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RENATA CRISTINA DA PENHA FRANÇA

CARACTERIZAÇÃO BIOQUÍMICA E MOLECULAR DA TRIPSINA DOS CECOS PILÓRICOS DO BIJUPIRÁ (*Rachycentron canadum*) E SUA COMPATIBILIDADE COM FORMULAÇÕES DE DETERGENTES

Recife
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Orientador: Prof. Dr. Ranilson de Souza Bezerra

Coorientador: Prof. Dr. Luiz Bezerra de Carvalho Júnior

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Aprovada em 26/02/2013

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Prof. Dr. Ian Porto Gurgel do Amaral/UFPB

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Mas onde se achará a Sabedoria? E onde está o lugar do entendimento? O homem não lhe conhece o caminho; nem se acha ela na terra dos viventes. O abismo diz: Não está em mim; e o mar diz: Ela não está comigo. Não pode ser comprada com ouro fino, nem a peso de prata se trocará. Donde, pois, vem à Sabedoria? Onde está o lugar do entendimento? Está encoberta aos olhos de todo vivente, e oculta às aves do céu. Deus entende o seu caminho, e ele sabe o seu lugar. Porque ele perscruta até as extremidades da terra, sim, ele vê tudo o que há debaixo do céu. E disse Deus ao homem: Eis que o temor do Senhor é a Sabedoria, e o apartar-se do mal é o entendimento.

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A todos vocês, o meu, muito obrigada!!!!

LISTA DE ABREVIATURAS

AZT - Azidotimidina

BApNA - N_{α}-benzoyl-$_{DL}$-arginine ρ-nitroanilide hydrochloride

N_{α}-benzoil-$_{DL}$-arginina ρ-nitroanilida hidrocloreto

BPTI - Bovine Pancreatic Trypsin Inhibitor

Inibidor Pancreátíco de Tripsina Bovino

CAISAN - Câmara Interministerial de Segurança Alimentar e Nutricional

CETENE - Centro de Tecnologias Estratégicas do Nordeste

DMSO - Dimethylsulphoxide

Dimetilsulfóxido

EDTA - Ethylenediamine tetraacetic acid

EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária

FAO - Food and Agriculture Organization

Organização das Nações Unidas para Alimentação e Agricultura

IUBMB - International Union of Biochemistry and Molecular Biology

União Internacional de Bioquímica e Biologia Molecular

kDa - Unidade de massa atômica (dálton)

MALDI/TOF-MS - Matrix Assisted Laser Desorption Ionization/Time of Flight - Mass Spectrometry (Matriz de Ionização/Dessorção Assistida por Laser por Tempo de Vôo/Espectrômetro de Massa)

MCT – Ministério da Ciência e Tecnologia

MPA - Ministério da Pesca e Aquicultura

MS - Ministério da Saúde

NCBI - National Center for Biotechnology Information

Centro Nacional de Informações sobre Biotecnologia Ciência e Saúde

OECD - Organisation for Economic Co-Operation and Development

Organização para a Cooperação e Desenvolvimento Econômico

pH - Potencial hidrogeniônico

PMSF - Phenylmethylsulphonyl Fluoride

Fluoreto de fenilmetilsulfonil

SApNA - N-Succinyl-Ala-Ala-Pro-Phe ρ-nitroanilide

N-Succinil-Ala-Ala-Pro-Phe ρ-nitroanilida

SDS - **S**odium Dodecyl Sulfate

Dodecil Sulfato de Sódio

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio

SEBRAE - Serviço Brasileiro de Apoio às Micro e Pequenas Empresas

SEAP - Secretaria Especial de Aquicultura e Pesca

TFA - Trifluoroacetic acid

Ácido trifluoroacético

TLCK- N-ρ-tosyl-L-lysine chloromethyl ketone

N-ρ-tosil-L-lisina clorometil cetona

TPCK - N-ρ-L-phenylalanine cholromethyl ketone

N-ρ-L-fenilalanina clorometil cetona

UFRPE - Universidade Federal Rural de Pernambuco

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RESUMO

Com a diminuição dos estoques pesqueiros e a estagnação das capturas observada desde o final da década de 1980, a aquicultura converteu-se atualmente em uma atividade consolidada capaz de abastecer e suprir à incessante demanda por produtos pesqueiros e proteicos de alto valor nutricional por seu crescente aporte na produção mundial de pescados. O bijupirá (*Rachycentron canadum*), conhecido internacionalmente como cobia é uma espécie nativa da costa brasileira e tem sido apontada como excelente candidato para desenvolver a piscicultura marinha nacional. Visando um melhor conhecimento sobre as exigências nutricionais e a fisiologia digestiva desta espécie. O objetivo do presente trabalho foi realizar a caracterização bioquímica e molecular da tripsina presente nos cecos pilóricos do bijupirá (*Rachycentron canadum*), testar a compatibilidade da tripsina purificada com sabões em pó e a sua estabilidade através de ensaios *in vitro* na presença de agentes surfactantes e oxidantes e comparar as peptidases digestivas presentes entre espécimes selvagens e cultivados no início do processo de domesticação em Pernambuco. Peptidases presentes nos cecos pilóricos de bijupirás selvagens e cultivados (*R. canadum*) foram caracterizadas quanto as suas propriedades físico-químicas através de ensaios de pH, temperatura ótima, estabilidade térmica, SDS-PAGE, efeito de inibidores e zimogramas utilizando substratos específicos para tripsina e quimotripsina (BApNA e SApNA), respectivamente. A temperatura e o pH ótimos obtidos para a tripsina-símile dos animais selvagens e cultivados foram de 55 e 60°C e 8,0 e 10,0, respectivamente, enquanto que para quimotripsina-símile foram de 50 e 45°C e 9,5 e 8,0 respectivamente. A tripsina-símile dos bijupirás selvagens e cultivados foi fortemente inibida com a utilização de inibidores clássicos como o PMSF (inibidor de serino proteases), TLCK (inibidor clássico de tripsina) e benzamidina (inibidor clássico de tripsina). O mesmo padrão foi observado para a quimotripsina-símile utilizando o TPCK (inibidor clássico de quimotripsina). O perfil eletroforético dos animais selvagens e cultivados revelaram bandas que variaram entre 6 kDa e 195 kDa. As bandas caseinolíticas observadas no zimograma revelaram pequenas variações que podem ser indicativos do processo inicial de adaptação enzimática do bijupirá frente às novas condições de cultivos quando comparados aos animais selvagens. O processo de purificação da tripsina do bijupirá utilizando-se a cromatografia de afinidade por BPTI-sepharose demonstrou ser um método eficiente, com alta reprodutibilidade, rápido e viável que permitiu o isolamento da tripsina presente no ceco pilórico e apresentou pH ótimo de 8,5, temperatura ótima de 50ºC, estabilidade térmica até 55ºC, K_m de 0,38mM, K_{cat} 3,14 s^{-1} e K_{cat}/K_m 8.26 s^{-1} mM^{-1} utilizando BApNA (8mM) como substrato. Estas caracterísiticas físico-químicas e cinéticas foram semelhantes a outras

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Palavras-chave: Peptidases digestivas, piscicultura marinha brasileira, *Rachycentron canadum*, tripsina, cromatografia de afinidade.

ABSTRACT

With the depletion of fish stocks and natural stagnation of catches observed since the late 1980´s, aquaculture has become a consolidated activity currently able to supply and meet the incessant demand for fish products and protein of high nutritional value by increasing its contribution in world production of fish. Cobia (*Rachycentron canadum*) is a native species of the Brazilian coast and has been identified as an excellent candidate to develop the national marine aquaculture. The lack of knowledge about the nutritional requirements of tropical fish and digestive physiology of this species make the diets available in the market not suitable for the proper development of the animal. The aim of this study was to characterize biochemical, molecular and of trypsin present in the pyloric caeca from cobia (*R.canadum*), determined commercially soap powder and evaluate the stability of purified enzyme through *in vitro* assay by incubation with oxidizing agents, surfactants and compare this activity with that of others commonly used commercial enzymes in the detergent industry compare the digestive peptidases present among wild and farmed specimens at the beginning of the domestication process in Pernambuco. Digestive peptidases present in the pyloric caeca of wild and farmed cobia (*R. canadum*) were characterized by physical-chemical properties through pH, optimum temperature, thermal stability assays, SDS-PAGE, effect of inhibitors and zymograms using specific substrates for trypsin-like and chymotrypsin-like (BApNA and SApNA), respectively. The optimum temperature and optimum pH obtained for trypsin-like from wild and farmed animals were 55 and 60°C and 8.0 and 10.0, respectively, while for chymotrypsin-like were 50 and 45°C, 9.5 and 8.0 respectively. The trypsin-like enzymes from wild and farmed cobia were strongly inhibited by the use of classical inhibitors such as PMSF (serine protease inhibitor), TLCK (classic trypsin inhibitor) and benzamidine (classic trypsin inhibitor). The same pattern was observed for chymotrypsin-like where the employee was TPCK (classic chymotrypsin inhibitor) using SApNA as specific substrate. The electrophoretic profile of wild and farmed specimens revealed bands with a molecular mass ranging from 6 kDa to 195 kDa. The caseinolytic bands present in zymograms showed small variations that may be indicative of the initial enzymatic adaptation from cobia forward to new rearing conditions compared to wild animals. The purification process of trypsin from *R. canadum* using BPTI-sepharose affinity chromatography shown to be an efficient method, with high reproducibility, rapid, and feasible that allowed the isolation of trypsin present in the pyloric caeca with optimum pH of 8.5, optimum temperature at 50ºC and thermostability until 55ºC, K_m value of purified trypsin was 0.38 mM, K_{cat} value was 3.14 s^{-1} and K_{cat}/K_m was 8.26 s^{-1} mM^{-1} for BApNA (8mM) as substrate. The physical-chemical and kinetic parameters

similar to other trypsins fish reported in literature and can be employed in the detergent industry as an alternative source of new enzymes and was stable in the presence of oxidants and agesntes compatible with surfactants and soaps and commercial powder that can be used in the detergent industry as an alternative source of new enzymes.

Keywords: Digestive peptidases, Marine fish farming, *Rachycentron canadum,* trypsin and affinity chromatography.

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1. INTRODUÇÃO

A aquicultura, definida como a produção de organismos com hábito predominantemente aquático, inclui principalmente os peixes, moluscos, crustáceos, anfíbios e plantas aquáticas (VALENTI, 2000) e é atualmente a atividade de maior crescimento entre os setores de produção animal (FAO, 2011).

A aquicultura é, portanto, uma atividade bastante antiga onde há registros do cultivo de carpas em viveiros por volta do século V a.C. onde as civilizações antigas do Oriente tinham o peixe como importante componente de sua alimentação e, por esta razão, o cultivavam em viveiros para diminuir a incerteza da pesca (SEBRAE, 2008).

As atividades da pesca e da aquicultura assumem papel fundamental no processo de desenvolvimento econômico especialmente para países em desenvolvimento como o Brasil, pois contribuem para a segurança alimentar através de rigoroso gerenciamento destas atividades como também no combate à pobreza, representando fonte vital de alimentos, geração de mão de obra qualificada e renda para as populações locais. A variedade de peixes é um diferencial para o Brasil atingir novos mercados, além de contar com um clima que é um trunfo adicional a favor do país, cujas condições para o cultivo de diversos organismos aquáticos é excelente (LOPES et al. 2010; SIDONIO et al. 2012).

Atualmente a aquicultura converteu-se em uma atividade consolidada capaz de abastecer a incessante demanda por produtos pesqueiros e proteicos de alto valor nutricional, frente ao estancamento das capturas observado desde o final dos anos 80.

O aumento da demanda no consumo por produtos pesqueiros tem sido impulsionado principalmente pelos mercados emergentes, portanto países como China, Índia, Brasil, entre outros, que apresentam elevados quantitativos populacionais, obtiveram aumento do poder aquisitivo da população de baixa renda implicando em mudanças no perfil nutricional que pode ser traduzido pelo aumento da inserção de proteínas nobres e saudáveis em sua alimentação. Estas mudanças econômicas, sociais e comportamentais observadas nos últimos anos constitui nítida valorização dos atributos do pescado em relação a outras fontes de proteína animal e representará um grande desafio ao setor agropecuário, sobretudo a aquicultura brasileira (CAISAN, 2011).

Na atualidade, cerca de 150 espécies diferentes de peixes, crustáceos, moluscos e plantas (onde se incluem as algas), têm seus cultivos bem estabelecidos sendo que a maior participação na produção comercial encontra-se na piscicultura (acima de 50%), sendo os exemplos de cultivo mais representativos no mundo: a tilápia, a carpa (a espécie mais antiga cultivada), o bagre de canal, a truta, o salmão e algumas espécies ornamentais. No Brasil além

da tilápia e da carpa, o tambaqui, o tambacu e o pacu são as espécies mais expressivas com uma produção estimada em 102 mil toneladas em 2010, das quais 80.800 toneladas corresponderam ao cultivo do tambaqui e seus híbridos (MPA, 2012).

O cultivo do bijupirá no Brasil encontra-se em sua fase inicial e os diversos aspectos referentes ao seu manejo são ainda pouco conhecidos, por esta razão é importante acompanhar e gerenciar o início desta atividade visto que esta espécie vem despertando a atenção de vários maricultores brasileiros e já conta com a liberação da concessão das águas da União para alguns empreendimentos ao longo do nosso litoral.

Visando agregar valor aos resíduos oriundos da aquicultura (escamas, cabeças, espinhas, pele, restos de filé e vísceras) que são descartados indiscriminadamente, diversos estudos têm focado na utilização destes insumos como fonte para extração de biomoléculas que podem ser empregadas na síntese de diversos bioprodutos. Estudos sobre as enzimas envolvidas no processo digestório principalmente a tripsina, contribuirão para o entendimento da fisiologia digestiva da espécie em tela e servirão de subsídio que permitirão avaliar os efeitos do processo inicial de confinamento desta espécie através do processo de adaptação enzimática, além de testar a compatibilidade da mesma na indústria de detergentes como fonte alternativa de proteases alcalinas.

2. REVISÃO DE LITERATURA

2.1 Aquiculturas Mundial e Brasileira

A demanda mundial por pescados vem crescendo de forma acelerada em decorrência do aumento demográfico e pela busca por alimentos mais saudáveis.

De 2004 a 2009, o crescimento do consumo de pescados foi de aproximadamente 13% no acumulado. Os países asiáticos continuam dominando o setor aquícola com uma produção de 89,1% da produção mundial de produtos pesqueiros, sendo a China o principal produtor da produção global de pescados. (Tabela 1).

Tabela 1. Produção mundial de pescado (t) dos dezoito maiores produtores em 2008 e 2009

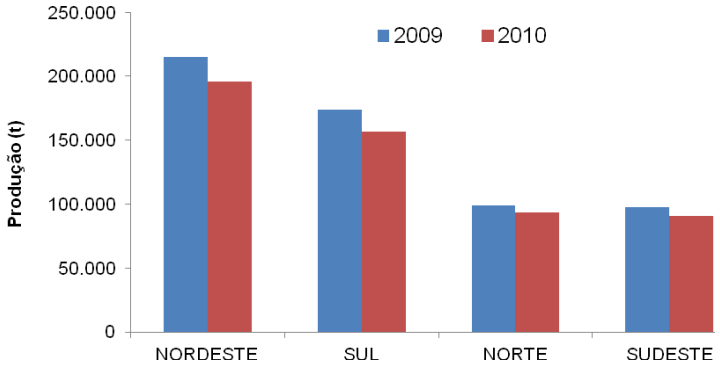
	País	**2008**		**2009**	
Posição		**Produção**	**%**	**Produção**	**%**
1°	China	57.827.108	40,64%	60.474.939	41,68%
2°	Indonésia	8.860.745	6,23%	9.815.202	6,76%
3°	Índia	7.950.287	5,59%	7.845.163	5,41%
4°	Peru	7.448.994	5,23%	6.964.446	4,80%
5°	Japão	5.615.779	3,95%	5.195.958	3,58%
6°	Filipinas	4.972.358	3,49%	5.083.131	3,50%
7°	Vietnã	4.585.620	3,22%	4.832.900	3,33%
8°	Estados Unidos	4.856.867	3,41%	4.710.453	3,25%
9°	Chile	4.810.216	3,38%	4.702.902	3,24%
10°	Rússia	3.509.646	2,47%	3.949.267	2,72%
11º	Mianmar	3.168.562	2,23%	3.545.186	2,44%
12º	Noruega	3.279.730	2,30%	3.486.277	2,40%
13º	Coréia do Sul	3.358.475	2,36%	3.199.177	2,20%
14º	Tailândia	3.204.293	2,25%	3.137.682	2,16%
15º	Bangladesh	2.563.296	1,80%	2.885.864	1,99%
16º	Malásia	1.757.348	1,23%	1.871.971	1,29%
17º	México	1.745.757	1,23%	1.773.644	1,22%
18º	Brasil	1.156.423	0,81%	1.240.813	0,86%

Fonte: Adaptado de MPA, 2012.

De acordo com a FAO (2012), a produção mundial de pescado em 2011 foi de 154 milhões de toneladas enquanto que no Brasil, para o ano de 2010, foram produzidos

1.264.765 toneladas de pescado, registrando-se um incremento de 2% em relação a 2009, quando foram produzidas 1.240.813 toneladas de pescado e ocupando a 18° posição no ranking mundial para os anos de 2009 e 2010 respectivamente. A pesca extrativa marinha continuou sendo a principal fonte de produção de pescado nacional, sendo responsável por 536.455 toneladas (42,4% do total de pescado), seguida, sucessivamente, pela aquicultura continental (31,2%), pesca extrativa continental (19,7%) e aquicultura marinha (6,7%) e sendo a região Nordeste a responsável pela maior parcela da produção nacional como pode ser observado na Figura 1.

Figura 1. Produção nacional de pescado (t) oriundos da pesca extrativa marinha em 2009 e 2010 discriminada por região.

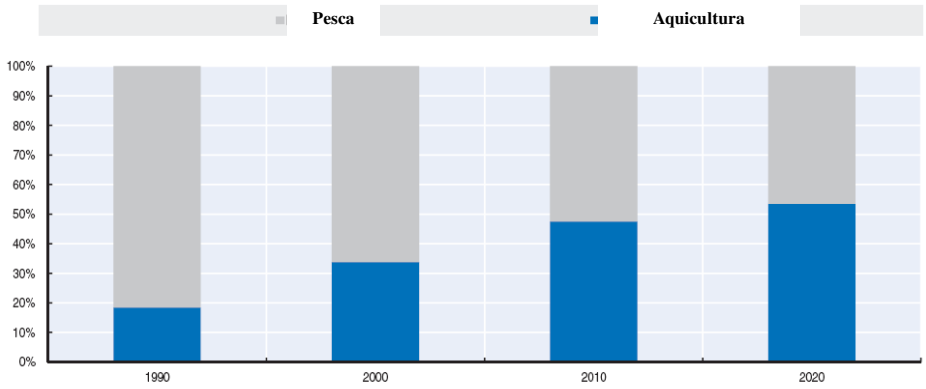


Fonte: Ministério da Pesca e Aquicultura (MPA, 2012)

De acordo com a FAO (2011), para o ano de 2012, mais de 50% da produção global de pescado destinada ao consumo humano foi proveniente da aquicultura e esta será a única forma de acompanhar o crescimento da demanda mundial por produtos pesqueiros, sobretudo a dos países desenvolvidos para os próximos anos (Figura 2).

Diante deste contexto de crescimento, estímulos e investimentos governamentais, a aquicultura juntamente com o setor pesqueiro necessita buscar alternativas viáveis para o gerenciamento racional de seus resíduos que seja de forma sustentável, economicamente viável e que contribua para a redução do impacto ambiental. As grandes quantidades de resíduos gerados por estas atividades são compostas principalmente por cabeças, vísceras, pele, escamas, nadadeiras, cauda, além de restos de filés, e podem chegar a representar até 50% da matéria prima utilizada, variando conforme a espécie (PESSATI, 2001).

Figura 2. Projeção sobre o papel da aquicultura no aumento e no consumo de pescado no mundo



Fonte: OECD-FAO Agricultural Outlook, 2011-2012.

O Brasil apresenta grande capacidade para o desenvolvimento da aquicultura, visto que possui clima favorável, 8,4 mil quilômetros de costa marítima, disponibilidade de terra, mão de obra especializada e grande demanda por parte dos consumidores no mercado interno e externo pelos produtos oriundos desta atividade, mas ainda assim, o potencial para a expansão desta atividade é pouco aproveitado (EMBRAPA, 2002; VALENTI, 2000).

