PURIFICATION AND CHARACTERIZATION OF TRYPSIN FROM INTESTINAL AND PYLORIC CAECAL TISSUES OF THE SILK SNAPPER, *LUTJANUS VIVANUS (CUVIER, 1828)*

by

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ABSTRACT

In an effort to better understand the chemical digestion of proteins in the silk snapper, *Lutjanus vivanus* (Cuvier, 1828), and thereby obtain a deeper understanding of how this fish meets its dietary needs for amino acids through its trophic environment, trypsin was purified 10-fold from intestinal and pyloric caecal tissues by sequential application of ammonium sulfate fractionation, size exclusion chromatography, and affinity chromatography. Standard kinetic characteristics of this enzyme were determined. The enzyme displayed optimal activity at pH 8 and 60^{o}C. It was completely or nearly completely inhibited by soybean trypsin inhibitor (SBTI) at a concentration of 0.1% or higher. SDS-PAGE analysis of the purified enzyme revealed a single band with an estimated molecular weight of 26.1 kDa.

RESUMEN

Tripsina proveniente del tejido intestinal y ciego pilórico del Chillo de ojo amarillo, *Lutjanus vivanus* (Cuvier, 1828) fue purificada y caracterizada. Dicho estudio se realizó en el esfuerzo por un mejor entendimiento sobre la digestión química de las proteínas y cómo este pez suple su necesidad de aminoácidos a través de hábitat donde se alimenta. La enzima fue purificada 10 veces mediante proceso de precipitación con sulfato de amonio, seguido por cromatografía de exclusión por tamaño y cromatografía de afinidad. Características sobre la cinética de tripsina, incluyendo el efecto de la temperatura, pH e inhibidor sobre la hidrólisis del sustrato artificial TAME, fueron determinadas. La enzima presentó actividad óptima a pH 8.0 y a 60^{o}C. Tripsina fue parcialmente o completamente inhibida, por el inhibidor de soya de tripsina (SBTI por sus siglas en ingles) a concentración de 0.1% o más. En el análisis de la electroforesis SDS-PAGE de la proteína purificada, se pudo observar una banda sencilla con un peso molecular estimado de 26.1 kDa.

DEDICATION

To my mother and father, Marylyn Santos Onoda and José M. Rivera, for their unconditional love, patience and support. To my baby and my grandmother, Kiyomi Alexandra and Kiyomi Onoda Ochi, for giving me the inspiration and strength to finish what I started.

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INTRODUCTION

Digestion is the process whereby an organism utilizes chemical and mechanical processes to convert food molecules into forms that are nutritionally useful to that organism. In the case of animals possessing an alimentary canal, numerous extracellular enzymes are secreted into the lumen of this structure so that ingested food items may be transformed into forms that can be absorbed through the epithelium (Randall et al., 1997). Among these digestive enzymes is a class known as the proteases. These enzymes attack peptide bonds of proteins and polypeptides, thereby converting large peptide chains into shorter polypeptide segments. There are two basic types of proteases, the endopeptidases and the exopeptidases. In the case of the former, the enzyme attacks bonds located deep within the substrate protein, thus transforming large peptide chains into shorter peptide segments. Exopeptidases function by converting these shorter segments, located near the end of a peptide chain, into free amino acids, dipeptides, and tripeptides.

Trypsin, chymotrypsin, and elastase are endopeptidases occurring in the alimentary canal of perhaps virtually all invertebrates and vertebrates organisms possessing this digestive structure (Solomon et al., 1996). Trypsin is specific for digestion of peptides and esters of the amino acids lysine and arginine (Ferscht, 1985). Although elastase and chymotrypsin are very similar to trypsin in their tertiary structure (the polypeptide backbones of these three enzymes may be superimposed upon one another), chymotrypsin is specific for digestion of the hydrophobic amino acid side chains of phenylalanine, tyrosine, and tryptophan while elastase is specific for small hydrophobic side chains such as alanine. Collectively, these three endopeptidases play

essential roles in the process that converts ingested proteins into individual amino acids, which may then be absorbed across the gut epithelium.

Trypsin, chymotrypsin, and elastase are kinetically very similar to one another. Indeed, all three catalyze the hydrolysis of peptides and synthetic ester substrates. All display highest catalytic activity at a pH of approximately 7.8. In addition, the pK_a of each is around 6.8.

Aquatic habitats contain a wide variety of organisms and thus enormous potential for the discovery of different enzymes. Fish viscera are known to be a rich source of enzymes, including trypsin, many of which present high activity at low concentrations. Fish digestive enzymes exhibit optimal activity at temperatures much higher than the ambient temperature of fish (Fereidoon and Janak-Kamil, 2001).

With a view towards better understanding trypsin in the silk snapper, *Lutjanus vivanus* (Cuvier, 1828) (Figure 1 by Allen, 1996), and thereby providing a foundation for potential future studies examining the relationship of this enzyme with growth rates in this and perhaps other fish species, this study identifies standard kinetic (i.e., pH effect, temperature effect, inhibitor effect, time effect) and molecular weight characteristics of trypsin obtained from silk snapper intestinal and pyloric caecal tissues. A silk snapper's digestive tract, including the fish's stomach, intestine and pyloric caeca similar to those used as a source of trypsin in this study, is illustrated in figure 2.

The silk snapper is indigenous to the Gulf of Mexico, Bahamas, and the Caribbean (Figure 3 by Allen, 1996). This species inhabits mainly rocky bottoms between 120 and 180 meters. It has been reported to feed on fishes, shrimp, crabs, isopods, and other invertebrate organisms (Brownell and Rainey, 1971).

