PURIFICATION AND KINETIC CHARACTERIZATION OF TRYPSIN FROM THE INTESTINE AND PYLORIC CAECA OF THE WHITE GRUNT, *Haemulon plumierii*, (Lacepède, 1801)

By

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ABSTRACT

Trypsin is one of several animal digestive proteases whose activities collectively breakdown ingested proteins to facilitate gut amino acid absorption. The goals of this research were to purify and characterize trypsin from intestinal and pyloric caecal tissues of white grunt, *Haemulon plumierii* (Lacepède, 1801). Trypsin was purified 11-fold from intestinal and pyloric caecal tissues of the white grunt, through the sequential application of ammoniun sulfate fractionation, size exclusion chromatography, and affinity chromatography. The enzyme displayed optimal activity at pH 8 and 40 ^{o}C. It was completely inhibited by the presence of soybean trypsin inhibitor at a concentration of 100 %. SDS-PAGE analysis of the purified enzyme revealed a single band with an estimated molecular weight of 24 kDa. This white grunt trypsin was similar to those from other fishes in terms of its molecular weight, kinetic properties, and response to the presence of soybean trypsin inhibitor.

RESUMEN

Tripsina es una de las varias proteasas animales digestivas que rompen colectivamente las proteínas ingeridas, para facilitar la absorción de aminoácidos en el intestino. Las metas de esta investigación fueron purificar y caracterizar tripsina del tejido intestinal y ciego pilórico del pez cachicata, *Haemulon plumierii* (Lacepède, 1801) mediante la aplicación secuencial de fraccionamiento con sulfato de amonio, cromatografía de exclusión por tamaño y cromatografía de afinidad. La enzima desplegó actividad óptima a pH 8 y a 40 ^{o}C. Tripsina fue completamente inhibida por la presencia de inhibidor de tripsina de soya a concentración de 100 %. El análisis de SDS-PAGE de la enzima purificada reveló una banda con un peso molecular estimado de 24 kDa. Esta tripsina de cachicata fue similar a aquellas obtenidas de otros peces en términos de su peso molecular, propiedades cinéticas y respuesta a la presencia de inhibidor de tripsina de soya.

DEDICATION

To God, to my parents, Nilda R. Muñoz del Moral and Edwin M. Rodríguez Gomez, my brothers Edwin F. Rodríguez Muñoz and Dennis A. Rodríguez Muñoz, my grandmother Ana Del Moral Ortiz and my nephew Fabiola Rodríguez Fonseca for their unconditional love, support, patience, and for giving me the inspiration and strength to reach my goals.

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INTRODUCTION

The white grunt, *Haemulon plumierii,* is a fish in Order Perciformes and Family Haemulidae that occurs throughout the western Atlantic from Chesapeake Bay, through the Gulf of Mexico and Caribbean, southward to Brazil, including the Antilles. It inhabits mainly subtropical to tropical (39^{o} N – 23^{o} S) reef-associated environments of 3 – 40 m depth (Smith, 1997). Matos-Caraballo (2002) established that during 1998 - 2001 various grunt species, mainly the white grunt, collectively comprised 4 % of the Puerto Rican commercial fish catch. As such, the white grunt is of some economic and nutritional significance to Puerto Rico, and there is interest to characterize trypsin from this and other marine fish species of Puerto Rico in an effort to better understand western Atlantic, tropical fish digestive physiology. Indeed, elevated levels of proteases in the alimentary canal have been found to be an effective indicator of increased dietary protein intake in freshwater fishes (Horn, 1998). Perhaps a similar correlation exists for marine species such as the white grunt.

Digestion consists of the chemical and mechanical process that break down foodstuffs to substances that are metabolically useful for the maintenance of life. Animal digestion is facilitated by enzymes that degrade molecules into metabolically useful forms. The proteases (also called proteinases) are a class of digestive enzymes that degrade proteins through hydrolysis of peptide bonds and fall in two basic types, the exopeptidases and the endopeptidases.

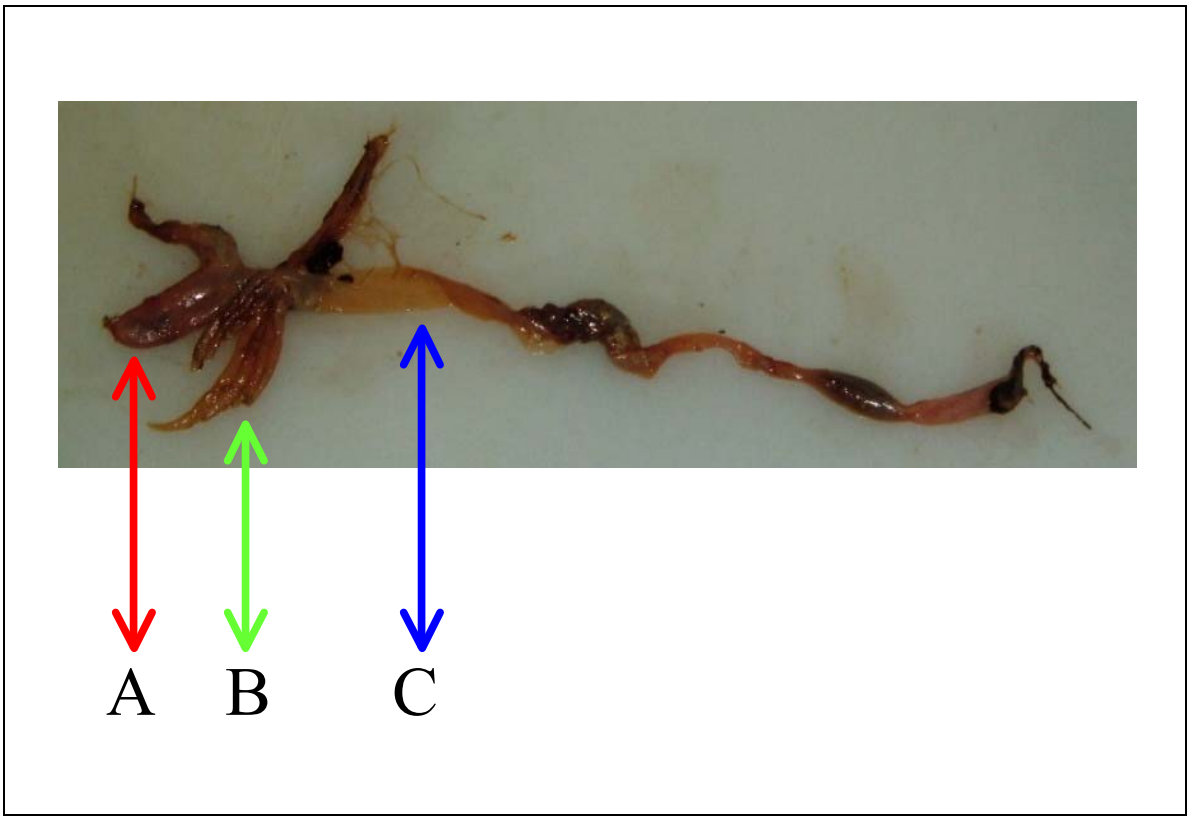
Exopeptidases function by catalysing the removal of terminal amino acids from peptide chains (including those as small as dipeptides, or tripeptides). By contrast, endopeptidases attack bonds located deep within the substrate protein, thus transforming large amino acid chains into shorter peptide segments. Examples of common endopeptidases include trypsin, chymotrypsin and elastase.