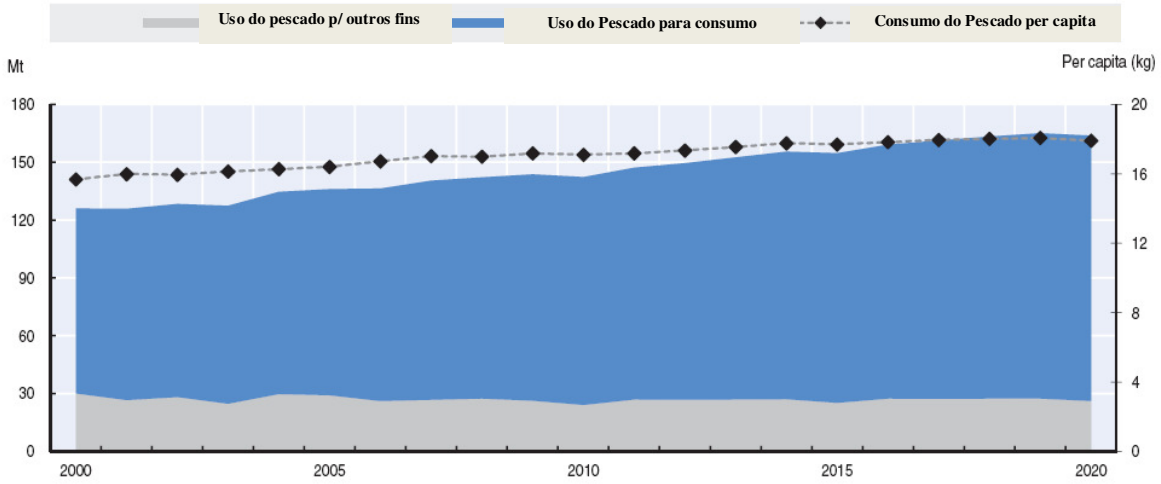
A criação da Secretaria Especial de Aquicultura e Pesca (SEAP), em 2003, pelo então presidente Sr. Luiz Inácio Lula da Silva, representou um grande marco para o desenvolvimento da pesca e aquicultura em nosso país porque a aquicultura não existia como atividade regulamentada e nesse âmbito a situação brasileira era de total estagnação neste setor.

Em junho de 2009, com a transformação da SEAP em Ministério da Pesca e Aquicultura (MPA), foi consolidada a implantação de políticas públicas voltadas para a estruturação da cadeia produtiva da pesca extrativa e da produção aquícola brasileira, onde foi sancionada a Lei da Pesca e Aquicultura, que tem por objetivo reconhecer o trabalho e direitos dos pescadores artesanais como também a legalização da atividade dos aquicultores que até então exerciam suas atividades sem o devido apoio do governo quase que de forma clandestina (MPA, 2008).

Estimativas sugerem que o aumento demográfico mundial até 2020 resultará em uma ampliação do consumo de pescado na ordem de 30 milhões de toneladas por ano. Este aumento só poderá ser suprido pela aquicultura visto que a pesca comercial já demonstrou ter atingido seu limite máximo de exploração. No Brasil, houve um crescimento no consumo de pescado de 6 kg para 9,75 kg por habitante/ano entre 2003 e 2010, representando um aumento

de 39,78% neste período, estimativas sugerem que esse aumento será gradativo e se expandirá para os anos seguintes, sendo este contexto uma tendência mundial (Figura 3).

Figura 3. Crescimento geral no consumo de pescado no mundo.



Fonte: OECD-FAO Agricultural Outlook, 2011-2012.

A exploração indiscriminada do estoque pesqueiro e a crescente diferença entre a quantidade de pescado capturado e a demanda de consumo, tornaram a aquicultura um das alternativas mais viáveis no mundo para produção de alimento de alto valor proteico. Nas últimas décadas, o perfil epidemiológico e nutricional do Brasil vem sendo modificado, com uma população que vem experimentando grandes transformações sociais que resultaram em mudanças no seu padrão de saúde, comportamento, consumo alimentar e, portanto, tem se tornado cada vez mais exigente e preocupada com a qualidade de vida que se reflete em seus hábitos alimentares saudáveis.

Graças à variedade de ambientes interiores e costeiros, que se dividem em estuários, represas, açudes, rios, baías e enseadas, estes contribuem para o potencial de expansão da aquicultura no Brasil, mas apesar da enorme potencialidade dos nossos recursos hídricos para a área de biotecnologia, boa parte das fontes nele encontrados ainda permanece desconhecida. A aquicultura, portanto, apresenta-se como uma atividade com grande oportunidade para alavancar o potencial biotecnológico brasileiro e colocar o Brasil no ranking mundial como um dos maiores países produtores de pescado (MPA, 2012).

Em comparação a outras fontes proteicas, pois apresentam menor teor de gordura saturada, sendo ricos em ácido graxo ômega 3, proteínas (possuem todos os aminoácidos essenciais), vitaminas (A, E, D e complexo B), além de grande quantidade de minerais como

ferro, potássio, magnésio, sódio, zinco, fósforo, iodo, cálcio, flúor, manganês e cobalto, entre outros (LANKE et al. 2003).

De acordo com Chuang et al. (2010), o conteúdo de lipídios nos bijupirás cultivados é cerca de duas vezes maior que nos espécimes capturados na natureza (selvagens), e esta característica é considerada como uma das principais determinantes do sabor da carne desta espécie o que justifica a sua procura por parte de restaurantes especializados na culinária japonesa.

Além dos benefícios acima citados, vários bioprodutos marinhos têm aplicação comercial na atualidade e potencial industrial para o futuro o que coloca o Brasil em uma posição favorável por apresentar um dos mais ricos e diversificados ecossistemas aquáticos do planeta (Brasil, MS/MCT, 2010). Como um bom exemplo da importância da descoberta de novas moléculas com aplicação na indústria farmacêutica pode-se citar os derivados sintéticos da espongouridina e da espongotimidina (Ara-A – adenina-arabinosídeo – e AraC – citosina-arabinosídeo), que foram substâncias isoladas de esponjas marinhas na década de 50, e que serviram de modelo para o desenvolvimento de inúmeros antivirais como o AZT, remédio básico no tratamento da AIDS, além de colágeno extraído a partir de resíduos de pescado que pode ser empregado em vários setores da indústria farmacêutica, cosmética como também na medicina (COSTA-LOTUFO et al. 2009; HAEFNER, 2003; POMPONI, 2001; ZENG et al. 2012).

2.2 Piscicultura Marinha no Brasil: Breve panorama histórico

A atividade da piscicultura é um dos ramos da aquicultura, destinada à criação de peixes (marinhos ou de água doce), em ambiente com condições propícias para o seu melhor desenvolvimento. Registros históricos relatam que no Brasil, a criação de peixes marinhos não é uma atividade recente, sendo introduzida no início no século XVII no Estado de Pernambuco durante o governo holandês de Maurício de Nassau.

De acordo com Von Ihering (1932), as principais espécies criadas extensivamente em viveiros de maré nos municípios de Recife e Olinda eram robalos (*Centropomus*), tainhas (*Mugil*) e carapebas (*Eugerres* e *Diapterus*).

Nas décadas de 70 e 80, outras espécies de hábito alimentar carnívoro tais como: surubim (*Pseudoplatystoma coruscans*), pirarucu (*Arapaima gigas*) e tucunaré (*Cichla sp*.) passaram a ser exploradas, pelo alto valor comercial, bom desempenho zootécnico e a excelência de suas carnes, atendendo aos anseios de criadores. Entretanto, nos anos 80, os dados obtidos de cultivo de espécies de água dulcícolas enfatizam a criação extensiva ou semi-intensiva em barragens, açudes e represas localizados na região Nordeste e Sudeste (SOARES, 2008).

Apesar desse início promissor, a piscicultura marinha não é considerada uma atividade comercial no Brasil, estando praticamente limitada às iniciativas das instituições de pesquisa (ROUBACH et al. 2003).

Segundo a FAO (2012), a piscicultura de água doce em 2010 continuou a expressar grande representatividade na aquícultura mundial com uma produção estimada em 33.7 milhões de toneladas (56,4%) e de acordo com o Ministério da Pesca e Aquicultura (MPA, 2012), a produção aquícola brasileira em 2009 foi de 415.649 toneladas sendo a tilápia o peixe mais cultivado no país representando 39% do total de pescado proveniente da piscicultura continental (MPA, 2012).

Tradicionalmente, no Brasil a criação de peixes desenvolveu-se com a introdução de espécies exóticas, prática esta que pode implicar em graves riscos ambientais através da possibilidade de transmissão de agentes patógenos, como também pelas consequências imprevisíveis nos ecossistemas aquáticos, razão pela qual esta prática deveria ser submetida a um rigoroso controle e monitoramento por parte dos órgãos e instituições responsáveis por esta atividade em nosso país. A contribuição das espécies nativas na piscicultura brasileira encontra-se abaixo dos 20% enquanto que na Ásia, onde está concentrada a maior produção mundial de peixes, cerca de 95% dos cultivos estão baseados em espécies nativas daquele continente. O Brasil apresenta no cenário atual excelentes condições para o desenvolvimento

da piscicultura marinha como disponbilidade de tecnologia de criação, potencial de crescimento além de diversificado mercado de espécies nativas de peixes naturalmente encontradas em todo o litoral brasileiro cujas pesquisas no Brasil que buscam viabilizar a este ramo da aquicultura nacional utilizando espécies nativas de valor comercial e com aptidão para o cultivo ainda são insuficientes (AZEVEDO et al. 1961; SANCHES et al. 2006; VITULE et al. 2009).

A avaliação do potencial das espécies nativas servirá para definir a rentabilidade de um sistema de piscicultura intensivo, direcionando desta maneira os futuros investimentos dos recursos públicos, humanos e financeiros para pesquisa e desenvolvimento tecnológico da atividade. Portanto, informações mercadológicas e econômicas devem ser utilizadas como critério de seleção destas espécies candidatas e aptas para o cultivo comercial avaliando-se parâmetros como fácil e rápido ganho de peso, adaptação à alimentação artificial, alta resistência a doenças, facilidade de produção de larvas e alevinos, entre outros. (SANCHES et al. 2006).

Neste contexto surge o bijupirá (*Rachycentron canadum*), peixe nativo da costa brasileira, considerado uma espécie com grande potencial para a criação intensiva apresentando características de interesse para a criação, como facilidade de reprodução em cativeiro, rápido crescimento, tolerância das larvas às mudanças na salinidade, aceitação de dietas extrusadas e produção de filés de alta qualidade adequados ao consumo na forma de *sashimi* (CRAIG et al. 2006; FAULK e HOLT, 2006; LIAO e LEAÑO, 2007). Embora tenha havido grande interesse na criação do bijupirá, inclusive com iniciativas de empresas privadas e apoio governamental, são incipientes os estudos disponíveis sobre a biologia e tecnologia de criação desta espécie. A reprodução de animais capturados no ambiente e aclimatados ao cativeiro vem sendo realizada de forma rotineira no Brasil desde 2006, quando desovas espontâneas foram obtidas na Bahia e Pernambuco (PEREGRINO JÚNIOR, 2009).

A atividade da piscicultura marinha brasileira se divide entre produtores rurais, que têm na piscicultura um incremento da renda e da alimentação familiar, e pequenos empresários do ramo. Em 13 de fevereiro de 2009 foi inaugurada a Fazenda Marinha Aqualider, na presença do então presidente da república Ilmo. Sr. Luís Inácio Lula da Silva, do governador do estado de Pernambuco Ilmo. Sr. Eduardo Campos e demais autoridades do corpo executivo, legislativo e judiciário dos governos federal, estadual e municipal, bem como empresários e personalidades de expressão da sociedade brasileira (Figura 4).

Figura 4. Inauguração da Fazenda Marinha Aqualider.

Fonte: www.aqualider.com.br

Esta solenidade tornou a Aqualider como a primeira empresa brasileira a obter a concessão de águas da União para a aquicultura por um período de 20 anos, tornando-se marco inicial da aquicultura legal no Brasil e esteve localizada em mar aberto na plataforma continental do Estado de Pernambuco, a 11 km da costa na prumada da praia de Boa Viagem, Recife. A empresa organizou a tecnologia e buscou funcionários além de contar com a participação da UFRPE e pescadores de área de Brasília Teimosa à Barra de Jangada.

O empreendimento visou produzir 10 mil toneladas/ano de bijupirá quando da conclusão dos 48 tanques-redes previstos em seu projeto inicial. Inaugurada inicialmente com 4 tanques-redes quando uma das gaiolas produziu 40 toneladas, a empresa cessou temporariamente suas operações em virtude de problemas como a inexistência de seguro para esta atividade no mercado brasileiro, a baixa qualidade das rações disponíveis no mercado nacional, a escassez de mão de obra qualificada, a instabilidade na produção de alevinos em laboratório (CAVALLI et al. 2011).

O Bijupirá (*Rachycentron canadum*) (LINNAEUS, 1766), conhecido internacionalmente como cobia (Figura 5), é um peixe marinho pelágico, migratório, de hábito alimentar carnívoro e que se encontra amplamente distribuído no mundo, principalmente em regiões que possuem águas tropicais e subtropicais com exceção para a região leste do oceano Pacífico sendo uma espécie nativa do litoral brasileiro, conhecido na região nordeste pelos pescadores como cação de escamas, bijupirá, beijupirá e/ou pirambijú) e que se encontra distribuído ao longo de todo o litoral brasileiro (CARVALHO FILHO, 1999; SUN, et al. 2006;). De acordo com o MPA (2012), a produção da pesca extrativa marinha brasileira no ano de 2010 para o bijupirá foi de 923 toneladas.

Sendo este, o único representante da família *Rachycentridae.* O bijupirá está classificado taxonomicamente da seguinte forma segundo Kaup, (1826):

Figura 5. Bijupirá (*Rachycentron canadum*)

Fonte: South Atlantic Fishery Management Council (Disponível em: <http://www.safmc.net/FishIDandRegs/FishGallery/Cobia/tabid/280/default.aspx) Acesso em: 08/05/2013

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).

Os indivíduos adultos podem alcançar 2m de comprimento máximo e podem alcançar o peso de até 60kg. Por ser uma espécie de hábito alimentar carnívoro, sua dieta natural constitui-se principalmente de crustáceos bentônicos e epibentônicos, pequenos peixes e eventualmente moluscos (CAVALIN, 2005).

Este peixe tem sido cultivado em Taiwan desde a década de 90 e representa um importante papel na indústria pesqueira comercial deste país, despertando a atenção dos criadores pela excelente qualidade de sua carne muito apreciada pela culinária oriental (LIAO et al. 2004; LIN et al. 2006).

O processo de domesticação de animais é uma prática bastante antiga e tem por objetivos principais a utilização destes indivíduos com fins lucrativos para a humanidade além

de servirem de fonte alimentar para os homens, como um meio de conservação de espécies selvagens e de maneira geral, é uma fonte causadora de estresse que pode se refletir em alterações no crescimento, resposta imune, taxa reprodutiva, entre outros. De acordo com Price (1984), pode ser definida como "o processo pelo qual uma população de animais torna-se adaptado para os seres humanos e para o ambiente cativo por alguma combinação de alterações genéticas que ocorrem ao longo de gerações e ambientalmente induzidos pelos eventos de desenvolvimento durante cada geração" (BALON, 2004; MAIRESSE et al. 2007; TYMCHUK e DEVLIN, 2005).

A piscicultura assim como outros ramos da produção animal, é uma atividade intensiva de produção de alimentos que tem por finalidade a obtenção de elevados índices de produtividade através da máxima redução dos custos de produção num menor tempo de cultivo possível. Esta atividade vem sendo apontada como forte causadora de um componente inevitável nos sistemas de cultivo intensivo que é o "estresse em peixes" caracterizado como um dos principais problemas enfrentados nas atuais práticas de criação de animais em cativeiro (VOLPATO et al. 2007).

O ambiente aquático por ser extremamente dinâmico contribui diretamente para o desenvolvimento do estresse em peixes e este pode ser proveniente de várias fontes, como por exemplo, da manipulação dos animais, do emprego de altas densidades de estocagem, do transporte, interações biológicas, da qualidade da água (variações de pH, oxigênio dissolvido, níveis de nitratos, nitritos, amônia, etc) e do manejo alimentar, entre outros (OBA et al. 2009; PALERMO et al. 2008).

A resposta ao estresse vem sendo apontada como fonte causadora de impacto negativo nos animais de cultivo e tem sido objeto de estudo por parte de muitos pesquisadores que tem por finalidade principal investigar seu efeito não apenas no contexto evolucionário, como também nas consequências diretas que esta produz no bem estar dos animais submetidos às condições de confinamento e que pode induzir diferentes respostas fisiológicas no organismo e acarretar sérios prejuízos de ordem econômica (ARAKI et al. 2007; DUARTE, 2007; OSURE et al. 2006). Estas pesquisas juntamente com o conhecimento da condição ambiental na qual os peixes estão inseridos tem contribuido para o desenvolvimento e aprimoramento de técnicas que visa obter sucesso da atividade da pisicultura em todos os níveis e tem sido relevantes para o desenvolvimento e obtenção da qualidade do produto final (BORGHETTI et al. 2003; CAIRNS et al. 2008; MILLA et al. 2010; OBA et al. 2009).

De acordo com Barton et al. (2002), a resposta ao estresse nos peixes pode ser dividida em três categorias: primária, secundária e terciária As respostas primárias são as hormonais,

as secundárias são mudanças nos parâmetros fisiológicos e bioquímicos e as terciárias são o comprometimento no desempenho, mudanças no comportamento e aumento da suscetibilidade a doenças.

Atualmente diversos estudos têm demonstrado que durante o confinamento dos peixes, a atividade e a expressão gênica de diversas enzimas digestivas proteolíticas podem sofrer alterações em função do manejo alimentar bem como na qualidade da composição do alimento fornecido aos animais em um processo conhecido e denominado de adaptação enzimática (MUHLIA-ALMAZÁN et al. 2003). Este processo de adaptação enzimática foi relatado por Zambonino-Infante e Cahu (1994) que observaram mudanças nos níveis das enzimas amilase, fosfatase alcalina e leucinoaminopeptidase em resposta à adaptação do organismo dos animais em virtude das alterações nas dietas administradas aos indivíuos submetidos a esta pesquisa. Olli et al (1994), relataram um aumento na secreção de proteases como compensação à presença de inibidores de proteases no alimento fornecido, como também foi possível observar mudanças na modulação de enzimas e em mRNAs devido aos níveis de proteína e carboidratos das dietas em peixes reportado por Peres et al. (1998).

Portanto, em virtude da problemática discutida acima, é necessário avaliar cuidadosamente vários aspectos referentes ao processo de domesticação do bijupirá no litoral brasileiro para que a piscicultura desta espécie atenda aos mínimos requisitos para que se torne bem sucedida e produtiva.

2.3 Enzimas

As enzimas são biomoléculas proteicas conhecidas por agirem como catalisadores biológicos de diversas reações bioquímicas mais notáveis e altamente especializadas e que desempenham um papel fundamental para a manutenção da vida, pois sem a sua existência, as reações de catálise não existiriam em tempo hábil ou ocorreriam de forma tão lenta que não atenderiam às necessidades de um determinado organismo (HARVEY e CHAMPE et al. 2009; NELSON e COX, 2011). A eficiência das enzimas em catalisar reações é tal que a velocidade de uma reação pode ser aumentada em até 10^{20} vezes (BERG, TYMOCZKO et al., 2004; CAMPBELL e FARRELL, 2007).

A forma de atuação das enzimas ocorre através da promoção de um ambiente específico adequado onde as reações possam acontecer rapidamente sem que haja alterações no equilíbrio das reações. De maneira geral, as reações catalisadas por uma enzima ocorrem em um bolsão tridimensional formado por grupamentos que vêm de diferentes partes da sequência linear de aminoácidos denominado sítio ativo que se liga ao seu substrato formando um complexo Enzima-Substrato (ES), de caráter transitório (BERG, TYMOCZKO et al., 2004).

De acordo com a reação catalisada pelas enzimas, a IUBMB adotou um sistema racional e prático de nomenclatura identificando as enzimas em seis grandes classes devido ao número cada vez maior de enzimas que são descobertas pelos bioquímicos (NELSON e COX, 2011) (Tabela 2).