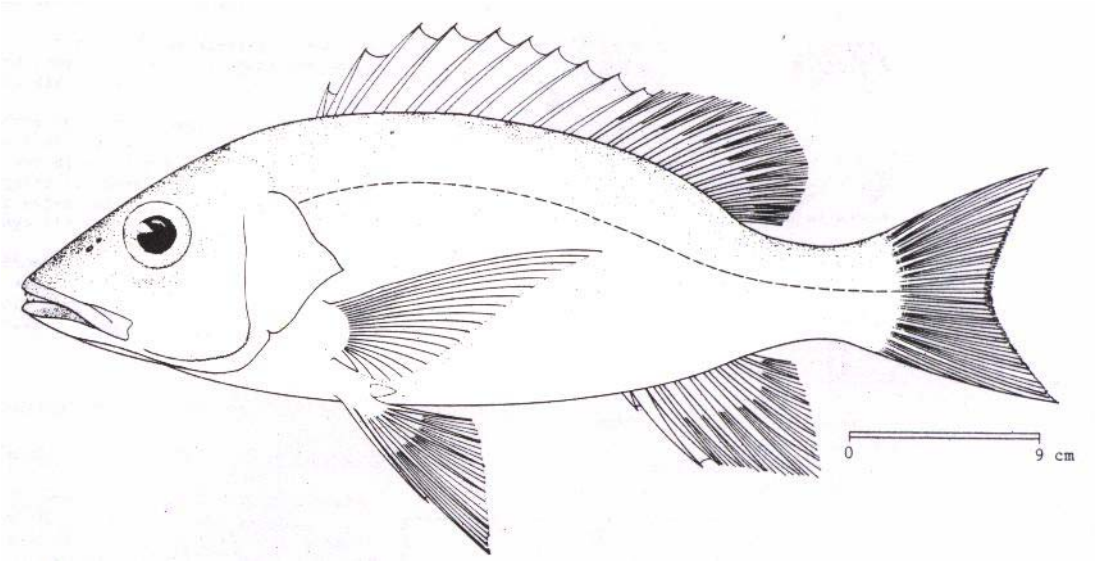

Figure 1. Silk snapper, *Lutjanus vivanus* (Cuvier, 1828) from Allen(1996).

Figure 2. Digestive tract of the silk snapper, *Lutjanus vivanus* (Cuvier, 1828).

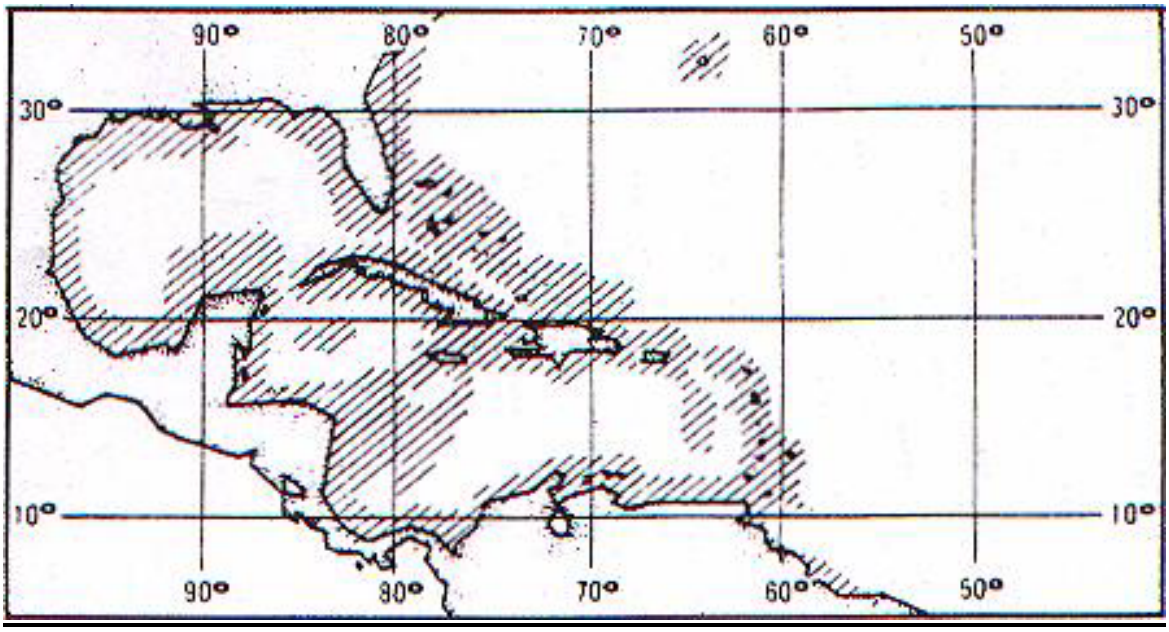

Figure 3. Geographical distribution (Allen, 1996) of the silk snapper, *Lutjanus vivanus* (Cuvier, 1828).

LITERATURE REVIEW

During the past four decades, both trypsin and trypsin-like enzymes from many fish species have been identified and characterized. Different fish digestive and digestion-related organs, including pancreas, intestine and pyloric caeca, are common sources of these proteases (Phillips, 1969).

According to Keil (1971), trypsin enzymes have a molecular weight ranging from 20,000 to 24,000 daltons. However, trypsin isolated from marine animals, including both invertebrates and vertebrates, generally have been found to have molecular weights somewhat outside of this range. It is also notable that these marine trypsins are anionic proteins at neutral pH (Martínez and Serra, 1989).

In 1960, Bradford, among the first to characterize a fish trypsin-like enzyme activity, identified proteolytic hydrolysis to be present in homogenates of pyloric caeca from the Chinook salmon, *Onchorhynchus tshawytscha* (Walbaum, 1792). 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^{o}C.

Prahl and Neurath (1966) described an anionic form of pancreatic trypsinogen, as well as other digestive enzymes, in the spiny dogfish, *Squalus acanthias,* Linnaeus, 1758. The dogfish enzyme was found to have a molecular weight of 24,000-25,000 daltons 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 annectens,* Heckel, 1851. One form was later purified and

characterized (Reeck and Neurath, 1972). It displayed a molecular weight of 24,000 daltons 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 that obtained from bovine and dogfish sources. Its properties, including pH optimum, 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* (Günther, 1877), the stone flounder, *Platichtys bicoloratus* (Basilewsky, 1855), and the olive flounder, *Paralichtys olivaceus* (Temminck and Schlegel, 1846). 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,* Linnaeus, 1758. Activities of the following were identified: trypsin, chymotrypsin, carboxypeptidase A and B, leucine aminopeptidase, ribonuclease, amylase, acid phosphatase, and alkaline phosphatase. The trypsin-like enzyme showed a molecular weight of 18,000 to 22,000 daltons. Its pH optimum ranged from 8.0 - 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 11,000 daltons and the other of 23,000 daltons. 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* (Bloch, 1782). 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°C - 50°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,* Linnaeus, 1758. The enzyme showed an approximate molecular weight of 25,000 daltons. The carp trypsin was found to be an anionic protein that is unstable at low pH.

Hjelmeland and Raa (1982) purified two trypsin-like enzymes from the gut of the arctic fish capelin, *Mallotus villosus* (Müller, 1776). Both enzymes had a molecular weight of about 28,000 daltons. The enzymes were inhibited by standard trypsin inhibitors and displayed a pH optimum of 8.0 – 9.0

In 1984, Simpson and Haard purified and characterized trypsin from the pyloric caeca of greenland cod, *Gadus ogac,* Richardson, 1836. 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 23,500 daltons. Characterization of the

enzyme included identifying its catalytic specificity for amide or ester bonds involving the carboxyl group of arginine, capacity to hydrolyze the trypsin-specific synthetic substrate αN-p-tosyl-L-arginine methyl ester (TAME), its sensitivity to serine protease inhibitors, and the lowering or cessation of enzyme activity when in the presence of soybean trypsin inhibitor.

