Characterization of Trypsin-like Activity From Pyloric Caeca of the Red Hind Grouper, *Epinephelus guttatus*: Considerations of Use as an Environmental Indicator

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ABSTRACT

Trypsin-like activity was detected, purified 48 fold, and characterized from pyloric caeca of the red hind grouper, *Epinephelus guttatus*. Groupers were obtained from southwestern coastal waters of Puerto Rico. Enzyme purification procedures consisted of ammonium sulfate fractionation of pyloric caeca homogenate followed by size exclusion chromatography of protein from the resuspended 45% ammonium sulfate pellet. The trypsin-like enzyme has a molecular weight between 17,000 and 44,000 and displays optimal activity in the pH range of 7.0 - 8.0.

Because changes in diet can affect the abundance of a particular digestive enzyme in a number of fish species (Jobling 1995, Kapoor et al. 1975), it is possible that red hind groupers and other fish species of the Gulf and Caribbean will display levels of certain digestive enzymes that are characteristic of specific trophic environments. The potential for red hind grouper trypsin-like activity to be utilized as such an environmental indicator is thus considered.

KEY WORDS: Pyloric caeca, red hind grouper, trypsin

INTRODUCTION

Investigations into how animals chemically digest food deepens our understanding of how their nutritional needs are met. Central to such investigations are enzymes, the catalysts of biochemical reactions. Trypsin, a proteolytic enzyme, has been studied from a wide range of vertebrate organisms, including mammals, birds, reptiles and fishes. The grouper, family Serranidae, a commercially important food fish that occupies a significant predatorial niche in ecosystems of Puerto Rican and other marine tropical waters, is one fish for which there are practically no published data regarding trypsin or other digestive proteases. This study is a partial purification and characterization of trypsin from pyloric caeca of the grouper *Epinephelus guttatus*. Besides providing a better understanding of how *Epinephelus guttatus* chemically processes ingested protein, this basic research will help to provide a foundation for the following potential future investigations:

i) Analysis of trophic habitat: here, the abundance of trypsin and/or other proteases detected in digestive organs of red hind grouper may be correlated with changes in habitat and thus function as an environmental indicator
ii) Comparative digestive enzyme studies, in which digestive proteases of other fish species are examined
ii) Better defining red hind grouper trophic environments that optimize growth and reproductive success.

MATERIALS AND METHODS

Fish Organs

Red hind groupers, *Epinephelus guttatus,* were caught in the Caribbean Sea by fishermen using hook and line gear. At the time of their capture, the fish were gutted and the organs were placed in plastic bags maintained on ice. Upon their arrival at a fishmarket in the Puerto Real district of Cabo Rojo, Puerto Rico, the organs were placed in a -30°C freezer for a period ranging from several days to one month. The frozen organs were then transferred to a -80°C laboratory freezer.

Chemicals

$(NH_4)_2SO_4$ (ammonium sulfate), NaCl, bovine serum albumin (BSA), trizma, trizma base, N-p-tosyl-L-arginine methyl ester (TAME), pancreatic porcine trypsin, soybean trypsin inhibitor (SBTI), were all purchased from Sigma Chemical Co. (St. Louis, MO). BioGel P-60 fine (45 - 90 µm [wet]), gel filtration standards, 30 x 1.5 cm glass column, DEAE2 column, and anion exchange chromatography standards were purchased from Bio-Rad (Richmond, CA). BCA protein reagents were from Pierce Chemical Company (Rockford, IL).

Crude Homogenate Preparation

Frozen digestive organs were thawed and pyloric caecal tissues were separated, pooled, and weighed (wet weight). A total of 150 g pyloric caeca tissue was immersed in 1000 ml of 4°C deionized water, placed in a blender, and homogenized at low speed. The resulting crude homogenate was filtered through gauze to remove large cellular debris and particulate matter.

Centrifugation/Ammonium Sulfate Fractionation

The entire filtered crude homogenate (550 ml) was distributed equally into 50 ml Oak Ridge polycarbonate centrifuge tubes (Nalgene Corp., Rochester, NY). The tubes were capped and placed in a Beckman (Palo Alto, CA) JA-20 rotor and centrifuged for 30 min at 48,400 g in a Beckman J2-MC centrifuge set at 0 °C. The resulting supernatants were filtered through gauze (to remove the

fatty layer), pooled and stored at -80 °C. Each pellet was resuspended in 3 ml deionized water. All resuspended pellets were then pooled and stored at 4 °C.