Tabela 2. Classificação internacional das enzimas

Número e Nome das Classes		Tipo de reação catalisada
1.	Oxirredutases	Transferências de elétrons (íons hidreto ou átomos de H)
2.	Transferases	Reações de transferências de grupos
3.	Hidrolases	Reações de hidrólise (transferências de grupos funcionais para a água)
4.	Liases	Adição de grupos a ligações duplas, ou formação de ligações duplas por remoção de grupos
5.	Isomerases	Transferências de grupos dentro de uma mesma molécula produzindo formas isoméricas
6.	Ligases	Formação de ligações C-C, C-S, C-O e C-N por reações de condensação acopladas à hidrólise de ATP ou cofatores similares

C – carbono, S- enxofre, O- oxigênio e N- nitrogênio. (Adaptado de NELSON e COX, 2011)

Historicamente, o uso de enzimas na indústtria de detergentes teve seu registro no início da década de 1930 pelos cientistas alemães Otto Röhm e Otto Haas que introduziram em suas formulações de detergentes enzimas pancreáticas extraídas de porcos/suínos abatidos e era constituído principalmente por proteases (tripsinas e quimotripsinas), carboxipeptidases, alfa-almilases, lactases, sucrases, maltases e lipases (WOLFGANG, 2007).

Dentre as diversas classes de enzimas utilizadas nos mais diversos setores industriais, as proteases alcalinas apresentam-se como o grupo mais representativo e estudado atualmente e responde por cerca de 40% do mercado mundial de enzimas (GUPTA et al. 2002). Estimativas realizadas para o ano de 2013 indicam que o mercado de enzimas crescerá em torno de 6,3% em média e este crescimento será alavancado principalmente pelas indústrias farmacêutica, cosmética e alimentícia movimentando cerca de US$3,74 bilhões até 2015 (FREEDONIA, 2009; LI et al. 2012).

Atualmente o mercado de proteases é amplamente representado por subtilisinas e/ou proteases alcalinas pertencentes à bactérias do género *Bacillus* e correspondem a 40% do mercado mundial de enzimas e devido à promissora aplicabilidade destas biomoléculas nos mais diversos processos biotecnológicos, sua obtenção deve ser intensificada para suprir à atual demanda do mercado (FREEDONIA, 2009; GUPTA et al. 2002).

A justificativa para a utilização desses biocatalisadores nos mais diversificados processos biotecnológicos industriais se deve principalmente ao fato destes atingir velocidades de reação superiores àquelas obtidas na presença de catalisadores químicos convencionais. Este comportamento característico das enzimas (que necessitam de condições específicas para seu melhor funcionamento como pH, temperatura, entre outros), permite significativa redução no custo final dos processos biotecnológicos, além de evitar a formação de subprodutos indesejáveis nos diversos segmentos industriais. Portanto, neste contexto, o enfoque biotecnológico vem se apresentando como excelente respaldo que justifica a exploração, o estudo e a utilização das enzimas nos mais diversos tipos de reações especialmente em relação à melhoria das características físico-químicas de matérias-primas e produtos como também dos processos biotecnológicos para a obtenção dos mesmos (KRAJEWSKA, 2004).

O Brasil importa grande parte das enzimas utilizadas nos mais diversos segmentos industriais, e como a atividade aquícola vem demonstrando efetivo crescimento no cenário nacional e mundial, a quantidade de vísceras oriundas do processamento do pescado (pode chegar a corresponder até 5% do peso total do animal), representará em grave problema ambiental se descartadas de forma indiscriminada no meio ambiente (SIMPSON e HAARD,

1987). Dentre os principais subprodutos oriundos da aquicultura destacam-se as vísceras (intestino, cecos pilóricos, estômagos, entre outros) que são ricas fontes de enzimas digestivas e possuem grande potencial de aplicação biotecnológica como fonte alternativa de enzimas em substituição às enzimas de origem bacterianas (ALI et al. 2009; SHAHIDI et al. 2001).

Portanto, é necessário buscar alternativas que sejam economicamente viáveis ao descarte desses resíduos que são ricos em biomoléculas como o colágeno, enzimas, entre outros, e que podem ser utilizados nos mais diversos processos biotecnológicos, representando uma importante e vantajosa fonte alternativa de enzimas em substituição às enzimas de origem microbiana (BEZERRA et al. 2001b; GUPTA et al. 2002; ORLANDELLI et al. 2012).

2.4 O Processo Digestivo dos Organismos Aquáticos

A digestão é a combinação dos processos mecânicos, físicos, químicos e microbianos que atuam sobre o alimento ingerido, promovendo sua quebra em componentes que são absorvidos pelo organismo ou que permanecem na luz do intestino até serem excretados. O processo digestivo é um processo fisiológico complexo que depende da ativação molecular, reconhecimento e hidrólise do alimento em tempo e locais específicos (MUHLIA-ALMAZÁN, 2002).

De maneira geral, compreende duas etapas mecânicas que são a mastigação e as contrações do tubo digestivo, uma etapa química, efetuada por enzimas digestivas secretadas pelo animal, nos diversos sucos digestivos e a atividade microbiana dos alimentos também é enzimática, porém é realizada por bactérias e protozoários presentes geralmente na porção final do tubo digestivo (SEIXAS FILHO, 2003). As informações disponíveis na literatura sugerem que os peixes são, de uma maneira geral, semelhantes aos outros vertebrados quanto aos processos digestivos. No entanto, o conhecimento sobre as enzimas digestivas em peixes são ainda mais complexos do que em outros animais domésticos, pelo simples fato destes apresentarem inúmeras espécies, com diferentes composições anatômicas digestivas e hábitos alimentares diversos e, por isso, os estudos realizados em uma determinada espécie não podem ser extrapolados para outras. Consequentemente, a ciência da nutrição de peixes está longe de estabelecer um padrão geral de exigências nutricionais. Peixes são animais pecilotérmicos, com dependência direta e indireta do ambiente, portanto mais afetados pelas variações de condições ambientais que animais terrestres (CYRINO et al. 2010; ROTTA, 2003).

Durante o desenvolvimento larval dos peixes, tanto nas espécies herbívoras como nas carnívoras, elas passam por uma mudança no hábito alimentar, que inicialmente é planctônico e, posteriormente, se especializando na ingestão de organismos animais ou vegetais. Portanto, se tornar muito especializado quanto ao hábito alimentar pode ser uma estratégia arriscada à sobrevivência de determinada espécie (ROTTA, 2003).

Apesar da grande diversidade das estruturas de alimentação e de digestão dos peixes, algumas generalizações são possíveis. Os peixes podem ser divididos, basicamente, em três grandes categorias, de acordo com o tipo de alimento consumido:

- Os herbívoros ingerem itens de origem vegetal - a maioria se alimenta de poucas espécies de plantas e, frequentemente, possuem estruturas de mastigação

especializadas, obtendo o máximo valor nutricional através da completa trituração do alimento.

- Os onívoros que se alimentam de itens de origem animal e vegetal - possuem uma dieta mista e estruturas pouco especializadas. Frequentemente consomem pequenos invertebrados, plantas e frutos.

- Os carnívoros ingerem predominantemente itens de origem animal - se alimentam de invertebrados de maior tamanho e outros peixes, podendo se especializar em algum tipo em particular. Essas preferências podem mudar com a disponibilidade sazonal dos alimentos.

Vários cultivos de peixes encontram-se bem estabelecidos no Brasil como, por exemplo, tilápia-do-nilo (*Oreochromis niloticus*), tambaqui (*Colossoma macropomum*) e carpa (*Cyprinus carpio*), entre outros. Por se tratarem de espécies cultivadas e difundidas em todo o mundo, o número de pesquisas relativas à biologia e fisiologia destes peixes são cada vez mais crescentes e significativos, gerando informações que proporcionam a racionalidade de seus cultivos (BEZERRA et al. 2001; BEZERRA et al. 2005, SOUZA et al. 2007).

Por se tratar de um processo complexo, os mecanismos digestivos e fisiológicos vêm sendo estudados em diversas espécies de peixes e em diferentes estágios de desenvolvimento com o intuito de compreender as necessidades nutricionais como também os componentes da dieta fornecida (SUZER, 2006). Nos peixes, as enzimas digestivas são secretadas dentro do lúmen do canal alimentar originados da mucosa gástrica, dos cecos pilóricos, do pâncreas e da mucosa intestinal com a função de hidrólise de proteínas, carboidratos e gorduras. Os cecos pilóricos (invaginação do intestino) excretam essencialmente as mesmas enzimas do intestino e do pâncreas, as quais são ativadas em ambientes neutros ou fortemente alcalino. A participação dos cecos pilóricos como órgão secretor de enzimas digestivas é bastante contraditória, sendo que alguns autores atribuem ao refluxo a presença destas (SEIXAS FILHO, 2003).

O pâncreas é essencialmente rico em protease básica, amilase, maltase e lipase. Os estudos de enzimas digestivas presentes nesse órgão, em peixes que possuem pâncreas compacto, tornam-se difíceis, uma vez que este se apresenta com tamanho reduzido e, nas

espécies com pâncreas difuso, formando o hepatopâncreas, aumenta essa dificuldade, uma vez que a sua separação é bastante complexa.

Dentro deste tema, torna-se fundamental o entendimento da fisiologia desses peixes, que consiste no estudo do funcionamento dos diferentes sistemas do organismo, como eles se interagem e respondem às diversas alterações ambientais e métodos de criação, permitindo que se estabeleçam melhores condições para o cultivo de uma determinada espécie (ROTTA, 2003).

Os peixes são mantidos em confinamento, onde o alimento natural se torna escasso necessitando de uma ração nutricionalmente completa e balanceada (ROTTA, 2003). A proteína é o macronutriente mais nobre e oneroso, fundamental na dieta de peixes e outros organismos aquáticos, pois é a partir dela que os animais obterão os aminoácidos essenciais que serão absorvidos pelo trato digestório e depois utilizados pelo organismo em todas as fases de desenvolvimento para a formação de novas proteínas fundamentais para o seu crescimento e sobrevivência (SANTOS et al. 2010).

A habilidade na utilização dos nutrientes da dieta depende da distribuição das enzimas ao longo do trato digestivo e a sua quantidade no conteúdo enzimático do suco digestivo é crucial para o processo de digestão química. Sabe-se também que fatores como idade do animal, estação do ano, temperatura de aclimatação, frequência alimentar, qualidade da dieta, entre outros são parâmetros conhecidos por influenciarem na secreção enzimática dos peixes (CÔRREA et al. 2007).

Dentre as principais enzimas digestivas estudadas encontra-se a tripsina (EC 3.4.21.4), que se apresenta como enzima chave no processo digestivo dos organismos aquáticos (Figura 6).

Figura 6. Estrutura tridimensional da tripsina do Bacalhau (*Gadus morhua*) complexada com benzamidina.

Fonte: (NCBI, 2012)

A tripsina é uma endopeptidase da classe das serino-proteases, que cliva ligações peptídicas na extremidade carboxi-terminal dos resíduos de aminoácido arginina e lisina (MANCHADO, 2008). Apresenta atividade de endopeptidase sendo responsável também pela ativação do tripsinogênio e de outros zimogênios, como por exemplo, o quimotripsinogênio (KLOMKLAO et al. 2007). A utilização de metodologias que determinam a atividade tríptica no sistema digestivo dos peixes, principalmente das larvas, serve como um indicador do condicionamento dos animais a alterações na qualidade e quantidade de alimento ingerido (GUDMUNDSDÓTTIR e PÁLSDÓTTIR, 2005).

Segundo Torrissen et al. (2006), diferentes isoformas de tripsina estão presentes nos cecos pilóricos e intestinos de peixes e a sua distribuição pode indicar variações genéticas individuais a respostas proteicas e alimentares que ocorrem durante o cultivo.

3. OBJETIVOS

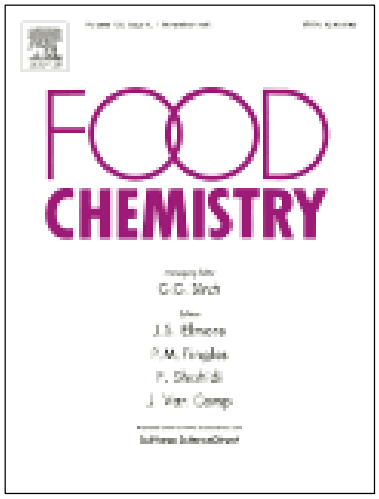
3.1 Objetivo geral

- Realizar a caracterização bioquímica e molecular da tripsina presente nos cecos pilóricos do bijupirá (*Rachycentron canadum*) e testar a compatibilidade desta enzima purificada com formulações de sabões em pó comercialmente disponíveis no mercado através de ensaios *in vitro* e comparar a atividade das peptidases digestivas entre espécimes selvagens e cultivados durante o processo inícial de domesticação desta espécie realizado no litoral de Pernambuco.

3.2 Objetivos específicos

- Purificar a tripsina presente no ceco pilórico do bijupirá (*Rachycentron canadum*);
- Caracterizar a enzima purificada com relação aos parâmetros físico-químicos e cinéticos;
- Estudar o efeito de íons e inibidores sobre a enzima purificada;
- Obter a estrutura primária da tripsina purificada;
- Testar a enzima purificada frente a sabões em pó, agentes surfactantes e agentes oxidantes e comparar com enzimas comerciais;
- Comparar as propriedades físico-químicas e cinéticas das peptidases digestivas presentes nos cecos pilóricos de espécimes selvagens e cultivados de bijupirás frente ao estresse inicial do processo de domesticação;
- Obter o final da sequência do gene que codifica a enzima tripsina e realizar estudos de modelagem estrutural.

CAPÍTULO 1: PURIFICATION OF A TRYPSIN FROM COBIA (*Rachycentron canadum*) USING BPTI-SEPHAROSE AFFINITY CHROMATOGRAPHY



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PURIFICATION OF A TRYPSIN FROM COBIA (*Rachycentron canadum*) USING BPTI-SEPHAROSE AFFINITY CHROMATOGRAPHY

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Abstract

A trypsin from the pyloric caeca of cobia (*Rachycentron canadum)* was purified in two steps: ammonium sulfate (0 - 80% saturation) precipitation and bovine pancreatic trypsin inhibitor (BPTI)-sepharose affinity chromatography. The purified trypsin had an apparent molecular mass of 24 kDa using SDS-PAGE and mass spectrometry. Optimum temperature and pH were 50ºC and 8.5, respectively. The enzymatic activity was strongly inhibited by TLCK (92.0 ± 4.65), benzamidine (87.1 ± 6.67): trypsin inhibitors and PMSF (49.1 ± 2.81), serine protease inhibitor. Apparent K_m value of purified trypsin was 0.38 mM, K_{cat} value was 3.14 s^{-1} and K_{cat}/K_m was 8.26 s^{-1} mM^{-1} for BApNA as substrate. The purified trypsin activity was inhibited by the following metal ions (final concentration at 0.15mM) in decreasing order: $Hg^{2+} > Cu^{2+} > Cd^{4+} > Mg^{2+} > Na^{+} > Ba^{2+} > K^{+} > Ca^{2+} = Al^{3+}$. The N-terminal amino acid sequence of 25 residues of trypsin was IVGGYECTPHSQAHQVSLNSGYHFC which is highly homologous with trypsin from other fish species. The affinity chromatography using BPTI-sepharose proved to be an efficient, rapid, fast and viable method for purification of trypsin from cobia (*R. canadum*) and which can be applied on large scale purification.

Highlights:

- A rapid and efficient method for trypsin purification from fish is proposed with using bovine pancreatic trypsin inhibitor affinity chromatography.
- Trypsin from cobia was purified by using this affinity chromatography approach and further characterized.
- The N-terminal sequence for the first 25 amino acids of cobia trypsin was determined.

Keywords: *Rachycentron canadum*, digestive peptidase, pyloric caeca, fish culture

1. Introduction

Cobia (*Rachycentron canadum*) is a marine fish species distributed in tropical and subtropical warm waters except in the eastern Pacific Ocean. This species is the only member of the *Rachycentridae* family, has a carnivorous feeding habit and is currently considered the most important candidate for marine aquaculture in the world including Brazil, and has gained momentum in Asian countries (Shaffer & Nakamura, 1989). Recently, Australia, Marshall Island, Gulf of Mexico, the Caribbean Sea and the United States have started developing hatchery technology and farming of cobia. The expanding interest in cultivation of this specie is due to characteristics like adaptability to captivity, high fecundity, good growth performance, resistance to diseases and excellent fillet quality that is suitable for sashimi, broiled, fried or steamed dishes (Benetti et al. 2008a; Liao et al. 2004). Cobia cultivation was initiated in the early 1990 in Taiwan and is one of the most economically important species in this country.

A series of research about proteolytic enzymes in fish has corroborated the knowledge regarding the importance of trypsin as the key enzyme for feed utilization and growth through its role in the protein digestion processes in aquatic animals (Rungruangsak Torrissen & Male, 2000). Among the digestive enzymes found in pyloric caeca and intestine of fish, trypsin (EC 3.4.21.4) is one of the most studied. This enzyme which is synthesized as an inactive precursor (trypsinogen), is a serine endoprotease characterized by a catalytic triad composed of a histidine, an aspartic acid, and a serine residue that specifically cleaves peptide bonds at the carboxylic end of the amino acid residues arginine (R) and lysine (K) and play major roles in biological process, including the breakdown of polypeptides chains and activation of zymogens of chymotrypsin and other enzymes (Khantaphant & Benjakul, 2010). Therefore, this enzyme has become the focus of several studies in recent years and has been endlessly studied and purified from the pyloric caeca of various species of fish using different

chromatographic methods (Freitas-Júnior et al. 2012; Kanthaphant et al. 2010; Kishimura et al. 2010; Fuchise et al. 2009).

Affinity chromatography is an efficient protein separation method based on the interaction between target proteins and specific immobilized ligands. Successful affinity purification requires a certain degree of knowledge and understanding of the nature of interactions between the target molecule and the ligand to help determine the selection of an appropriate affinity ligand and purification procedure with a minimum number of steps. With the growing popularity of affinity purification, many of the commonly used ligands coupled to affinity matrices are now commercially available and are ready to use (Urh et al. 2009).

Bovine pancreatic trypsins inhibitor (BPTI), also known as aprotinin, is a small basic peptide of 58 amino acids, member of the Kunitz-type inhibitor family and is generally found in high amounts in organs from ruminants, where they have been located in the mast cells. This peptide possesses the ability to inhibit several enzymes among others trypsins, chymotrypsin, kallikrein and plasmin and this inhibitor have been extremely useful to separate peptidases during purification process with great efficiency. Due to its high selectivity, this method has often been used in separation of proteins and peptides, both naturally occurring as well as those obtained by chemical or by genetic engineering (Polanowski et al. 2003).

Thus, the aim of this study was to evaluate the use of BPTI-sepharose affinity chromatography as a tool to purify a trypsin from the pyloric caeca of cobia (*Rachycentron canadum*) as well as provide all basic information including biochemical properties and determine N-terminal sequence and molecular mass on this purified trypsin.

2. Materials and methods

Reagents

BPTI (bovine pancreatic trypsin inhibitor), DMSO (dimethylsulphoxide), PMSF (phenylmethylsulphonyl fluoride), TPCK (N-ρ-L-phenylalanine cholromethyl ketone), EDTA (ethylenediamine tetra acetic acid), TLCK (N-ρ-tosyl-L-lysine chloromethyl ketone), benzamidine, BApNA (N_{α}-benzoyl-DL-arginine ρ-nitroanilide hydrochloride) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The resin (CNBr - activated Sepharose 4B) and PD-10 Desalting Column were purchased from GE, Healthcare. The salts and acid solutions were purchased from Merck (Darmstadt, Germany) and all SDS–PAGE reagents and molecular mass marker were from Bio-Rad Laboratories (Ontario, Canada). All other reagents were of analytical grade.

2.1 Immobilization process

The resin (CNBr - activated Sepharose 4B, GE, Healthcare) was extensively washed using 1mM HCl (500 mL) for 15 minutes with the use of a porous funnel. Then, the ligand (2 mg of BPTI – bovine pancreatic trypsin inhibitor) was dissolved in 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl buffer for 2h at 25ºC under stirring. After this step, the mixture was resuspended in 1 M ethanolamine, pH 8.0, for blocking the remaining active groups, and was stirrers for 2 hours at 25ºC. At the end, the resin was filtered and washed exhaustively with 0.1 M sodium acetate, pH 4.0, as washing buffer and after the latest step, the resin was ready to be packed and used in purifications process.