Yoshinaka et al. (1984) isolated an anionic trypsin from the pancreas of the amur catfish, *Silurus asotus*, Linnaeus, 1758. This enzyme had a molecular weight of 26,000 daltons. 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* (Linnaeus, 1758). 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* (Burchell, 1822). 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* (Linnaeus, 1758), 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 27,000 and 28,000 daltons. Their isoelectric points were between 4.6 and 4.9. The enzymes displayed optimal activity in a pH range of 8 - 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*, Linnaeus 1758. 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 7.5-9.0. 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 24,000 daltons.

Trypsin from the pyloric caeca of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), was purified and characterized by Kristjansson (1991). The isolated enzyme had an estimated molecular weight of 25,700 daltons. The enzyme was stable at temperatures in the range 40^{o}C - 50^{o}C and at a pH range of 5.4 - 8.0. However, this thermal stability was shown to be calcium-dependent. Hydrolysis of substrate was maximal at approximately 60^{o}C.

Sabapathy and Teo (1993) conducted a comparative study of the rabbitfish, *Siganus canaliculatus* (Park, 1797), and sea bass, *Lates calcarifer* (Bloch, 1790), digestive tract and digestive enzyme distribution and activity. 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, in 1995, Sabapathy and Teo identified some properties of the rabbitfish's intestinal enzymes.

Comparison of trypsin and chymotrypsin from the viscera of anchovy, *Engraulis japonicus,* Temminck and Schlegel, 1846*,* was undertaken by Heu et al. (1995). The

molecular weight of the trypsin was estimated to be 25,600 daltons. Maximal activity was found at pH 9.0 and 45°C for casein, and at pH 8.0 and 45°C for TAME.

Four differently charged trypsins were purified from pyloric caeca of Atlantic salmon, *Salmo salar*, Linnaeus, 1758 (Outzen et al. 1996). The four isoforms of trypsin were differentiated as anionic trypsin I, II and III, and cationic trypsin. All were found to have a molecular weight of approximately 25,000 daltons. Cationic salmon trypsin displayed optimal activity at a pH range of 8.5 - 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 a marine species.

The proteolytic activities in the gut of three carnivorous fish species, the deepwater redfish, *Sebastes mentella* (Travin, 1951), the turbot, *Scophthalmus maximus* (Linnaeus, 1758), and the gilthead bream, *Sparus aurata,* Linnaeus, 1758*,* were compared by Munilla-Morán and Saborido-Rey (1996). Optimum stomach trypsin activity was detected at pH 2.0 while such activity in the intestinal forms of the enzyme showed optimal activity at a pH range of 9.5 - 10.0. The temperature range at which both enzymes displayed maximal activity was 35 °C - 40°C.

Diaz (1999) obtained a 50-fold level of purification for trypsin from pyloric caeca of the red hind grouper, *Epinephilis guttatus* (Linnaeus, 1758). 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°C. The enzyme's molecular weight was somewhere within the range of 17,000 to 44,000 daltons. This trypsin was inhibited by soybean trypsin inhibitor.

Quiñones (2000) obtained a 20-fold level of purification for trypsin from pyloric caeca and intestinal tissues of the queen snapper, *Etelis occulatus* (Valenciennes, 1828). 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 17,000 to 44,000 daltons and was inhibited by soybean trypsin inhibitor.

Sekizaki et al. (2000) purified an anionic trypsin from the pyloric caeca of chum salmon, *Onchorhynchus keta* (Wallbaum, 1792). 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 isoeletric point of 5.10. The effect of temperature on the hydrolysis o 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 a similar amino acid composition to that of bovine trypsin.

We can glean from this literature review that trypsin is a characteristic digestive enzyme of fishes and that it may be readily characterized from digestive fish tissues.

These trypsins are optimally active at pH ranges of 7.5 – 9.0. In addition, at its optimal pH each fish trypsin displayed greatest catalytic rate at temperatures of 45°C – 60°C.

Most of the published literature pertaining to the biology of the silk snapper, *Lutjanus vivanus* (Cuvier, 1828), has focused on this species' ecology and behavior (Thompson and Munro, 1983). While several post-1996 articles have examined the silk snapper's commercial importance, as yet there remains little in the way of investigations that have examined this fish's physiology and/or biochemistry. In order to better understand the digestive physiology/biochemistry of the silk snapper, this study identified, purified, and characterized trypsin from pyloric caeca and intestinal tissues from this species.

MATERIAL AND METHODS

Fish Organs

Twenty-five silk snapper were captured in La Parguera Bay, Lajas by commercial fishermen using hook and line gear. The fish were gutted at sea and the digestive organs (stomach, pyloric caeca and intestines) were placed in sealed plastic bags. These organs were stored for up to one week in a -20^{o}C laboratory freezer prior to use.

Chemicals

$(NH_4)_2SO_4$ (ammonium sulfate), NaCl, serum albumin (BSA), Trizma base 0.2M, calcium chloride ($CaCl_2$*$2H_2O$), Tris Hydrochloride, HCl, acetic acid, methanol, Nα-p-tosyl-L-arginine methyl ester (TAME), N,N,N',N'-tetramethylethylenediamine (TEMED), soybean trypsin inhibitor (SBTI), bromophenol blue, brilliant blue R, and Tris buffer were purchased from Sigma Chemical Company (St. Louis, MO). Bicinchoninic acid (BCA) reagents (reagent A- BCA-Na_2, 2% Na_2CO_3 * H_2O, Na_2 tartrate, 4% NaOH, and 0.95% NaHC O_3) (reagent B- CuS O_4 * 5 H_2O) were purchased as part of a protein quantification kit (Pierce Chemical Company, Rockford, IL). Gel filtration standards, P-60 polyacrylamide gel, glycine, Precision plus protein standards, precast gel for polyacrylamide electrophoresis, Trisbase /Glycine/ SDS running buffer and Laemilli sample buffer were obtained from BioRad Laboratories (Hercules, CA).