The supernatant was brought to 20% saturation with $(NH_4)_2SO_4$ and centrifuged in a manner identical to that described above for the crude homogenate. Again, the resulting supernatants were pooled and stored at 4 °C. Pellets were also resuspended, pooled, and stored at 4 °C as described above. The 20% $(NH_4)_2SO_4$ saturated supernatant was brought to 45% saturation with $(NH_4)_2SO_4$ and centrifuged. The resulting supernatant and pellet were treated as described previously. Additional 65% and 85% $(NH_4)_2SO_4$ saturated supernatants, produced respectively from the 45% and 65% supernatants, were again centrifuged as described above and the resulting pellets and supernatants were treated as described previously.

Dialysis

Dialysis tubing of 10mm diameter and 3,500 MWCO was purchased from Spectra Medical Inc. (Houston, TX) and prepared as per the manufacturer=s directions. The pooled, resuspended pellets of each ammonium sulfate fractionation were twice dialyzed for 12 hours against distilled water at 4 °C. The dialyzed, resuspended pellet material was pipetted out of the tubing and material that had precipitated in the tubing was discarded. The dialyzed solutions were stored at 4 °C.

Protein Quantification

Protein concentrations in supernatant, resuspended pellet solutions, and collected size exclusion chromatography and ion exchange fractions were determined using a BCA protein assay (Rockford, IL) in conjunction with a Pharmacia (Piscataway, NJ) Ultrospec 4000 computer-assisted spectrophotometer.

Size Exclusion Chromatography

Resuspended 45% ammonium sulfate fraction pellet proteins were loaded onto the top of a 30 X 1.5 cm column packed with p-60 biogel (Bio-Rad) and run through the column at a flow rate of 0.18 ml/min. The column running solvent was deionized water. After discarding the fist 10 ml of solution to pass through the column, the next 110 ml to pass was collected as 55 equal fractions (2 ml/fraction). All fractions were examined for trypsin activity as described below.

Assessing Trypsin-like Activity

Trypsin-like activity was determined according to a modified version of the assay described by Hummel (1959). In this modification, buffer was replaced

with deionized water. Rates of trypsin-like hydrolitic activity upon the substrate TAME were determined with a Pharmacia (Piscataway, NJ) Ultrospec 4000 spectrophotometer set at 247 nm maximum absorption. Reactions were performed in quartz cuvettes in all cases.

RESULTS

Our results document the presence of a trypsin-like enzyme activity in pyloric caecal tissues of the red hind grouper, *Epinephelus guttatus*. Table 1 shows the trypsin activity detected in crude homogenate, 25%-, 45%-, 65%-, and 85%- ammonium sulfate fractions obtained from crude homogenate, as well as the 34-36 ml and 36-38 ml fractions obtained from 45%-ammonium sulfate fraction proteins subjected to size exclusion chromatography.

Table 1. Trypsin-like activity detected in crude homogenate, ammonium sulfate fractions, and 45% ammonium sulfate fraction subjected to size exclusion chromatography.

Protein Source	Total Protein (mg)	Enzyme Activity (Δabs/min/mg at 247 nm)	Total Activity (Δabs/min at 247 nm)	Fold Level of Purification
Crude Homogenate	1777.0	0.061	108.40	1.00
20% Resuspended Pellet	15.1	0.085	1.29	1.39
45% Resuspended Pellet	172.5	0.487	84.00	7.98
65% Resuspended Pellet	49.2	0.393	19.30	6.44
85% Resuspended Pellet	105.0	0.085	3.60	1.39
Size Exclusion Chromatography of 45% Pellet Resuspended Proteins				
34 - 36 ml Fraction	0.1	2.858	0.04	48.86
36 - 38 ml Fraction	0.4	0.973	0.40	15.96

Among the four ammonium sulfate fractions produced, the highest concentration of activity was detected in the 45% resuspended pellet proteins. This was therefore selected for further purification via size exclusion chromatography. Highest trypsin-like activity (on a min/mg basis), and therefore highest level of purification, was detected in the 36 - 38 ml fraction collected from resuspended 45%-ammonium sulfate fractionated proteins. These proteins, representing an almost 50-fold level of trypsin-like enzyme purification over the crude homogenate, were thus chosen for all further analyses.

Figure 1 illustrates the levels of trypsin-like activity associated with the different protein fractions collected following size exclusion chromatography of the pellet proteins obtained through the 45% ammonium sulfate fractionation procedure. This fraction corresponds to a molecular weight between 17,000 and 44,000 daltons and therefore indicates that the molecular weight of the trypsin-like enzyme is also within this size range.