2.2 Enzyme purification

Specimens of cobia (*R. canadum*) were kindly provided by AQUALIDER (Ipojuca, Pernambuco, Brasil) and Projeto Cação de Escama (UFRPE, Recife, Brasil). The specimens

(n = 3) were sacrificed in an ice bath and had their pyloric caecum (52.3 ± 1.53 g per fish for media) collected and homogenized in 0.1M Tris–HCl containing 0.15 mM NaCl, pH 8.0 (5g of tissue/mL). The resulting homogenate was centrifuged (Herolab UniCen MR Centrifuge, Germany) at 10,000 x g for 20 min at 4ºC to remove cell debris and nuclei. The enzyme was purified from the homogenate supernatant (crude extract) through two steps procedure: (1) Ammonium sulphate fractionation for 1 hour at 4ºC with the final salt saturation of 0-80% and the final supernatant (protein soluble in 80% salt concentration) used in the following step; (2) affinity chromatography (2cm^3 with 5 mL of BPTI-sepharose) at a flow of 1mL/min of 0.1 M Tris–HCl, pH 8.0 as the binding buffer and then 0.5 M KCl–HCl, pH 2.0, as the elution buffer. For each 1 mL fraction collected, 70 µL of 0.1 M Tris–HCl, pH 9.0, buffer was added to alkalinize the sample. The fractions that present tryptic activity were pooled and desalted using PD-10 Desalting Columns (GE, Healthcare) previously equilibrated with 0.1 M Tris–HCl, pH 8.0, as the buffer. Protein concentration of the samples was estimated following the procedure described by Smith et al. (1985), using bovine serum albumin (BSA) as the standard protein.

2.3 Reverse-phase chromatography on C8 column

The purified trypsin from cobia (1 mL) was applied on a reverse-phase Sephasil C8 column from Amersham–Pharmacia Biotech (Uppsala, Sweden) pre equilibrated with 0.1% trifluoroacetic acid (TFA) in Milli-Q water (ÄKTA system). The proteins were eluted using an acetonitrile linear gradient (0 - 90%) in 0.1% TFA (45 min) at a flow rate of 0.7 mL/min and monitored at 215 nm. The collected fractions containing the trypsin activity were dried and stored at - 20ºC.

153 **2.4 N-terminal amino acid sequencing and mass spectrometry**

154 The N-terminal amino acid sequence analysis was performed by automated Edman
155 degradation (Edman & Begg, 1967) on a PPSQ-23 model protein sequencer (Tokyo, Japan)
156 using an online phenylthiohydantoin derivative identification. The molecular mass and purity
157 of the purified enzyme was also checked by matrix-assisted laser desorption/ionization mass
158 spectrometry (MALDI-TOF, TofSpec-E, Micromass).

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160 **2.5 Trypsin activity assay**

161 Trypsin activity was assayed using a specific substrate BApNA at 8 mM prepared in DMSO
162 according to Bezerra et al. (2001). The release of ρ-nitroaniline (product) was monitored at λ
163 405nm in a microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA). One
164 unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing one
165 µmol of BApNA per min (ε = 8.800 M^{-1} cm^{-1}) under the established experimental conditions.
166 Specific activity was expressed as units per mg of protein.

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168 **2.6 SDS-PAGE and zymograms**

169 The purified trypsin from cobia (*R. canadum*) was separated by sodium dodecyl sulphate
170 polyacrylamide gel electrophoresis (100 µg of protein to SDS-PAGE) according to Laemmli
171 (1970) using a 4% (w/v) stacking gel and a 15% (w/v) separating gel. The gel was stained for
172 protein using Comassie Brilliant Blue R250. Electrophoresis was performed at a constant
173 current of 11 mA per gel, at 4°C.

174 Zymogram was carried out following the method described by Garcia-Carreño et al. (1993).
175 After electrophoresis (4°C), gels were immersed in 2.5% Triton X-100 in 0.1 M Tris-HCl, pH
176 8.0, to remove SDS. The Triton X-100 was removed by washing the gels three times with 100
177 mL of 0.1 M Tris-HCl, pH 8.0, as the buffer 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 37°C for 90 min in order to allow the casein digestion by the active proteinases. Finally, gels were stained for protein using Coomassie Brilliant Blue R250.

2.7 Effect of pH and temperature

The effect of pH on purified trypsin was evaluated with BApNA (8 mM) at 25°C in the range of 4 – 11.5 (100 mM citrate – phosphate, Tris–HCl and Glycine – NaOH). The effect of temperature was evaluated in the range of 25 – 75°C using 0.1 M Tris–HCl, pH 8.0, as the 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).

2.8 Effect of synthetic inhibitors and metal ions

The inhibition tests were performed using a methodology adapted from Bezerra et al. (2001). The purified enzyme (30 µL) was incubated in microplates for 30 min with 30 µL of different peptidase inhibitors maintaining a final concentration at 1.2 mM. The inhibitors used in this test were EDTA (chelating compound), PMSF (serine peptidases inhibitor), benzamidine (trypsin inhibitor), TLCK (trypsin inhibitor) and TPCK (chymotrypsin inhibitor). After incubation, 110 µL of buffer 0.1 M Tris–HCl, pH 8.0, and 30 µL of BApNA were then added. After 10 min, the absorbance reading was performed in microplate reader at a wavelength of 405 nm. Controls were performed without the enzyme or substrate solution. The effect of metal ions ($BaCl_2$, $CaCl_2$, $MgCl_2$, $CdSO_4$, KCl, NaCl, $AlCl_3$, $HgCl_2$, $CuCl_2$) was assayed according to Souza et al. (2007) using an initial solution at 1 mM (final concentration at 0.15 mM). After 30 min of incubation, 0.1 M Tris-HCl, pH 8.0, (110 µL) and 8 mM BApNA (30 µL) were added. The ρ-nitroaniline produced was recorded in a microplate reader (Bio-Rad 680) at 405 nm after 15 min of reaction at 25°C.

203 **2.9 Kinetics parameters**

204 The assay was carried out using BApNA as substrate in the range of initial concentration from
205 0.25 to 32 mM and under the same conditions (pH 8.0 and 25°C) as described above. The
206 reaction (triplicates) was started by adding 30 µL of purified enzyme solution (170 µg of
207 protein/mL). Reaction rates were fitted to Michaelis–Menten kinetics, using Origin 6.0
208 Professional.

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210 **2.10 Statistical analysis**

211 The statistical program used was Microcal™ Origin™ version 6.0 (Software, Inc., US).

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3. Results and discussion

The purification of trypsin present in the pyloric caeca from cobia (*R. canadum*) is summarized in Table 1. The precipitate (0 - 80% saturation) ammonium sulfate precipitation showed specific trypsin activity (2.09U/mg) lower than BPTI-sepharose affinity chromatography (28.01U/mg) and one single trypsin peak in the BPTI-sepharose affinity chromatographic profile was measured specific activity using 8 mM BApNA as substrate (Fig. 1A). It can be observed in Table 1, that the purification of trypsin from cobia (*R. canadum*) exhibited a low yield but a high degree of purity (50.8, and yield of 8.6% respectively) and no specific trypsin activity was detected in the final supernatant. In comparison with other trypsins fish reported in the literature, Ktari et al. (2012) obtained after the final purification step, the trypsin was purified 4.2 - fold from zebra blenny (*Salaria basilisca*) while Freitas, Jr. et al. (2012), obtained a yield of 24.9% and 17.4 degree of purification for *Arapaima gigas.* Although other authors found higher values for the yield (%) of fish trypsins, they utilized more steps in the purification process of obtaining samples with less purity degree (fold) as compared to affinity chromatography employed in this study This difference is an indication that despite the loss of enzyme during the purification process, the methodology allows the obtention of a protein with purity higher than those purified from other fishes (Ktari et al. 2012; Freitas-Jr. et al. 2012; Kishimura et al. 2010; Kanno et al. 2010).

The same column (BPTI-sepharose affinity chromatography) was used more than 10 times at an interval of 5 months and remained the same efficiency and quality of purification ensuring the safety and experimental reproducibility in a short time. This justify the use of this chromatography technique for use on an industrial scale is a way to reduce production costs as well as the time spent for obtaining these new enzymes with biotechnological potential. The use of an inhibitor in the purification procedure relies entirely of the

association–dissociation rates of the ligand–protein complex as well as the amount of inhibitor which is coupled to the resin. Therefore, the yield (%) of the purification is directly related to the amount of inhibitor which is coupled to the resin, that is, the higher amount of bound inhibitor, the greater formation of ligand-protein complex and consequently yields obtained at the end of the purification process as well as careful choice of the elution buffer. Therefore, in this study was used of 0.5 M KCl-HCl, pH 2.0, as elution buffer which allowed for a selective recovery of target enzyme.

The reverse-phase chromatography was used to determine the purity and the apparent molecular mass of purified trypsin from cobia (*R. canadum*) and in this study, the physicochemical properties was determined using several assays. The majority single peak fractions obtained for reversed-phase profile were pooled to be used as the starting material for MALDI-TOF (Fig 1B) and was determined to be 24034.273 Da (Fig. 1C). Based in SDS-PAGE, the molecular mass was estimated at 24 kDa as shown in Fig 1C. Other trypsin from different fish species have similar molecular mass values, such as 24 kDa to *Eleginus gracilis* and 23.2 kDa to *Balistes capriscus*, respectively and this result found to trypsin of cobia (*R. canadum*) is consistent with other fish trypsins reported in the literature that may have molecular weight ranging from 21 to 29 kDa (Fuchise et al. 2009; Jellouli et al. 2009; Klomklao et al. 2007).

The N-terminal amino acid sequence of purified trypsin from cobia (*R. canadum*) was aligned to compare with those of other trypsins as depicted in Fig. 2. The N-terminal amino acid sequence of the first 25 amino acids of purified trypsin from cobia was determined to be IVGGYECTPHSQAHQVSLNSGYHFC. Of all marine fish used in alignment of the N-terminal, *Salmo salar* (Male et al. 1995), *Gadus morhua* (Gudmundsdottir et al. 1993), *Paranothotenia magellanica* (Genicot et al. 1996) and *Oncorhynchus keta* (Toyota et al. 2009) were the species that have more similarity to *R. canadum* except for the *Dicentrarchus*

283 *labrax* (Kuhl et al. 2011) that was similar in only 10 amino acids residues. It was found that
284 the sequence of the purified trypsin displayed high homology to other trypsin especially the
285 conservation of consensus IVGG and the sequence from the first to eight residues
286 (IVGGYECT) was similar with *Gadus morhua*, while for *Salmo salar* and *Oncorhynchus*
287 *keta,* only the first to seven amino acid residues (IVGGYEC) were similar to *R. canadum.*
288 Moreover, other important observation for trypsin of cobia (*R. canadum*) was the
289 conservation of Cys (C) residue at position 7 which is common to trypsin pancreatic bovine
290 and this result is in agreement with others fish trypsins reported in literature where is an
291 evidence of formation of disulphide bond to the corresponding residues between Cys-7 and
292 Cys-142 (Roach et al. 1997). The residue sequence (QVSLN) located between amino acid at
293 position 15 to 19 showed conserved for all vertebrate and mammalian trypsins used in this
294 alignment except for *Dicentrarchus labrax* which showed a glutamine (Q) instead of
295 asparagine (N). Generally, the N-terminal region of trypsin-like proteinase, especially from
296 first to seventh residues demonstrates high homology. However, all fish trypsins had a
297 charged Glu (E) residue at position 6, whereas Thr (T) is more common in pancreatic trypsin
298 of mammals such as *Bos taurus* (Mikes et al. 1966) and *Homo sapi*ens respectively (Wiegand
299 et al. 1993) (Fig. 2).

300 Optimum temperature of purified trypsin from cobia (*R. canadum*) was 50ºC using
301 BApNA as substrate (Fig. 3A). This value was similar to other species of fish reported in
302 literature: *D. rhombeus* (Silva et al. 2011), but different to *S. basilisca* and *P. maculatus*
303 which showed an optimum temperature of 60ºC and 55ºC respectively (Ktari et al. 2012;
304 Souza et al. 2007). These differences found for the results of optimum temperature may be
305 due to adaptations to environments colder and warm waters as these fish live, respectively.
306 Among the environmental factors known to play a role in the adaptation of organisms,
307 temperature is an important one as it affects the structure and therefore the stability of proteins

308 along with the catalytic properties of enzymes. This fact could explain the differences
309 between enzymes and their respective substrate used in enzyme kinetics assays (Genicot et al.
310 1996).

311 The purified trypsin from cobia (*R. canadum*) was highly active, ranging from 8.0 to
312 10.0 with optimum pH at 8.5 (Fig. 3B). All purified trypsin of fish reported in literature show
313 optimum pH range from 7.0 to 10.0. This result was lower than *B. capriscus*, *S. basilisca*, *A.*
314 *gigas* and *P. maculates* that showed optimum pH at 10.5, 9.5, 9.0 and 9.0, respectively
315 (Jellouli et al. 2009; Ktari et al. 2012; Freitas-Jr et al. 2012; Souza et al. 2007) and similar to
316 *C. pectoralis* and *D. rhombeus* (8.5) (Klomklao et al. 2009; Silva et al. 2011). At acidic pH
317 ranging from 4 to 6, the activity was almost negligible for purified trypsin from *R. canadum*
318 which is commonly observed in other fish trypsins due to conformational changes under the
319 acid conditions due to enzymatic denaturation which prevents an appropriate binding to the
320 substrate (Klomklao et al. 2011; Klomklao et al. 2009; Kanthaphant et al. 2010).

321 The thermal stability profile of the purified trypsin showed that this enzyme is highly
322 stable at temperatures below 55ºC, and above 60ºC about 20% of activity was maintained but
323 was inactivated at higher temperatures. This result was confirmed by zymogram of thermal
324 stability which is a more sensitive technique employed to confirm the actual activity *in vitro*.
325 Caseinolytic bands of purified trypsin from *R. canadum*, showed loss of band intensity in
326 temperatures higher than 60ºC (Fig. 3C). Currently, most publications focused on the area of
327 the purification of trypsin from fishes are focused on the study of cold-adapted trypsins fish
328 for presenting interesting features that could be very useful in the biotechnological process
329 mainly when compared with mammalian's trypsin or warm blood animals due to better
330 catalytic efficient these enzymes that support low temperatures and exhibit high molecular
331 flexibility allowing interactions with their substrates (Kishimura et al. 2010; Fuchise et al.
332 2009; Klomklao et al. 2009; Gudmundsdóttir & Pálsdóttir, 2005). However, several author

found enzymes of tropical fish with interesting characteristics to biotechnological process in industries of detergent and food (Ktari et al. 2012; Kanno et al. 2011; Silva et al. 2011).

The sensitivity of proteases can be determined by employing synthetic inhibitor and their nature could be confirmed. As show in Table 2, purified trypsin from cobia (*R. canadum*) was strongly inhibited by specific trypsin inhibitors TLCK and benzamidine (92% and 87.1% respectively), EDTA and PMSF (56.3% and 49.1%). These results confirm that the purified enzyme assayed in this work was a serine protease, most likely trypsin.

The effect of various metal ions on trypsin activity from cobia (*R. canadum*) is detailed in Table 2. No metal ion at the final concentration of 0.15 mM was capable of activating the enzyme activity including Ca^{2+}, which is described in the literature as a trypsin activator and in moderate concentrations increase the activity and stability of vertebrate trypsins. The ions Al^{3+}, Ca^{2+}, K^{+}, Ba^{2+} and Na^{+} did not exerted strong inhibition on enzyme activity. The trivalent ion (Al^{3+}) strongly inhibited the trypsin of *A. gigas* and *D. rhombeus* respectively (Freitas-Jr et al. 2012; Silva et al. 2011). However, the activity was strongly affected by Hg^{2+}, Cu^{2+} and Cd^{4+} with 52.2%, 42.7 and 34.5% of inhibition respectively. Hg^{2+} has been reported to bind to SH groups of the target enzyme and subsequently inhibit the enzymatic activity (Klee, 1988). Inhibition of trypsin by Hg^{2+} suggests that the active site of these proteases might also contain cysteine.

The Kinetics parameters such as Michaellis-Menten constant (K_m), molecular catalytic constant (K_{cat}) and catalytic efficiencies (K_{cat}/K_m) for purified trypsin from cobia (*R. canadum*) were evaluated using BApNA as substrate (Table 3). The value of K_m (0.38 mM) represent that the enzyme studied in this work have a higher affinity for BApNA as specific substrate. The latest publications in this area have reported purified trypsin from fishes showing great affinity when employing BApNA as substrate: *Arapaima gigas* (Freitas-Júnior et al. 2012), *Salaria basilisca* (Ktari et al. 2012), *Diapterus rhombeus* (Silva et al. 2011),

358 demonstrating the use of this reagent suitable for tests with trypsin marine fish as well as
359 freshwater fish. The catalysis rate (k_{cat} – enzymatic reactions catalyzed per second) of the
360 purified enzyme was higher than the values found for trypsin from other animals. However,
361 the k_{cat}/K_m (the capability of catalyze the transformation of substrate into product) varied to
362 different extents, as it possible to observe that the purified trypsin from cobia (*R. canadum*)
363 showed the highest catalytic efficiency (k_{cat}/K_m) 8.26 s^{-1} mM^{-1} than other tropical species
364 evaluated in this study using BApNA as the specific substrate except to *L. vitta* (Table 3). In
365 generally, several works have reported that trypsins from fish adapted to cold environments
366 showed higher catalytic efficiencies especially at low temperatures than mammalian trypsins
367 (Kristjansson, M. 1991; Simpson and Haard 1984). This feature is due to high molecular and
368 conformational structures flexibilities which appear to play important role in enzyme activity
369 as well as substrate specificity allowing the adequate interaction and transformation (Aghajari
370 et al. 2003; Gudmundsdóttir et al. 2005). This property is a desirable feature and has attracted
371 the interest of the commercial use of these enzymes usually present more suitable for
372 biotechnological process.
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4. Conclusions

The use of BPTI-Sepharose affinity chromatography proved to be a suitable and efficient method for the purification of trypsin from cobia (*R. canadum*). The purified enzyme was strongly inhibited by benzamidine and TLCK, showed an apparent molecular mass of 24 kDa and exhibited a conserved trypsin NH2-terminal amino acid sequence. The physicochemical properties were similar to other species of fish reported in literature and these results suggest that this enzyme may be classified as a trypsin. This source of trypsin for presenting characteristics as high optimum temperature, optimum pH and catalytic efficiency can also be an attractive alternative to meet the growing demand of additives for detergent industry.

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573 FIGURE CAPTIONS

574 Fig. 1. Purification of trypsin from the pyloric caeca of cobia (*R. canadum*). (A) Elution
575 profile of trypsin on affinity chromatography column (BPTI-sepharose): (●) specific tryptic
576 activity (□) protein measurement (A280 nm). Fig. 1(B) Elution profile of trypsin on reverse-
577 phase chromatography: The sample was applied on a Sephasil Peptide C_8 column previously
578 equilibrated with 0.1% trifluoroacetic acid (TFA) solution. Protein was eluted (indicated by a
579 black arrow) with linear gradient of acetonitrile (0 - 100%) and constant flow rate of 0.7
580 mL/min. Fig. 1(C) MALDI/TOF-MS spectrum of purified trypsin from the pyloric caeca of
581 cobia (*R. canadum*) and SDS-PAGE: Lane 1: standard molecular weight, lane 2: purified
582 trypsin.

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584 Fig. 2. Alignment of the NH_2-terminal amino acid sequence of trypsin from cobia
585 (*Rachycentron canadum*) compared to trypsins sequences to other species of fish and
586 vertebrates. *Salmo salar* (Male et al. 1995), *Gadus morhua* (Gudmundsdottir et al. 1993),
587 Hybrid catfish (Klomklao et al. 2011), *Paranotothenia magellanica* (Genicot et al. 1996),
588 *Dicentrarchus labrax* (Kuhl et al. 2011), *Oncorhyncus keta* (Toyota et al. 2009), *Bos taurus*
589 (Mikes et al. 1966), *Homo sapiens* (Wiegand et al. 1993).