Trypsin, chymotrypsin, and elastase occur in the alimentary canal of virtually all-invertebrate and vertebrate organisms (Solomon et al., 1996). Trypsin is an enzyme specific for peptides and esters of the amino acids lysine and arginine. Although elastase and chymotrypsin are very similar to trypsin in their tertiary structure, elastase is specific for

hydrolysing small hydrophobic side chains, such as alanine, while chymotrypsin is specific for the hydrophobic amino acid side chains of phenylalanine, tyrosine and tryptophan. Each of these enzymes displays the highest rate of catalytic activity at pH 7.8. All have a pKa of 6.8. Collectively, these three endopeptidases play essential roles in converting ingested proteins into individual amino acids, all of which are subsequently absorbed across the gut epithelium.

The goals of this research were to purify and characterize, according to standard kinetic parameters, trypsin from intestinal tissues of the white grunt, *Haemulon plumierii*, including a determination of its molecular weight. A white grunt'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 1.

Figure 1. Digestive tract of the white grunt, *Haemulon plumierii* (Lacepède, 1801). **A** represents the stomach, **B** represents the pyloric caeca, and **C** represents the intestinal tract extending from the stomach.



PREVIOUS WORK

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

According to the definition proposed by Keil (1971), trypsins have a molecular weight ranging from 20 – 24 kDa. However, trypsin isolated from marine invertebrates and vertebrates have, in most cases, been found to possess molecular weights outside of this range. Thus, molecular weights around 27 – 30 kDa have been reported. Additionally, it has also been found that marine trypsins are anionic at neutral pH. It seems that during evolution trypsins have decreased their molecular weights as they have lost some of the surface negative charges (Martinez et al., 1988).

In 1960, Bradford characterized a fish trypsin-like enzyme activity. He identified proteolytic hydrolysis from pyloric caeca of the chinook salmon, *Onchorhynchus tshawytscha*. The enzyme was found to have similar properties to mammalian trypsin and chymotrypsin. This trypsin-like enzyme exhibited optimal activity at pH 9.0 and a temperature of 49 ºC.

Reeck et al. (1970) identified three forms of trypsinogen in the African lungfish, *Protopterus aethiopicus*. One form was later purified and characterized (Reeck and Neurath, 1972). It displayed a molecular weight of 24 kDa and optimal 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 or dogfish sources. The properties of this enzyme were found to lie between those trypsins isolated from invertebrate and mammalian sources. It resembled invertebrate enzymes in being anionic and stable at neutral pH in 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 the digestive organs from three

flatfish species, the marbled flounder, *Limanda yokohamae*, the stone flounder *Kareius bicoloratus*, and the olive flounder, *Paralichtys olivaceus*. He found this activity to be optimal at pH of 8.0 and a temperature of 40 ºC.

Overnell (1973) made a study of the digestive enzymes from pyloric caeca and its associated mesentery in cod, *Gadus morhua*. The following enzymatic activities were identified: trypsin, chymotrypsin, carboxypeptidase A and B, leucine aminopeptidase, ribonuclease, amylase, acid phosphatase and alkaline phosphatase. The trypsin-like enzyme had a molecular weight of 18 – 22 kDa while its pH optimum ranged from 8.0 to 9.0. It is notable that the principal pancreatic enzymes were found between the mesentery of the caeca and not in the caeca themselves.

Titani et al. (1975), utilizing electrophoresis, purified pancreatic trypsins from the spiny dogfish, *Squalus acanthias*. This trypsin presented two bands, one of 11 kDa and the other of 23 kDa. These data suggested a potential mixture of a single chain and double chain form of trypsin. In addition, the amino acid sequences of these trypsins were determined. These revealed that each contained one less amino acid residue than the amino acid sequence counterparts of bovine and porcine trypsins.

Klaus-Dieter (1976) found a trypsin-like activity in the intestine and hepatopancreas of the bonefish, *Carassius auratus*. He determined its pH optimum to be 9.0. Optimal activity occurred within a temperature range of 30 to 50 ^{o}C. He also found that the trypsinogen was synthesized by the hepatopancreas. Subsequently, this zymogen, upon being secreted into the intestine, became activated through enterokinase activity.

Cohen et al. (1981) purified pancreatic proteolytic enzymes, including trypsin, from the carp, *Cyprinus carpio.* The enzyme displayed an approximate molecular weight of 25 kDa. The carp trypsin was found to be anionic and unstable at low pH.

Clark et al. (1985) identified protease activities from intestinal tissues of the marine flatfish, *Solea solea.* Experiments using synthetic substrates suggested the presence of a trypsin-like enzyme. This enzyme activity revealed a pH optimum of 8.0.

Pancreatic enzymes, including trypsin, were characterized from the sharptooth catfish, *Clarias gariepinus* (Uys and Hecht, 1987). This trypsin displayed optimal activity at pH 8.2 and at temperatures ranging from 30 to 40 ºC.

