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CENTRO DE CIÊNCIAS BIOLÓGICAS/CCB
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS/PPGCB

OBTENÇÃO DE PROTEASES A PARTIR DO TRATO DIGESTIVO DE PEIXES NEOTROPICAIS PARA APLICAÇÃO NA PRODUÇÃO DE PEPTÍDEOS DE COLÁGENO

VAGNE DE MELO OLIVEIRA

RECIFE
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Orientadora: Prof. Dra. Ana Lúcia Figueiredo Porto
Co-orientador: Prof. Dr. Ranilson de Souza Bezerra

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“...cada um de nós compõe a sua história
cada ser em si carrega o dom de ser capaz
de ser feliz...”

(Tocando em Frente, Renato Teixeira)

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RESUMO

Este trabalho objetivou obter proteases com propriedades colagenolíticas a partir dos resíduos de três espécies de peixes neotropicais (arabaiana *Seriola dumerili*; pescada-branca *Cynoscion leiarchus*; e tucunaré *Cichla ocellaris*), através de extração por sistema de duas fases aquosas (SDFA), visando aplicá-lo para produção de peptídeos de colágeno. Inicialmente, serino proteases foram extraídas e caracterizadas físico-quimicamente quanto à temperatura e pH ótimos, bem como estabilidade à variação desses parâmetros e verificação de seus parâmetros cinéticos (K_m e $V_{máx}$). A sensibilidade aos íons metálicos e aos inibidores naturais e sintéticos também foi avaliada. Em seguida, a atividade colagenolítica dos extratos foi mensurada, obtendo-se 106,82 U/mg (*C. ocellaris*), 42,44 U/mg (*S. dumerili*) e 98,08 U/mg (*C. leiarchus*). Nos testes com inibidores de serino (PMSF) e metaloproteases (EDTA) obteve-se 18,53 e 23,29 U/mg, 14,09 e 14,94 U/mg e de 37,45 e 15,55 U/mg, como atividades enzimáticas de *C. ocellaris*, *S. dumerili* e *C. leiarchus*, respectivamente. Proteases com propriedades colagenolíticas de *C. leiarchus* e de *C. ocellaris* obtiveram pH ótimo de 8,0 e 7,5, mantendo-se estável entre 6,5 e 11,5; temperatura ótima de 55°C, termoestável entre 25 e 60°C, respectivamente. Os íons Ca^{2+} e Mg^{2+} funcionaram como ativadores, enquanto Zn^{2+} e Pb^{2+} atuaram como inibidores, assim como a ação da Benzamidina e TLCK, inibidores específicos de tripsina. No teste de especificidade ao colágeno, a enzima de *C. leiarchus*, após 48 horas, clivou todos os tipos de colágeno testados, sendo eficaz na seguinte ordem: colágeno da pele de *P. corruscans* > colágeno da pele de *O. niloticus* > colágeno bovino tipo I; enquanto que, para a enzima de *C. ocellaris*, após 36 horas, colágeno tipo I > colágeno de pele de *P. corruscans* > colágeno de pele de *O. niloticus*. As características físico-químicas da colagenases de *C. ocellaris* mostraram-se mais interessantes para prosseguimento das análises. Dessa forma, foram obtidos o K_m e o $V_{máx}$ como 5,92 mM e 294,40 U/mg, respectivamente. Esta enzima mostrou especificidade aos colágenos testados, durante o intervalo de 1 h a 55°C, na seguinte condição: colágeno tipo I > colágeno de pele de *O. niloticus* > colágeno de pele de *P. corruscans*. A colagenase intestinal de *C. ocellaris* foi recuperada por meio do sistema de duas fases aquosas (SDFA) para obtenção da enzima purificada, obtendo-se coeficiente de partição de 8.24, usando 20,0% (w/w) de PEG 8000 e 12,5% (w/w) de sal de fosfato a pH 8,0. A enzima PEG-colagenolítica ainda foi capaz de clivar o colágeno do tipo I e do colágeno extraído da pele de *C. ocellaris* (rendimento 2.9% de peso seco). A técnica de SDFA permitiu a remoção de contaminantes por um processo rápido, simples e econômico e funcionou como etapa principal de purificação capaz satisfazer a necessidade de isolamento para a produção de peptídeos de colágeno, como descritos no presente trabalho. A simplicidade e alto rendimento, somando investimentos mais baixos, tornam o SDFA uma opção promissora de extração de colagenases a partir de resíduos do processamento do pescado para a produção de peptídeos de colágeno, visando aplicação na indústria alimentícia, farmacêutica e de cosméticos.

Palavras-chave: Extração líquido-líquido (ELL), *Cichla ocellaris*, *Cynoscion leiarchus*, peptídeos de colágeno, *Seriola dumerili*, resíduos, vísceras digestivas.