Crude Homogenate Preparation

Digestive organs were thawed at room temperature. The intestinal and attached pyloric caecal tissues were selected, weighed (wet weight), and homogenized. Stomach contents were noted and consisted of partially-digested fishes, crabs, squids, octopi, and/or coral fragments. Intestinal and caecal lumens were flushed/cleaned by forcing a stream of distilled, deionized water through them. In the case of the caecal tissue, the last, approximately 5 mm of the blind end was cut away to facilitate this flushing/cleaning process. The cleaned tissues were subsequently immersed in 540 ml of deionized, distilled water at room temperature and homogenized in an Osterizer® blender set at medium high speed for 5 min. The resulting homogenate was filtered through gauze to remove large cellular debris. This filtered solution was then stored at 4^{o}C.

Crude Homogenate Centrifugation

The crude homogenate was distributed into 50-mL Oak Ridge polycarbonate centrifuge tubes (Nalgene Corp, Rochester, NY). The tubes were placed in a JA-20 Beckman rotor and then centrifuged in a J2-21 Beckman centrifuge set at 20,000 rpm for 30 min at a temperature of 0^{o}C. The resulting supernatants were filtered through gauze to eliminate fatty particles, pooled, and then stored at 4^{o}C. The total volume of such pooled samples was measured and recorded.

Ammonium Sulfate Precipitation Procedures

Ammonium sulfate precipitations were performed as described by Deutscher (1990). 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°C. The resulting supernatants were pooled and stored at 4°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. The 50% ammonium sulfate supernatant and pellet solutions were produced as described for the 25% ammonium sulfate forms. Subsequently, the above-described procedure was repeated to obtain 75% ammonium sulfate solution. The total volume and protein concentration of each supernatant and pellet fraction were quantified and recorded.

Dialysis

Regenerated cellulose dialysis tubing of 3500 MWCO was purchased from Fisher Scientific (Pittsburgh, PA) and prepared as per the manufacturer's instructions. The ammonium sulfate-precipitated fractions, including all supernatants and pellet solutions, were dialyzed twice for 24 hours/dialysis against distilled water at 4°C. The dialyzed solution were pipetted out of the tubing and stored. The volume of each fraction was measured and recorded.

Protein determination

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

Trypsin activity assay and standard conditions

The spectrophotometric trypsin assay described by Hummel (1959) was utilized in order to determine hydrolysis rates of this enzyme obtained from silk snapper digestive tissues. Standard temperature was 27^{o}C. The source, and therefore quantity, of partially purified trypsin proteins varied in accordance with the particular step of the purification procedure that was being examined at a given time. However, in all cases the activity obtained was, through extrapolation, reported as total absorption change/mg/min. The trypsin assay was monitored with a Pharmacia (Piscataway, NY) Ultrospec 4000 spectrophotometer set at 247 nm maximum absorption. Reactions were performed in quartz cuvettes. Reaction time varied in accordance with the level of trypsin activity detected.

Source of trypsin proteins used at different steps of the purification procedure

In all cases, the source of trypsin selected for further purification or for kinetic characterization of the enzyme was that source displaying highest activity on a mg/min basis

Chromatographic Procedures

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 deionized

water. 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). Fraction # 37 displayed highest trypsin activity and was therefore selected for further purification analysis.

Affinity chromatographic procedures were performed using a Pharmacia High Trap Benzamidine 1.0 cm column fitted with a BioRad Econosystem peristaltic pump. The binding and washing buffer used was 0.05 M Tris HCl, 0.5 M NaCl, pH 7.4. Elution buffer was 0.05 M glycine, pH 3.0. Flow rate was 1 ml/min. All fractions were 1.0 ml in volume. A total of 2.0 ml solution collected in size exclusion chromatography fraction tube #37 was diluted six times by adding binding and washing buffer solution, thus producing a total volume to 12 ml. This solution was then loaded onto the HiTrap Benzamidine column through the assistance of the peristaltic pump. A total of 10 ml binding and washing buffer was run through the column and ten fractions were collected. Subsequently, 35.0 ml of elution buffer was run through the column and 35 fractions were collected. Finally, 10 ml of binding and washing buffer were run through the column and 10 more fractions were collected. All fractions were obtained using a BioRad Model 2128 collector (Hercules, CA). A total of 55 fractions were collected. Tubes #30 - #33 displayed highest trypsin activity and were thus pooled for use in subsequent analyses.

Temperature effect

Temperature effect upon 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^{o}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 dry bath or immersing the cuvette in ice water.

pH effect

Analysis of pH effect upon hydrolysis of artificial substrate TAME was determined at pH values ranging from 2.0 to 10.0. All other aspects of the trypsin assay were as described for our standard assay conditions.

Inhibitor effect

The effect of soybean trypsin inhibitor (SBTI) upon silk snapper trypsin activity was examined at each of four different inhibitor concentrations (i.e., 0.1%, 1%, 10%, or 100%) in the otherwise standard trypsin assay buffer (Hummel, 1959). All other aspects were identical as described for our standard assay conditions. Partially purified trypsin from 75% resuspended intestinal protein pellet or from pooled affinity chromatography fractions #30 - #33 were utilized in all of these assays.

Electrophoresis

Molecular weight determination of silk snapper trypsin was realized through SDS PAGE gel elctrophoretic procedures. All fish protein used for this procedure were obtained by pooling affinity chromatography tubes #30 – #33 and then concentrating the resulting solution through use of concentration Eppendorf 5415D centrifuge tubes (Eppendorf Corp., Hamburg, Germany). SDS PAGE electrophoresis was performed by Laemmli gel method utilizing a Biorad (Hercules, CA) Mini-Protean 3 cell apparatus as

per manufacturer's instructions. A 15% Tris-HCl ready gel (BioRad, Hercules, CA) was used for all SDS PAGE analyses. BioRad Blue Precision Plus protein standards (BioRad, Hercules, CA) were used for electrophoretic determination of molecular weight. The SDS gel was run at 170 volts for one hour. Proteins, including molecular weight standards, purified porcine pancreatic trypsin (Sigma, St. Louis, MO), as well as partially purified silk snapper trypsin and other intestinal tissue proteins, were identified in gel lanes through visualization coomassie blue staining procedure (BioRad, Hercules, CA). Molecular weights of unknown proteins were calculated using the protein's RF values.

RESULTS

Trypsin Purification Procedure Results

Table 1 presents the levels of purification obtained from crude homogenate sequentially subjected to ammonium sulfate precipitation fractionation, size exclusion chromatographic procedures, and affinity chromatographic procedures. The highest activity, based upon a mg/min basis (such a basis should also be assumed in all such trypsin activity cases referred to hereafter), among the resuspended ammonium sulfate pellet proteins was detected in the 75% fraction. This was a 4.29 fold level of purification over the crude homogenate.