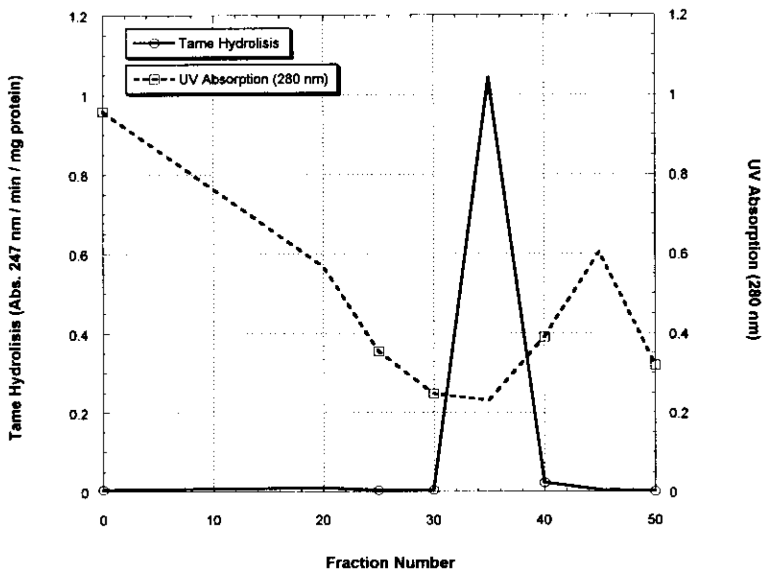

Figure 1. Size Exclusion Chromatography of Resuspended 45% Ammonium Sulfate Fractionated Proteins. The solid line indicates trypsin-like activity detected while the broken line indicates relative levels of protein as indicated by absorption at 280 nm.

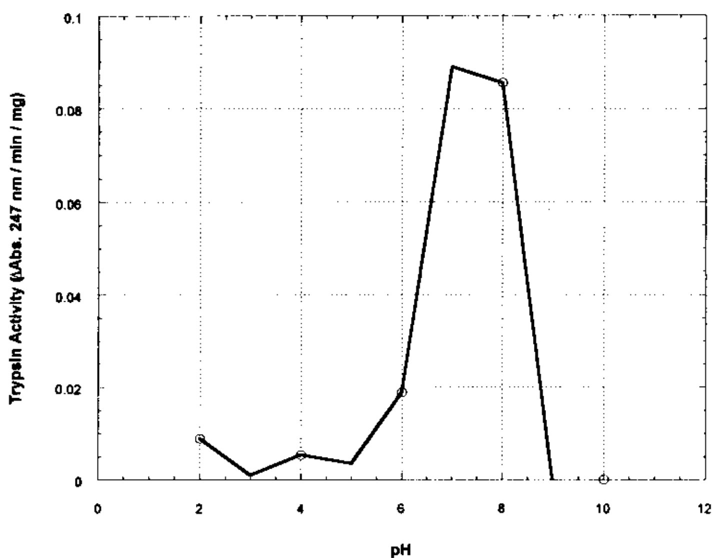

Figure 2. The Effect of Different pH on Trypsin-like Activity.

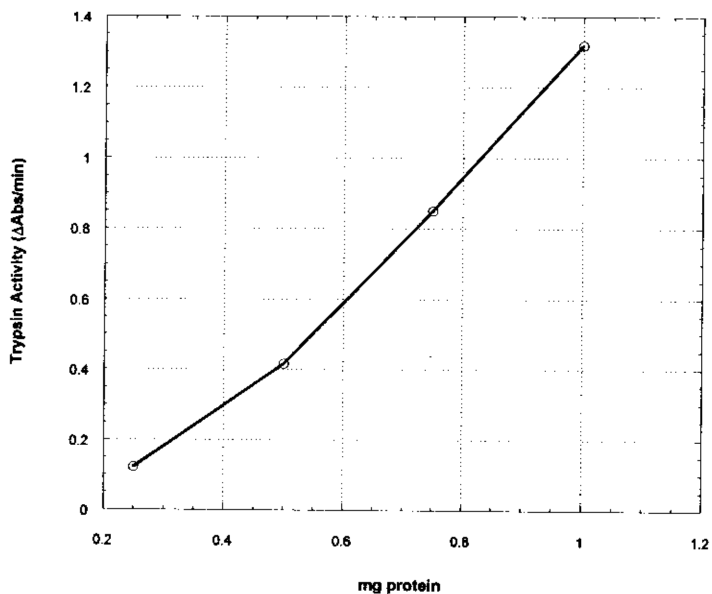

Figure 3. Effect of Amount of Protein Upon Trypsin-like Activity.

