes were placed in a JA-20 Beckman® rotor and spun in a J2-21 Beckman® centrifuge set at 20,000 rpm for 30 min at 4°C. The resulting supernatants were filtered through 0.8 µm and 0.45 µm filters (Millipore Corp.), pooled, and then stored at -20ºC.

Protein determination

Protein concentrations of each group were determined using a BCA protein assay (Pierce, Rockford, IL) as per manufacturer's directions (Smith et al., 1985) in conjunction with a Pharmacia Ultrospec 4000 computer-assisted spectrophotometer (Pharmacia, Piscataway, NY).

Fluorometric assay preparation

Employing the methodology described by Ueberschaer et al. (1992), a Turner® BioSystems TD-700 Laboratory Fluorometer was used in conjunction with a Molecular probes' EnzChek™ Protease Assay Kit to provide direct fluorescence-based assays for detecting protease activity from pooled homogenates of tilapia.

Solutions were prepared according to manufacturer's directions. This entailed preparing a 1.0 mg/mL stock solution of BODIPY FL casein (kit component A) by adding 0.2 mL of PBS (Phosphate buffer Saline) directly to one of the vials containing the lyophilized substrate. The resulting solution was then mixed via repeated inversions to fully dissolve the substrate. This was subsequently incubated for 10 min at room temperature. This constituted the stock solution.

An adequate volume of 1X digestion buffer (kit component B) was prepared as per manufacrurer's directions by using deionized water (because this volume varied from one group analysis to another, the precise volume was determined as per dictates of samples to be analyzed in a given group). The protease assay solution, having a casein concentration of 10 ug/mL, was prepared by adding 0.2 mL of the stock solution (see above) to 19.8 mL of the 1X digestion buffer.

Total Intestinal Proteases activity determination in *Oreochromis niloticus* (gray tilapia) and *Oreochromis hornorum* (red tilapia)

Total protease activity determination was made following the procedure standardized by Puerta-Martínez (2007). 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 and 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, respectively. Values were recorded and plotted, and the enzymatic activity was expressed in µmol/minute using the 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):

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

Where: U = Units of Enzymatic Activity/mL

C_E = Enzyme Concentration

Determining the amount of protease activity attributable to the enzyme trypsin

A special assay was developed to determine the amount of protease activity specifically attributable to the enzyme trypsin. Trypsin contained in intestinal homogenate was totally and specifically inhibited through the addition of 10 μg soybean trypsin inhibitor, SBTI (Sigma Aldrich), 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 manufacturer's directions. Observed values were recorded, plotted, and compared with those values obtained in absence of the SBTI.

Partial Purification of *Oreochromis niloticus* and *Oreochromis hornorum* trypsin

Trypsin was partially purified using the ammonium sulfate precipitation procedure as described by Deutscher (1990) in conjunction with size exclusion chromatography. Supernatant of red and gray tilapia produced through crude homogenate centrifugation was brought to 25% saturation with ammonium sulfate and subsequently centrifuged at 20,000 rpm for 30 min at 4 ^{o}C. The resulting supernatants were pooled and stored at 4 ^{o}C. Each pellet was resuspended in 2 ml of deionized water. Resuspended pellet solutions were then pooled. 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, and 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 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°C for further analysis.

Kinetic Characterization of the partially purified *Oreochromis niloticus* and *Oreochromis hornorum* 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 were identical to those already described for the standard trypsin assay. Cuvette reaction temperatures were obtained through use of either a hot bath or via 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 tilapia trypsin activity was examined at different inhibitor concentrations (100, 200, 300, 400, 500, 600 and 700 ng) in the otherwise standard fluorometric assay buffer (Hummel, 1959). All other aspects were identical as described for our standard assay conditions.

Molecular weight determination was performed through SDS PAGE gel electrophoretic procedures. SDS PAGE electrophoresis was performed under non-denaturing conditions in a 12% acrylamide gel. BioRad Blue Precision Plus protein

standards (BioRad, Hercules, CA) and low-range marker proteins (Roche Diagnostics) were used for electrophoretic determination of molecular weight. The SDS gel was run at 120 volts for 2 hour. Proteins, including molecular weight standards, purified porcine pancreatic trypsin (Sigma, St. Louis, MO), as well as partially purified tilapia trypsin, fractions were identified in gel lanes through visualization with coomassie blue staining procedure (BioRad, Hercules, CA).

Effect of protease inhibitors present in processed soybean meal homogenate upon protease activity from intestinal homogenate from *Oreochromis niloticus* and *Oreochromis hornorum* (inhibition *in vitro*)

A stock of 100 mg/ml solution of processed soybean was prepared with distilled water and manual homogenization using a standard laboratory mortar and pestel. The resulting solution was then centrifuged at 1500G for 10 min (rotor Eppendorf 5415). The inhibitory effect of soybean homogenate on digestive proteases of tilapia was determined by measuring the reduction of proteases activity assay as described by García-Carreño et al. (1997). Each red or gray tilapia intestinal homogenate was treated as per this methodology. The effect of pre-incubating extracts (60 min at room temperature) with different soybean solution concentrations (specifically, 10, 20, 30, 40, 50, 100, 200, 250, 500, 750, and 1000 μg) was determined through use of a Biosystems TD-700 Laboratory Fluorometer. In all such assays, an equivalent volume of water was added to the tube that was used as a100% of activity.