Purification and characterization of two trypsin-like enzymes from the digestive tract of the anchovy, *Engraulis encrasicholus*, was realized through the use of affinity and subsequent ion exchange chromatographic procedures (Martinez et al., 1988). The enzymes had molecular weights in the 27 – 28 kDa range. Their isoelectric points were between 4.6 and 4.9. These enzymes exhibited optimal activity at a pH range of 8 to 9. Overall, these anchovy trypsins resembled those from other fish in terms of their molecular weights, kinetic properties, and instability at low pH.

Dimes et al. (1994) isolated and partially purified trypsin from the pyloric caeca of rainbow trout, *Oncorhynchus mykiss*, wild chinook salmon, *Oncorhynchus tschawytscha*, and the coho salmon, *Oncorhynchus kisutch*. These enzymes were least stable at pH values below 5.0 and most stable under alkaline conditions up to pH 10.0.

Guizani et al. (1991) purified and characterized a trypsin from the pyloric caeca of mullet, *Mugil cephalus*. The enzyme exhibited optimal activity at pH 8.0 and at a temperature of 55 ºC. It was stable within a pH range of 7.5 - 9.0. This stability is similar to that reported for most marine trypsins, which are highly unstable under acidic conditions but very stable at neutral to somewhat alkaline conditions. Electrophoretic analysis determined the molecular weight of the enzyme to be 24 kDa.

Comparison of trypsin and chymotrypsin from the viscera of anchovy, *Engraulis japonicus*, was conducted by Heu et al. (1995). The molecular weight of the trypsin was estimated to be 25.6 kDa. Maximal activity was found at pH 9.0 and a temperature of 45 ºC for casein substrate while maximal activity for synthetic substrate, TAME (Nα-p-toluenesulphonyl-L-arginine methyl ester), was identified at pH 8.0 and 45 ºC.

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

The proteolytic activities in the gut of three carnivorous fish species, the deepwater redfish, *Sebastes mentella,* the gilthead seabream, *Sparus aurata* and turbot, *Scophthalmus maximus,* 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 a pH range from 9.5 to 10.0. The temperature at which both enzymes displayed maximum activity ranged from 35 ˚C to 40 ˚C.

Díaz (1999) obtained a 50-fold level of purification for trypsin from pyloric caeca of the red hind grouper, *Epinephelus guttatus*. This study revealed that the red hind trypsin displayed optimal activity at a pH range of 6 to 8. Activity was also found to be temperature-dependent, with highest catalysis occurring at 40 ˚C. The enzyme had a molecular weight in the range of 17- 44 kDa and 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*. This study showed that the queen snapper trypsin displayed optimal activity in a pH range of 8 to 9. The temperature-dependent activity was highest at 50 ˚C for pyloric caeca trypsin and 60 ˚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 – 44 kDa. The enzyme was inhibited by soybean trypsin inhibitor.

Beirao et al. (2001) purified and characterized a trypsin like-enzyme from the pyloric caeca of cod through affinity chromatography utilizing CHOM sepharose 4B. In this work the enzyme displayed catalytic activity upon TAME yet was inhibited by the presence of serine protease inhibitors. The cod trypsin displayed isoelectric points of 5.3 and 5.89 and was found to have a similar amino acid composition to that of bovine trypsin.

Rivera (2003) obtained a 10-fold level of purification for trypsin from intestinal and pyloric caecal tissues of the silk snapper, *Lutjanus vivanus*. This trypsin displayed optimal activity at pH 8 and a temperature of 60 ˚C. The SDS-Page analysis revealed a single band with an estimated molecular weight of 26.1 kDa. The enzyme was inhibited by soybean trypsin inhibitor.

The molecular weights, pH optima, and temperature optima for trypsin activities from all of the fish species referred to above are summarized in Table 1, Table 2, and Table 3, respectively. Four broad fish trypsin characteristics can be gleaned from this literature review: 1) trypsin is a common digestive enzyme that can be expected to occur in digestive tissues of virtually all fish species; 2) trypsin is most active at a pH of approximately 8.0; 3) trypsin has a molecular weight in the range of approximately 20 – 30 kDa; and 4) trypsin may be readily characterized from tissues.

Very little previous research has focused on the protein digestive biochemistry/physiology of the white grunt. A notable exception to this is the study undertaken by Smith (1967), in which peptide cleavage of gelatin fed to aquarium-raised white grunts was observed, suggesting the presence of a tryspin activity. In an effort to better understand proteolytic biochemistry and associated physiology in this species, which is becoming increasingly important as a commercial food fish in Puerto Rico, this investigation seeked to purify white grunt trypsin and subsequently determine both its kinetic characteristics and molecular weight.

Table 1: Molecular weights of trypsins from various fish species

Fish	Molecular weight (kDa)	Autor
Cod, *Gadus morhua*	18 – 22	Overnell, 1973
African lungfish, *Protopterus aethiopicus*	24	Reeck et al., 1970
Carp, *Cyprinus carpio*	25	Cohen et al., 1981
Mullet, *Mugil cephalus*	24	Guizani et al., 1991
Anchovy, *Engraulis encrasicholus*	27 – 28	Martínez et al.,1988
Anchovy, *Engraulis japonicus*	25.6	Heu et al., 1995
Atlantic salmon, *Salmo salar*	25	Outzen et al., 1996
Silk snapper, *Lutjanus vivanus*	26.1	Rivera, 2003

* The range of molecular weight is 18-28 kDa.