Subjecting the 75% ammonium sulfate proteins to further purification via size exclusion chromatography resulted in a 2.28 and 9.80 fold purification, respectively, over the 75% ammonium sulfate fraction proteins themselves and the crude homogenate proteins. Figure 4 presents the elution profile of the silk snapper's proteins obtained through size exclusion chromatography. The highest trypsin activity was found in the size exclusion chromatography fraction tube number 37 (Figure 5).

The proteins from tube number 37 of the size exclusion chromatographic procedure were subjected to affinity chromatography and resulted in a 10.43 level of purification over crude homogenate. The affinity chromatography yielded a 1.06 fold increase in trypsin purification over that obtained in the size exclusion step. The highest trypsin activity obtained through affinity chromatography was in tubes #30 – #33. Figure 6 illustrates the relative trypsin activity obtained from tubes #20 – #55 for the affinity chromatographic procedure.

Trypsin Molecular Weight Determination via SDS PAGE Electrophoresis

Figure 7 presents the SDS PAGE electrophoretic analysis results for each of the following: protein size standards (lane STD), purified porcine pancreatic trypsin (lane TS), crude homogenate proteins (CH), and the pooled proteins obtained in tubes #30 – #33 of the affinity chromatographic procedure used in this study (lane AFF). Lane AFF reveals a band corresponding to a molecular weight of 26.1 kDa for the silk snapper trypsin. Figure 8 represents the relative mobility factor (Rf) values confirming this determination based upon the molecular weight standards from lane STD. The 22 kDa porcine pancreatic trypsin migrated to a position between that of the 20 kDa size standard (see lane STD) and the silk snapper trypsin (see lane AFF).

Kinetic Analysis Results

The pH optimum for the silk snapper trypsin was determined to be 8.0 (Figure 9). Lower levels of activity were detected at pH levels of 4.0 and 6.0. The enzyme was inactive at pH 2.0 and 10.0.

The results of temperatures effects upon silk snapper trypsin activity are shown on Figure 10. Highest trypsin activity was observed at 60^{o}C. Above 60^{o}C, trypsin activity decreased markedly or ceased altogether. Although trypsin activity at 50^{o}C was only slightly lower than that at 60^{o}C, considerably lower levels of activity were obtained at temperatures below 50^{o}C extending down to 10^{o}C.

Figure 11 shows the results of soybean trypsin inhibitor effect on TAME hydrolysis by the purified silk snapper trypsin. Increased soybean trypsin inhibitor (SBTI) concentration corresponded with decreased trypsin activity.

Table 1. Purification of trypsin extracted from the intestine and pyloric caeca of the silk snapper, *Lutjanus vivanus* (Cuvier, 1828).

Protein source	Total protein (mg)	Trypsin Activity (Δabs/mg/min)	Total Enzyme Activity (Δabs/mg/min)	Level of Purification (fold)
Crude Homogenate	**1544**	**0.164**	**253.22**	**1**
25% Resuspended Pellet	**2**	**0.162**	**0.324**	**0.99**
50% Resuspended Pellet	**36**	**0.632**	**22.75**	**3.85**
75% Resuspended Pellet	**91**	**0.704**	**64.06**	**4.29**
Size Exclusion Chromatography of 75% Resuspended Pellet				
37 ml fraction	**1.109**	**1.607**	**1.78**	**9.80**
Affinity Chromatography of 75% Resuspended Pellet				
30 – 33 ml elution fraction	**1.822**	**1.711**	**3.12**	**10.43**

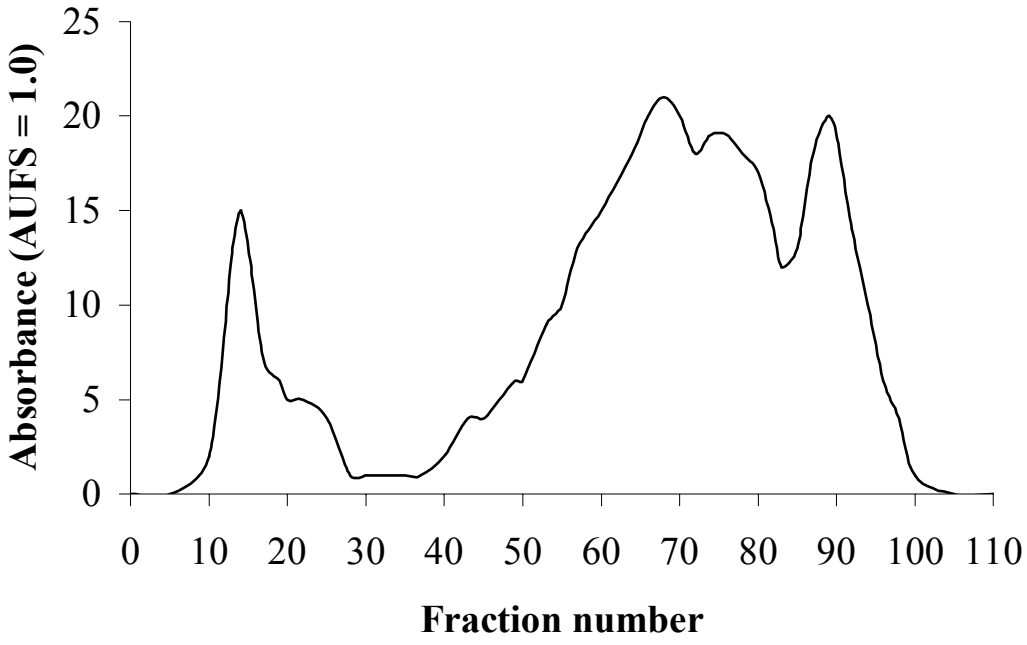

Figure 4. Elution profile of the silk snapper's, *Lutjanus vivanus* (Cuvier, 1828), proteins obtained through size exclusion chromatography.