The pre-incubated samples were tested for protease activity using the fluorometric assay under standard conditions as described by Puerta-Martínez (2007). Briefly, 98 µl of digestion buffer were added to a mini borosilicate glass cuvette containing 100 µl of fluorogenic substrate solution and 2 µl of pre-incubated extract. The resulting solution was fluorometrically read at 485 +/- 12.5 nm and 530 +/- 15 nm, respectively. Results were used to determine total protease activity (pre-incubation with water was taken as 100% of activity) via the Beer-Lambert law equation and plotted as per percentage of inhibition.

Zymograms of enzymatic extracts

The SDS-PAGE was made following the protocol described by Laemmli (1970) for a 10% polyacrylamide gel. Intestinal homogenate from gray or red tilapia was pre-incubated (60 min at room temperature) containing one of seven different processed soybean solutions (i.e., 25, 50, 75, 130, 200, 300, and 500 ug/Unit of activity). The zymograms produced to determine protease activities were made according to the methods described by García-Carreño et al (1993). The SDS-PAGE was run at 100 Volts for 1 hour at 4 ºC. The gel was subsequently washed and incubated in a 0.5% pH 9.0 casein solution for 30 min in a refrigerator set at 5 ºC. It was then transferred to the bench top in the same solution and incubated at 25 ºC for 90 min and without agitation. After this incubation time the gels were washed and fixed with 12% TCA, stained with coomassie blue (BBC R-

250)-methanol-acetic acid solution (50:20:50), and then destained with methanol-acetic acid:water solution (35:10:55).

Statistical Analysis of Results

The results obtained for total gut protease activity as a function of variable raw soybean content in fines was analyzed with an Infostat Software Program (InfoStat versión 2007).

RESULTS

Influence of the levels of soybean on the tilapia weight

The average biomass of each group of tilapia from each feeding regimen was measured at both the beginning and end of study. The percentage of biomass increase was determined (Table 3) for each group. No clear linear relationship between amount of raw soybean content and biomass was detected for gray tilapia (Table 3A). A potential relationship between increased raw soybean protein and increased biomass was revealed for red tilapia (Table 3B)

Table 3: Average percentage of biomass change as a function of increasing raw soybean content in gray tilapia (A) and red tilapia (B) feed.

(A)

Dietary Protein (% Raw Soybean)	Gray Tilapia Feeding				
	Before		After		
	T	A	T	A	I
0	2.41	0.301	2.40 (n=7)	0.342	14
25	2.40	0.300	2.20 (n=7)	0.314	5
50	2.55	0.318	2.85 (n=8)	0.356	12
75	2.45	0.306	2.60 (n=8)	0.325	6
100	2.40	0.300	2.65 (n=8)	0.331	10

(B)

Dietary Protein (% Raw Soybean)	Red Tilapia Feeding				
	Before		After		
	T	A	T	A	I
0	2.65	0.331	2.00 (n=5)	0.400	21
25	2.75	0.343	2.90 (n=7)	0.414	21
50	2.90	0.362	3.75 (n=8)	0.468	29
75	2.45	0.306	2.90 (n=7)	0.414	35
100	2.95	0.368	3.25 (n=7)	0.464	26

T= Total weight (kg)
A= Average weight (kg)
I= Percentage of Biomass Increase (kg): (weight final - weight original /weight original)

Total intestinal protease activity in tilapia as a function of variable raw soybean content in feed

Table 4 illustrates the effect of increasing levels of raw soybean content, in fines fed to gray and red tilapia, upon total gut protease activity. ANOVA analysis revealed significant differences ($p < 0.05$) (Appendix 1) between level of dietary raw soybean feed content and protease activity with respect to species, with gray tilapia consistently attaining higher levels of protease activity than red tilapia for all raw soybean levels examined. Within each tilapia species there were significant differences between protease activity and amount of raw soybean content in fines. However, this relationship was not linear for either species (Fig. 3). Lowest protease activity for red tilapia was obtained at 0% raw soybean content while that for gray tilapia was obtained at 75%. Highest protease activity for red tilapia was obtained at 75% raw soybean content while that for gray tilapia was obtained at 100%.

Table 4: Total intestinal protease activity obtained for gray and re tilapia as a function of increasing raw soybean content in fines

Dietary Protein (% Raw Soybean)	Species of Tilapia	Total Protease Activity (µmol/ml/min)	Specific Enzymatic Activity (U/mg)
0	RT	0.109	20.123
	GT	0.152	28.396
25	RT	0.118	20.877
	GT	0.172	31.464
50	RT	0.131	22.410
	GT	0.155	28.832
75	RT	0.144	23.688
	GT	0.142	26.060
100	RT	0.115	20.149
	GT	0.197	35.994