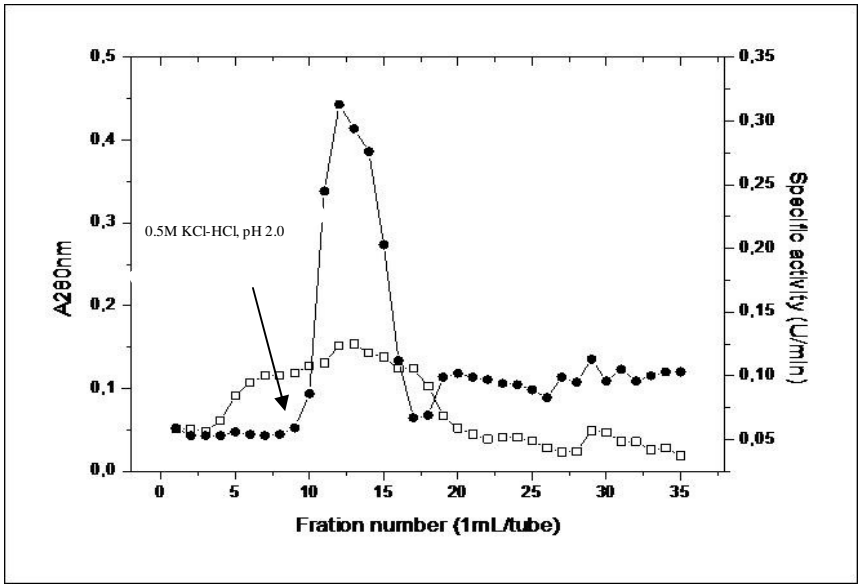
590

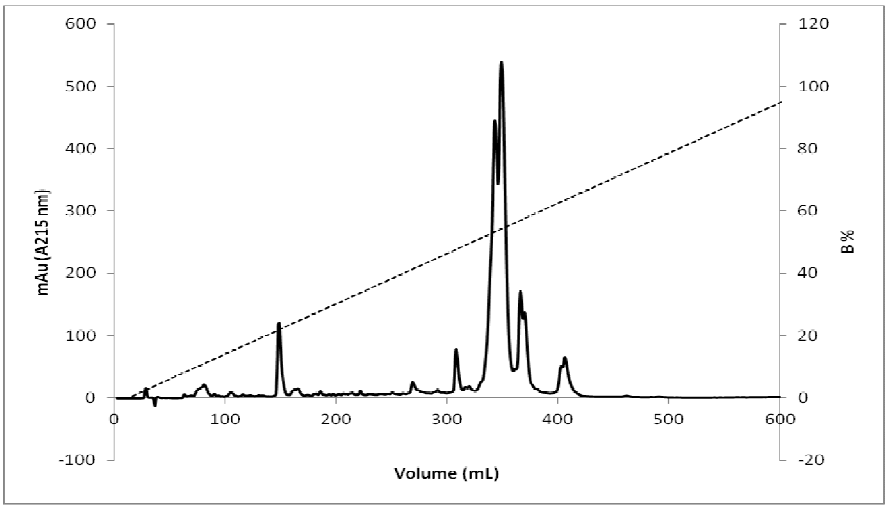
591 Fig. 3. Effect of temperature, pH and thermal stability on purified trypsin from the pyloric
592 caeca of cobia (*R. canadum*). (A) The activity was evaluated at temperatures ranging from
593 25ºC to 70ºC using BApNA (8 mM) as specific substrate at final concentration of 1.2 mM.
594 (B) Optimum pH for activity of enzyme, utilizing various buffers in the pH range 4 – 11.5.

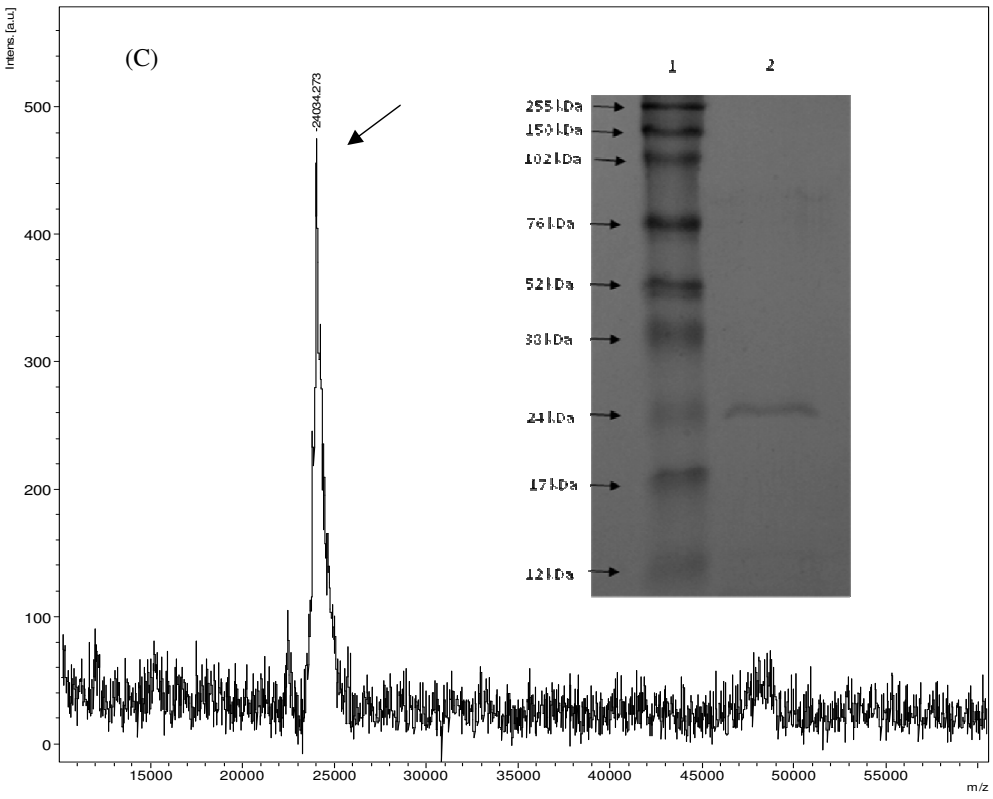
(C) Thermal stability of enzyme, after 30 min of incubation in the temperature range 25 – 70ºC.

FIGURES

Fig. 1(A), 1(B) and 1(C).



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662 Fig. 2

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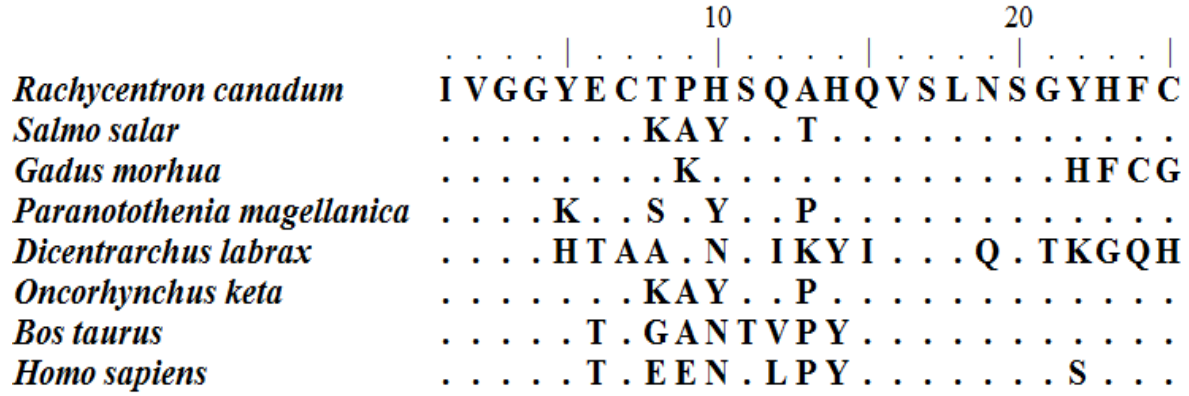

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686 Fig. 3(A), 3(B) and 3(C).

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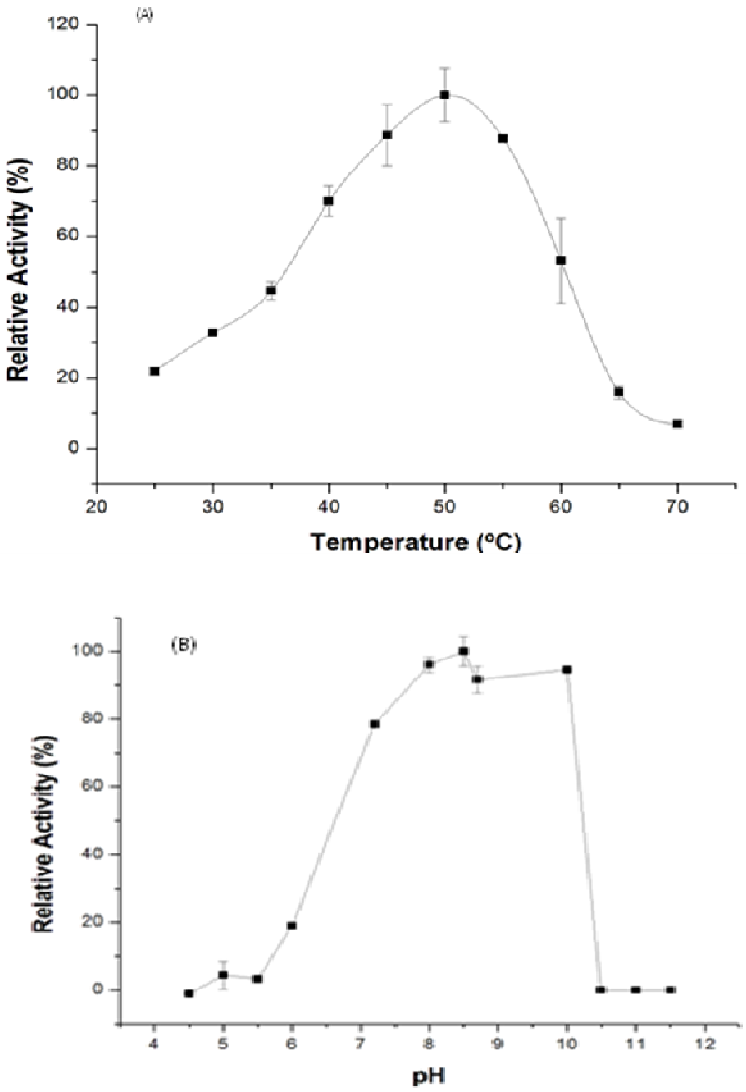

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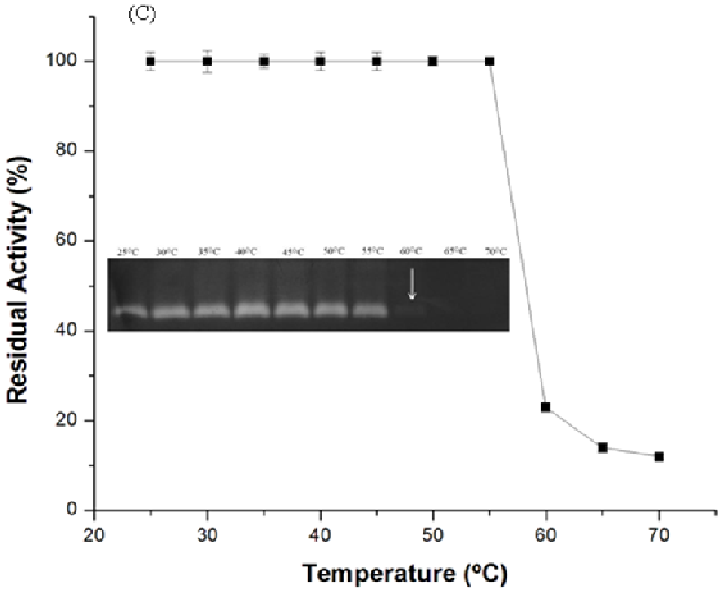
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Residual Activity (%)
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TABLES

Table 1. A summary of the purification process of trypsin from cobia (*R canadum*)

Procedure	Protein (mg)	Trypsin activity (U)	Specific Trypsin activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	446.2	246	0.55	100.0	1.0
Ammonium sulphate (0-80%)	79.7	166.8	2.09	67.8	3.8
BPTI-Sepharose	0.8	21.09	28.01	8.6	50.8

All steps were carried out at 4 ºC.

782 Table 2. Effect of inhibitors and metal ions on purified trypsin from the pyloric caeca of cobia
783 (*Rachycentron canadum*)
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Inhibitors/Ions	Inhibition (% ± SD)
PMSF	49.1 ± 2.81
TLCK	92.0 ± 4.65
Benzamidine	87.1 ± 6.67
TPCK	29.6 ± 1.25
EDTA	56.3 ± 4.65
Ions (final concentration 0.15mM)	
Control 100%	0
Hg^{2+}	52.2 ± 5.35
Cu^{2+}	42.7 ± 3.34
Cd^{4+}	34.5 ± 1.25
Mg^{2+}	28.8 ± 7.21
Na^{+}	22.7 ± 5.11
Ba^{2+}	22.6 ± 5.46
K^{+}	17.1 ± 5.71
Ca^{2+}	15.9 ± 0.16
Al^{3+}	15.5 ± 2.18

785 All inhibitors were prepared in DMSO (dimethylsulfoxide) with a final
786 concentration at 1.2 mM.
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790 Table 3. Kinetic parameters of purified trypsin from *Rachycentron canadum* and other
791 species of fish
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Species	Parameters			References
	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})	
Rachycentron canadum	0.38	3.14	8.26	This study
Arapaima gigas	0.47	1.37	2.83	Freitas-Jr. et al. 2012
Salaria basilisca	0.6	1.38	2.3	Ktari et al. 2012
Diapterus rhombeus	0.266	0.93	3.48	Silva et al. 2011
Lutjanus vitta	0.507	4.71	9.27	Khantaphant et al. 2010
Bovine	2.017	0.5	0.248	Simpson and Haard 1984

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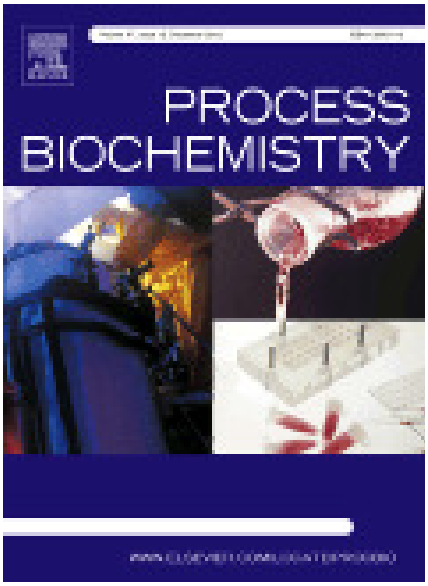
CAPÍTULO 2: TRYPSIN FROM COBIA (*Rachycentron canadum*) AS DETERGENT ADDITIVE: A BIOCHEMICAL AND STRUCTURAL STUDY



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TRYPSIN FROM COBIA (*Rachycentron canadum*) AS DETERGENT ADDITIVE: A BIOCHEMICAL AND STRUCTURAL STUDY

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Abstract

A trypsin from the pyloric caeca of cobia (*R. canadum)* was purified by two steps process: ammonium sulfate (0-80% saturation) precipitation and affinity chromatography (bovine pancreatic trypsin inhibitor, -BPTI-sepharose). The physical-chemical properties, stability toward surfactants and compatibility with solid commercial laundry detergents were compared with Alcalase®, Flavourzyme®, Commercial Trypsin® and Commercial Chymotrypsin® (Sigma-Aldrich). The purified enzyme from cobia showed an apparent molecular mass of 24 kDa. Optimum temperature and pH were 55ºC and 10.0 respectively, using 1% azocasein as substrate. The enzyme also demonstrated to be relatively stable toward various surfactants: Tween 20, Tween 80, Triton X-100, Sodium Choleate with exception of the anionic SDS (Sodium Dodecyl sulfate), which strongly inhibited the enzyme activity. All enzymes were also compatible with solid commercial laundry detergents (7mg/mL), such as, Omo®, Ace®, Surf® and Ala®, retaining more than 70% of their activity after 40 min of incubation at 25ºC. The only exception was the Commercial Chymotrypsin® that was completely inhibited by all solid detergents. From these characteristics showed by trypsin from cobia, this enzyme could be a potential candidate to be used as an additive in laundry industry.

Keywords: laundry detergents, *Rachycentron canadum*, surfactants, circular dichroism

1. Introduction

The production of soap is a human practice that dates back to 2200 BC, and while ancient soaps were mainly composed of oil, alkali and water [1], cleaning products today are composed of complex mixtures of surfactants, bleaching agents, enzymes, dispersing agents, fabric softening, optical brighteners and perfumes [2,3]. Even though the basic principles of cleaning agents have been kept the same throughout the years, new additives such as alkaline proteases, have made them much more efficient.

The advantage of adding enzymes to the washing process is mainly due to their hydrolytic capabilities, which increase in about 35-40% the cleaning efficiency of detergents. Moreover, the use of enzymes allows for reduction in the consumption of chemicals products, water and energy. This leads to a reduction in waste generation, thus making those products more environmentally friendly, what is a desirable characteristic for all industries [6, 7, 8, 9].

Proteases were used for the first time in laundry detergent formulation in 1913, when Otto Röhm and Otto Haas introduced porcine pancreatic extract to their detergents [5]. However, it was only in 1963, with the application of Alcalase (a subtilisin Carlsberg from *Baccilus licheniformis*), that the use of proteases in detergents became more effective [1]. Currently, the protease market intended for this purpose is largely represented by sutilisins and/or alkaline proteases belonging to bacteria of the genus *Bacillus*. Due to this development, proteases today correspond to 40% of the world enzymes market [4].

Since the introduction of Alcalase, other sources of enzyme have been sought as alternatives for detergents incorporation. There are several ways to assess whether a new found enzyme is a suitable detergent additive, being biochemical and structural analysis the most common. Studies of secondary structure using the non-destructive techniques, like circular dichroism (CD), is one way to observe the ideal conditions in which proteins perform their biological function. This helps researchers to understand how a biological molecule

behaves in certain situations of environmental disturbance like changes in pH, temperature, and addition of surfactants [17].

Currently, a well known rich source of enzymes is the fisheries and aquaculture processing waste, mainly fish viscera [10, 11], in which the most important group of alkaline proteases found is trypsin (EC 3.4.21.4). Trypsin-like enzymes have been isolated from several species and evaluated with respect to their compatibility with various commercial ingredients of detergent formulations [11, 12, 13]. In this context, marine animals have an advantage to other organisms, because they live in ecosystems that is constantly changing, leading these animals to develop the ability to adapt to these environments and, therefore, are these adaptations together with inter and intra genetic variations contribute to the unique properties of certain proteases its marine animals, particularly fish, thereby providing a demand for new sources of alkaline enzymes [14].

Cobia (*R. canadum*) is a marine fish species distributed worldwide in warm waters with the exception of eastern of Pacific Ocean [15]. This fish has been considered as an excellent candidate species for offshore farming system and some projects already being developed along the Brazilian coast [16]. Thus, the amount of viscera generated by cultivation of cobia can be used as an alternative source for extraction of alkaline proteases (especially trypsin) and contribute to the reduction of environmental pollution making this activity more profitable and economically.

The aim of this study was to observe the changes in secondary structure of purified trypsin obtained from the pyloric caeca of cobia and determine the compatibility with commercially avaiable soap powder formulations and evaluate the stability of this biomolecule through *in vitro* assay by incubation with oxidizing agents, surfactants and compare this activity with that of others commonly used commercial enzymes in the detergent industry.

2. Materials and Methods

2.1 Enzyme purification

Specimens of Brazilian cobia (*R. canadum*) were kindly provided by AQUALIDER (Ipojuca, Pernambuco, Brasil) and Projeto Cação de Escamas (UFRPE). The specimens were sacrificed in an ice bath and had their pyloric caecum (52.3 ± 1.53 g per fish in average) collected and homogenized in 0.1M Tris–HCl containing 0.15mM NaCl, pH 8.0, at a ratio of 1:5 (w/v). The resulting homogenate was centrifuged (Herolab UniCen MR Centrifuge, Germany) at 10,000 x g for 20 min at 4ºC to remove cell debris and nucleii. The enzyme was purified from the homogenate supernatant (crude extract) through a two steps procedures: (1) Ammonium sulphate fractionation: incubation for 1 hour at 4ºC with final salt saturation salt of 80%, followed by centrifugation at 10,000 x g for 20 min at 4ºC; (2) affinity chromatography (2cm^3 with 5mL of BPTI-sepharose) at a flow of 1mL/min of 0.1M Tris–HCl, pH 8.0 as the binding buffer and 0.5M KCl–HCl, pH 2.0, as the elution buffer. For each 1mL of fraction collected, 70µL of 0.1M Tris–HCl, pH 9.0, buffer was added to alkalinize the sample. These fractions that presented tryptic activity (monitored using BApNA 8mM as specific substrate) were pooled and dessalted using PD10 columns (GE Healthcare) previously equilibrated with the buffer 0.1M Tris–HCl, pH 8.0. Protein concentration of the samples was estimated following the procedure described by Smith et al. [18], using the Pierce® BCA Protein Assay Kit (Thermo Scientific), as well as bovine serum albumin (BSA) as the standard protein.