ABSTRACT

This study aimed to obtain proteases with collagenolytic properties from the waste of three species of Neotropical fish (greater amberjack *Seriola dumerili*; hake *Cynoscion leiarchus*, and peacock bass *Cichla ocellaris*), through extraction by aqueous two-phase system (ATPS), to apply it in the production of collagen peptides. Initially, serine proteases were extracted and physicochemically characterized for optimum temperature and pH, as well as stability to the variation of these parameters and verification of its kinetic parameters (K_m and V_{max}). The sensitivity to the metal ions and natural and synthetic inhibitors was also assessed. Then, the collagenolytic activity of the extracts was measured, yielding 106.82 U/mg (*C. ocellaris*), 42.44 U/mg (*S. dumerili*) and 98.08 U/mg (*C. leiarchus*). Tests with inhibitors of serine (PMSF) and metalloproteinases (EDTA) yielded 18.53 and 23.29 U/mg, 14.09 and 14.94 U/mg and 37.45 and 15.55 U/mg such as enzymatic activities of *C. ocellaris*, *S dumerili* and *C. leiarchus*, respectively. Collagenolytic proteases with collagenolytic properties from *C. leiarchus* and *C. ocellaris* obtained optimum pH of 8.0 to 7.5, and is stable between 6.5 and 11.5; optimum temperature 55°C, thermostable between 25 and 60°C, respectively. Ca^{2+} and Mg^{2+} functioned as activators and Zn^{2+}, and Pb^{2+} acted as inhibitors, as well as the action of Benzamidine and TLCK, specific inhibitors of trypsin. In the collagen specificity test enzyme of *C. leiarchus*, after 48 hours, cleaved all collagen types tested being effective in the following order: *P. corruscans* skin collagen > *O. niloticus* collagen skin > bovine collagen type I; while for the enzyme from *C. ocellaris*, after 36 hours, type I collagen > *P. corruscans* skin collagen > *O. niloticus* skin collagen. The physicochemical characteristics of the collagenase of *C. ocellaris* were more interesting for further analysis. Thus, there was obtained a Michaelis-Menten constant (K_m) and V_{max}, 5.92 mM and 294.40 U/mg, respectively. The enzyme was specific to the tested collagens, during the interval of 1 h at 55°C in the following condition: type I collagen > *O. niloticus* skin collagen > *P. corruscans* skin collagen. The intestinal collagenase *C. ocellaris* was recovered by means of aqueous two-phase system (ATPS) to obtain the enzyme purified to give 8.24 partition coefficient, using 20.0% (w/w) PEG 8000 and 12.5% (w/w) of pH 8.0 phosphate salt. PEG-collagenolytic enzyme was still able to cleave collagen type I and collagen extracted from skin of *C. ocellaris* (yield 2.9% dry weight). The ATPS allowed the removal of contaminants by a fast, simple and cost effective technique and worked as main stage of purification, meeting the need for isolation, as in the production of collagen peptides, as described in this work. The simplicity and high performance, adding lower investments, make the ATPS a promising option collagenase extraction from waste from the fish processing for the production of collagen peptides, aiming at application in the food industry, pharmaceutical and cosmetics.

Keywords: Liquid-liquid extraction (LLE), *Cichla ocellaris*, collagenase, *Cynoscion leiarchus*, collagen peptides, *Seriola dumerili*, waste, digestive viscera.

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LISTA DE ABREVIAÇÕES E SIGLAS

Azocoll – Azo dye-impregnated collagen.

BApNA - Benzoil arginina ρ-nitroanilida

IUBMB – International Union of Biochemistry and Molecular Biology

kDa – Quilo Dalton

PMSF– Fenil-metil-sulfonil fluoreto

SBTI – Inibidores de tripsina de soja

SDS - Sódio dodecil sulfato

SDS-PAGE - Eletroforese em gel de poliacrilamida utilizando SDS

SAPNA – succinil- alanina-fenilalanina-p-nitroanilina

SUCPHEPNAN - Succinil fenilalanina ρ-nitroanilida

TAME – Tosil-Arginina-Metil-Éster

TCA - Ácido tricloroacético

TPCK – tosil-lisina clorometil cetona

ZEE – Zona Econômica Exclusiva

ZPCK – carbobenzoxi-fenil-clorometilcetona

(**t**) – toneladas

PEG – polietilenoglicol

SDFA – sistema de duas fases aquosas.

ATPS – aqueous two-phase system.

RIISPOA – Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal.