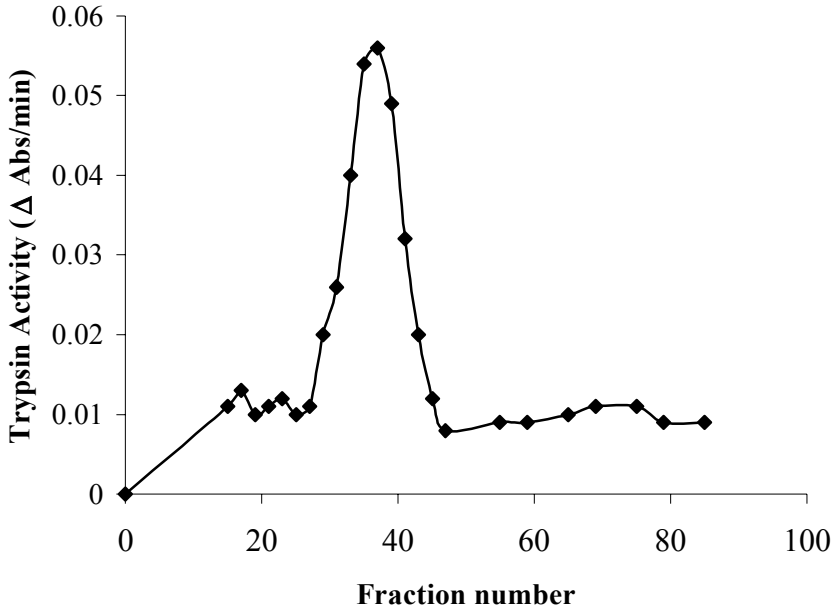

Figure 5. Trypsin activity obtained through size exclusion chromatography of resuspended 75% ammonium sulfate fraction from the intestine and pyloric caeca of the silk snapper, *Lutjanus vivanus* (Cuvier, 1828).

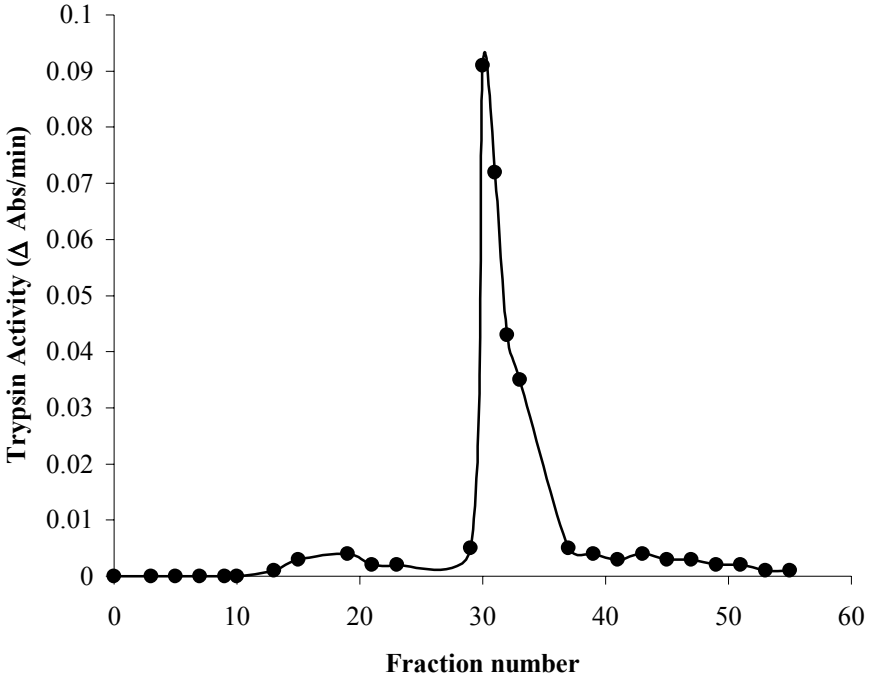

Figure 6. Trypsin activity obtained through affinity chromatography of size exclusion protein fractions obtained from the resuspended 75% ammonium sulfate fraction from the intestine and pyloric caeca of the silk snapper, *Lutjanus vivanus* (Cuvier, 1828).

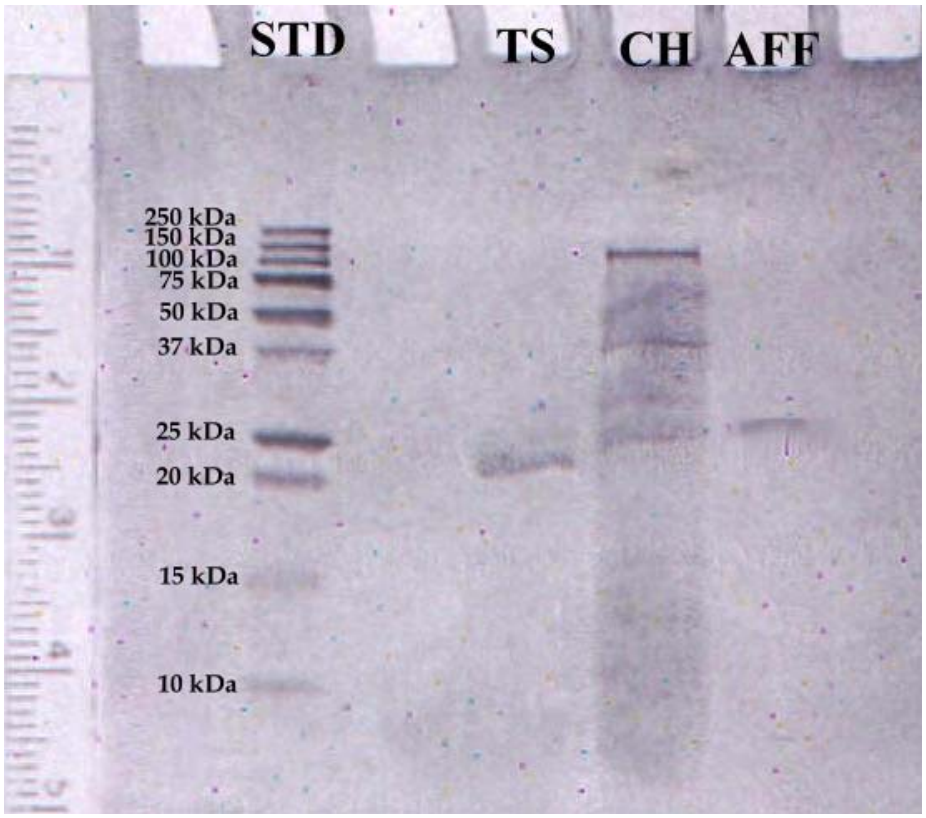

Figure 7. SDS PAGE electrophoresis of protein size standards (STD), purified porcine pancreatic trypsin (TS), crude homogenate (CH) and silk snapper's, *Lutjanus vivanus* (Cuvier, 1828), **trypsin obtained from affinity chromatography pooled sample (AFF).**