Table 2. pH values at which optimal activity was obtained for trypsins of various fish species

Fish	pH	Author
Chinook salmon, *Onchorhynchus tshawytscha*	9.0	Bradford, 1960
African lungfish, *Protopterus aethiopicus*	8.0	Reeck et al., 1972
Flatfish species, *Limanda yokohamae*, *Kareius bicoloratus*, and *Paralichtys olivaceus*.	8.0	Yasunaga, 1972
Cod, *Gadus morhua*	8.0 to 9.0	Overnell,1973
Bonefish, *Carassius auratus*	9.0	Klaus-Dieter,1976
Marine flatfish, *Solea solea*	8.0	Clark et al., 1985
Sharptooth catfish, *Clarias gariepinus*	8.2	Uys and Hecht, 1987
Anchovy, *Engraulis encrasicholus*	8.0 to 9.0	Martinez et al.,1988
Mullet, *Mugil cephalus*	8.0	Guizani et al., 1991
Anchovy, *Engraulis japonicus*	8.0	Heu et al., 1995
Carnivorous fish species, *Sebastes mentella, Sparus aurata* and *Scophthalmus maximus*	9.5 to 10.0	Munilla-Morán and Saborido-Rey, 1996
Red hind grouper, *Epinephelus guttatus*	7.0 to 8.0	Díaz, 1999
Silk snapper, *Lujanus vivanus*	8.0	Rivera, 2003

* The range of optimal pH for typsin activity is 7-10.

Table 3. Temperatures at which optimal activity was obtained for trypsins from various fish species

Fish	Temperature (ºC)	Author
Chinook salmon, *Onchorhynchus tshawytscha*	49	Bradford, 1960
Flatfish species, *Limanda yokohamae*, *Kareius bicoloratus*, and *Paralichtys olivaceus*.	40	Yasunaga, 1972
Bonefish, *Carassius auratus*	30 to 50	Klaus-Dieter, 1976
Sharptooth catfish, *Clarias gariepinus*	30 to 40	Uys and Hecht, 1987
Mullet, *Mugil cephalus*	55	Guizani et al., 1991
Anchovy, *Engraulis japonicus*	45	Heu et al., 1995
Carnivorous fish species, *Sebastes mentella, Sparus aurata* and *Scophthalmus maximus*	35 to 40	Munilla-Morán and Saborido-Rey, 1996
Red hind grouper, *Epinephelus guttatus*.	40	Díaz, 1999
Silk snapper, *Lujanus vivanus*	60	Rivera, 2003

* The range of optimal temperature for typsin activity is 30-60 ºC.

MATERIALS AND METHODS

Fish Organs

A total of 72 white grunt, *Haemulon plumierii,* intestines with attached pyloric caeca were obtained from commercial fishermen, who had earlier that day captured the fish using single and multiple handlines. Within one hour of their capture, the fish were gutted by the fishermen and the digestive organs, including intestines and pyloric caeca, were placed in plastic bags and maintained on ice. Upon delivery to our laboratory facility, these tissues were kept for up two months in a -20 ^{0}C freezer.

Chemicals

Bovine serum albumin (BSA) and bicinchoninic acid (BCA) reagent were part of a protein quantification kit obtained from Pierce Chemical Company, Rockford, Il.. Trizma base, calcium chloride ($CaCl_2$ * $2H_2O$), ammonium sulphate ($(NH_4)_2SO_4$), NaCl, Tris hydrochloride, HCL, Nα-p-tosyl-inhibitor (soybean trypsin inhibitor or SBTI), glycine, NaOH, N, N, N', N'-tetramethylethylenediamine (TEMED), Nα-p-toluenesulphonyl-L-arginine methyl ester (TAME), medical grade polypropylene, acrylamide, bisacryamide, bromophenol blue, N' N'- bis - methylene-acrylamide|glycerol, glycerol, and brilliant blue R were obtained from Sigma (St. Louis, MO). Gel filtration standards, SDS page gel standards, ion exchange standards, and Bio-Gel P-60-Gel (medium grade) size exclusion chromatography medium were obtained from Bio-Rad Laboratories (Hercules, CA).

Buffers

Trypsin assay solution, containing 0.001 M TAME as substrate, was identical to that described by Hummel (1959). Size exclusion running buffer was 0.1 M Trizma base, pH 7.0. Affinity chromatography buffers/solutions were prepared as suggested by the manufacturer (Amersham-Pharmacia, Piscataway, New Jersey) for use with High Trap Benzamidine columns. Electrophoresis buffers were the same as those described in the instructions for the Mini-Protean 3 Cell gels (Bio-Rad, Hercules, CA).

Tissue Homogenization

Fish organs (intestines and pyloric caeca) were thawed at room temperature for three hours. Intestinal tissues were cleaned via flushing the lumens with a stream of distilled water. All tissues were weighed collectively and then immersed in 454 ml of room temperature distilled water and placed in a blender (osterizer). The blender was run at high speed for two minutes and then low speed for one additional minute. The resulting homogenate was filtered once through gauze to remove cellular debris and excess connective tissue. Its volume was then determined and subsequently stored at 4 ^{o}C.

Crude Homogenate Preparation

The tissue homogenate was distributed into 50 ml Oak Ridge polycarbonate centrifuge tubes (Nalgene Corp, Rochester, NY). The tubes were then placed in a JA-20 Beckman rotor and centrifuged in a J2-21 Beckman Centrifuge set at 20,000 rpm for 30 minutes at 0 ^{o}C. The resulting supernatants were filtered through gauze (to eliminate fatty particles) and pooled. The entire obtained solution, referred to as *crude homogenate,* was then stored at 4 ^{o}C.

Ammonium Sulphate Precipitation Procedures

Crude homogenate (423 ml total volume) was brought to 25 % saturation with ammonium sulphate as described by Deutscher (1990), and subsequently centrifuged at 20, 000 rpm for 30 minutes at 0 ^{o}C. The resulting supernatant was pooled and maintained in a parafilm-covered beaker at 4 ^{o}C. Each centrifuge tube pellet was resuspended in 1 ml of distilled water. These were pooled and the resulting solution was then filtered once though a 5.0 micron filter and, subsequently, once through a 0.45 micron filter. This filtered solution was maintained in a capped plastic tube at 4 ^{o}C. The 25 % supernatant solution was brought to 50 % saturation with ammonium sulphate and treated in a manner identical to that just described so as to produce similar resuspended pellet proteins and supernatant. In a like

manner, the 50 % supernatant solution was subsequently brought to 75 % ammonium sulphate saturation and treated so as to produce resuspended pellet proteins.

Protein Quantification

Protein concentrations of crude homogenate and resuspended pellet solutions were determined using a BCA protein assay kit (Pierce, Rockford, IL) as per the manufacturer's instructions. All readings were obtained with a Pharmacia Ultrospec 4000 computer-assisted spectrophotometer (Pharmacia, Piscataway, NY).