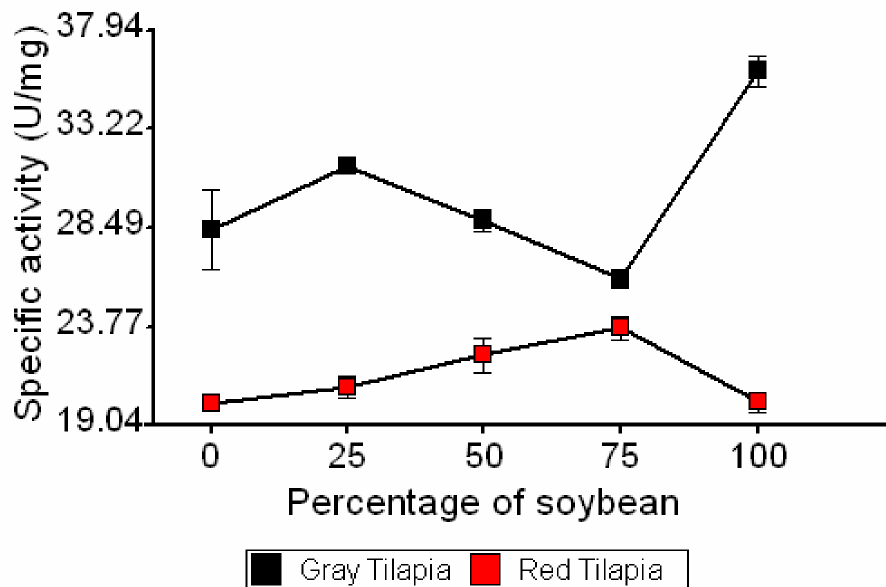

Figure 3: Average of specific activity (U/mg) for gray tilapia (black square) and red tilapia (red square) to as a function of increasing raw soybean content in fines (bars represent SDs).

Effect of processed soybean homogenate upon red and gray tilapia intestinal protease activity (*in vitro* assay)

Figure 4 shows the effect of processed soybean homogenate upon red and gray tilapia gut protease activity. Both species of tilapia exhibited lower protease activity as a function of increasing levels. However, whereas 200 μg and higher levels of processed soybean homogenate abolished > 95% of gut protease activity in gray tilapia, these levels only inhibited approximately 50% of activity in red tilapia. These results suggest that the processed soybean homogenate is somehow inhibiting tilapia gut protease activities.

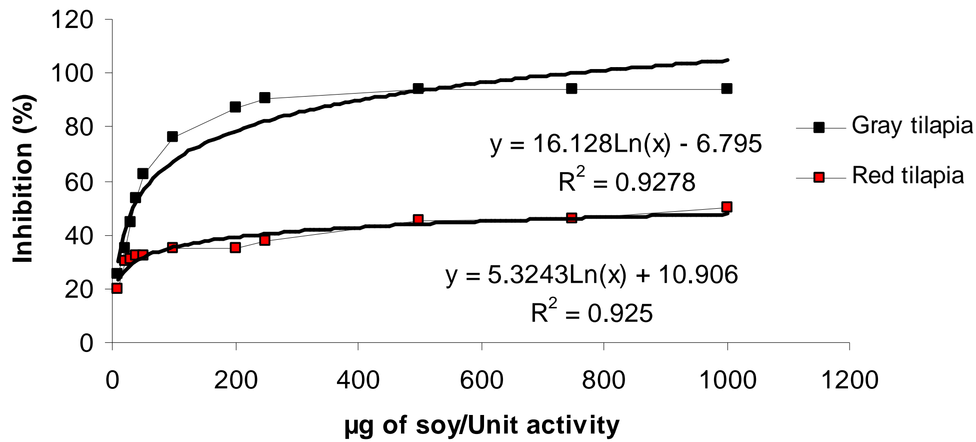

Figure 4: Effect of increasing levels of processed soybean homogenate upon gut protease activities obtained from gray and red tilapia

The results of SDS-PAGE zymograms (Figures 5 and 6) indicate that increased levels of soybean content correlate with decreased levels of protease activity. The result is apparent as a decrease in band intensity (from left to right), this result is more apparent in gray tilapia (Fig. 5) than in red tilapia (Fig. 6). It is notable that the number of zymogram bands was greater in red tilapia than gray tilapia.

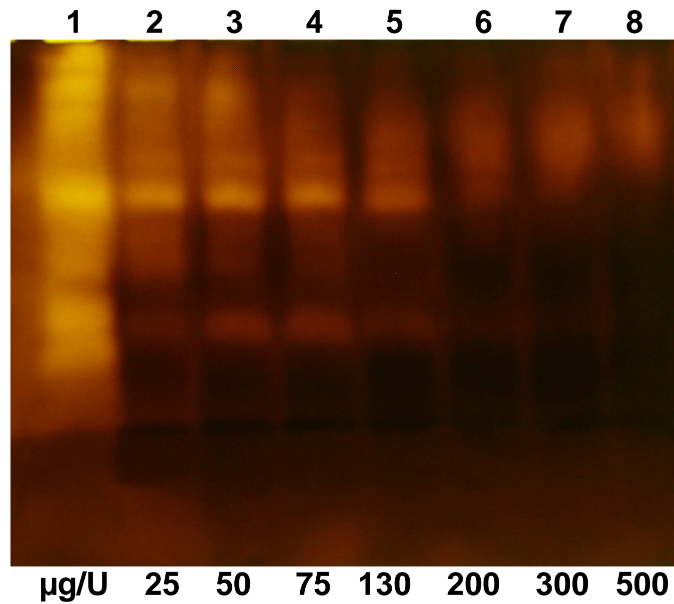

Figure 5: SDS-PAGE zymogram of gray tilapia digestive proteases visualized after 1 hour of incubation of extracts with increasing processed soybean concentrations (µg of soybean /Units of activity). Line 1: control without inhibition.

