UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
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PEPTIDASES DIGESTIVAS DO PEIXE BIJUPIRÁ (*Rachycentron canadum*) SELVAGENS E CULTIVADOS.

WERLAYNE MENDES DE SANTANA

Recife
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Dissertação apresentada para o cumprimento parcial das exigências para a obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Pernambuco (UFPE).

Orientador: Prof. Dr. Ranilson de Souza Bezerra
Co-orientador: Prof. Dr. Luiz Bezerra de Carvalho Júnior

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Esta dissertação foi julgada para a obtenção do título de Mestre em Ciências Biológicas e aprovada em 23/02/11 pelo Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco em sua forma final.

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RESUMO

Peptidases dos cecos pilóricos de bijupirás selvagens e cultivados (*Rachycentron canadum*) foram caracterizados utilizando substratos específicos de tripsina e quimotripsina, inibidores e íons metálicos. Além disso, o perfil protéico foi determinado empregando SDS-PAGE e zimogramas. Para tanto pH ótimo e temperatura dos bijupirás selvagens e cultivados foi 7,0-10,0 e 40-60 ° C para a tripsina, como 7,0-9,5 e 40-55 ° C para quimotripsina. A tripsina e quimotripsina foram estáveis por 30 min a 50 ° C. O perfil eletroforetíco e zimograma foram similares para bijupirás selvagens e cultivados com faixas que variam de 195 kDa a 6 kDa. A atividade da enzima (BApNA como substrato) foi fortemente inibida por benzamidina e TLCK, sintéticos inibidores de tripsina, onde, como, a atividade de quimotripsina (SApNA como substrato) foi ligeiramente inibido por um inibidor de quimotripsina específico (TPCK). O uso de substratos e inibidores específicos sugerem a presença de tripsina e quimotripsina no cecos pilóricos de ambos animais selvagens e cultivados (*Rachycentron canadum*). Além disso, observou-se que os zimogramas dos animais selvagens e cultivados foram semelhantes, sugerindo que peptidases digestiva não foram influenciadas pela dieta artificial empregada e nem pela gestão de cultivo.

Palavras-chave: *Rachycentron canadum*, bijupirá, piscicultura marinha, peptidases, tripsina e quimotripsina.

ABSTRACT

Peptidases from the pyloric caeca of wild and farmed cobia (*Rachycentron canadum*) were characterized using specific trypsin and chymotrypsin substrates, inhibitors and metal ions. Also the protein profile was determined employing SDS-PAGE and zymograms. To both wild and farmed cobia the optimum pH and temperature ranges from 7.0 to 10.0 and from 40 to 60 °C for trypsin, where as, from 7.0 to 9.5 and from 40 to 55 °C for chymotrypsin. The trypsin and chymotrypsin were stable for 30 min at 50°C. The SDS-PAGE and zymogram profiles were similar for wild and farmed cobia with bands ranging from 195 kDa to 6 kDa. The enzyme activity (BApNA as substrate) was strongly inhibited by benzamidine and TLCK, synthetic trypsin inhibitors, where as, the chymotrypsin activity (SApNA as substrate) was slightly inhibited by a specific chymotrypsin inhibitor (TPCK). The use of specific substrates and inhibitors suggest the presence of trypsin and chymotrypsin in the pyloric caeca of both wild and farmed cobia (*Rachycentron canadum*). Also, it was observed in the zymograms for wild and farmed animals were similar, suggesting that digestive peptidases were not influenced by both the employed artificial diet and the management of cultivation.

Keywords: *Rachycentron canadum*, cobia, marine fish, peptidases, trypsin and chymotrypsin.

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LISTA DE ABREVIAÇÕES

BApNA – *N*α-benzoyl-DL-arginine-*p*-nitroaniline

FAO – Food and Agriculture Organization

IUBMB – International Union of Biochemistry and Molecular Biology

PMSF – fluoreto de fenil-metil-sulfonil

SApNA – N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide

SDS – sódio dodecil sulfato

SDS-PAGE – eletroforese em gel de poliacrilamida utilizando SDS

SEAP/PR – Secretaria Especial de Aqüicultura e Pesca/Paraná.

TPCK – tosil - fenilalanina clorometil cetona

TLCK – tosil-lisina clorometil cetona

SUMÁRIO

1. INTRODUÇÃO

O cultivo racional de organismos aquáticos mais conhecido como aquicultura converteu-se em uma atividade consolidada capaz de abastecer a incessante demanda por produtos pesqueiros frente à estagnação e estancamento das capturas observado desde o final da década de 80. A aquicultura é uma das alternativas mais viáveis para a produção de alimento para consumo humano que apresenta alto valor protéico. De acordo com dados FAO (2008) foram produzidos 159 milhões de toneladas de pescado das quais 93 milhões são oriundas da pesca e 66 milhões da aquicultura.

Desde a metade da década de 50, a produção da aquicultura está concentrada principalmente nos países em desenvolvimento. Esta situação ficou mais acentuada durante a década de 80, quando a contribuição dos países desenvolvidos na produção aquícola mundial se reduziu ainda mais. O Brasil assumiu a segunda posição entre os produtores latinos americanos a partir de 1999, quando ocorreu uma diminuição brusca na produção aquícola do Equador em virtude de problemas sanitários que dizimaram a carcinicultura equatoriana (SEAP/PR 2005).

O Brasil possui condições extremamente favoráveis para o desenvolvimento da aquicultura por apresentar 8,4 mil quilômetros de costa marítima, mão de obra especializada, terra e clima extremamente favoráveis para o crescimento e desenvolvimento de organismos aquáticos, além de inúmeras espécies nativas com potencial para o cultivo.

Dentre várias espécies de peixe marinho disponíveis para o cultivo, o bijupirá (*Rachycentron canadum*) é uma espécie marinha com a maior possibilidade de alavancar a piscicultura marinha no Brasil, pois apresenta interessantes aspectos zootécnicos e econômicos (LIAO *et al*., 2004; BENETTI *et al*., 2007). Possui uma carne branca, consistente e saborosa muito apreciada no mercado asiático, rica em ômega 3, ômega 6, taurina, ornitina, vitamina E e outros componentes nutricionais essenciais para uma vida saudável. Além do mais é reconhecida como uma excelente espécie para a aquicultura por apresentar um rápido crescimento em cativeiro, podendo alcançar de 6 a 8 kg em um ano (CHANG, 2003; PEREGRINO JR. 2009). Estas características atrativas estimulam o interesse ao estudo da espécie, para isso, faz-se necessário investigar dados sobre a sua biologia e fisiologia digestiva (BROWN-PETERSON *et al*., 2001). A falta de informações referentes a estes dados na literatura serve de incentivo para o desenvolvimento de estudo que subsidiará o conhecimento do efeito do processo de domesticação sobre as proteases digestivas.

Estudos relacionados às enzimas digestivas vêm sendo realizados em peixes de importância para a aquicultura como a tilápia nilótica (*Oreochromis niloticus*) (JUN-SHENG *et al*., 2006), o tambaqui (*Colossoma macropomum*) (ALMEIDA *et al*., 2006), a carpa comum (*Cyprinus carpio*) (YANBO *et al*., 2006) de forma a maximizar o desempenho do cultivo no Brasil e no mundo (BEZERRA *et al*., 2005).