Trypsin Activity Assays

Trypsin activity was determined using the assay described by Hummel (1959) in conjunction with an Amersham-Pharmacia (Piscataway, NY) Ultraspec 4,000 spectrophotometer. Absorbance readings were obtained every 10 seconds at 247nm for a total assay time of 2 minutes. All assays were performed in quartz cuvettes. Assays for determining kinetic aspects of the enzyme routinely used 230 μg of white grunt proteins from pooled size exclusion chromatography fractions of tubes 38, 39, 43, and 44.

In those kinetic assays examining the effect of soybean trypsin inhibitor (SBTI), inhibitor concentration in standard assay solution was one of the following: 1 mg/ml, 0.1 mg/ml or 0.01 mg/ml.

Chromatographic Procedures

Proteins subjected to size exclusion chromatography were those from the pooled, resuspended 50 % ammonium sulfate fraction pellets. Size exclusion chromatographic procedures were performed using a Bio-Rad Econosystem chromatographic system equipped with a 250 ml, 100 cm column loaded with P-60 polyacrylamide gel prepared as per manufacture's instructions (Bio - Rad, Hercules, CA). The absorption wavelength was 280 nm. Flow rate was 0.18 ml/min. Fraction size was 3.0 ml each for 80 total collected fractions obtained with a Bio-Rad 2 128 fraction collector. Running buffer was 0.1 M Trizma Base, pH 7.0.

Proteins subjected to affinity chromatography procedures were those of pooled size exclusion chromatography fractions from tubes 40, 41, and 42. Affinity chromatographic procedure was realized through use of a High Trap Benzamidine 1.0 cm column (Amersham Biosciences) fitted with a Bio-Rad Econosystem peristaltic pump and model 2 128 fraction collection apparatus. The binding and washing buffer characteristics were 0.05 M Tris-HCL, 0.5 M NaCl, pH 7.4. The elution buffer was 0.05 M glycine, pH 3.0. Flow rate was 1ml/min. Prior to their introduction to the affinity column, selected size exclusion chromatography protein fractions were diluted 2.5-fold in a buffer of the following characteristics: 0.06 M Tris-HCl, 0.6 M NaCl, pH 7.4. Before beginning the procedure, all fraction-collecting tubes received 0.2 ml of the following buffer: 1.0 M Tris-HCl, pH 9.0. A total of 45 elution fractions were collected, each containing 1.2 ml total volume (1.0 ml elution buffer + 0.2 ml 1.0 M Tris-HCl, pH 9.0).

Electrophoresis

Proteins analyzed via electrophoresis included those from crude homogenate, 50 % ammonium sulfate resuspended pellets, size exclusion chromatography of pooled contents from fraction tubes 36 and 37, and affinity chromatography pooled contents of fraction tubes 19 and 20. SDS PAGE electrophoresis was performed as per the modified Laemmli method described in the instructions provided with the Ready Gel Precast gel (15 % Tris- HCl, 10 wells, 30 µl) (Bio-Rad, Hercules, CA) that was used in this analysis. Prestained SDS-PAGE molecular weight protein standards (including myosin, ß-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and aprotinin) (Bio-Rad, Hercules, CA) constituted molecular weight markers. Non-prestained, purified porcine pancreatic trypsin (mw 23.8 kDa) (Sigma, St. Louis, MO) was also run by itself as a size standard in a separate gel lane. Proteins were visualized through Coomassie blue staining procedure.

RESULTS

Trypsin Purification Procedure

Table 4 presents the levels of purification obtained from crude homogenate sequentially subjected to ammonium sulfate precipitation fractionation, size exclusion chromatographic procedures, and affinity chromatographic procedures. Highest activity, based upon a mg/min basis (such a basis should also be assumed in all such trypsin activity cases referred to here after), among the resuspended ammonium sulfate pellet proteins was detected in the 50 % fraction. This was a 4.05 fold level of purification over the crude homogenate.

Subjecting the 50 % ammonium sulfate proteins to further purification via size exclusion chromatography resulted in a 7.42 fold trypsin purification over that from 50 % crude homogenate. Highest activity was found in size exclusion chromatography fraction tube numbers 40 – 42 (Fig. 2). Thus, these tubes were selected for further purification via affinity chromatography (see below). Tubes 36 and 37 were pooled for later use in gel electrophoretic analysis (see below).

The proteins from pooled size exclusion chromatography fraction tubes 40 – 42 were subjected to affinity chromatographic analysis and resulted in a 11.26 level of purification over crude homogenate. This was obtained in tubes 19 and 20 (Fig. 3).

Trypsin Molecular Weight Determination via SDS PAGE Electrophoresis

Figure 4 presents the SDS-PAGE electrophoretic analysis results for each of the following: protein size standards (lanes 1 and 7), purified porcine pancreatic trypsin (lane 2), crude homogenate proteins (lane 3), 50 % ammonium sulfate precipitate resuspended pellet proteins (lane 4), proteins obtained in fraction tubes 40 – 42 of the size exclusión chromatographic procedure (lane 5), and proteins obtained in fraction tubes 19 and 20 of the affinity chromatographic procedure (lane 6). It is notable that lane 6, containing proteins representing the highest level of trypsin purification obtained in this study, revealed a single band that corresponds very closely to the single 23.8 kDa band produced in lane 2 for purified, porcine pancreatic trypsin as well as the 23 kDa molecular weight band for soybean trypsin inhibitor size standard (lanes 1 and 7). These findings indicate that the lane six band, the purified trypsin itself, indicates an aproximate molecular weight of 24 kDa.

Kinetic Analysis Results

The pH optimum for the white grunt trypsin was determined to be 8.0 (Figure 5). Lower levels of activity were detected at pH levels of 4.0, 6.0, and 10.0. The enzyme was inactive at pH 2.0 and 12.0.

The results of temperature effect upon white grunt trypsin activity are shown in Figure 6. Highest trypsin activity was observed at 40 ^{o}C. Above 40 ^{o}C, trypsin activity decreased continuously at 10 ^{o}C intervals until no activity was detected at 70 ^{o}C. Similarly, levels of trypsin activity at temperatures below 40 ^{o}C also decreased continuously until no catalysis was detected at 4 ^{o}C.