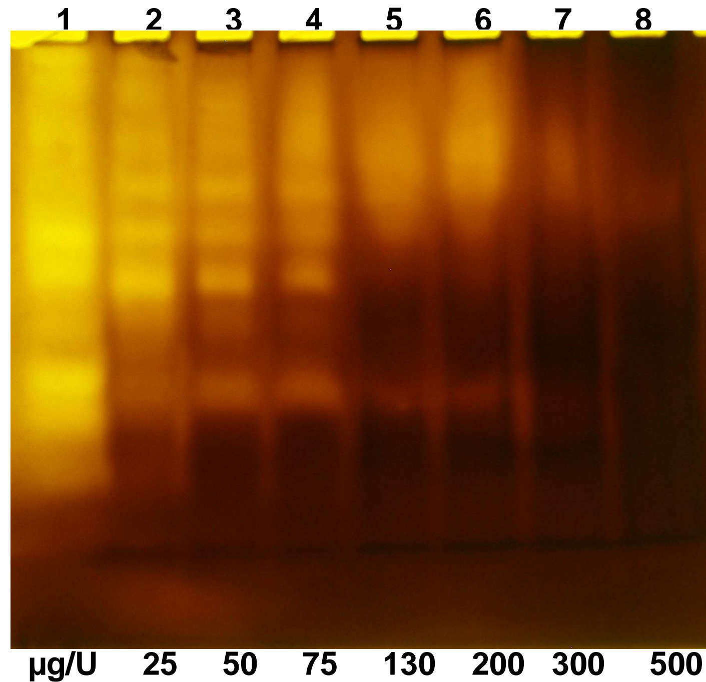

Figure 6: SDS-PAGE zymogram of red tilapia digestive proteases visualized after 1 hour incubation of extracts with increasing processed soybean concentrations (µg of soybean /Units of activity). Line 1: control without inhibition.

Proportion of total protease activity attributable to trypsin

The assay to determine the amount of intestinal protease activity directly attributable to trypsin (Table 5) revealed that 82-86 % of this activity resulted from trypsin.

Table 5: Percentage of total protease activity attributable to trypsin

Dietary Protein (% Raw Soybean)	Tilapia Species	Specific Enzymatic Activity before trypsin inhibition (U/mg)	Specific Enzymatic Activity after trypsin inhibition (U/mg)	Percent of inhibition
0	RT	20.123	3.421	83
	GT	28.396	4.543	84
25	RT	20.877	3.340	84
	GT	31.464	5.664	82
50	RT	22.41	3.137	86
	GT	28.832	4.613	84
75	RT	23.688	3.316	86
	GT	26.06	3.909	85
100	RT	20.149	2.821	86
	GT	35.994	6.479	82

Partial purification of *Oreochromis niloticus* and *Oreochromis hornorum* trypsin

Table 6 summarizes the levels of trypsin activity obtained at the five different steps in the partial purification procedure. As the 50% ammonium sulfate fraction displayed highest activity for both gray and red tilapia, this fraction was selected for further purification via size exclusion chromatography. The Hummel (1959) trypsin-specific assay revealed that highest gray tilapia activity was obtained in collection tube # 55 (i.e.,

163 - 165 ml fraction) (Fig. 7) and highest red tilapia activity was obtained in tube # 44 (i.e., 130 – 132 ml fraction) (Fig. 8). Both of these fractions produced a band that was in the 21 – 24 kDa range (Fig. 9).

Table 6: Partial purification of trypsin from gray and red tilapia intestinal tissues

Samples	Species of Tilapia	Protein concentration (mg/mL)	Total Enzyme Activity (µmol/ml/min)	Specific activity of Trypsin (U/mg of P)
Crude Homogenate	RT GT	7.409 10.584	0.105 0.132	0.366 0.213
25% Resuspended Pellet	RT GT	2.79 2.40	0.034 0.056	0.243 0.471
50% Resuspended Pellet	RT GT	6.88 8.48	0.784 0.739	4.560 3.488
75% Resuspended Pellet	RT GT	3.73 5.71	0.034 0.147	0.183 0.514
Size exclusion chromathography of 50% resuspended pellet	RT fraction 44 GT fraction 55	0.077 0.133	0.063 0.069	8.178 5.172

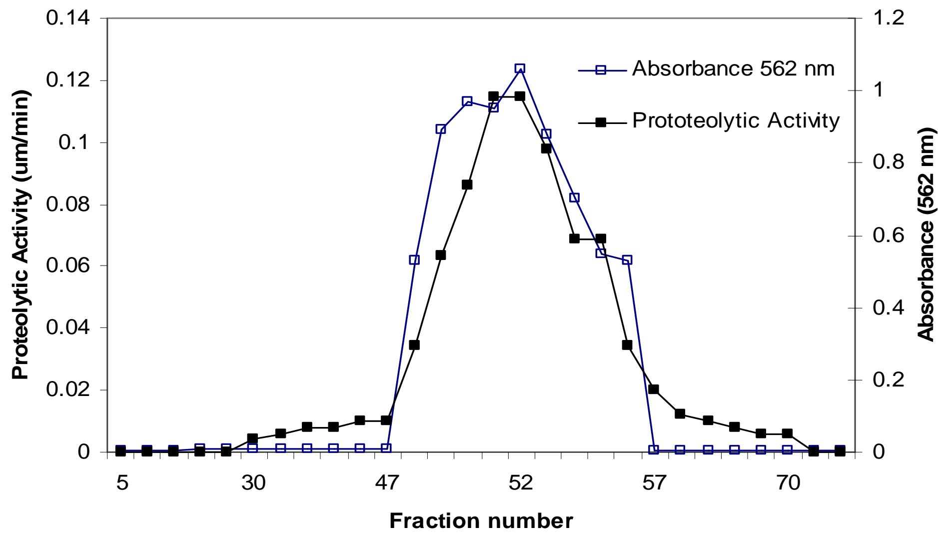

Figure 7: Elution profile of the ammonium sulphate fraction (50%) subjected to size exclusion chromatography from gray tilapia. Trypsin activity was determined via Hummel (1959) assay for each fraction collected.