As enzimas digestivas são estudadas como uma maneira de compreender as exigências nutricionais, o efeito dos constituintes da dieta e a capacidade das espécies em utilizar os nutrientes de determinados alimentos a fim de otimizar o seu fornecimento (MAMUN *et al*., 2007). O entendimento do mecanismo de atuação enzimática é importante para a elaboração de uma ração com os componentes adequados para o cultivo da espécie alvo (FERNÁNDEZ *et al*., 1997; MUHLIA-ALMAZÁN *et al*., 2003).

Em animais aquáticos a tripsina, a quimotripsina e a elastase compreendem entre os teleósteos as proteases do trato digestivo mais citadas e estudadas (BEZERRA *et al*., 2000). De acordo com Hjelmeland & Jorgensen (1985), a tripsina apresenta-se como enzima-chave na regulação e no processo digestivo em peixes e esta enzima tem sido isolada, purificada e caracterizada dos cecos pilóricos de espécies como: tambaqui (*Colossoma macropomum*) (MARCUSCHI *et al.* 2010), salmão (*Oncorhynchus masou*) (KANNO *et al*. 2009), *Lutjanus vitta* (KHANTAPHANT *et al.* 2010) entre outros.

A habilidade na utilização dos ingredientes alimentares depende da capacidade do animal em utilizar o arsenal enzimático presente no seu trato digestivo, sendo este um fator limitante para o processo de digestão química (CORRÊA, *et al*., 2007). Com o auxilio das proteases, as proteínas adquiridas nas dietas são degradadas até que seus peptídeos e aminoácidos constituintes possam ser utilizados para a síntese de novas proteínas que contribuíram para o desenvolvimento e saúde dos animais (BERG *et al*. 2004).

Os gastos com alimentação na aquicultura pode representar cerca de 50 a 80% do total da produção e para que se otimize a relação custo/benefício é necessário considerar os aspectos qualitativos e quantitativos da alimentação. A matéria-prima utilizada nas formulações de rações implica em altos custos, por isso o interesse nas pesquisas tem sido cada vez mais direcionado a fontes protéicas alternativas que visa aumentar a digestibilidade dos alimentos e o desempenho dos animais (CAVERO, 2004).

Tendo em vista a aprovação de um projeto para criação de pescado em alto-mar com bijupirá em Pernambuco e, estando este em fase inicial no Brasil, com as matrizes sendo capturadas do meio ambiente, e assim em processo de domesticação, surge a necessidade de investimento em pesquisas científicas que tenham como foco a racionalidade de seu cultivo. Considerando ser a alimentação um dos itens de maior custo em sistemas de cultivo, o conhecimento da fisiologia digestiva da espécie em estudo poderá contribuir para o desenvolvimento de manejos adequados visando à diminuição de custos, possibilitando maior produtividade e uma sustentabilidade econômica e ambiental.

Portanto, a caracterização das enzimas digestivas de bijupirás selvagens e cultivados representa um estudo básico que subsidiará o conhecimento do efeito do processo de domesticação

sobre as peptidases digestivas, enzimas chave no processo de absorção da proteína da dieta. Sendo assim, esse trabalho será uma referência para futuros estudos de digestibilidade e nutrição, que contribuirão para o desenvolvimento de rações mais apropriadas no mercado

2. OBJETIVO

2.1. GERAL

- Caracterizar as peptidases dos cecos pilóricos do bijupirá (*Rachycentron canadum*) de espécimes selvagens e cultivados, visando à contribuição para o conhecimento da fisiologia digestiva da espécie.

2.2. ESPECÍFICOS

- Definir as propriedades físico-químicas das proteases alcalinas, utilizando substratos específicos e comparar os dados obtidos entre os diferentes sistemas de cultivo;
- Caracterizar as enzimas digestivas utilizando SDS-PAGE e zimograma;
- Avaliar o efeito de íons e inibidores sobre as enzimas caracterizadas.

3. REVISÃO DE LITERATURA

3.1. A ESPÉCIE

Rachycentron canadum (Figura 1) (LINNAEUS, 1766), espécie nativa do litoral brasileiro, conhecido na região nordeste pelos pescadores como cação de escamas, bijupirá, beijupirá e/ou pirambijú (CARVALHO FILHO, 1999). Sendo este, o único representante da família Rachycentridae. O bijupirá está classificado taxonomicamente da seguinte forma segundo Kaup (1826):

REINO A*nnimalia*
FILO *Chordata*
SUBFILO V*ertebrata*
SUPERCLASSE *Gnathostomata*
CLASSE *Osteichthyes*
SUPERORDEM *Acanthopterygii*
ORDEM *Perciformes*
SUBORDEM *Percoidei*
FAMILIA *Rachycentridae*
GÊNERO *Rachycentron*
ESPÉCIE *Rachycentron canadum,* Linnaeus 1766.

Figura 1. Bijupirá, *Rachycentron canadum*. (Fonte:http://www.fishbase.org).

O bijupirá é um peixe nerítico, epipelágico, de hábito natatório ativo devido à ausência da vesícula gasosa, e que apresenta comportamento migratório. Possui um achatamento na parte anterior do corpo, abrangendo, principalmente, a cabeça, e uma coloração amarronzada, com duas faixas longitudinais, de coloração prata bem definidas nos flancos. A espécie normalmente ocorre em grupos pequenos ou isolados, sendo comumente encontrado em associação a várias estruturas no

mar (bóias, naufrágios, recifes artificiais, etc.) ou com animais de grande porte (tubarões, tartarugas e raias) (HAMMOND *et al*., 1977; SHAFFER & NAKAMURA, 1989).

De hábito alimentar carnívoro, reflexo de sua exigência por proteínas, alimenta-se de zoobentos e o nécton constituídos principalmente por peixes, caranguejos e moluscos bivalves e durante as fases larvais, sua dieta é composta principalmente por copépodos (SHAFFER & NAKAMURA, 1989). Estudos realizados na costa pernambucana apontam como principais itens alimentares dessa espécie peixes ósseos de pequeno porte, como xirás (*Haemulon aurolineatum*), mariquitas (*Holocentrus adscensionis)*, sóias (*Bothus ocellatus)* e saramunetes (*Pseudupeneus maculatus*) (PEREGRINO JR., 2005).

A espécie distribui-se amplamente em águas tropicais e subtropicais de todos os oceanos, com exceção da porção leste do Oceano Pacífico (Figura 2) (SHAFFER & NAKAMURA, 1989), no Brasil ele ocorre ao longo de toda costa marítima, sendo mais abundante em águas tropicais (FIGUEIREDO & MENEZES, 1980). É um peixe de grande porte, pelágico e pode atingir até 60 kg e mais de 2 m de comprimento.

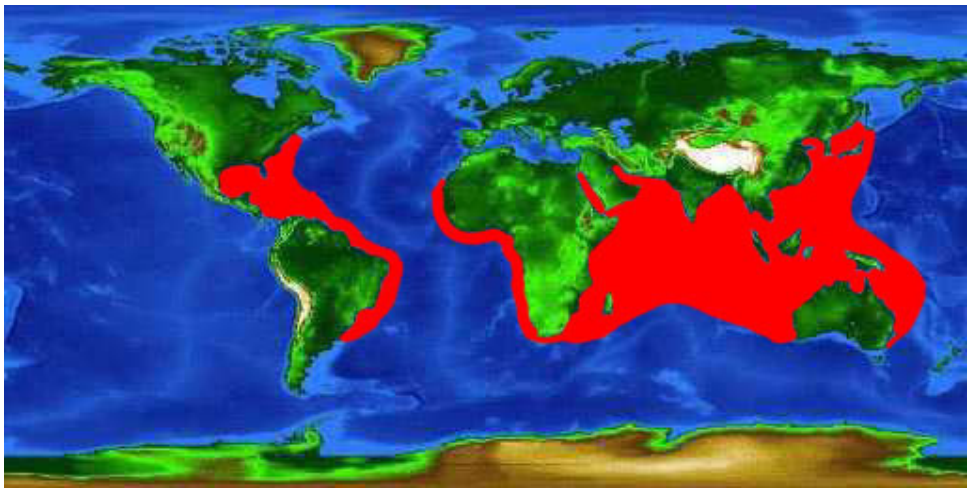

Figura 2. Área de ocorrência (em vermelho) do *Rachycentron canadum* no mundo (Florida Museum of Natural History, EUA).