Figure 7 shows the results of soybean trypsin inhibitor effect upon TAME hydrolysis via purified white grunt trypsin. The soybean trypsin inhibitor (SBTI) caused a decrease in enzyme activity with a corresponding increase in inhibitor concentration until no trypsin activity was detected at a 1 mg/ml inhibitor concentration.

Table 4: Purification of trypsin from intestinal and pyloric caecal tissues of the white grunt, *Haemulon plumierii,* (Lacepède, 1801)

Protein source	Trypsin Activity (Δ abs/mg/min)	Level of Purification (fold)
Crude Homogenate	0.15	1
25 % Resuspended Pellets	0.06	0.39
50 % Resuspended Pellets	0.61	4.05
75 % Resuspended Pellets	0.29	1.93
Size Exclusion Cromatography of 50 % Resuspended Pellets (Fractions 40-42)	1.12	7.42
Affinity Cromatography of 50 % Resuspended Pellets (Fractions 19-20)	1.70	11.26

Figure 2. Trypsin activity detected in size exclusion fractions (3 ml/fraction)

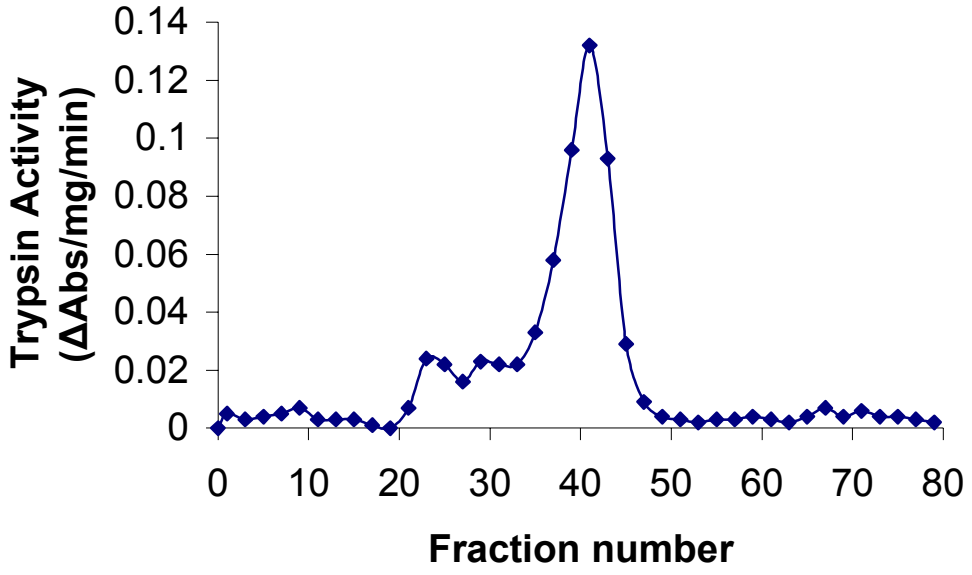


Figure 3. Trypsin activity detected in affinity chromatography fractions

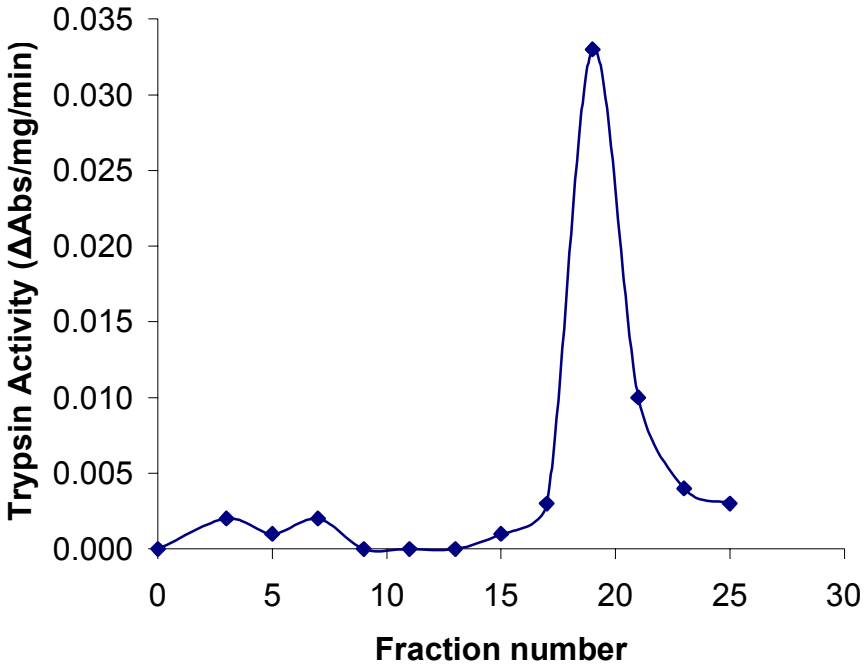


Figure 4. SDS-PAGE electrophoresis of proteins. The electrophoresis shown is from the following sources: BioRad size standards (lanes 1 and 7), purified porcine pancreatic trypsin (lane 2), crude homogenate (lane 3), resuspended pellet from 50 % ammonium sulfate fractionation procedure (lane 4), pooled size exclusion chromatography fractions 36 and 37 (lane 5), and pooled affinity chromatography fractions 19 and 20 (lane 6). Numbers across the top indicate gel lanes. Numbers along the left hand side of gel indicate molecular weight (in kDa) of BioRad size standard proteins