2.2 Immobilization process

The resin (CNBr-activated Sepharose 4B) was extensively washed using 1mM HCl (500 mL) for 15 minutes with the use of a porous funnel. Then, the ligand (2mg of BPTI) was dissolved in 0.1M $NaHCO_3$, pH 8.3, containing 0.5M NaCl buffer for 2h at 25ºC under stirring. After this step, the mixture was resuspended in 1M ethanolamine, pH 8.0, for blocking the active

groups, and stirring for 2 hours at 25ºC. At the end, the resin was filtered and washed exhaustively with 0.1M sodium acetate, pH 4.0, as buffer and used in purifications process

2.3 Assay for Alkaline Proteases

Protease activity was assayed using 1% azocasein (w/v) as substrate according to Bezerra et al. [19]. The samples were incubated in triplicate for 60 minutes at 25ºC as well as 40°C, to simulate the temperature usually seen during a washing process in a washing machine. After incubation of enzyme with substrate, 240µL of trichloroacetic acid (TCA) was added to stop the reaction, and after 15 min, the tubes were centrifuged for 5 min at 8,000 x g. The supernatant (70µL) was mixed with 1M NaOH in a microtiter plate and the absorbance was measured at 450nm against a blank, which consisted of distilled water instead of the enzyme. One unit (U) of enzyme activity was defined as the amount of enzyme able to hydrolyze 1% azocasein to produce a change of 0.001 of absorbance per minute.

2.4 SDS-PAGE

The purified trypsin from cobia was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (100µg of protein to SDS-PAGE) according to Laemmli [20] using a 4% (w/v) stacking gel a 15% (w/v) separation gel. The gel was stained for protein using Comassie Brilliant Blue R250. Electrophoresis was performed at a constant current of 11mA per gel, at 4ºC.

2.5 Effect of pH

Protease activity was measured at different pH values under standard assay conditions. The enzymatic activity was assayed using 1% azocasein prepared in each specific buffers: 0.1M phosphate buffer (pH 6.5-7.5), 0.1M Tris–HCl buffer (pH 7.2-9.0) and 0.1M NaOH/Glycine

buffer (pH 8.6 - 12.5). The effect of pH on enzyme stability was studied by incubating the enzyme for 30 min at 25°C.

2.6 Effect of oxidizing agents

Hydrogen peroxide stability of the proteases from cobia was investigated by incubating samples (600µL) with H_2O_2 (600µL) at concentrations of 5%, 10% and 15% at room temperature. Samples (150µL) were withdrawn at 15, 30, and 75 min intervals to establish their activities (triplicates) on 1% azocasein and to compare them to the non treated sample Moreira et al. [21].

2.7 Effect of Surfactants

Stability with regard to anionic (SDS and sodium choleate) and nonionic surfactants (Tween 20, Tween 80 and Triton X-100) was investigated by incubating the purified enzyme in a 1% concentration of surfactant solution (w/v) for 30 and 60 min at 40ºC, after which enzyme activity was assayed Moreira et al. [21].

2.8 Compatibility with Commercial Detergents

The purified trypsin from cobia at a concentration of 0.20 mg/mL^{-1} was incubated at 40°C with commercial detergents: Ala® (Procter & Gamble); Ace® (Procter & Gamble); Omo Multi Ação® (UniLever) and Surf® (UniLever) in a final concentration of $7mg/mL^{-1}$. Samples were collected at 10 min intervals for 40 min. The residual proteolytic activity in each sample was determined at room temperature and compared with the control sample incubated in 0.1M Tris-HCl, pH 9.0. Protease activity was assayed in triplicate using 1% azocasein (w/v) as substrate according to Bezerra et al. [19].

3. Results and discussion

The purification of trypsin present in the pyloric caeca from cobia (*R. canadum*) is summarized in Table 1. The 0-80% saturation- ammonium sulfate precipitation showed specific trypsin activity (2.09U/mg) lower than BPTI-sepharose affinity chromatography (28.01U/mg) and the chromatogram showed a single trypsin activity peak. The BPTI-sepharose step allowed a purification fold of trypsin from cobia (*R. canadum*) of 50.8, and yield of 8.6%. Comparing these results with those found in the literature for fish trypsins, is possible to observe that the purification of the trypsin from cobia showed a low yield, but a high degree of purity. Freitas, Jr. et al. [22], obtained a yield of 24.9% and 17.4 degree of purification for *Arapaima gigas*, Fuchise et al. [23] obtained a 17% yield and degree of purification of 28 for *Eleginus gracilis*, and Souza et al. [24] reported a 26.1% yield and degree of purification of 57.7 for *Pseudupeneus maculatus*. This difference is an indication that despite the loss of enzyme during the purification process, the methodology allows the obtention of a protein with purity higher than those purified from other fishes [10, 25].

The SDS-PAGE showed one single protein band, which estimated molecular mass of 24 kDa (Fig. 1). This value is consistent with other fish trypsins reported in the literature that may have molecular weight ranging from 21 to 29 kDa such as *Siniperca chuatsi* trypsin A and B (21 e 21.5 kDa), *Eleginus gracilis* (24 kDa), *Balistes capriscus* (23.2 kDa), *Sarda sarda* (29 kDa) [23, 26, 27, 28] .

In the pH experiment, the purified trypsin from cobia retained more than 80% of with maximum activity at 10.0. At pH 7.0 - 8.5, more than 55% of activity was observed for purified trypsin from cobia and after pH 11.5, the purified trypsin activity was reduced to 59% (Fig. 2a). The Flavourzyme® (Fig .2d), showed optimum pH at 7.0, and Alcalase® (Fig. 2e), showed the highest optimum pH at 10.5. Jellouli et al. [26], found optimum pH at 10.5 from *Balistes capriscus* and the similar result were found to *Lithognathus mormyrus* that showed

optimum pH at 10.0 [12]. Espósito et al. [29], found an optimum pH at 11.0 for the partially purified alkaline protease from *Cyprino carpius* L. another important representative species of Brazilian fish. Generally, trypsins and alkaline proteases isolated from fish viscera have shown optimum activity to pH ranging from 7.0 to 11.0. The remarkable activity of purified trypsin from cobia over a wide pH range reinforces the alkaline nature of this protease, which has desirable characteristic to be applied to the detergent formulation.

The purified trypsin from cobia was active and at the different temperatures used in the present study. The optimum temperature observed for purified trypsin from cobia was 55ºC (Fig. 3a) while Commercial Chymotrypsin® (Fig. 3c) and Flavourzyme® (Fig. 3d), showed optimum temperatures at 45ºC and 40ºC respectively. At 60ºC and 65ºC, the purified trypsin from cobia showed a residual activity at 33.2% and 25.1% respectively. Sila et al. [11], found similar result from *Barbus callensis* (55ºC) and Ali et al. [12], found from *Lithognathus mormyrus* an optimum temperature at 50ºC. According to Klomklao et al. [30], the use of alkaline proteases from viscera fish, especially trypsin, greatly increased, since this enzyme is active and stable in harsh conditions, such as at temperatures of 50 to 60°C.

As additive detergent, trypsin from cobia was analyzed in presence of surfactants at 25ºC and 40ºC for 1 hour using 1% azocasein as substrate (Fig 4a). The highest activities were observed for trypsin from cobia at 25ºC: Tween 20 (129% ± 0.3), Tween 80 (139.25 ± 0.2), SDS (45% ± 0.2), Sodium Choleate (196.74 ± 0.2) and Triton X-100 (87.7 ± 0.3). Commercial Trypsin® and Chymotrypsin ®, were strongly inhibited by anionic surfactant SDS (Fig. 4b and 4c), SDS is a strong denaturant agent [31].

The incubation of trypsin from cobia with solid commercial detergents for 1 hour at 40ºC was not enough to drastically affect its activity that remained around 45% of residual activity. In this assay, SDS was the surfactant agent that strongly inhibited trypsin from cobia which showed a residual activity of 18.2% and the same behavior was observed for the

226 commercial enzymes tested in this study: Commercial Trypsin® (8.1% ± 0.4), Commercial
227 Chymotrypsin® (9.2% ± 0.2), Alcalase® (0.5% ± 0.3) and Flavourzyme® (18.3% ± 0.4).
228 Esposito et al. [13], found that SDS strongly inhibited the trypsin from the lane snapper
229 (*Lutjanus sinagris*). Therefore, the stability of trypsin from cobia suggests that it is a potential
230 candidate for a detergent additive.

231 The compatibility of a purified trypsin from cobia was tested with commercial solid
232 laundry detergents available in local Brazilian markets. The results presented in Fig. 5,
233 showed that a purified trypsin from cobia was extremely compatible and stable to all solid
234 laundry detergents used in this assay after incubation for 40 minutes at 25ºC. It maintained
235 more than 50% of residual activity. Espósito et al. [13] obtained similar results for trypsin
236 from *L. synagris* after 1 hour of incubation with Surf® and Ala® at 25ºC. During the
237 incubation with Omo®, the purified trypsin from cobia showed the highest residual activity
238 (97.44% ± 0.2) as well as Alcalase® (80.54% ± 0.3), while Flavourzyme®, was the only
239 commercial enzyme that had its commercial activity inhibited by Omo®. The Commercial
240 Chymotrypsin® was completely inhibited by all commercial solid laundry detergents. To be
241 considered an excellent enzyme to be used as an additive in detergent industry, it is important
242 to have the ability to remain active in the presence of laundry detergents during washing
243 conditions and purified trypsin from cobia showed to be a suitable additive for laundry
244 detergent formulations.
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Conclusions

The results of this study suggest that the viscera of cobia (*R. canadum*) is a potential source of trypsin with interesting properties such as high activity and stability at high alkaline pH (9.5-11.0) and temperature (40-55ºC), as well as relative stability in the presence of surfactants and commercial solid detergents, which can make this enzyme as a potential candidate for the development of industrial process waste based sustainable as alternative to use of microbial enzymes.

Acknowledgments

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405 **Figure legends**

406 Fig. 1. SDS-PAGE of purified trypsin from cobia (*R. canadum*). 1. Molecular weight
407 standard. 2. Purified Trypsin from cobia (100µg).

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409 Fig. 2. Effect of pH on the purified trypsin from cobia (*R. canadum*) and commercial enzymes
410 using 1% azocasein as substrate. (a) Trypsin from *R. canadum.* (b) Commercial trypsin®. (c)
411 Commercial Chymotrypsin®. (d) Flavourzyme®. (e) Alcalase®.

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413 Fig. 3. Effect of temperature on the purified trypsin from cobia (*R. canadum*) and commercial
414 enzymes using 1% azocasein as substrate. (a) Trypsin from *R. canadum.* (b) Commercial
415 trypsin®. (c) Commercial Chymotrypsin®. (d) Flavourzyme®. (e) Alcalase®.

416

417 Fig. 4. Stability of purified trypsin from cobia (*R. canadum*) in the presence of various
418 surfactants agents: 1: 1% Tween 20. 2: 1% Tween 80. 3: 1% SDS. 4: 1% Sodium Choleate. 5:
419 Triton X-100.

420

421 Fig. 5. Stability of purified trypsin from cobia (*R. canadum*) in the presence of various solid
422 commercial laundry detergents: Ala®, Ace®, Surf® and Omo® (7mg/mL).

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Figures

Fig. 1.

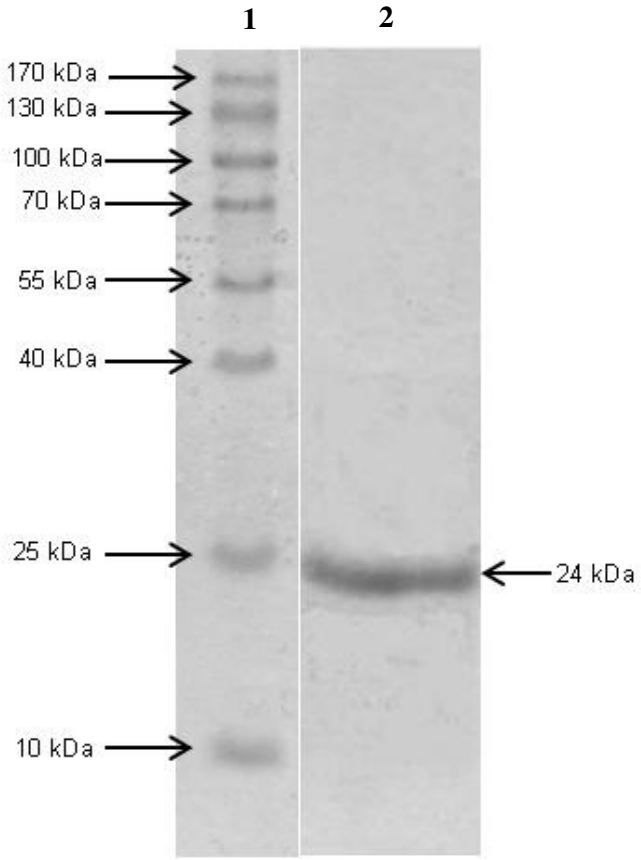
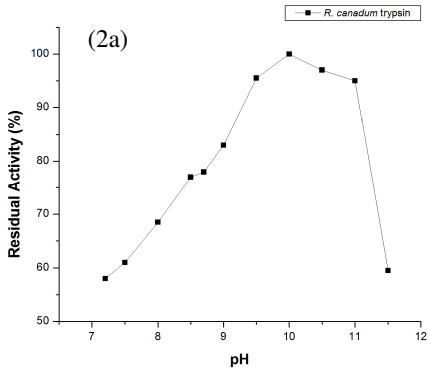
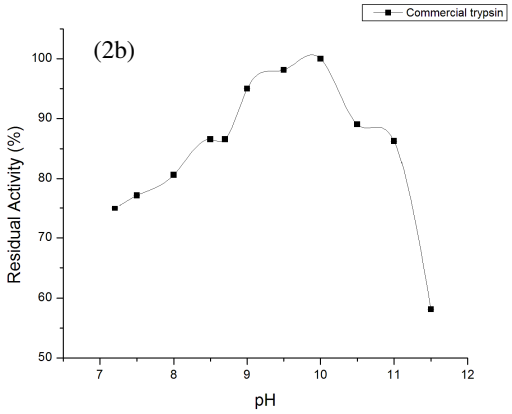
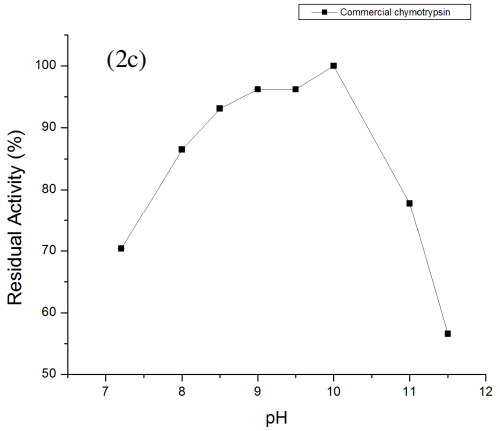
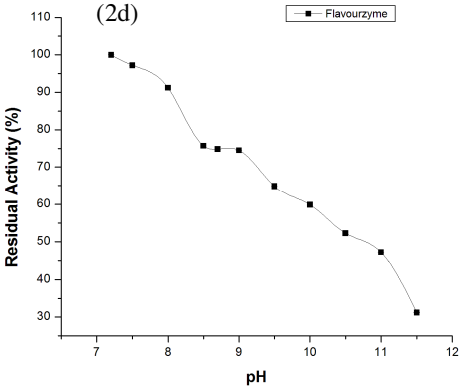


Fig. 2(a), 2(b), 2(c), 2(d) and 2(e)









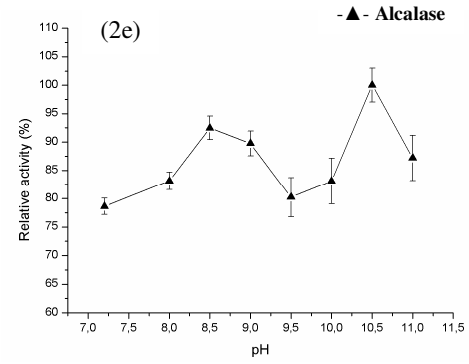
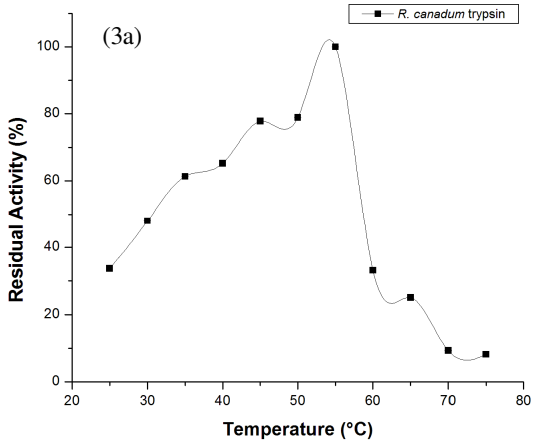
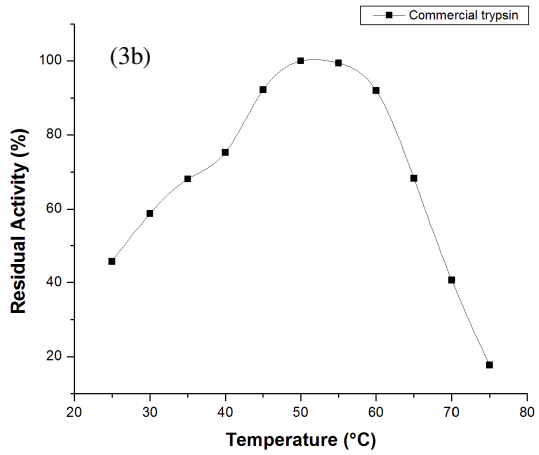
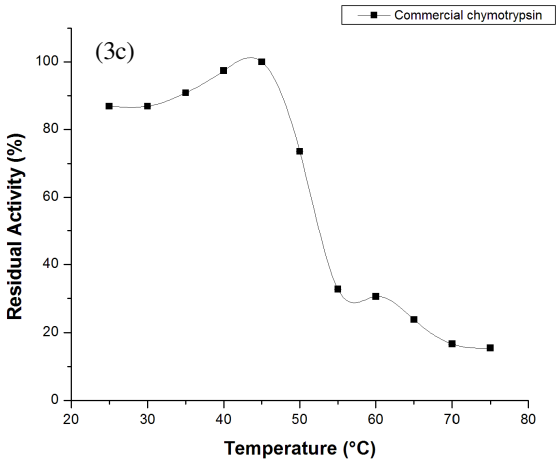
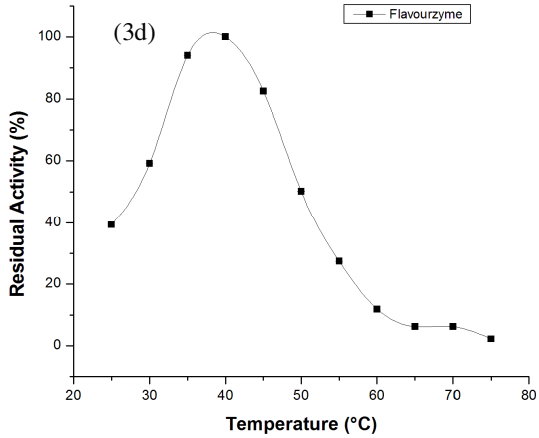


Fig. 3(a), 3(b), 3(c), 3(d) and 3(e)









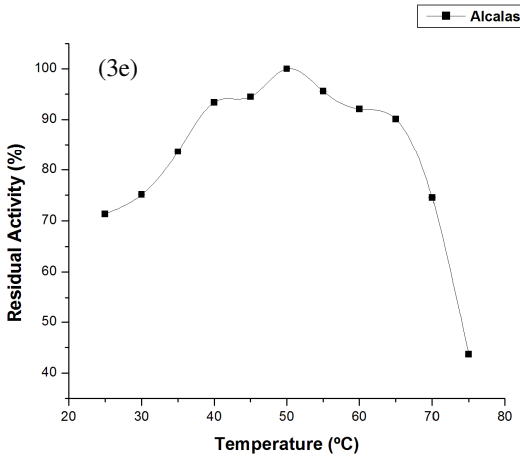
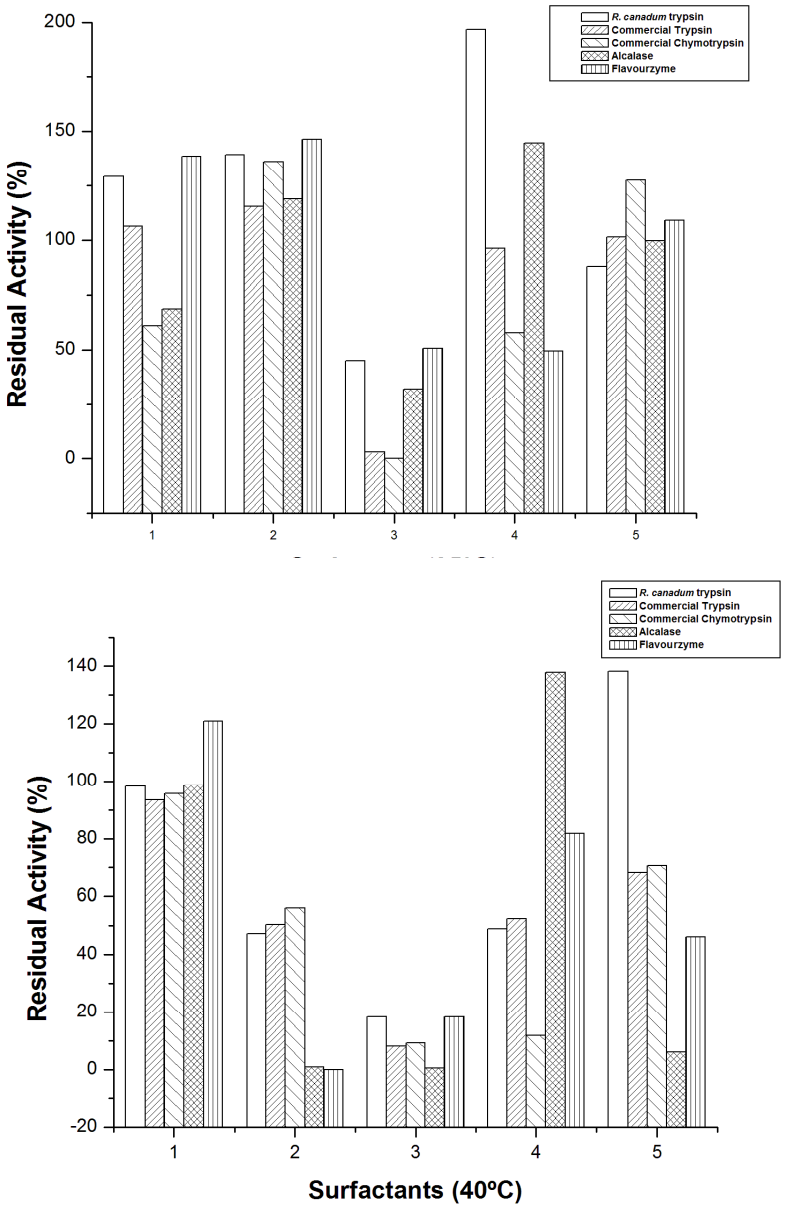


Fig. 4.