Segundo a FAO (2009), a captura mundial de bijupirá em 2007 foi de 10.484 toneladas, sendo o Paquistão o maior produtor com 2.253 toneladas, seguido das Filipinas. O Brasil é o quinto produtor mundial, com 898 toneladas. De acordo com Ibama 2008, o bijupirá é capturado com linha-de-mão, covos e rede de emalhar, representando apenas 0,2% da produção pesqueira. O bijupirá é capturado também durante atividades recreativas da pesca submarina, capturas estas que, normalmente, não estão inclusas nas estatísticas oficiais.

A espécie apresenta uma boa adaptação ao confinamento, entretanto, Chou *et al*. (2001) relata que um dos maiores entraves está relacionado na necessidade de desenvolvimento e formulação de dietas específicas para o bijupirá. Existe uma escassez de informações sobre as exigências nutricionais principalmente, em relação aos aminoácidos essenciais, ácidos graxos e outros nutrientes importantes para o sucesso produtivo e viabilidade econômica em nível comercial (CRAIG *et al*., 2006).

3.2. ENZIMAS DIGESTIVAS

As enzimas são biomoléculas protéicas fundamentais que desempenhando o papel de catalisadores, ou seja, de acelerar a velocidade das reações químicas nos organismos, diminuem o nível de energia de ativação das reações (HARVEY *et al.*, 2009).

O estudo das enzimas tem uma grande relevância prática. A indústria biotecnológica produz várias enzimas para diferentes usos. Na indústria, detergentes para na remoção de manchas de origem biológica, em restaurantes para amaciar a carne, em cervejas para eliminar a turvação, na textura do pão, em indústria de luvas para amaciar o couro, é muito importante no diagnóstico de certas doenças, muitas drogas exercem seu poder biológico através de interações com enzimas. Essas moléculas são importantes ferramentas práticas não só na medicina, mas também na indústria química, alimentícia, têxtil, do couro, do papel, na agricultura (MURRAY e*t al.*, 2002; NELSON *et al.*, 2005b; DAMHUS *et al.*, 2008) e na pecuária (SOUZA *et al.*, 2007).

Com a descoberta de grande número de enzimas, houve a necessidade de sistematização da nomenclatura. De acordo com a reação especifica que catalisa, a IUBMB adotou um sistema racional e prático de nomenclatura identificando as enzimas em seis classes (NELSON & COX, 2004) (Tabela 1). Para cada enzima, são atribuídos dois nomes e um número de classificação de quatro dígitos que identificam as classes, as subclasses e as sub-subclasses.

Tabela 1. Classificação das enzimas segundo a IUBMB.

CLASSE	REAÇÕES QUE CATALISAM
1. Oxidorredutases	Reações de oxidação-redução
2. Transferases	Reações de grupos contendo C, N ou P -
3. Hidrolases	Clivagem das reações adicionando água
4. Liases	Clivagem de C-C, C-S e certas ligações de C-N
5. Isomerases	Racemização de isômeros ópticos ou geométricos
6. Ligases	Formação de pontes entre C e O, S, N acoplados a hidrólise de fosfatos de alta energia.

C, carbono; N, nitrogênio; P^-, íon fosfato; S, enxofre; O, oxigênio. Fonte: NELSON & COX, 2004.

Constituindo um dos grupos mais importantes da indústria de enzimas, as peptidases são responsáveis por 50% de toda enzima comercializada (GUPTA *et al*., 2002; RAO *et al*., 1998), além de apresentarem diversas aplicabilidades nas indústrias cosmética, farmacêutica, biotecnologia, entre outras, (BEZERRA *et al*., 2005). São derivadas principalmente de fontes vegetais, animais e microrganismos, enquanto as derivadas de fontes aquáticas, não têm sido muito utilizadas (HAARD & SIMPSON, 1994).

Atualmente, há uma crescente demanda por enzimas proteolíticas de peixes na transformação de alimentos. As vísceras dos peixes, um dos subprodutos mais importantes da indústria pesqueira, têm grande potencial biotecnológico como fonte de enzimas digestivas, especialmente peptidases com alta atividade em uma ampla faixa de pH e condições de temperatura (GILDBERG, 1992; SHAHIDI & JANAK KAMIL 2001), exibem alta atividade catalítica a concentrações relativamente baixas (HAARD, 1998). O processamento destas, considerando suas características especificas está sendo utilizada atualmente para extração de enzimas.

Em animais aquáticos, tripsina, quimotripsina e elastase, que atuam em grupamentos lisina ou arginina, fenilalanina e elastina, respectivamente, compreendem, entre os teleósteos, as proteases do trato digestivo mais citadas e estudadas dessa família (KOLODZIEJSKA & SIKORSKI, 1996; DE VECCHI & COPPES, 1996).

Proteases são enzimas que quebram ligações peptídicas entre os aminoácidos das proteínas, são responsáveis pela digestão de proteínas dos alimentos ingeridos, liberando os aminoácidos essenciais ao crescimento (SÁNCHEZ-PAZ *et al.*, 2003). Dentre as peptidases de maior importância encontram-se a tripsina, a quimotripsina e as aminopeptidases.

A tripsina é uma enzima proteolítica, responsável pela hidrólise de proteínas, é um membro da família das serinoproteases caracterizada por um mecanismo catalítico comum, envolvendo a presença de uma tríade catalítica composta de resíduos específicos: serina, histidina e ácido aspártico. Esta enzima cliva peptídeos no lado carboxila de residuos aminoacidos carregados de lisina e arginina (KLOMKLAO *et al.*, 2007), sendo importantes em muitos processos biológicos como: digestão protéica propriamente dita, ativação de zimogênios e mediação entre a ingestão do alimento e a assimilação dos nutrientes (SAINZ *et al.*, 2004). Destacando também a ampla aplicabilidade industrial de tripsinas (KLEIN *et al.*, 1996). Tais características têm feito das tripsinas as enzimas mais estudadas em organismos aquáticos.

A quimotripsina é uma protease que catalisa a hidrólise de ligações peptídicas. Esta enzima é específica para quebrar ligações adjacentes a resíduos de aminoácidos aromáticos. Ela é formada por três cadeias de polipeptídeos ligados entre si por ligações de dissulfeto. A quimotripsina cliva ligações entre aminoácidos desde que extremidade carboxila do polipeptídio seja um aminoácido aromático, ou seja, triptofano, fenilanina e tirosina e também substratos sintéticos, tais como

SApNA (DE VECCHI et al. 1996; VIPARELLI *et al.*, 2001; ABUIN *et al.*, 2004; CASTILLO-YAÑEZ *et al.*, 2006). A reação catalisada por esta enzima ilustra o principio da estabilização do estado de transição e também fornece um exemplo clássico de catálise ácido-base geral e catálise covalente.