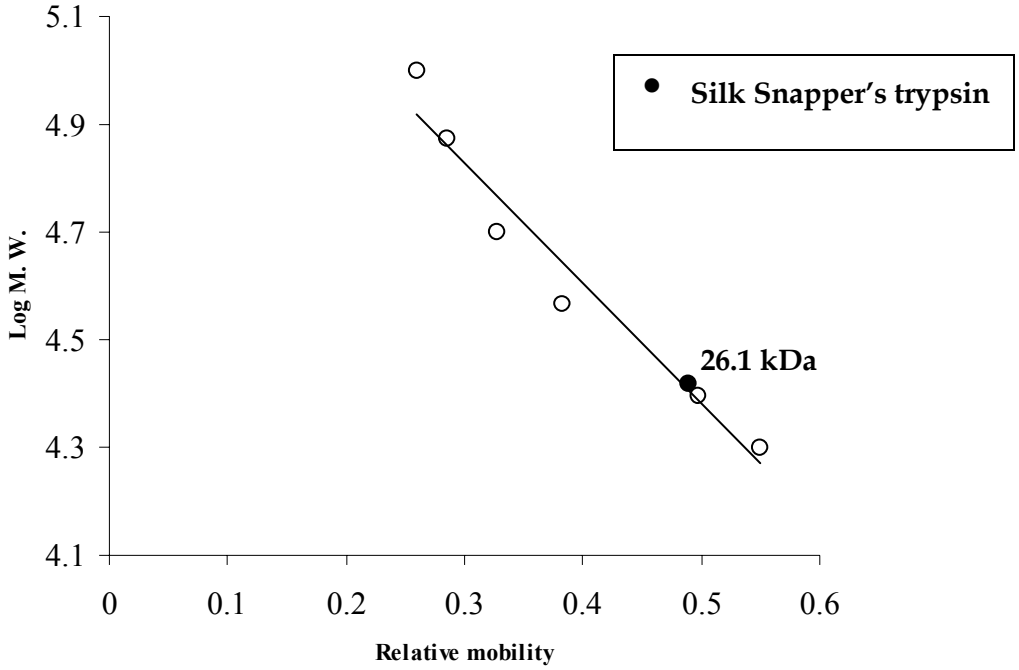

Figure 8. Molecular weight of silk snapper's, *Lutjanus vivanus* (Cuvier, 1828), **trypsin determined by SDS PAGE electrophoresis.**

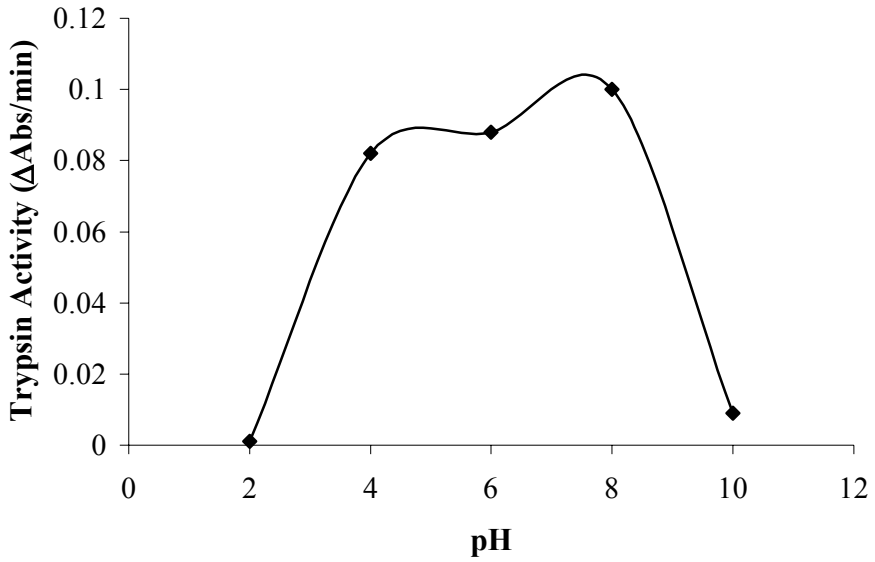

Figure 9. Effect of pH on silk snapper's, *Lutjanus vivanus* (Cuvier, 1828), **trypsin activity upon hydrolysis of TAME.**

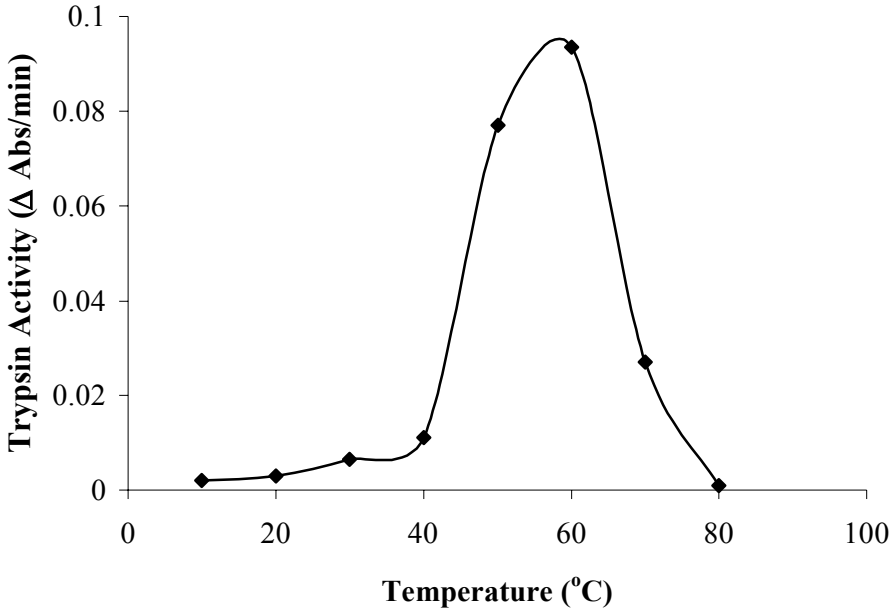

Figure 10. Effect of temperature upon silk snapper's, *Lutjanus vivanus* (Cuvier, 1828), **trypsin activity.**