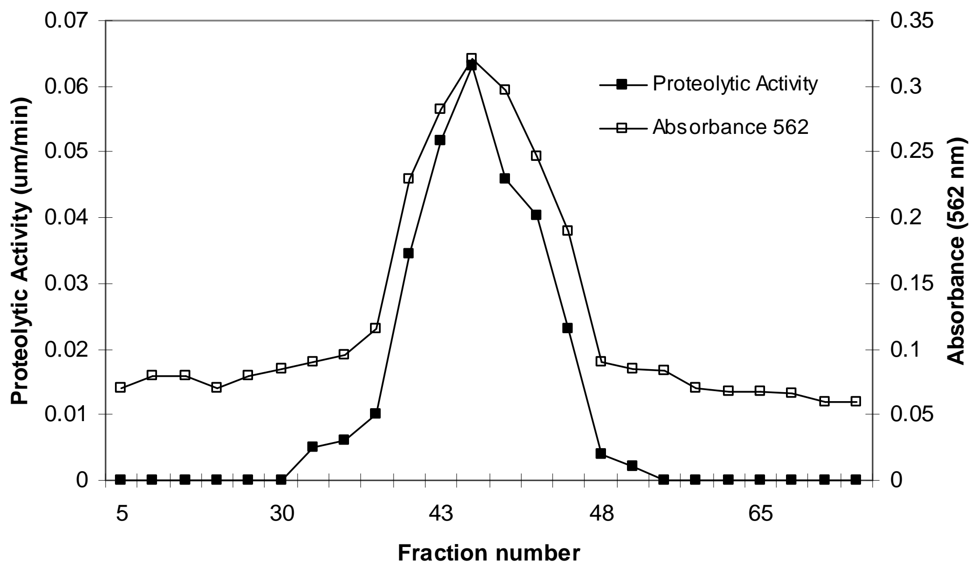

Figure 8: Elution profile of the ammonium sulphate fraction (50%) subjected to size exclusion chromatography from red tilapia. Trypsin activity was determined via Hummel (1959) assay for each fraction collected.

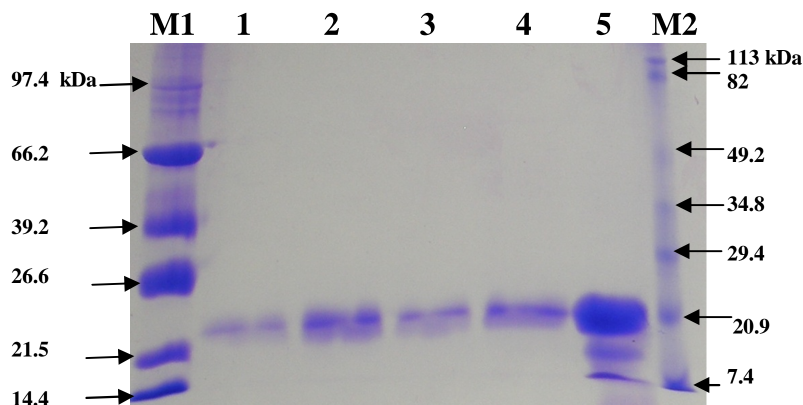

Figure 9: SDS polyacrylamide gel electrophoresis of partially purified trypsin from red and gray tilapia: protein size standards (Lines M1 and M2); gray tilapia size exclusion chromatographic fractions 54– 55 (lanes 1 and 2); red tilapia size exclusion chromatographic fractions 43–44 (lanes 3 and 4); purified pancreactic porcine trypsin (5)

In vitro Kinetic analysis of trypsin activity

Analysis of temperature effect upon gray and red tilapia trypsin activity revealed that both enzymes displayed highest activity at 50 ^{o}C (Fig.10). Activity fell off completely at 80 ^{o}C while activity levels decreased by approximately 40% at 10 ^{o}C. Examination of pH effect indicated that highest trypsin activity from gray and red tilapia was obtained at a pH 8 (Fig.11). The results of inhibition analysis (Fig. 12) indicate a more or less linear decrease in trypsin activity from 0 to 700 ng SBTI/3 ml total assay volume as SBTI concentrations increased at intervals of 100 ng/3 ml.