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5. ARTIGO

5.1. PEPTIDASES FROM THE PYLORIC CAECA OF WILD AND FARMED COBIA (*Rachycentron canadum*)

A ser submetido

AQUACULTURE NUTRITION

To be submitted

AQUACULTURE NUTRITION

PEPTIDASES FROM THE PYLORIC CAECA OF WILD AND FARMED COBIA (*Rachycentron canadum*)

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Running Title: Peptidases digestive from Cobia (*Rachycentron Canadum*)

Keyword: *Rachycentron canadum*, marine psiculture, peptidases, trypsin and chymotrypsin.

1. ABSTRACT

Peptidases from the pyloric caeca of wild and farmed cobia (*Rachycentron canadum*) were characterized using specific trypsin and chymotrypsin substrates, inhibitors and metal ions. Also the protein profile was determined employing SDS-PAGE and zymograms. To both wild and farmed cobia the optimum pH and temperature ranges from 7.0 to 10.0 and from 40 to 60 °C for trypsin, where as, from 7.0 to 9.5 and from 40 to 55 °C for chymotrypsin. The trypsin and chymotrypsin were stable for 30 min at 50°C. The SDS-PAGE and caseinolytic zymogram profiles were similar for wild and farmed cobia with bands ranging from 195 kDa to 6 kDa. The enzyme activity (BApNA as substrate) was strongly inhibited by benzamidine and TLCK, synthetic trypsin inhibitors, where as, the chymotrypsin activity (SApNA as substrate) was slightly inhibited by a specific chymotrypsin inhibitor (TPCK). The use of specific substrates and inhibitors suggest the presence of trypsin and chymotrypsin in the pyloric caeca of both wild and farmed cobia (*Rachycentron canadum*). Also, it was observed in the zymograms that the caseinolytic profile for wild and farmed animals were similar, suggesting that digestive peptidases were not influenced by both the employed artificial diet and the management of cultivation.

Keyword: Wild and farmed marine fish, cobia *Rachycentron canadum*, digestive peptidases, trypsin and chymotrypsin.

2. INTRODUCTION

During the last three decades, technological advances in capture fisheries and aquaculture, globalization trends, market and consumer demands have resulted in the continued growth of the production and trading from fish and fisheries products (Shann-Tzong Jiang 2010). Therefore, aquaculture is considered to be the sector of food production further developed in several countries, one of the most effective ways to reduce the deficit of fish in the world market and an important tool for increasing fish stocks (Hiddink et al. 2008).

Brazil has ideal characteristics for the development of aquaculture (skilled labor, appropriate climate, large territory, etc.) but with the challenge of using their potential in a sustainable manner. The Brazilian aquaculture has established itself as a profitable activity for fish production, since different segments of the sector is developing very fast in comparison to Brazilian marine psiculture (Roubach et al. 2003).

Cobia (*Rachycentron canadum*) (Linnaeus, 1766) as is known in Brazil is a specie with great possibility to leverage to marine psiculture in this country because present some interesting economics and husbandry aspects. Since February 2009 was the species chosen for the first crop offshore in Northwest of Brazil (Sanches et al. 2008; Benetti et al. 2003). *Rachycentron canadum*, the only species in the family *Rachycentridae* is a large and migratory marine pelagic fish, with carnivorous feeding habits. This fish is widely distributed worldwide, mainly in tropical and subtropical regions with warm waters with exception of the eastern Pacific Ocean (Shaffer & Nakamura, 1989). In Brazil, the cobia can be found along the coast, but is more abundant in tropical waters. This specie is considered to be a promising candidate for commercial production, since it is a rustic fish, with white flesh disease of good quality, resistant to diseases, fast growing, with ability to spawn in captivity, presenting high fecundity, and great ability to adapt to different conditions of cultivation (especially salinity) (Chen et al. 2009; Benetti et al., 2008).

Several crops of fish are well established around the world, and the number of searches on the biology and physiology of these fish are very significant and ever increasing. The increase in

research regarding the digestive physiologic mechanisms and enzymatic species (especially trypsin and chymotrypsin) generates information that provide the rationale for their crops, respecting the physiological differences between all aquatic animals (Souza et al. 2007; Bezerra et al. 2005; Bezerra et al. 2001).

Trypsin and chymotrypsin are serine endoproteases (EC 3.4.21.x) with proteolytic activity that hydrolyzes peptide bonds (beyond of protein degradation) at the carboxylic end of the amino acid residues arginine (R) and lysine (K). Trypsin is one the main enzyme present in pyloric caeca of fish and presents itself as a key enzyme in the digestion of aquatic organisms (Khantaphant & Benjakul 2010; Klomklao et al. 2007). Fish chymotrypsins are endopeptidases that cleave the peptide bond of proteins on the carboxyl side of phenylalanine, tyrosine, and tryptophan (Castillo-Yañez et al. 2009).

Feed is one of the most costly items in the process of protein creation and one of the noblest ingredients, studies about proteolytic enzymes involved in the digestive process mainly trypsin and chymotrypsin, will contribute to understanding the digestive physiology of the species and form the basis for the preparation of specific diets that promote better nutrient utilization, reflected in better growth and a lower release of pollutants to water resources. Therefore, the aim of this study was to characterize digestive enzymes present in pyloric caeca from cobia and obtain valuable information necessary to enable the rationality of its cultivation especially as regard the use of protein in feed formulation for its be more valuable and expensive ingredient used in diets for aquatic animals.

3. MATERIALS AND METHODS

3.1 REAGENTS

N_{α}-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA), N-succinil-alanine-alanine-alanine *p*-nitroanilide (SApNA), tosyl phenylalanine chlorometyl ketone (TPCK), tosyl lysine chloromethyl ketone (TLCK), phenylmethylsulfonylfluoride (PMSF), benzamidine, soybean trypsin inhibitor (SBTI), were purchased from Sigma Aldrich Sigma-Aldrich Chemical Co. (St. Louis, MO, USA.),

molecular weight standard was purchased from Biorad. All other reagents used in this study were of analytical grade.

3.2 PREPARATION OF CRUDE EXTRACT

Specimens of farmed cobia were kindly provided by Aqualider (Brazil) while wild cobia was captured in open sea. The animals were sacrificed in an ice bath and their pyloric caeca were removed collected and homogenized in 0.1M Tris–HCl pH 8.0 (5 mg/mL). The resulting homogenate was centrifuged (Herolab UniCen MR Centrifuge, Germany) at 10.000 g for 20 min at 4°C to remove cell debris and nucleii. The supernatant (crude extract) was frozen at -20ºC and used for further characterization studies.

3.3 PROTEIN DETERMINATION

Protein concentration of the samples was estimated following the procedure described by Bradford (1976), using bovine serum albumin (BSA) as the standard protein.

3.4 TRYPTIC ACTIVITY ASSAY

Trypsin activity was assayed using a specific substrate N_{α}-benzoyl-DL-arginine p-nitroanilide hydrochloride (BApNA at 8mM) prepared in dimethylsulfoxide (DMSO), according to Bezerra et al. (2001). The release of p-nitroaniline (product) was monitored at λ405nm in a microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA). One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing one mM of BApNA per min ($\varepsilon = 9.100\ M^{-1}\ cm^{-1}$) under laboratories the established conditions. Specific activity was expressed as units per mg of protein.

3.5 CHYMOTRYPTIC ACTIVITY ASSAY

Chymotrypsin activity was assayed using a specific substrate N-succinil-alanine-alanine-alanine *p*-nitroanilide (SApNA at 8mM), prepared in dimethylsulfoxide (DMSO), according to Bezerra et al. (2001). The release of p-nitroaniline (product) was monitored at λ405 nm in a microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA). One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing one mM of SApNA per min ($\varepsilon = 9.100\ M^{-1}\ cm^{-1}$) under the established conditions. Specific activity was expressed as units per mg of protein.