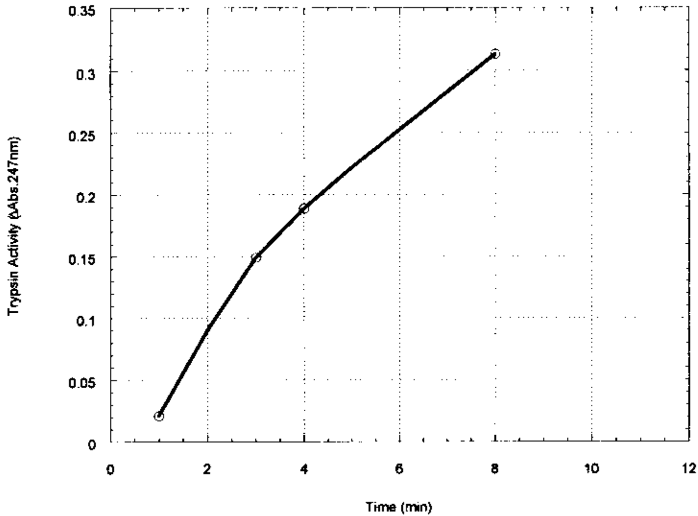

Figure 4. Time effect on trypsin-like activity.

DISCUSSION

Our data indicate that a trypsin-like activity is present in pyloric caeca of the red hind grouper, *Epinephelus guttatus*. The characteristics of the enzyme, including pH optimum, molecular weight, and substrate specificity, are similar to those reported for other trypsins from mammalian and fish sources.

Keil (1971) notes that trypsin in general has a molecular weight ranging from 20 - 24,000 daltons. However, trypsin isolated from both marine invertebrates and vertebrates has a molecular weight of 27 - 30,000 daltons. Because our size exclusion data have identified the red hind grouper trypsin activity as ranging from 17 - 44,000 daltons, we cannot yet conclude whether or not this enzyme is characteristic of either of the above trypsin size classes. Future work using affinity chromatography coupled with polyacrylamide gel electrophoresis will attempt to more precisely define the molecular weight of the grouper trypsin and thus settle this issue.

Our work with ion exchange chromatography (data not shown) indicates that the grouper trypsin is anionic. This finding compares favorably with those for other marine fish and invertebrate trypsins in that these are also anionic enzymes. By contrast, mammalian trypsins are cationic (Mallory and Travis 1973, Louvard and Puigserver 1974).

The supposition that levels of grouper trypsin activity may prove useful as a trophic, and therefore environmental, indicator is based upon the assertions of Jobling (1995) and Kapoor et al. (1975). These authors argue that intraspecific differences in the proteolytic enzyme levels of certain freshwater fish species reflect changes in protein dietary intake. Specifically, diets higher in protein cause the fishes to exhibit correspondingly higher levels of proteolytic digestive enzyme activity.

If a certain, unique level of red hind grouper pyloric caeca trypsin-like activity is found to be characteristic of each of several different trophic environments, then the enzyme would be useful as an environmental indicator. Although we have not yet begun this aspect of our investigation, some comments on this topic are in order at this time. First, we need to define what constitutes a *different* trophic environment. Red hind groupers inhabit the tropical western Atlantic, ranging north to North Carolina and South to Venezuela. Thus, as they occur throughout the West Indies and the Gulf of Mexico, their geographic range is sufficiently large that we may reasonably expect the fish to live within different trophic habitats (Heemstra and Randall 1993). This assumption is further supported by noting that *Epinephelus guttatus* both inhabits and thrives in shallow reef and rocky bottom (ranging to 200 m deep) environments.

Red hind grouper pyloric caeca trypsin-like activity may prove useful in assessing the impact of humans upon the trophic habitat of this fish and other marine species. In this case, it will be necessary to first characterize all such habitats for trypsin-like activity prior to occurrence of the human-induced changes. The time period after which such changes affect (if at all) the trypsin-like activity levels in red hind groupers will likely vary depending upon the nature and severity of the human impact.

Finally, more efficient management of the red hind grouper fishery may be possible through the suggested utility of the trypsin-like activity in functioning as a trophic indicator. Of course, all of the above suppositions are based on the assumption that levels of trypsin-like activity detected in red hind grouper pyloric caeca are affected by changes in trophic characteristics. This remains to be demonstrated and will be the focus of our future investigations.

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