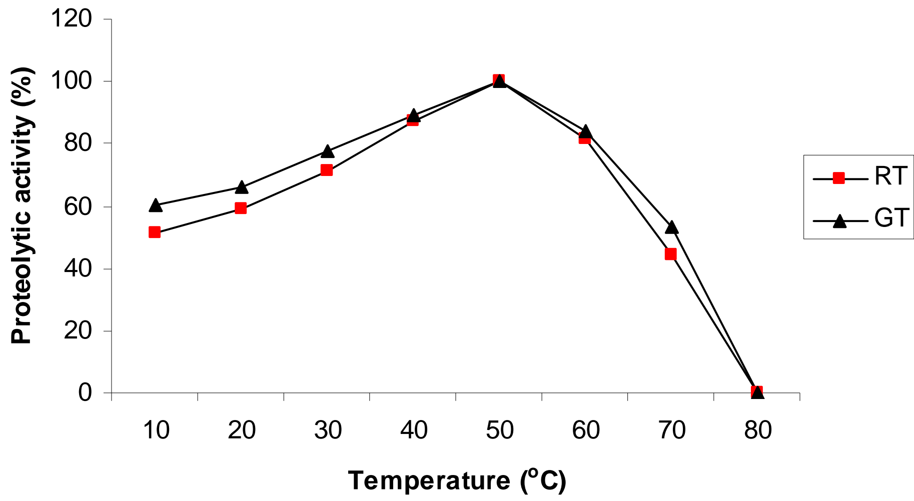

Figure 10: The effect of temperature upon red and gray tilapia trypsin activities.

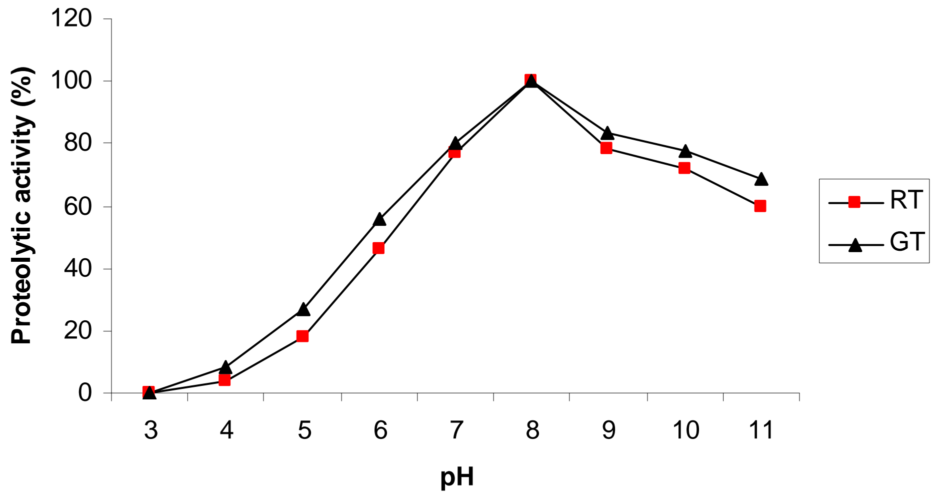

Figure 11: Effect of pH on gray and red tilapia intestinal trypsin activities.

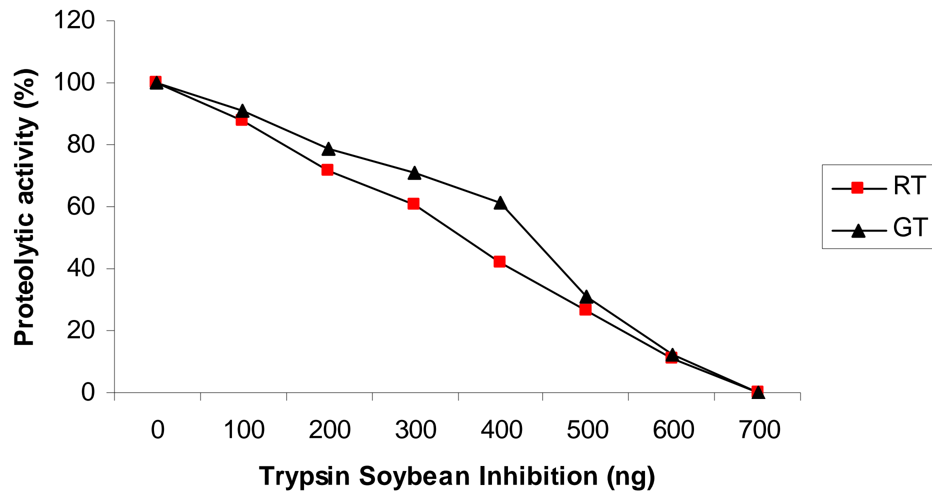

Figure 12: SBTI effect upon gray and red tilapia intestinal trypsin activities.

DISCUSSION

While raw soybeans have been used as an excellent protein source in a number of different feed preparations in aquaculture (El-Sayed, 1999), this plant product carries with it a natural trypsin inhibitor that can potentially block the chemical digestive capacity of an organism and hence negatively impact its ability to absorb amino acids from ingested proteins (Liener, 1994). Of course, this can result in decreased biomass production of the animal being fed, clearly a detriment to the farming/aquaculture industry. It is of interest, therefore, to assess just how crude soybean meal and its naturally occurring inhibitors affect both cultured animal biomass production and, at the biochemical level, the activities from proteases of these organisms.

Tilapia, a commercially raised, freshwater food fish, has steadily gained in worldwide popularity during the past twenty years (Naylor, 2000). Aquaculturists routinely rear these fish in commercial ponds. The feeds used often include significant levels of protein from soybeans. While some researchers contend that the effect of soybean trypsin inhibitor, SBTI, upon trypsin is abolished through treatment with heat, others have asserted that such treatment has little or no effect on the inhibitor (El-Sayed et al., 2000).