3.6 EFFECT OF pH AND TEMPERATURE

The effect of pH on crude extract was evaluated with BApNA and SApNA at 25°C in a range of 4–11.5 (100 mM citrate–phosphate, Tris–HCl and Glycine–NaOH). The effect of temperature was evaluated in a range of 25-75°C using 0.1M Tris–HCl pH 8.0 as buffer. Thermal stability was evaluated by assaying enzyme activity at 25ºC after pre-incubation for 30 min at temperatures ranging from 25 to 75ºC (Bezerra et al., 2001).

3.7 EFFECT OF SYNTHETIC INHIBITORS AND METAL IONS

The effect of protease inhibitors and metal ions on crude extract from the pyloric caeca of cobia was evaluated using BApNA (8mM), according to Bezerra et al. (2001). The inhibitors used in this assay were prepared in DMSO: 8mM tosyl lysine chloromethyl ketone (TLCK), 8mM phenylmethylsulfonylfluoride (PMSF), 8mM tosyl phenylalanine chloromethyl ketone (TPCK), benzamidine. Samples of the crude extract (30µL) and 0.1 mM Tris-HCl pH 8.0 (115µL) were transferred to a 96-well microtiter plate containing the previous inhibitors (25µL). After incubation for 15 min 8mM BApNA (30µL) was added in the tests on samples and SApNA (30µL) TPCK. The increase in absorbance at λ405nm was monitored using a microtiter plate reader. Controls were performed without the enzyme or substrate solution. The effect of metal ions was assayed using the methods described by Souza et al. (2007) using three different concentrations at 10mM, 5mM and

1mM. Samples of crude extract (30 µL) were added to a 96-well microtiter plate with a solution (70 µL) of $CdCl_2$, NaCl, KCl, LiCl and $CaCl_2$. After 30 min of incubation, Tris-HCl buffer (70 µL), pH 8.0, and 8 mM of BApNA (30 µL) were added. The p-nitroaniline produced was recorded in a microplate reader (Bio-Rad 680) at 405 nm after 30min of reaction.

3.8 ELECTROPHORESIS SDS-PAGE AND ZYMOGRAMS

Proteins from the pyloric caeca of cobia were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (100µg of protein to SDS-PAGE) according to Laemmli (1970) using a 4% (w/v) stacking gel a 12.5% (w/v) separation gel. The gel was stained for protein using Comassie Brilliant Blue R250. Electrophoresis was performed at a constant current of 11 mA per gel, at 4°C.

Zymogram was carried out following the method described by Garcia-Carreño et al. (1993). After electrophoresis (4°C), gels were immersed in 2.5% Triton X-100 in 0.1 M Tris-HCl pH 8.0 to remove SDS. The Triton X-100 was removed by washing the gels three times with 100mL of 0.1M Tris-HCl buffer, pH 8.0 and then incubated with 3% casein (w/v) prepared in 0.1 M Tris-HCl pH 8.0 for 30 min at 4°C. The temperature was raised to 37ºC for 90 min in order to allow the digestion of casein by the active proteinases. Finally, gels were stained for protein using Coomassie Brilliant Blue R250.

3.9. STATISTICAL ANALYSIS

The Microcal Origin 6.0™ software was used for statistical analysis. Data are expressed as mean ± standard deviation. Differences between means (effect of inhibitors and metal ions) were analysed using one-way ANOVA, followed by Tukey's multi-comparison test and considered to be significant when $P<0.05$ (Zar 1984).

4. RESULTS AND DISCUSSION

The aquatic environment contains a wide variety of genetic material representing an enormous potential for discovering different enzymes and main variations have been observed in aquatic animals in relation to molecular weight, amino acid composition, optima pH and temperature, thermal stability, inhibition characteristics and kinetic properties (França et al. 2010; Marcuschi et al. 2010; Espósito et al. 2010; Khantaphant & Benjakul 2010; Espósito et al. 2009; Buarque et al. 2009; Castillo-Yañez et al. 2009; Souza et al. 2007; Bougatef et al. 2007; Bezerra et al. 2005).

For live in different environments and feed of the most diverse sources, the digestive enzymes of aquatic animals have unique characteristics that make them interesting sources of employment in several biotechnological application and sectors such as food industry, textiles, detergents and others, so it is increasingly common studies about the characterization physical-chemical of these enzymes have adaptations that front the conditions that are submitted, process this known and denominated as enzymatic adaptation (Muhlia-Almazán, et al. 2005; Muhlia-Almazán et al. 2003; García-Carreño & Hernández 1996).

Specific tryptic activity present in pyloric caeca from wild and farmed cobia (*R. canadum*) was 1.772 $U.mg^{-1}$ and 2.064 $U.mg^{-1}$ respectively. Bougatef et al. 2007 described a specific trypsin activity from sardine (*Sardina pilchardus*) of 1011.86 $U.mg^{-1}$. In *Tisbe biminiensis* (a marine harpacticoida copepod) specific tryptic activity was 1.77 $U.mg^{-1}$ (França et al. 2010). Espósito et al. (2010) found to lane snapper (*Lutjanus synagris*) a specific tryptic activity of 850.76 $U.mg^{-1}$. These results are variable because different methods in under conditions are employed to determinate and quantify the proteins presents in an extract of different animals that have different feed habits and live in several ecosystems.

Thermal stability was evaluated by assaying enzyme activity at different temperatures (25-75°C) for 30 minutes (Fig. 1A) and showed the same temperature at 50°C for wild and farmed cobia (*R. canadum*) using BApNA as substrate. After 60°C, farmed cobia showed at 66.67% of

residual activity while wild cobia showed 68.17% of residual activity. Thermal stability using SApNA as substrate showed that at 50°C wild cobia kept 88.38% of residual activity while farmed cobia was thermal stable until 50°C with a residual activity of 71.09%.

Please insert Fig. 1A and 1B

These results are similar to other fish reported in literature (Castillo-Yañez et al. 2009; Yang et al. 2009; Ali et al. 2010).

The effect of temperature on tryptic and chymotryptic activity was determined by assaying enzyme activity at different temperatures (Fig. 2). Optima temperature using BApNA as substrate from wild and farmed cobia (*R. canadum*) was 55 and 60°C respectively.

Please insert Fig.2A and 2B

Similar results have been found for trypsin from other fish: *Pseudupeneus maculatus* (55°C) (Souza et al. 2007), *Sardinella aurita* (55°C) (Khaled et al. 2008) and higher than *Balistes capriscus* (40°C) (Jellouli et al. 2009) and *Lutjanus synagris* (45°C) (Espósito et al. 2010), lower than *Colossoma macropomum* that showed optima temperature at 70°C (Marcuschi et al. 2010) and higher than *Cyprinus carpio* that showed optima temperature at 50°C (Espósito et al. 2009). However, using SApNA as substrate, chymotrypsin from wild and farmed cobia (*R. canadum*) showed optima temperature at 50 and 45°C respectively. In relation to chymotryptic activity, several authors have reported similar results for other species of fishes in literature: *Lateolabrax japonicus* (45°C) (Jiang et al. 2010), *Sciaenops ocellatus* (50°C) (Aplebaum et al. 2001), *Lithognathus mormyrus* (50°C) (Ali et al. 2010), these values found in this study was lower than *Sardinella aurita* that showed optima temperature at 60°C (Hayet et al. 2011). These results are variable between aquatic animals demonstrating the potential of study and use of these digestive enzymes with their possible biotechnological applications. Most of the researches on fish chymotrypsins have shown that it is common to find two or more isoforms with similar specific activity (Jiang et al, 2010; Castillo-Yañez et al. 2006) and according to Shahidi & Kamil (2001),

compared to trypsin there are few researches that report the characterization of chymotrypsin from fish.