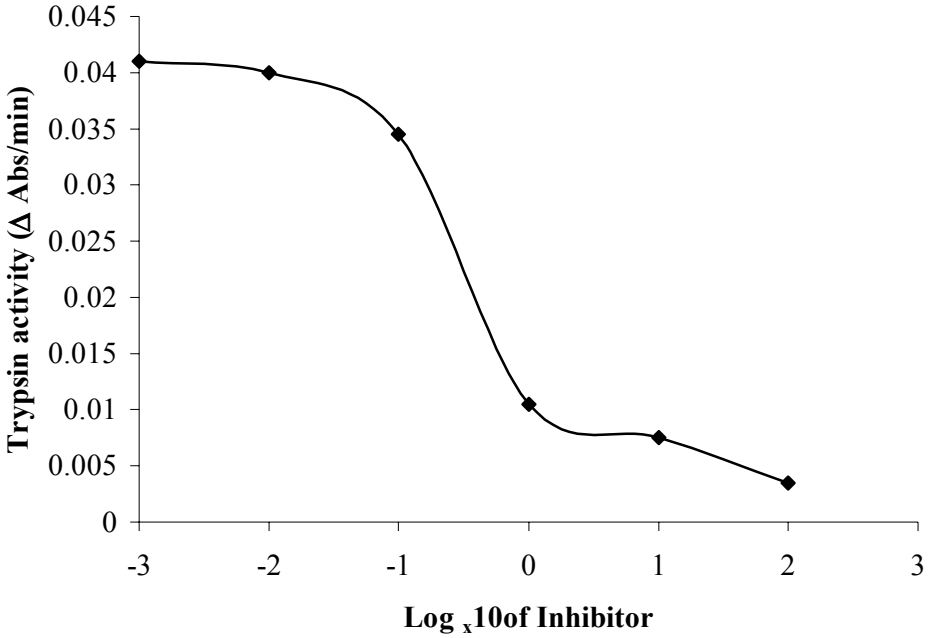

Figure 11. Effect of soybean inhibitor (SBTI) on silk snapper's, *Lutjanus vivanus* (Cuvier, 1828), **trypsin upon hydrolysis of TAME.**

DISCUSSION

Through a combination of chromatographic purification steps, a molecular weight determination procedure, and standard enzyme kinetics analyses, this study has documented the presence and characteristics of a digestive trypsin in intestinal tissues of the silk snapper, *Lutjanus vivanus* (Cuvier 1828).

As already established in the literature review, fish trypsins are characterized by having molecular weights within a range of 18,000 to 28,000 daltons. Therefore, my finding that the silk snapper trypsin is a 26.1 kDa protein is in agreement with this generalization. Silk snapper trypsin is also similar to other fish trypsins in that its optimal activity is approximately pH 8.0, with few exceptions, are optimally active within a pH range of 6.5 – 9.0. The finding that the silk snapper trypsin is optimally active at a temperature of 60^{o}C is also in accordance with fish trypsins being optimally active at temperatures between 30 – 60^{o}C. Finally, the SBTI effect, which is very specific for trypsin inhibition, confirms that the observed hydrolysis is actually from a trypsin.

One critical issue of this study that remains to be clarified is why the trypsin activity obtained after affinity chromatography is not considerably higher than the highest activity, tube # 37, obtained in the size exclusion chromatographic application. Indeed, affinity chromatography is a protein purification method whereby the target molecule tends to increase markedly in concentration. That this may have occurred is borne out in the SDS PAGE gel results (Fig. 6), where a single band is clearly visible and of a size within the predicted range for fish trypsins. This finding strongly suggests that, indeed, the silk snapper trypsin was purified through the affinity chromatography procedure.

There are several potential reasons why the yield of activity was not higher. First, the enzyme enterokinase, which occurs in intestinal epithelial cells of vertebrate organisms and functions to convert inactive trypsinogen into active trypsin, may have been separated from the trypsin during the affinity chromatography. One way to examine if this was the case would be to conduct future experiments with the addition of enterokinase to the pooled affinity chromatography fractions that displayed trypsin activity. Secondly, we cannot dismiss the possibility that the p-Aminobenzamidine column itself may have somehow permanently deactivated a certain percentage of the enzyme. Although other trypsins are routinely eluted from such affinity columns without significant loss of activity, the silk snapper trypsin may represent an exception. Another possibility is that in the preceding chromatographic step (i.e., passage of the 75% ammonium sulfate resuspended proteins through the size exclusion column), an approximately 10-fold level of trypsin purification had already been obtained. That this may have occurred is supported by the elution profile obtained from the size exclusion chromatographic step (Figure 4). Here, we note that a large amount of protein, as evidenced by higher UV absorption, eluted from the column in fractions #50 - #105. However, very low to no trypsin activity was detected in these fractions. By contrast, fraction tubes #30 – #40 contained high trypsin activity yet yielded little corresponding UV absorption, indicating that there was comparatively much less protein in these trypsin-rich fractions. Thus, the trypsin activity detected in the affinity chromatography fraction tubes (i.e., tube #s 30 –33) may simply represent the approximately same trypsin proteins at the approximately same concentration as those in size exclusion fraction tube #37. This possibility could be tested by collecting much smaller affinity chromatography

fractions (e.g., 50 µls instead of 1 ml) and noting whether or not the activity was appreciably higher, on a mg/min basis, than in the size exclusion tube #37.

Turning our attention to the potential for the information obtained in this study being applied in a practical manner, it is notable that Lemieux et al. (1999) reported that low trypsin activities in the Atlantic cod, *Gadus morhua* L., correlated with lower growth rates. This investigation stated that, at the level of digestion, trypsin appears to be the only enzyme that potentially limits Atlantic cod growth. We can consider that if such a relationship also exists between silk snapper trypsin and its growth rate, then methodologies might be developed that utilize the information obtained in this study to more effectively manage, perhaps through allowing harvesting at only certain times of the year or only during certain years, for this species. The potential for trypsin to be used as a fishery management tool remains to be determined.

CONCLUSIONS AND RECOMMENDATION

Considering the data presented in this study, we can assert that a 26.1 kDa trypsin is present in intestinal tissues of *Lutjanus vivanus*. Moreover, this enzyme can be purified and kinetically characterized. The results in this investigation demonstrated a degree of similarity between the silk snapper's trypsin and trypsin isolated from other fish species. This was the first time that trypsin obtained from the intestinal tissues and pyloric caeca of the silk snapper was purified and characterized. Future investigations, to better understand the contribution of trypsin and other serine proteases in helping fishes meet their nutritional requirement for amino acids, can be aimed at the following: 1) inter- and intraspecific studies comparing trypsin characteristics with a view towards better understanding the occurrence and function of this enzyme in fishes (such analyses could include comparing trypsin activities within a single species living in different trophic environments, or comparing among different fish species that exhibit different feeding habits [e.g., carnivorous vs. herbivorous species]), 2) potential utility of trypsin as an environmental indicator based upon differential presence of trypsin abundance as a function of trophic habitat, 3) development of oligonucleotide probes based upon trypsin amino acid sequence (such probes may provide a basis for evolutionary studies as gene sequences are analyzed and compared). It is clear that trypsin may prove useful not only in better understanding the biochemistry/physiology of the silk snapper, but in many other animal species as well.

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