R. canadum trypsin
Commercial Trypsin
Commercial Chymotrypsin
Alcalase
Flavourzyme
Residual Activity (%)
200
150
100
50
0
1
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3
4
5
R. canadum trypsin
Commercial Trypsin
Commercial Chymotrypsin
Alcalase
Flavourzyme
Residual Activity (%)
140
120
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80
60
40
20
0
-20
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3
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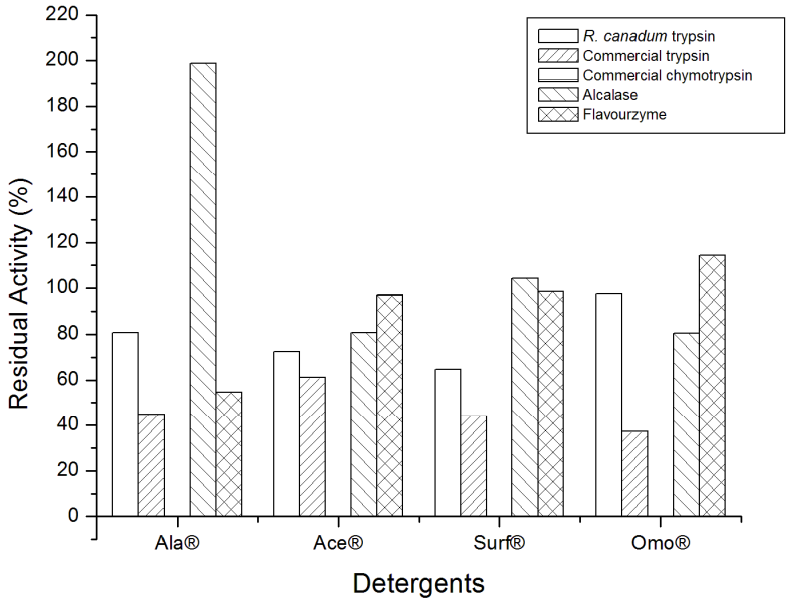
Surfactants (40ºC)

Fig. 5.


R. canadum trypsin
Commercial trypsin
Commercial chymotrypsin
Alcalase
Flavourzyme
Residual Activity (%)
0
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140
160
180
200
220
Ala®
Ace®
Surf®
Omo®
Detergents

599 Tables

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602 Table 1. A summary of the purification process of trypsin from cobia (*R canadum*)

603

Procedure	Protein (mg)	Trypsin activity (U)	Specific Trypsin activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	446.2	246	0.55	100.0	1.0
Ammonium sulphate (0-80%)	79.7	166.8	2.09	67.8	3.8
BPTI-Sepharose	0.8	21.09	28.01	8.6	50.8

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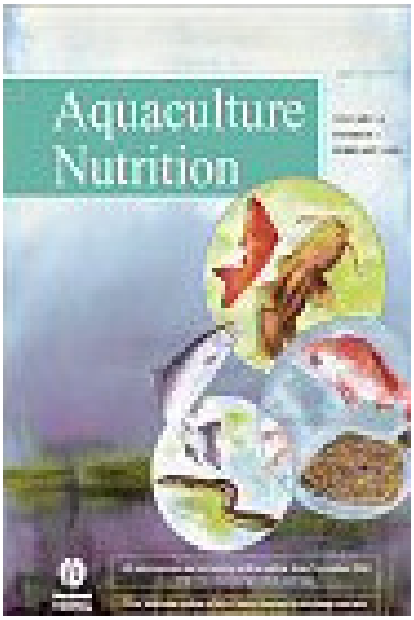
CAPÍTULO 3: PEPTIDASES FROM THE PYLORIC CAECA OF WILD AND FARMED (F1 GENERATION) COBIA (*Rachycentron canadum*)



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PEPTIDASES FROM THE PYLORIC CAECA OF WILD AND FARMED (F1 GENERATION) COBIA (*Rachycentron canadum*)

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Running Title: Digestive peptidases from Cobia (*Rachycentron Canadum*)

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

1. ABSTRACT

Digestive peptidases from the pyloric caeca of wild and farmed cobia (*Rachycentron canadum*) were characterized using specific trypsin and chymotrypsin substrates (BApNA and SApNA respectively) and inhibitors. Also the protein profile was determined employing SDS-PAGE and zymograms. To both wild and farmed Brazilian 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 195kDa to 6kDa. Peptidase activities were strongly inhibited by synthetic trypsin and chymotrypsin inhibitors, suggesting the presence of trypsin-like and chymotrypsin-like enzymes from the pyloric caeca of both wild and farmed cobia. Also, zymograms revealed that the caseinolytic profiles for wild and farmed animals were similar, suggesting that digestive peptidases were not influenced by either the employed artificial diet or the management of cultivation.

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

2. INTRODUCTION

The domestication process of animals is very important event in human history that have to purpose to economic profit for humanity that maintains total control over its breeding, organization of territory and food supply (Diamond, 2002).

During the past three decades, technological advances in fishing and aquaculture together with the trends of globalization, consumer requirements and demand caused by global population growth has resulted in continued growth in production and marketing of fishery products (Shann-Tzong Jiang 2010). Therefore, aquaculture is considered the sector of animal production more 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 populations of fish (Hiddink et al. 2008).

Cobia (*Rachycentron canadum*) (Linnaeus, 1766) is a specie with great possibility to leverage to marine psiculture because present some economic and husbandry interesting aspects. This specie is considered to be a promising candidate for commercial production, since it is a rustic fish, with white flesh 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). *R.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. Since February 2009 was the species chosen for the first crop offshore in Northwest of Brazil (Sanches et al. 2008; Benetti et al. 2003).

The stress caused by confinement is an inevitable component in modern practices of raising animals in captivity and could produce negative effects on growth, reproductive

function and immune function. During the domestication process of fish, digestive enzymes have adaptations under the conditions that are submitted the animals. Due to this fact, several studies about the characterization physical-chemical of these enzymes that suffer this process denominated as enzymatic adaptation has achieved over the last years.

Increased scientific research on the physiological mechanisms and digestive enzyme (mainly trypsin and chymotrypsin) generates information that provide the rationale for their crops, while respecting the physiological differences between aquatic animals as well as their welfare (Douxfils, et al. 2011; Millot et al. 2010; Vandeputte et al. 2009; Jentoft et al. 2005).

Trypsin and chymotrypsin are serine proteases (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).

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 weight gain. Therefore, the aim of this study was to characterize, compared and evaluate digestive peptidases present in pyloric caeca from wild and farmed cobia in initial process of domestication 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 (Ipojuca, Pernambuco, Brasil) while wild cobia was captured in open sea. The animals were sacrificed in an ice bath and their pyloric caeca removed 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 nuclei. The supernatant (crude extract) was frozen at -20 ºC and used for further characterization studies. Protein concentration of the samples was estimated following the procedure described by Bradford (1976), using bovine serum albumin (BSA) as the standard protein.

3.3 TRYPTIC ACTIVITY ASSAY

Trypsin-like 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 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 BApNA per min (ε = 9.100 M^{-1} cm^{-1}) under established conditions. Specific activity was expressed as units per mg of protein.

3.4 CHYMOTRYPTIC ACTIVITY ASSAY

Chymotrypsin-like 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 (ε = 9.100 M^{-1} cm^{-1}) under the established conditions. Specific activity was expressed as units per mg of protein.

3.5 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 25 ºC after pre-incubation for 30 min at temperatures ranging from 25 to 75 ºC (Bezerra et al., 2001).

3.6 EFFECT OF SYNTHETIC INHIBITORS

The effect of protease inhibitors on crude extract from the pyloric caeca of cobia was evaluated using BApNA (8 mM), 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) and benzamidine. Samples of the crude extract (30µL) and 0.1 mM Tris-HCl pH 8.0 (115 µL) were transferred to a 96-well microtiter plate containing the previous inhibitors (25 µL). After incubation for 15 min, 8mM BApNA or SApNA (30µL) was added in the tests on samples and SApNA (30 µL) TPCK. The increase in absorbance at λ 405nm was monitored using a microtiter plate reader. Controls were performed without the enzyme or substrate solution.

3.7 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) separating 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^{o}C.

Zymogram was carried out following the method described by Garcia-Carreño et al. (1993). After electrophoresis (4^{o}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 ^{o}C. The temperature was raised to 37 ºC 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.

3.8. STATISTICAL ANALYSIS

The Microcal Origin 6.0™ software was used for statistical analysis. Data are expressed as mean ± standard deviation. Differences between means were analyzed using one-way

ANOVA, followed by Tukey's multi-comparison test and considered to be significant when $P<0.05$.

4. RESULTS AND DISCUSSION

The stress response is first action an adaptative function responsible for benefic effects at short term (homeostasis and survival) allowing the animal to cope with a potentially hostile environment (Douxfils et al. 2011). For live in different environments and feed of the most diverse sources, the digestive enzymes of aquatic animals have unique characteristics (this variation is due to enzymatic adaptation process) that make them interesting biomolecules for several biotechnological application (Glover et al. 2009; Muhlia-Almazán, et al. 2005; Muhlia-Almazán et al. 2003).

The aquatic environment represents 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).

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. . Espósito et al. (2010) found to lane snapper (*Lutjanus synagris*) a specific tryptic activity of 850.76 $U.mg^{-1}$. Bougatef et al. 2007 described a specific trypsin activity from sardine (*Sardina pilchardus*) of 1011.86 $U.mg^{-1}$. These results could be 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 both wild and farmed cobia (*R. canadum*) using BApNA as substrate. After 60°C, farmed cobia showed ± 20% 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%. These results are similar to other fish reported in literature (Castillo-Yañez et al. 2009; Yang et al. 2009; Ali et al. 2010).

Please insert Fig. 1A and 1B

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) (Applebaum 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 activity 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 patterns of caseynolitic bands (Fig. 4).

Please insert Fig.4

To be a sensitive technique to observe the activity of peptidases caseynolitic present in the pyloric caeca from wild and farmed cobia Brazil, the zymograms showed that stress the crop did not promote abrupt changes in enzyme profile of clear bands.

This similarity of behavior of peptidases remained constant for all other physical-chemical parameters compared between wild and farmed cobia, with no significant differences. 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.

The presence of these peptidases 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).

The action of peptidases represents an important pathway for the absorption of amino acids, influencing the incorporation and formation of muscle proteins and hence muscle growth and consequently weight gain. According to Grigorakis K. 2007, muscle composition is a major quality aspect in fresh fish and changes in muscle composition of fish may have consequences for marketing. Therefore, the rapid growth and weight gain in a short period of cultivation are desirable parameters by producers because there are offsetting the high costs of production, mainly with food and high levels of dietary protein given to the animals. The rationale of cultivation from Brazilian cobia could be reached with the reduction of responsiveness to environmental stressors is arguably a key element of the drive to fully domesticate this specie.

In this study, the comparison between the physical-chemical properties of the peptidases of wild and farmed cobia (F1 generation) was not significant, indicating that the stress caused by the cultivation was not enough to promote changes or adaptations in enzyme profile of farmed animals. This fact may show some degree of influence on muscle growth. Both groups showed similar peptidase profiles, suggesting similar development rates. This is not interesting, mainly because fish that live in the open sea exhibit slower growth when compared with farmed animals. On the other hand, in an commercial culture, growth should

be faster and compatible with commercial needs, especially concerning the reduction in production costs for food and time of cultivation.

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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 30ºC 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

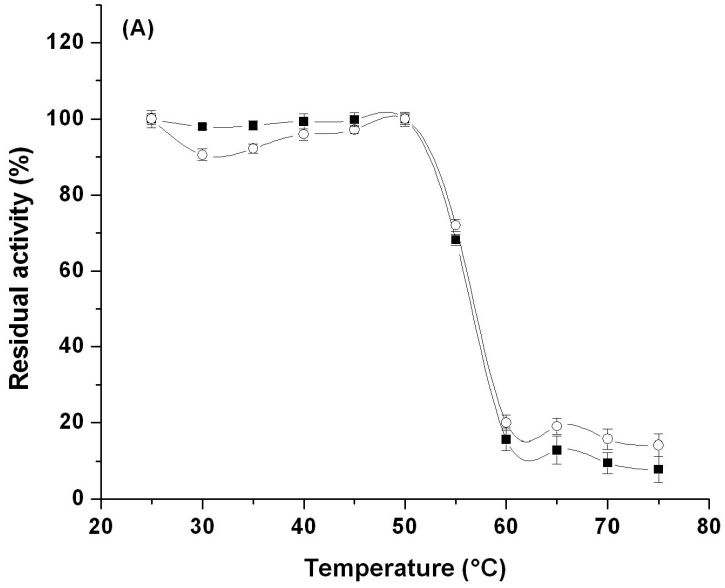
Table 2. Effect of inhibitors on trypsin and chymotrypsin activities from the pyloric caeca of cobia (*Rachycentron canadum*)

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.

10. FIGURES

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.



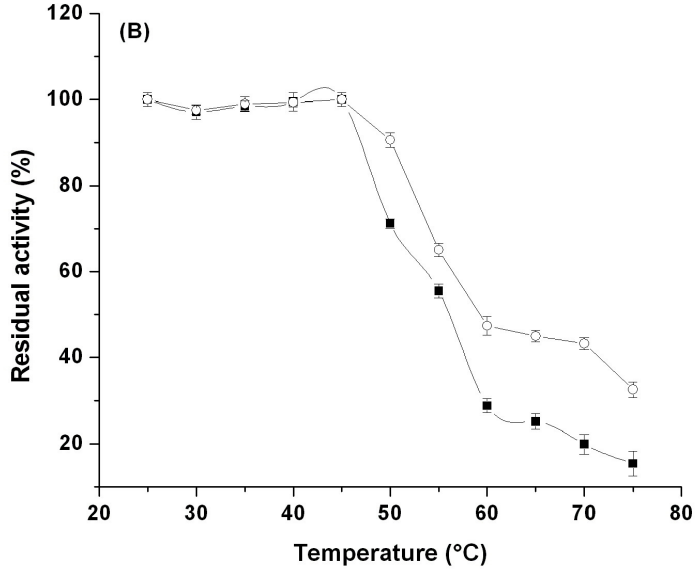


Figure 2. Effect of temperature in residual activity (tryptic and chymotryptic) from the pyloric caeca of wild and farmed cobia (*Rachycentron canadum*) using BApNA and SApNA as substrates.

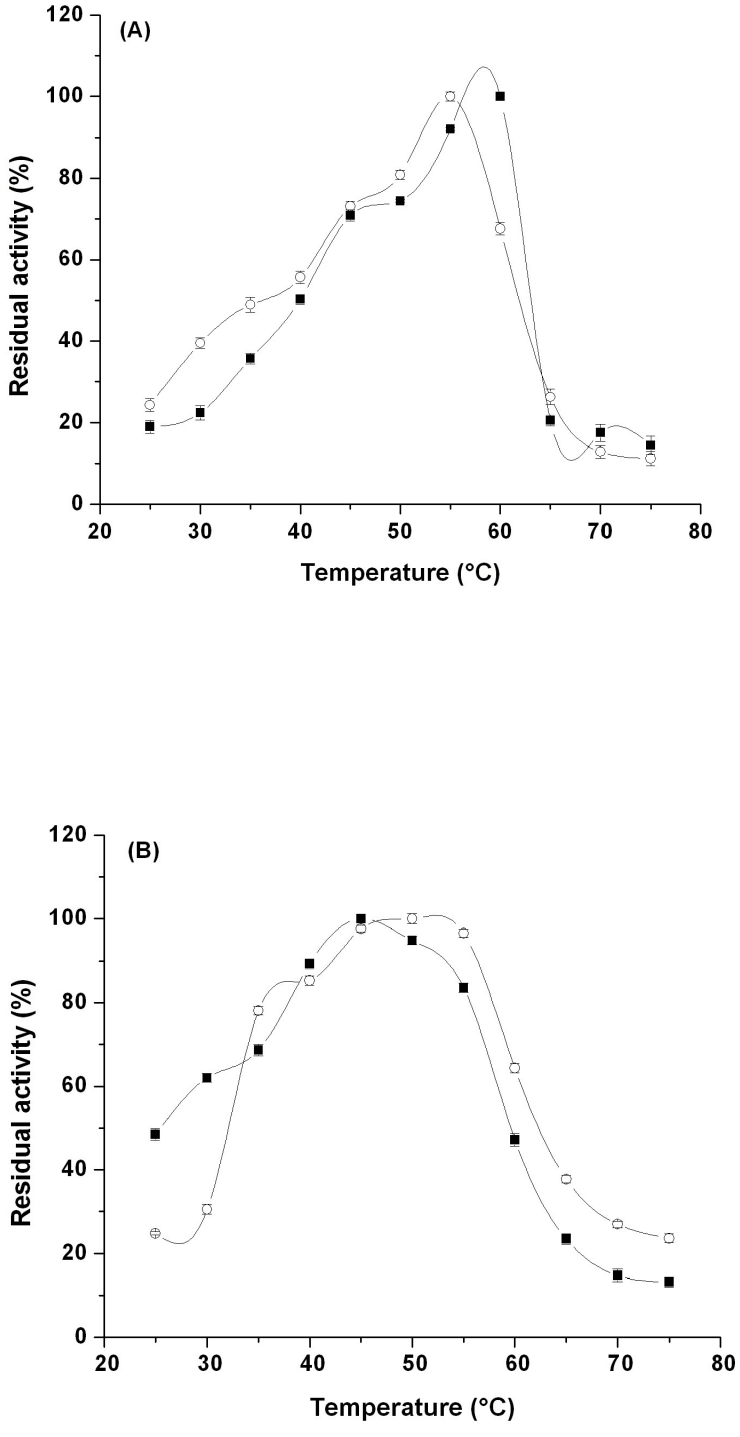
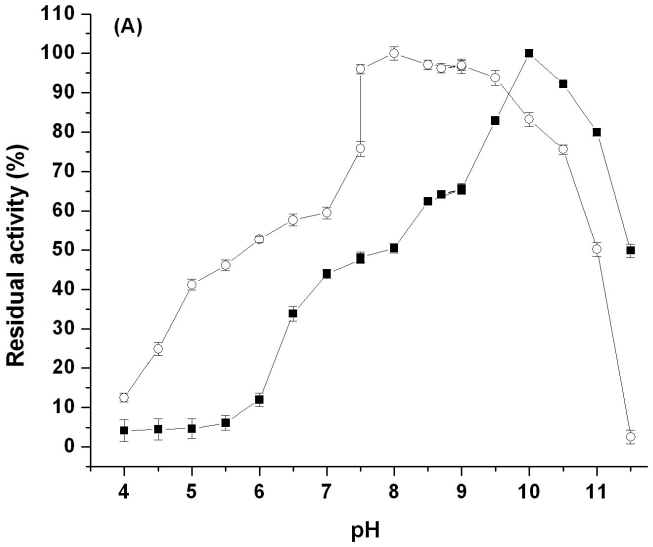


Figure 3. Effect of pH in residual activity (tryptic and chymotryptic) from the pyloric caeca of wild and farmed cobia (*Rachycentron canadum*) using BApNA and SApNA as substrates.