The effect of pH on tryptic and chymotryptic is illustrated in Fig. 3. Optimum pH was 10.0 and 8.0, 9.5 and 8.0 for wild and farmed cobia using BApNA and SApNA as substrates respectively. Trypsins and chymotrypsins from aquatic animals tend to be more stable at alkaline pH, but are unstable at acidic pH. Thermo stability, effect of temperature, and pH are important parameters to assess the ability of enzymes to support the conformational changes without losing their biological activities that should be elevated for a long periods of time simulating industrial processes.

Please insert Fig.3A and 3B

Proteins profile showed similar eletrophoretic and zymograms behavior with bands from 195 kDa to 6 kDa in wild and farmed cobia with similar standard of caseynolitic bands (Fig. 4).

Please insert Fig.4

Table 1 display the effect of inhibitors on tryptic and chymotryptic activities using BApNA and SApNA as specific substrates.

Please insert Table 1

The trypsin from the pyloric caeca of both wild and farmed cobia (*R. canadum*) was strongly inhibited by TLCK (91.39 and 81.01%), benzamidine (88.07 and 78.04%) which are synthetic inhibitors and PMSF (37.46 and 43.32%) that is a serine protease inhibitor. Chymotrypsin was slightly inhibited by TPCK (a chymotrypsin inhibitor) that showed 4.51 and 14.29% of inhibition.

Table 2 displays the effect of five different ions using three different concentrations on trypsin and chymotrypsin from the pyloric caeca of wild and farmed cobia.

Please insert Table 2

In a concentration at 10mM, trypsin from farmed cobia was sensitive to Cd^{2+} and Li^{+}, activated by Ca^{+} and less inhibitory effect by Na^{+} and K^{+}. At 5mM and 1mM trypsin was less inhibited by Na^{+}, K^{+}, Li^{+} and Ca^{2+} excepted by Cd^{2+} that maintained inhibitory effect. Bezerra et al. (2005) found from *Oreochormis niloticus* strongly inhibitory effect by Cd^{2+} at 10mM. It is known that Cd^{2+} act on sulphhydryl residues in proteins (Male et al. 1995). In all concentrations (10mM, 5mM and 1mM) all metal ions activated the activity of chymotrypsin from farmed cobia. For wild cobia, trypsin was less sensitive by Cd^{2+} at 10, 5 and 1mM and activated by Na^{+}, K^{+}, Li and Ca^{2+}. Chymotrypsin activity from wild cobia was activated by Na^{+}, K^{+}, Li and Ca^{2+} in all concentrations. The effect of Ca^{2+} is very contradictory to be a classical trypsin activator and in some cases, this ion exhibit inhibitory effect in digestive enzymes of aquatic animals (Souza et al. 2007; Espósito et al. 2010; Bezerra et al. 2005).

The presence of these proteolytic enzymes in the pyloric caeca from cobia and its carnivorous nature are important clues to confirm the nutritional requirements through the addition of high levels of dietary protein and for this reason, several studies have been developed that allows to understanding of specific diets for this specie (Webb et al. 2010; Saadiah et al. 2010; Mach et al. 2010).

In this study, the comparison between the physical and chemical properties of the digestive enzymes of animals wild and farmed cobia was not significant, these results may reflect the little knowledge we have about the management of the species.

5. CONCLUSIONS

Trypsin and chymotrypsin were present from the pyloric caeca of cobia and their physical-chemical properties are similar to other species of fish reported in literature. Based in their properties, these enzymes could be used for further studies and their properties could be compared to other enzymes used in food and laundry industries.

6. ACKNOWLEDGMENTS

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8. FIGURE LEGENDS

Figure 1. Thermal stability on tryptic and chymotryptic activities from wild (○) and farmed cobia (*R. canadum*) (■). Enzyme samples were pre-incubated at the indicated temperatures and after 30°C equilibration the activity was assayed (triplicates) using BApNA (A) and SApNA (B) as specific substrates at final concentration of 1.2 mM. The highest activity (100%) was at 50 ºC for both wild and farmed cobia using BApNA and SApNA respectively.

Figure 2. Effect of temperature on tryptic and chymotryptic activities from the pyloric caeca of wild (○) and farmed cobia (*R. canadum*) (■). The activity was evaluated at temperatures ranging from 25ºC to 75ºC using BApNA (A) and SApNA (B) as specific substrates at final concentration of 1.2 mM.

Figure 3. Effects of pH on tryptic and chymotryptic activities from the pyloric caeca of wild (○) and farmed cobia (*R. canadum*) (■). The activities were measured using Tris-HCl, NaOH-glycine and citrate-phosphate as buffers. Final concentration of BApNA (A) and SApNA (B) as specific substrate was 1.2 mM.

Figure 4. Eletrophoretic profile and zymograms of tryptic and chymotryptic activities present in crude extract from the pyloric caeca of wild (○) and farmed cobia (*R. canadum*) (■) using a 4% (w/v) stacking gel, 12.5% (w/v) separating gel for electrophoresis and 10% separating gel for zymogram. Mw – molecular weights standards, 1- wild cobia and 2- farmed cobia.

9. TABLES

Table 1. Effect of inhibitors on trypsin and chymotryptisin activities from the pyloric caeca of cobia (*Rachycentron canadum*)

Inhibitors	Farmed cobia Residual activity ± SD (%)	Wild cobia Residual activity ± SD (%)
PMSF	62.54 ± 1.8[a]	56.68 ± 1.6[a]
TLCK	8.61 ± 3.0[a]	18.99 ± 2.7[a]
Benzamidine	11.93 ± 2.2[a]	21.96 ± 2.3[a]
TPCK (using SApNA as substrate)	95.49 ± 1.7[a]	85.71 ± 1.9[b]

All inhibitors were prepared in DMSO (dimethylsulfoxide) with an initial concentration of 8mM.

Table 2. Effect of five metal ions on trypsin and chymotrypsin activities from the pyloric caeca of cobia (*Rachycentron canadum*)