In order to determine the effect of raw soybeans upon both tilapia biomass production and protease activity, this study examined both the growth and protease activity

of five different raw soybean-fed groups each of two tilapia species maintained in commercial-grade ponds in the Lajas, Puerto Rico Aquaculture Research Facility of the University of Puerto Rico. It was hoped that this study might shed light upon the utility of raw soybean meal as a protein source in commercial fish feeds.

The results of this study indicate that raw soybean meal, as an additive to fish fines, does not negatively impact biomass production in either gray or red tilapia (Table 3). This finding is especially apparent in the results obtained for gray tilapia. In the five different raw soybean amounts (i.e., 0, 25, 50, 75, and 100% of total protein feed content) used for preparing fish fines, the biomass production obtained at the end of the feeding period appears not to depend upon the presence or absence of raw soybean. While it is true that greatest biomass production (i.e., 14% above starting biomass) of gray tilapia occurred when 0% soybean meal was used in feed, that obtained for 50% and 100% raw soybean content was 12% and 10% respectively. These values are very near to the 14% value obtained for the 0% crude soybean content feed gray tilapia group.

Curiously, in red tilapia the percentage of biomass increase is lowest (i.e., 21%) in the groups fed 0 or 25% crude soybeans while it is highest for the groups fed 50 and 75% crude soybeans (29 and 35%, respectively). This is the exact opposite of what one would predict if one assumes that the SBTI in raw (i.e., untreated) soybeans will negatively impact trypsin and thereby lower levels of ingested amino acid assimilation and thus biomass production. Considered as a whole, the obtained data indicate that 1) gray tilapia

are unaffected by their feed containing either processed or raw soybean and 2) red tilapia actually increase their biomass production when fed a diet containing 75% raw soy protein.

These findings beg the question as to what precisely is occurring at the biochemical level in the intestine when raw soybeans in feeds are ingested by tilapia. More specifically, what is happening to protease activity as a result of this unprocessed soybean component, presumably laden with SBTI, in the tilapia intestine? The next round of experiments addressed this question by examining the effect of different raw soybean quantities in fines upon total protease activity obtained from gray or red tilapia intestinal homogenate.

The results of Table 4 indicate that while within red tilapia or within gray tilapia there is little difference, if any, among total protease activity of groups fed any of the five different raw soybean content fine preparations, there is a decided difference between the amount of total protease activity obtained for gray tilapia versus red tilapia. Indeed, the Tukey alpha test indicates that this difference is significant ($p < 0.05$) (Appendix 1). The conclusion that can be drawn from these results is that gray tilapia exhibit significantly higher overall protease activity than do red tilapia and that this activity is independent of the soybean content of the feed being either raw or processed.

To further analyze the effect of ingested soybeans upon gray or red tilapia protease activities, an *in vitro* experiment was undertaken in which homogenized, processed

soybean was incubated with gray or red tilapia intestinal homogenate to determine the effect upon total protease activity. While both gray and red tilapia intestinal protease activity was lowered by the presence of 200 μg or greater of processed soybean homogenate, that of red tilapia was only lowered approximately 50% while that of gray tilapia was lowered > 95%. This would suggest that the gray tilapia intestinal proteases are more susceptible to inhibition by some aspect of the processed soybean homogenate than are the red tilapia proteases. However, the fact that this inhibition occurs at all is expected in view of reports of similar studies on other fish species, including seabream, *Sparus aurata*, and African sole, *Solea senegalensis* (Moyano-Lopéz et al., 1999). Interestingly, however, the protein source in the fish meal preparation for these studies was wheat and soybean. Thus, there are effective protease inhibitors in other plant-derived foods and soybeans cannot thus be singled out for their potential negative impact upon gut protease activities.

Results obtained through zymogram analysis indicate that increased levels of processed soybean inhibit tilapia intestinal protease activities in a dose-dependent manner, with greater concentration of processed soybean resulting in greater protease inhibition. It is notable that gray tilapia protease activity was more sensitive to this treatment than was that from red tilapia (note the greater light band areas in red tilapia (Fig. 6) than gray tilapia (Fig. 5)). However, red tilapia displayed a greater number of bands than red tilapia. This finding suggests that red tilapia may secrete more active proteases into their gut than

gray tilapia. Such increased protease levels would presumably result in more rapid breakdown of ingested proteins.

With respect to natural inhibition of proteases as revealed through zymogram analyses, Moyano-López et al. (1999) obtained a similar result for seabream, tilapia, *Oreochromis niloticus*, and African sole. Results were attributed to natural protease inhibitors present in vegetable feed stuffs. It is therefore very likely that such inhibitors are also affecting protease activity in the two species of tilapia examined in this study.

Having examined the effect of soybean presence in feeds at both the whole organisms (i.e., biomass) and biochemical (i.e., total protease) levels, this study sought to gain some further insight into the contribution of trypsin to total protease activity. Indeed, trypsin is the protease that confers the greatest amount of protein degradation upon ingested proteins in fishes (Gawlicka and Horn, 2006; Puerta-Martínez, 2007). It is thus of interest to better characterize this enzyme with respect to fish digestive enzymology.