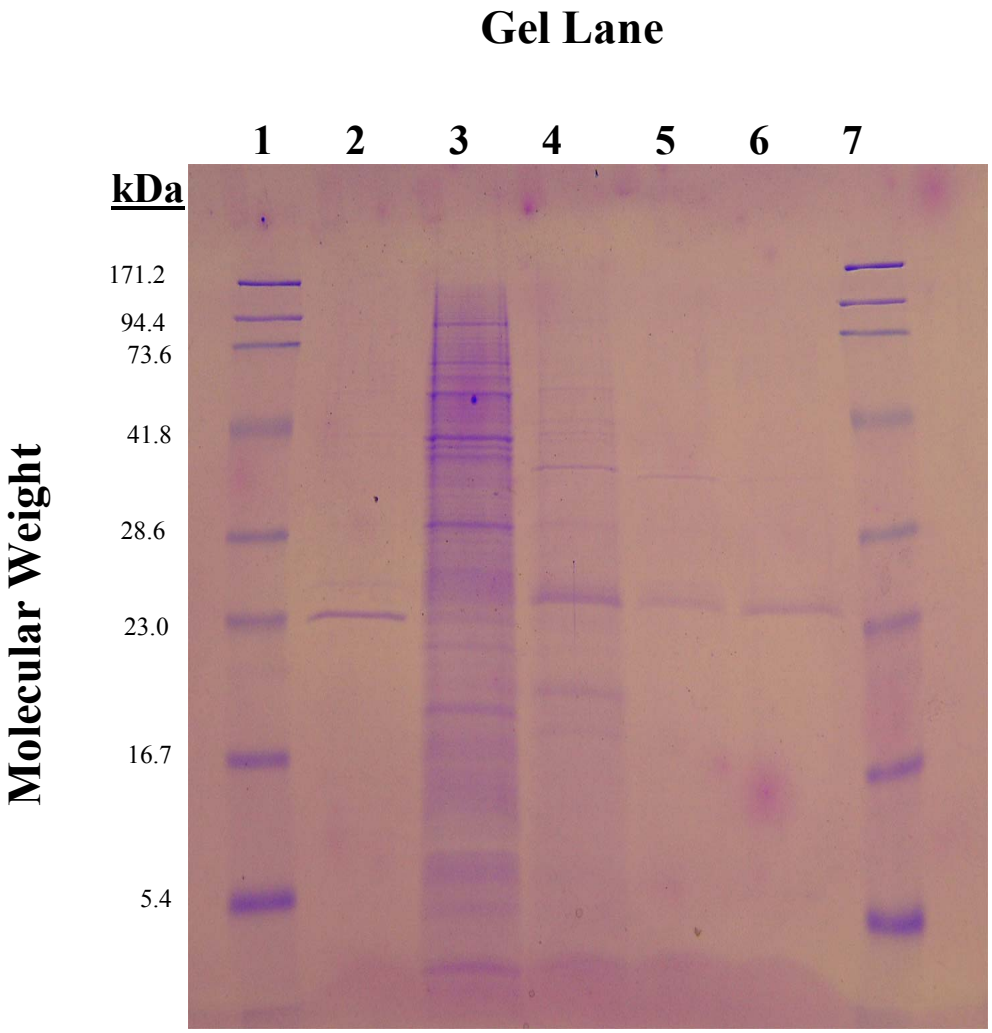


Figure 5. Effect of pH effect upon white grunt trypsin activity

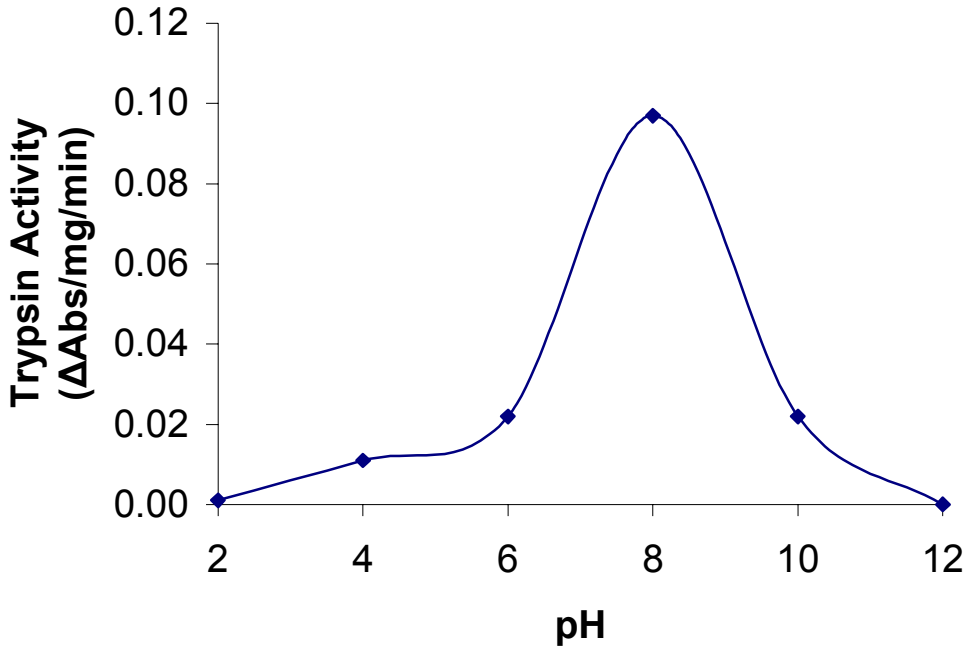


Figure 6. Effect of temperature upon white grunt trypsin activity

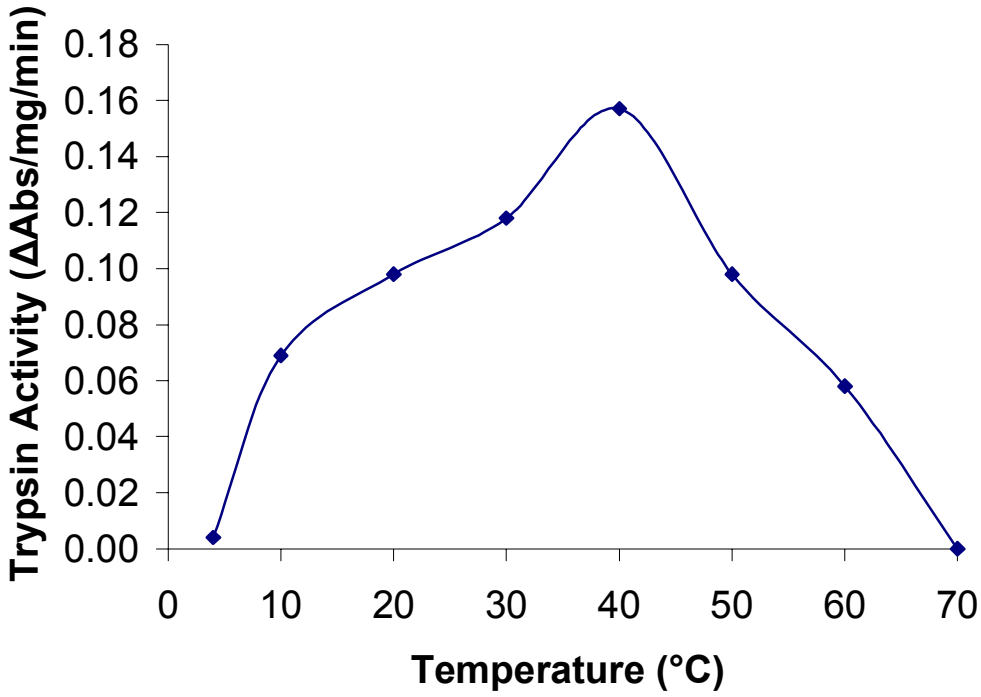
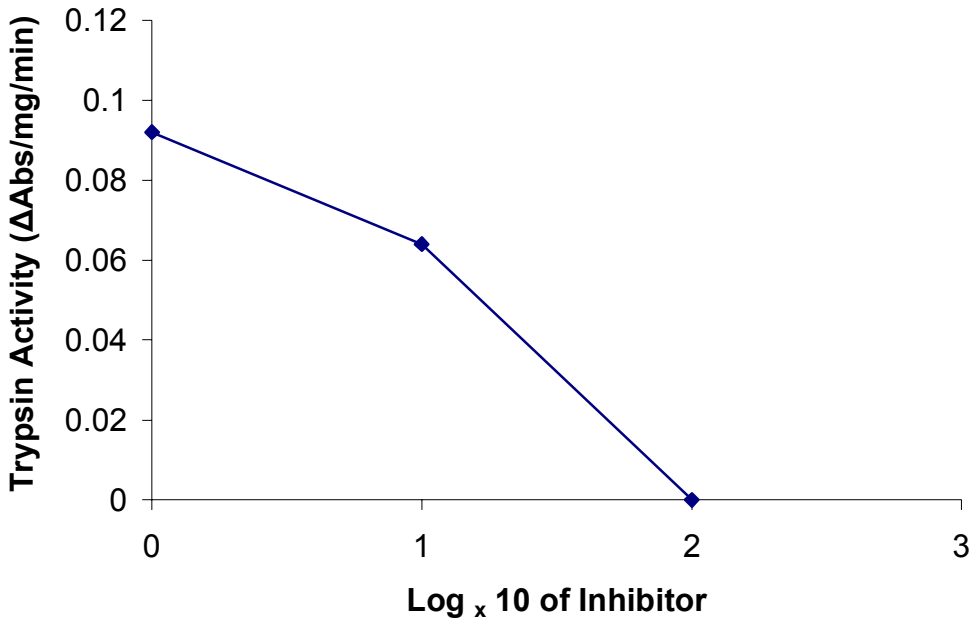


Figure 7. Effect of soybean inhibitor upon white grunt trypsin activity



DISCUSSION

The data collected in this work confirms Smith's results as a trypsin with the following characteristics was identified: 1) molecular weight of 24 kDa; 2) pH optimum of 8.0; 3) temperature optimum of 40 ^{o}C; 4) complete inhibition in the presence of a 100% SBTI solution containing a standard assay amount of the trypsin-specific substrate TAME.

The molecular weight of white grunt trypsin of approximately 24 kDa is expected in view of similar or identical molecular weights determined for trypsins of other fish species. This point is made clear by Table 1, which lists the molecular weights of trypsins from 9 different fish species. The molecular weight range of these trypsins is 18-28 kDa. It can be concluded that the determined 24 kDa molecular weight for white grunt trypsin is in the range of that reported for other fish species.

The optimal pH of 8.0 for white grunt trypsin activity is very close to that of 14 different fish species that have been similarly investigated (Table 2). The pH range for optimal trypsin activity is 7-10. Again, it can be noted that this is another, significant piece of data that supports the white grunt trypsin being similar that of other fishes.

The temperature at which the highest trypsin activity was obtained for the white grunt was 40 ˚C. Table 3 presents the temperature reported for optimal trypsin activity in 9 different fish species. The range of optimal temperature for these species was 30-60 ˚C. The 40 ˚C optimum for white grunt trypsin activity is between these values and supports the assertion that this species' trypsin is similar to that of other fish trypsins previously studied.