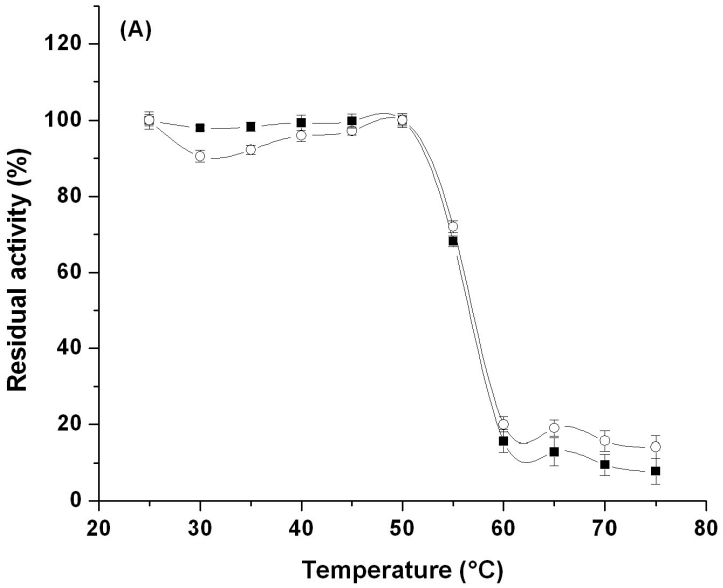
Íon		Farmed cobia		Wild cobia	
		Residual activity ± SD (%)		Residual activity ± SD (%)	
		BApNA	SApNA	BApNA	SApNA
Cd^{2+}	10 mM	37.44 ± 3.6	144.71 ± 1.9	83.10 ± 3.6	111.61 ± 1.5
	5 mM	31.50 ± 3.8	194.93 ± 1.5	76.06 ± 4.8	143.98 ± 1.7
	1 mM	30.40 ± 3.8	222.50 ± 2.4	69.01 ± 3.8	151.96 ± 0.4
Li^{+}	10 mM	66.30 ± 2.8	193.59 ± 1.5	124.41 ± 3.6	125.40 ± 1.8
	5 mM	64.54 ± 3.6	187.33 ± 1.5	112.21 ± 3.6	122.21 ± 2.4
	1 mM	58.15 ± 3.8	171.98 ± 1.0	91.55 ± 4.6	113.06 ± 0.8
K^{+}	10 mM	86.78 ± 4.6	189.57 ± 1.1	143.19 ± 4.8	127.43 ± 1.3
	5 mM	77.09 ± 3.8	184.65 ± 1.7	126.76 ± 2.8	120.03 ± 0.8
	1 mM	62.33 ± 5.6	184.20 ± 1.2	118.31 ± 4.6	115.97 ± 2.5
Na^{+}	10 mM	91.41 ± 4.6	161.25 ± 1.3	141.31 ± 5.6	107.40 ± 1.6
	5 mM	77.31 ± 2.8	149.93 ± 0.9	111.74 ± 4.6	97.68 ± 1.7
	1 mM	55.07 ± 3.6	145.16 ± 0.7	88.73 ± 4.8	92.60 ± 1.3
Ca^{2+}	10 mM	112.78 ± 5.6	205.51 ± 1.9	209.39 ± 4.8	166.18 ± 1.3
	5 mM	94.49 ± 1.8	200.30 ± 2.0	173.24 ± 4.6	162.99 ± 1.1
	1 mM	65.20 ± 4.6	189.27 ± 1.7	126.76 ± 5.9	130.33 ± 1.6

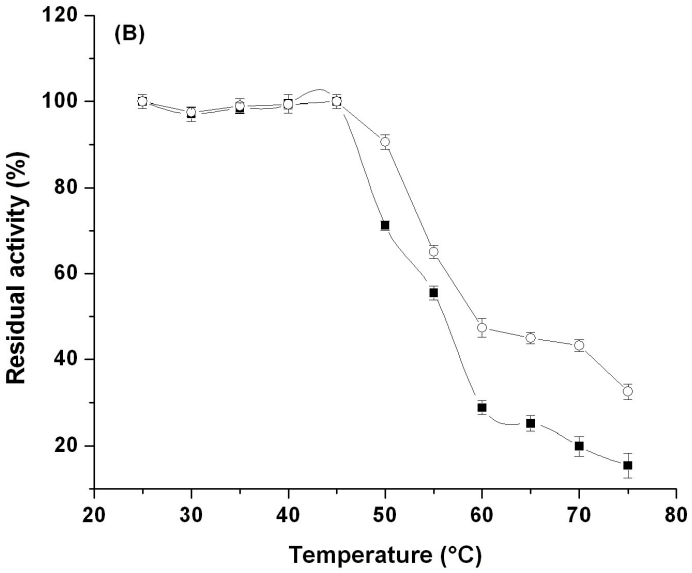
10. FIGURES

PEPTIDASES DIGESTIVE FROM COBIA (*Rachycentron canadum*)

Santana, W.M; França, R. C. P.; Santana, W.M; Carvalho Jr, L. B. and Bezerra, R. S.

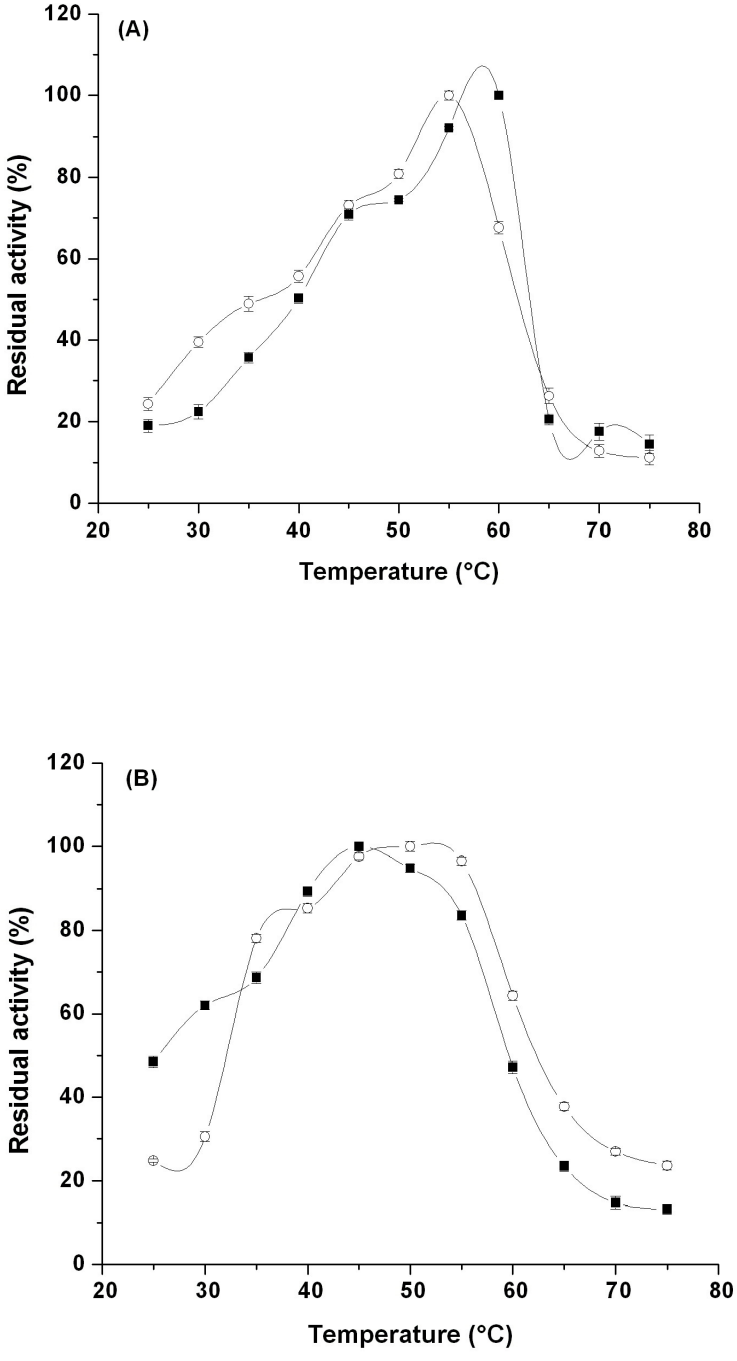
Figure 1. Thermal stability in residual activity (tryptic and chymotryptic) from the pyloric caeca of wild and farmed cobia (*Rachycentron canadum*) using BApNA and SApNA as substrates.