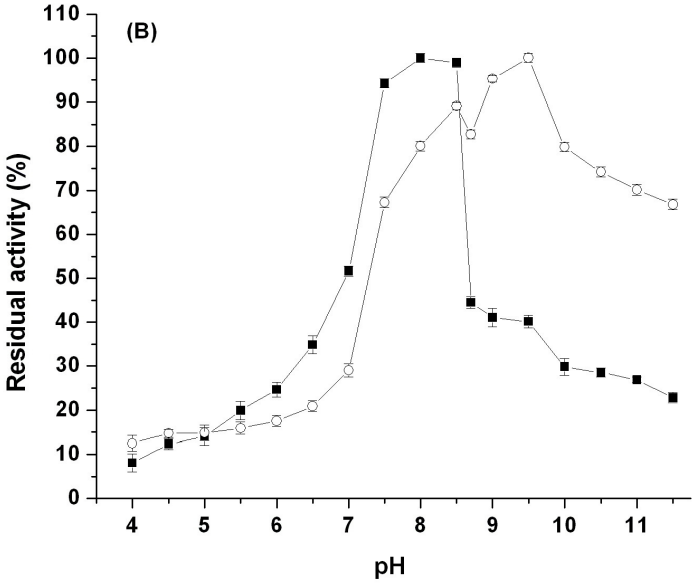
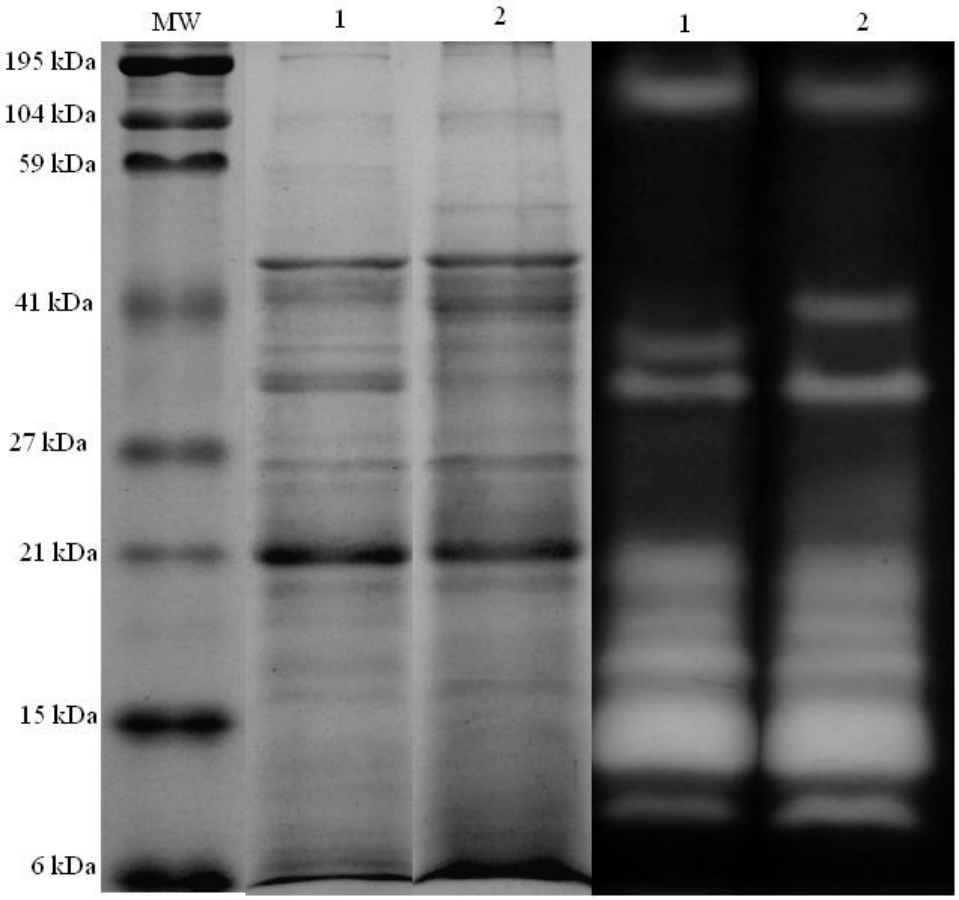


Figure 4. Eletrophoretic profile of digestive enzymes from the pyloric caeca of *Rachycentron canadum* using a 4% (w/v) stacking gel and a 12.5% (w/v) separating gel: MW (molecular weights standards), 1 (wild cobia) and 2 (farmed cobia).



4. CONCLUSÕES

- O processo de purificação da tripsina presente no ceco pilórico do bijupirá (*R. canadum*) utilizando-se a cromatografia de afinidade por BPTI-sepharose, demonstrou ser um método rápido, eficiente, viável e de alta reprodutibilidade experimental que permitiu o isolamento da tripsina de alto teor de pureza com características físico-químicas e cinéticas semelhantes a outras tripsinas de peixes descritas na literatura e que pode ser empregada na indústria de detergentes como nova fonte alternativa de enzimas.

- As propriedades físico-químicas e cinéticas da tripsina purificada do bijupirá (*R. canadum*) foram semelhantes a outras tripsinas de peixes reportadas na literatura: A tripsina purificada do bijupirá apresentou massa molecular de 24 kDa, temperatura ótima de 50ºC, pH ótimo de 8,5, K_m de 0,38mM, K_{cat} de 3.14 s^{-1} K_{cat}/K_m de 8.26 s^{-1} mM^{-1} e teve sua atividade fortemente inibida por inibidores clássicos de tripsina (TLCK e benzamidina) e pelos seguintes íons metálicos na ordem drecrescente: $Hg^{2+} > Cu^{2+} > Cd^{4+} > Mg^{2+} > Na^{+} > Ba^{2+} > K^{+} > Ca^{2+} = Al^{3+}$ utilizando-se o BApNA (8mM) como substrato específico. A determinação da sequência do N-terminal da tripsina do bijupirá para os 25 primeiros aminoácidos foi IVGGYECTPHSQAHQVSLNSGYHFC apresentando alta homologia com outros N-terminais de outras tripsinas de peixes.

- As vísceras do bijupirá (*R. canadum*), especialmente o ceco pilórico, são ricas fontes de tripsina que podem ser empregadas na indústria de formulação de detergentes por apresentar características como a atividade em ampla faixa de pH alcalino (9,5-11,0) e de temperatura (40ºC-55ºC), bem como relativa estabilidade na presença de agentes tensoativos e detergentes comerciais sólidos, o que tornam esta enzima como uma candidata em potencial como alternativa à utilização de enzimas microbianas.

- As peptidases (tripsina e quimotripsina) presentes nos cecos pilóricos do bijupirá (*R. canadum*) apresentaram pequenas diferenças nas propriedades físico-químicas investigadas (pH ótimo, temperatura ótima, estabilidade térmica, efeito de inibidores, SDS-PAGE e zimograma) entre os espécimes selvagens e cultivados o que pode ser

indicativo do processo de adaptação enzimática sofrido pelos animais em virtude do processo inicial de domesticação na costa Pernambucana e influenciado pela dieta fornecida.

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ANEXOS

EXTRAÇÃO, CLONAGEM E SEQUENCIAMENTO DO GENE QUE CODIFICA A TRIPSINA PRESENTE NO CECO PILÓRICO DO BIJUPIRÁ (*R. canadum*)

EXTRAÇÃO DO mRNA

Espécimes de bijupirás foram gentilmente concedidos pela empresa Aqualider (Ipojuca, Pernambuco, Brasil) e também pelo Projeto Cação de Escamas da Universidade Federal Rural de Pernambuco. Os animais foram encaminhados ao Laboratório de Enzimologia (LABENZ) da Universidade Federal de Pernambuco, onde foram sacrificados através de choque térmico em banho de gelo e seus cecos pilóricos foram conservados em nitrogênio líquido. A extração do RNA total do ceco pilórico foi realizada utilizando-se o reagente TRIZOL (SIGMA®) seguindo as instruções do fabricante. Após a maceração completa do tecido, o material foi centrifugado a 12,000 x g por 10 minutos a 4ºC. A precipitação do RNA foi realizada através da adição de 200µL de clorofórmio, o material foi vortexado e centrifugado a 12,000 x g por 15 minutos a 4ºC e o sobrenadante foi transferido para um novo tubo estéril para então ser adicionado 500µL de isopropanol e incubado por 10 minutos e ser realizada uma nova centrifugação. O sobrenadante foi descartado e foi adicionado 1mL de álcool 40% preparado em água DEPC. Após a formação do pellet, o mesmo foi ressuspenso em 20µL de água DEPC.

SÍNTESE DO cDNA

A síntese do cDNA foi realizada utilizando 1µg do RNA total previamente tratado com RNAsein (para evitar contaminação e degradação do mesmo), 1µL de random primer e água DEPC por 5 minutos a 70ºC. A reação de transcriptase reversa foi realizada nas seguintes condições: 4,5 uL de água DEPC + 4 µL de tampão 5x + 4 µL de $MgCL_2$ 25mM + 1µL de dNTP + 0,5µL de RNasein + 1µL de transcriptase reversa Após esse período, foi realizada uma PCR nas seguintes condições: 25ºC por 5 minutos + 42ºC por 1 hora + 70ºC por 15 minutos). A pureza e a concentração do cDNA foi avaliada através da relação A260/A280 e em gel de agarose a 1%.

REAÇÃO DA POLIMERASE EM CADEIA (PCR)

O protocolo de PCR foi desenhado de acordo com Sambrook & Russell (2001). Na Tabela 1 estão contidas as concentrações de cada reagente utilizado na PCR.

Tabela 1. Reagentes utilizados na PCR

Reagentes	**Concentração inicial**	**Concentração final**
Tampão da enzima	5x concentrado	1x concentrado
$MgCl_2$	7,5 mM	0,75 mM
DNTPs	100 mM	0,2 mM
Primer senso	10 pM	1 pM
Primer anti-senso	10 pM	1 pM
Template (cDNA)		1 µg
Taq polimerase gold		1 a 5 Unidades
Água		Completar para 25µL

CLONAGEM DO INSERTO

O vetor utilizado foi o pGEMT® easy da Promega utilizando-se a enzima T4 DNA ligase (Thermo Scientific®), na proporção de 1:3 de vetor:inserto. Células competentes de *E. coli* da linhagem DH5α foram transformadas por eletroporação com o produto de ligação e em seguida foram plaqueadas em placas de Petri com meio Luria Bertani (LB) sólido, contendo o antibiótico ampicilina.

EXTRAÇÃO DE DNA PLASMIDIAL POR LISE ALCALINA COM SDS (MINIPREP)

As *minipreps* foram realizadas de acordo com a metodologia descrita por Sambrook & Russell (2001). As colônias selecionadas foram inoculadas em tubos *Falcon* contendo 2 mL de meio LB (Luria Bertani Broth) líquido com ampicilina (50 µg/mL). Os tubos foram mantidos sob agitação (150 RPM), a 37 °C, *overnight.* Após crescimento, as células foram lisadas. Em seguida, o DNA plasmidial foi separado dos restos celulares, com o uso de precipitação por álcool isopropílico e lavagem com etanol 70%.

SEQUENCIAMENTO DE DNA

Para o sequenciamento, primeiramente, foi realizada uma reação de PCR, utilizando-se o kit Big Dye (Applied biosystems®). Após a reação de PCR, o DNA foi precipitado, utilizando-se acetato de sódio 3M e etanol 95% e lavado com etanol 70%. O sequenciamento foi executado no sequenciador ABI 3130 (Applied Biosystems).

RESULTADOS E DISCUSSÃO

Após a obtenção dos amplicons, utilizando pares de primers degenerados previamente desenhados baseados em espécies de peixes tropicais utilizando-se o NCBI (Nacional Center for Biotechnology Information), o mesmo foi purificado utilizando-se o Kit de purificação da Qiagen® através da excição do gel de agarose para em seguida ser clonado no plasmídeo pGEMT® easy da Promega.

Figura 1. Análise dos produtos da PCR em gel de agarose 1%

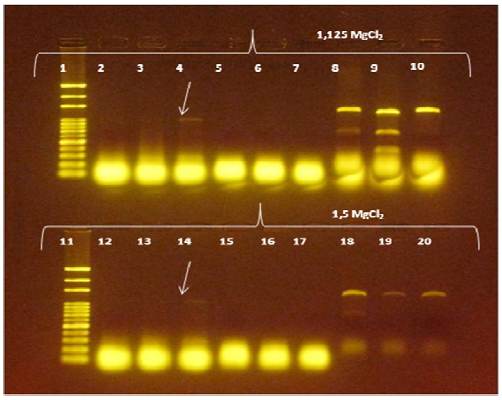
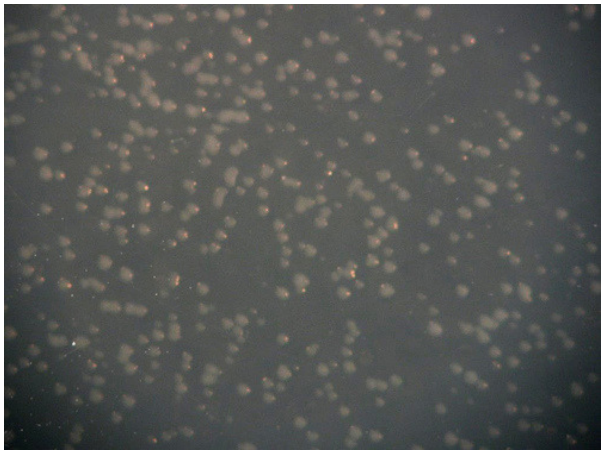


Figura 2. Colônias de Bactérias DH5α (*E. coli*) cultivadas em meio LB (Luria Bertani sólido) a 37ºC



Após a MiniPrep (mini-preparação plasmidial) dos clones positivos (Figura 2), os fragmentos que possuíam o inserto com a proteína de interesse foram avaliadas através de gel de agarose a 1% e em seguida, o material foi encaminhado para ser realizado o

sequenciamento dos fragmentos de cDNA para confirmar se a sequência obtida estava correta e se o fragmento foi inserido corretamente no vetor.

Em seguida, a sequência foi inserida contra um banco de dados (NCBI) usando o programa BLAST X, observou-se que a sequência obtida correspondia a outras sequências de tripsinas de peixes depositadas no banco de dados como mostra a Figura 3.

Figura 3. Sequência transcrita da tripsina do bijupirá a partir do sequenciamento realizado com o clone positivo ***Rachycentron canadum*** **(678pb)**

3´NTCCCCATATGNTCGACCNGCAGGNNGNCCGCGAATTCACCTAGTGATTGACA
AGATCGTCGGAGGGTATGAGTGCACACCTCATTCTCAGGCTCATCAGGTGTCTCT
GAACTCCGGCTACCACTTCTGTGGAGGCTCCCTGGTCAGCGAGAACTGGGTTGTG
TCTGCTGCTCACTGCTACAAGTCCCGTGTGGAGGTGCGTCTGGGTGAGCACAACA
TCAGGGTCAATGAGGGAAGCGAGCAGTACATCAGATCCTCCCGTGTCATCCGCCA
CCCCAACTACAGCTCCTACAACATCAACAATGACATCATGCTGATCAAGCTGAGC
GAGCCCGCCACCCTCAACCAGTACGTGCAGCCTGTGGCTCTGCCCACCAGCTGTG
CCCCCGCTGGCACCATGTGCTTAGTCTCTGGCTGGGGCAACACCATGAGCTCCTC
TGCTGACAGGAACAAGCTGCAGTGCCTGGACCTTCCCATCCTGTCCGACAAGGAC
TGTGATAACGCCTACCCCGGCATGATCACTAATGCAATGTTCTGCGCTGGATACC
TGGAGGGCGGCAAGGACTCTTGCCAGGGTGACTCCGGTGGCCCCGTTGTGTGCAA
TGGTGAGCTGCAGGGTGTTGTGTCCTGGGGCTACGGANNTNNNNNAAGAACCAC
CCTGGTGTTACGCCAAGGTNT´5

Figura 4. Sequência traduzida da tripsina do bijupirá com destaque em amarelo para o N-terminal que foi similar ao obtido pelo Método de Degradação de Endman.

SPYXRPAGXPRIHLVIDKIVGGYECTPHSQAHQVSLNSGYHFCGGSLVSENWVVSAA
HCYSRVEVRLGEHNIRVNEGSEQYIRSSRVIRHPNYSSYNINNDIMLIKLSEPATLNQY
VQPVALPTSCAPAGTMCLVSGWGNTMSSSADRNKLQCLDLPILSDKDCDNAYPGMI
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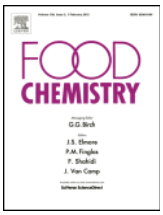
NORMAS DO PERIÓDICO FOOD CHEMISTRY

FOOD CHEMISTRY



AUTHOR INFORMATION PACK

GUIDE FOR AUTHORS

DESCRIPTION

Food Chemistry publishes original research papers dealing with the chemistry and biochemistry of foods and raw materials covering the entire food chain from `farm to fork.'

Topics include:

– Chemistry relating to major and minor components of food, their nutritional, physiological, sensory, flavour and microbiological aspects;

– Bioactive constituents of foods, including antioxidants, phytochemicals, and botanicals. Data must accompany sufficient discussion to demonstrate their relevance to food and/or food chemistry;

– Chemical and biochemical composition and structure changes in molecules induced by processing, distribution and domestic conditions;

– Effects of processing on the composition, quality and safety of foods, other bio-based materials, by-products, and processing wastes;

–Chemistry of food additives, contaminants, and other agro-chemicals, together with their metabolism, toxicology and food fate.

Analytical Section

Analytical papers related to the microbiological, sensory, nutritional, physiological, authenticity and origin aspects of food. Papers should be primarily concerned with new or novel methods (especially instrumental or rapid) provided adequate validation is described including sufficient data from real samples to demonstrate robustness. Papers dealing with significant improvements to existing methods, or data from application of existing methods to new foods, or commodities produced in unreported geographical areas, will also be considered.

– Methods for the determination of both major and minor components of food especially nutrients and non-nutrient bioactive compounds (with putative health benefits) will be considered.

– Results of method inter-comparison studies and development of food reference materials for use in the assay of food components;

– Methods concerned with the chemical forms in food, nutrient bioavailability and nutritional status;

– General authentication and origin [e.g. Country of Origin Labelling (COOL), Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), Certificate of Specific Character (CSC)] determination of foods (both geographical and production including commodity substitution, and verification of organic, biological and ecological labelling) providing sufficient data from authentic samples should be included to ensure that interpretations are meaningful.

INTRODUCTION

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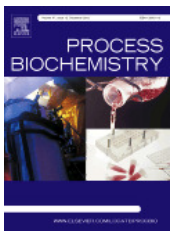

PROCESS BIOCHEMISTRY

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ISSN: 1359-5113

DESCRIPTION.

Process Biochemistry is an application-orientated research journal devoted to reporting advances with originality and novelty, in the science and technology of the **processes** involving **bioactive molecules** and living **organisms.** These processes concern the production of useful metabolites or materials, or the removal of toxic compounds using tools and methods of current **biology** and **engineering.** Its main areas of interest include novel **bioprocesses** and enabling technologies (such as nanobiotechnology, tissue engineering, directed evolution, metabolic engineering, systems biology, and synthetic biology) applicable in food (nutraceutical), healthcare (medical, pharmaceutical, cosmetic), energy (biofuels), environmental, and biorefinery industries and their underlying biological and engineering principles.

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• Cell, tissue and antibody engineering: animal and plant cells/tissues, algae, micro-algae, extremophile, antibody screening and production
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• Bioseparation, purification, protein refolding.
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Y.-H. Zhang, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

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GUIDE FOR AUTHORS.

INTRODUCTION

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[2] Stephanopoulos GN, Aristidou AA, Nielsen JE. Metabolic engineering: principles and methodologies. New York: Academic Press; 1998. p. 494

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