The inhibition of TAME hydrolysis obtained through the presence of soybean trypsin inhibitor show that the purified protein is an actual trypsin. This inhibitor has similarly been applied to blocking the activity of other fish trypsins, including those from chinook salmon, *Oncorhyncus tswawytcha* (Bradford, 1960; Dimes et al, 1994), rainbow trout, *Oncorhyncus mykiss* and coho salmon, *Oncorhyncus kisutch* (Dimes et al., 1994), mullet, *Mugil cephalus* (Guizani et al, 1991), grouper, *Epinephelus guttatus* (Díaz, 1999), queen snapper, *Etelis oculatus* (Quiñones, 2000), cod, *Gadus morhua* (Beirao et al, 2001), and silk snapper, *Lutjanus vivanus* (Rivera, 2003). Thus, the inhibition results obtained for the white grunt trypsin are similar to those reported for other fish species.

Horn (1998) observed that fish protease activity is very much influenced by the protein content present in ingested food items, with greater levels of protein correlating with greater levels of protease secretion and activity. Therefore, studies that link protein content of white grunt feeds with levels of trypsin/protease activities and related biomass production are of interest to better understanding the protein digestive biochemistry/physiology of the white grunt. In the event that future aquaculture practices involve the raising of white grunt, such studies could be of considerable interest. Presently, it can be summarized that the kinetic characteristics of the white grunt trypsin are very similar to those for fish trypsins in general. Future investigations aimed at elucidating its amino acid sequence, the nucleic acid sequence of the white grunt trypsin gene, and factors regulating its secretion into the gut lumen will help to deepen our understanding of the role played by trypsin in enabling fishes to meet their requirements for dietary amino acids.

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