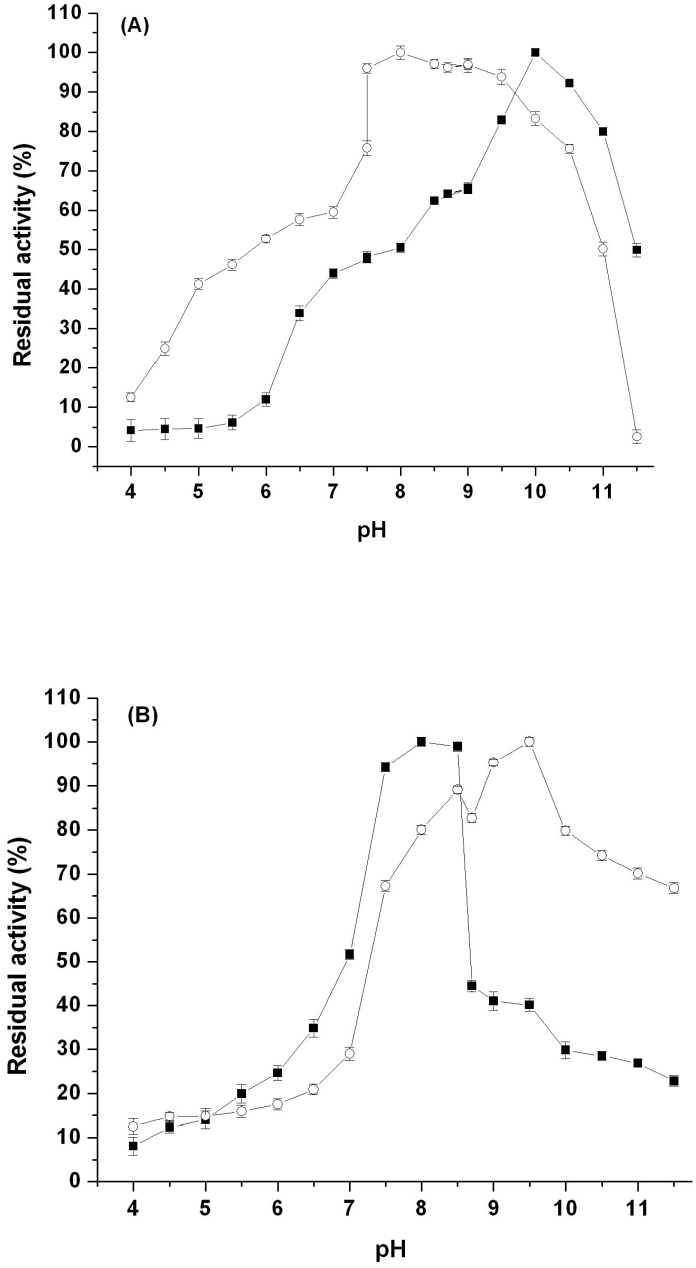
Santana, W.M; França, R. C. P.; Santana, W.M; Carvalho Jr, L. B. and Bezerra, R. S.

Figure 2. Effect of temperature in residual activity (tryptic and chymotryptic) from the pyloric caeca of wild and farmed cobia (*Rachycentron canadum*) using BApNA and SApNA as substrates.



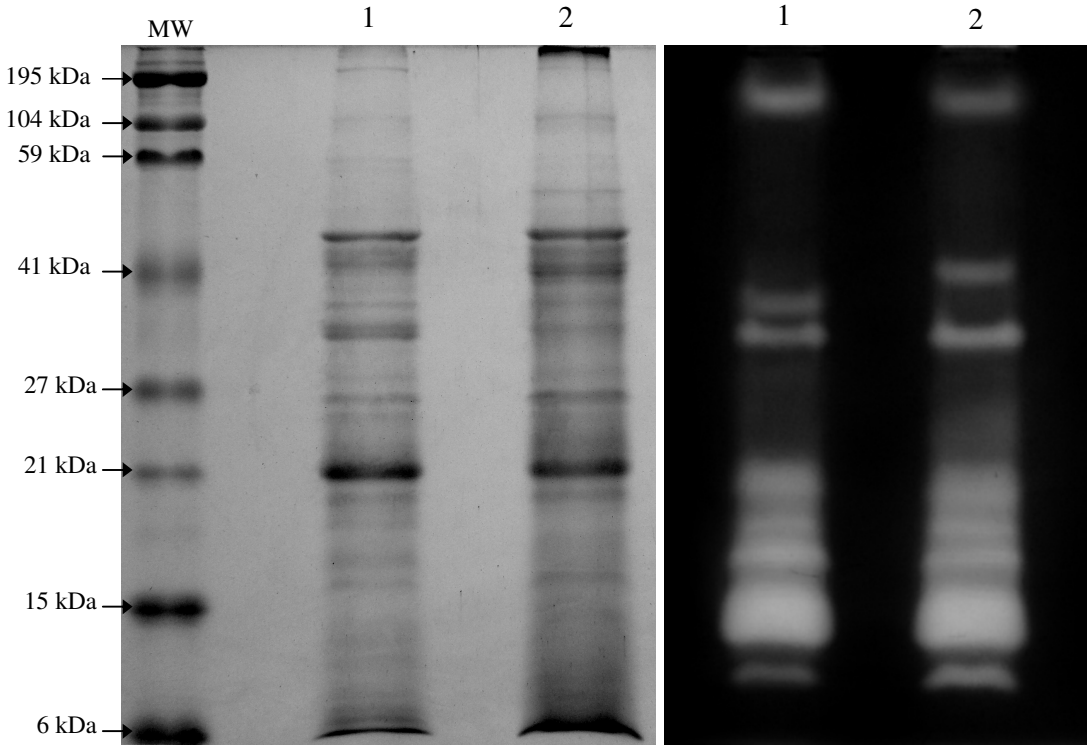
Santana, W.M; França, R. C. P.; Santana, W.M; Carvalho Jr, L. B. and Bezerra, R. S.

Figure 3. Effect of pH in residual activity (tryptic and chymotryptic) from the pyloric caeca of wild and farmed cobia (*Rachycentron canadum*) using BApNA and SApNA as substrates.



Santana, W.M; França, R. C. P.; Santana, W.M; Carvalho Jr, L. B. and Bezerra, R. S.

Figure 4. Eletrophoretic profile of digestive enzymes from the pyloric caeca of *Rachycentron canadum* using a 4% (w/v) stacking gel and a 12.5% (w/v) separating gel. MW: molecular weights standards



6. CONCLUSÕES

O presente estudo indicou que:

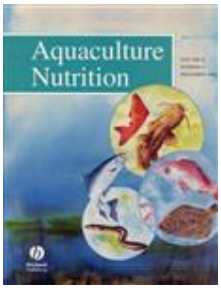
- ✓ Tripsina e quimotripsina estavam presentes no ceco pilórico do bijupirá, suas propriedades físico-químicas são semelhantes às outras espécies de peixes relatados na literatura;

- ✓ O perfil eletroforetíco dos animais selvagens e cultivados foi similar apresentando bandas de 195 kDa a 6 kDa. O zimograma apresentou bandas com atividade caseinolítica similares entre os animais;

- ✓ A atividade enzimática foi fortemente inibida pelo TLCK (91,39 e 81,01%), e benzamidina (88,07 e 78,04%), inibição moderada pelo PMSF (37,46 e 43,32%) e ou TPCK não apresentou uma inibição significativa (4,51 e 14,29%).

7. ANEXOS

7.1. NORMAS PARA REDAÇÃO DE ARTIGOS CIENTÍFICOS PARA A REVISTA "AQUACULTURE NUTRITION":



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