Table 5 illustrates the contribution of trypsin to total protease activity in both gray and red tilapia fed the same five different amounts of raw soybean. In all cases, trypsin constituted 82 – 86% of total protease activity because this was the amount of protease activity lost when the trypsin-specific inhibitor SBTI was added to the assay mixture. Considering this finding in conjunction with the zymogram and processed soybean homogenate inhibition studies discussed above, it appears that soybeans do indeed contain

a very effective trypsin-specific inhibitor(s). This would further imply that such inhibitors may adversely affect the capacity of fishes to chemically breakdown and assimilate amino acids of ingested proteins. However, while this conclusion is supported by all of this study's *in vitro* analyses, the initial fish feeding analysis (see Table 3) indicates that biomass production in gray and red tilapia is unaffected by the presence of raw soybeans versus processed soybeans. Thus, while one might conclude, based upon the *in vitro* analysis, that the natural soybean inhibitors, present in raw and perhaps also processed soybeans, would actually impair tilapia biomass production, this conclusion is not supported by the results obtained in the fish feeding component of this study (see Table 3). There are several possible explanations for this seeming contradiction.

First, it may be that the sample size in this study was insufficient to obtain reliable biomass production data. Thus, it might be found, through more extensive analysis, that biomass production is, in fact, impaired through some level of raw and/or processed soybean content in fines. Alternatively, it is possible that tilapia have some as yet unidentified mechanism for, in effect, neutralizing the naturally-occurring protease inhibitors.

With a view towards future research upon tilapia proteases and trypsin in particular, this study also undertook a partial purification of trypsin from both gray and red tilapia. The findings, including partial purification procedure, highest catalytic rate at 50 ^{o}C, a pH optimum of 8.0, and a molecular weight of approximately 23 kDa trypsin from both tilapia

species, are in accordance with what has been found for other fish species and, indeed, other vertebrates in general (Kolodziejska and Sikorski, 1996; El-Shemy and Levin, 1997; Bezerra et al., 2005; Rivera-Santos, 2003; Puerta-Martínez, 2007). These findings are presented in Figures 7, 8, Fig. 9, 10, 11, and 12.

In conclusion, this study has demonstrated the following concerning gray and red tilapia: 1) these fishes can increase their biomass by at least 20% over a 5 week period when fed a diet whose protein component is derived from soybeans, raw or processed; 2) protease activities from the intestines of fishes fed upon different levels of raw soybean-containing feed can be detected *in vitro*; 3) this *in vitro*-detected protease activity can be inhibited by the addition of soybean homogenate; 4) 82- 86% of protease activity is attributable to trypsin; 5) trypsin can be at least partially purified from intestinal tissues; and 6) the trypsin is similar to other vertebrate trypsins in terms of its kinetic parameters and molecular weight.

RECOMMENDATIONS

- Future *in vivo* analysis should examine the effect of raw soybean as a feed component upon larval tilapia stages and for a longer period of time. Indeed, larval fish, as opposed to adults, are passing through critical life developmental stages. Therefore, the effect of raw soybean consumption, if any, might be more readily detected by using larval tilapia. A more extended time period (e.g., 12 weeks rather than the 5 weeks used in this study) might provide the time needed to better evaluate the effects of raw soybean upon cultured fish.

- In order to better understand the relationship between the various *in vitro* assays and their implications, if any, with regard to the *in vivo* analyses, these assays should be performed at specific life stages, larval or otherwise, of the developing tilapia.

- Because different protease isozymes may display different levels of sensitivity to inhibitors and other factors related to digestive biochemistry, a more complete analysis of the total protease-activity-conferring enzymes (e.g., different forms of trypsin, chymotrypsin, and/or elastases) will be essential to obtain a deeper understanding of the enzymatic mechanisms underlying dietary protein assimilation in tilapia. Such a line of investigation may require the use of molecular techniques.

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APPENDIX I. ANOVA for specific protease activity as a function of variable raw soybean content in fines

Analysis of Variance

Variable	N	R^2	R^2 Adj	CV
Specific activity	30	0.95	0.93	5.35

Analysis of Variance table (Partial SS)

S.V.	S S	df	MS	F	p-value
Model	769.08	9	85.45	44.81	<0.0001
Percentage of soybean	51.36	4	12.84	6.73	0.0013
Specie	567.68	1	567.68	297.68	<0.0001
Percentage of soy*Species	150.05	4	37.51	19.67	<0.0001
Error	38.14	20	1.91		
Total	807.22	29			

Test:Tukey Alfa:=0.05 DMS:=2.38586

Error: 1.9070 gl: 20

Percentage of soybean	Means	n		
100.00	28.07	6	A	
25.00	26.17	6	A	B
50.00	25.62	6		B
75.00	24.87	6		B
0.00	24.26	6		B

Different letters indicate significant differences *(p<= 0.05)*

Test:Tukey Alfa:=0.05 DMS:=1.05184

Error: 1.9070 gl: 20

Specie	Means	n		
GT	30.15	15	A	
RT	21.45	15		B

Different letters indicate significant differences *(p<= 0.05)*

Test:Tukey Alfa:=0.05 DMS:=3.99281

Error: 1.9070 gl: 20

Percent of soy	Specie	Means	n					
100.00	GT	35.99	3	A				
25.00	GT	31.46	3		B			
50.00	GT	28.83	3		B	C		
0.00	GT	28.40	3		B	C		
75.00	GT	26.06	3			C	D	
75.00	RT	23.69	3				D	E
50.00	RT	22.41	3				D	E
25.00	RT	20.88	3					E
100.00	RT	20.15	3					E
0.00	RT	20.12	3					E

Different letters indicate significant differences *(p<= 0.05)*