ÍNDICE

1. INTRODUÇÃO

O pescado tem colaborado de forma significativa para segurança alimentar, contribuindo para a manutenção da boa saúde humana, por se constituir em uma fonte de proteína animal balanceada (FAO, 2012). Este alimento faz parte da dieta de muitos países, por apresentar, entre outras cacrteristicas, alta qualidade, proteínas de fácil absorção – a proteína de peixes, crustáceos e moluscos representa cerca de 20% das fontes alimentares de proteína animal consumidas –, e uma grande variedade de vitaminas (tiamina, riboflavina, niacina) e minerais (cálcio, ferro, fósforo, iodo), contribuindo com um quarto da oferta mundial de proteína de origem animal. Ainda comoem se tratando das características nutricionais, estes alimentos possuem altos níveis de ácidos graxos poliinsaturados, especialmente *ômega-3*, que diminui as taxas de colesterol no sangue. Além do que, peixes, moluscos, crustáceos e bivalves são fontes relevantes de emprego em indústrias de processamento e de lucro para pescadores artesanais e industriais (DELGADO et al., 2003; FELTES et al., 2010; FAO, 2012; 2014a).

O crescimento e a profissionalização do setor pesqueiro têm aumentado consideravelmente a geração de resíduos, devido ao forte potencial brasileiro em mananciais aquáticos (BEZERRA et al., 2006), com ônus ecológico gerado pela poluição oriunda de seu processamento (BOUGATEF, 2013). O processamento dos subprodutos da indústria pesqueira vem crescendo como uma alternativa rentável e a utilização de proteases em etapas da produção de biomoléculas ativas a partir de vísceras digestivas de peixes é um exemplo contundente do uso de enzimas no aproveitamento de resíduos, os quais poderiam ser descartados indevidamente, degradando o meio ambiente (BEZERRA et al., 2006). Vísceras digestivas representam uma fonte rica em peptidases, enzimas naturais presentes em todos os organismos aquáticos e que estão entre os grupos mais importantes de enzimas comerciais, podendo representar até 60% do total das enzimas comercializadas no mundo (BEZERRA et al., 2006; SILVA et al., 2011). Estas enzimas, geralmente, apresentam elevada atividade catalítica em concentrações relativamente baixas em variadas de condições de pH e de temperatura (KIM et al., 2002; PARK et al., 2002; BOUGATEF, 2013). O uso de enzimas digestivas como catalisadores de processos industriais é de fundamental importância para a obtenção de produtos de alta qualidade e de maior valor agregado através da utilização de tecnologias limpas, e em sintonia com as necessidades tecnológicas, de mercado e de preservação ambiental que norteiam os processos produtivos internacionais (POLITZER e BON, 2006).

Com o crescimento exponencial do marcado de enzimas nas últimas décadas, há uma demanda crescente por enzimas proteolíticas de peixes por poderem ser utilizadas em processos que visam a produção de alimentos, cosméticos e produtos farmacêuticos. Elas oferecem vantagens sobre as técnicas químicas, incluindo especificidade de substrato e uma elevada atividade. No entanto, o uso de enzimas em aplicações industriais, requer sua a produção em larga escala para atender as necessidades de mercado (BEZERRA et al., 2006).

Apesar do enorme potencial no uso comercial das proteases de vísceras de peixes tropicais, fica evidente também a enorme carência em pesquisas sobre o tema. Isto faz com que estas proteínas percam em competitividade em relação às de outras fontes disponíveis no mercado (BEZERRA et al., 2006). Um exemplo de proteases pouco exploradas, porém de potencial, são as pertencentes ao grande grupo das colagenases – embora nem todas as enzimas do grupo consigam decompor o colágeno como, por exemplo, a catepsina K e elastase (DABOOR et al., 2010).

A produção eficiente de enzimas colagenolíticas requer procedimentos de baixo custo e, portanto, deve-se evitar métodos sofisticados que aumentem os custos de produção (DABOOR et al., 2010). Nessa perspectiva, a presente pesquisa de tese intitulada "*Obtenção de proteases a partir do trato digestivo de peixes neotropicais para aplicação na produção de peptídeos de colágeno***"** propõe o emprego dessas proteases extraídas a partir de resíduos intestinais de peixes carnívoros, e submetidas a um sistema de extração líquido-líquido (ELL), um método eficiente, rápido e simples, para produzir peptídeos bioativos de colágeno como fonte alternativa para a indústria alimentícia. Para tanto, o presente trabalho está estruturado em um tripé teórico, a saber: PARTE I: *Produção de pescado e geração de resíduos*; PARTE II: *Trato digestivo - Fonte alternativa de proteases*; e, por fim, PARTE III: *Aplicação biotecnológica de colagenases verdadeiras e/ou de proteases que apresentem a propriedade colagenolítica na produção de peptídeos bioativos de colágeno*, como descritos em todos os itens do ponto 2, de que trata a fundamentação teórica desta pesquisa.

2. FUNDAMENTAÇÃO TEÓRICA

PARTE I: *Produção de pescado e geração de resíduos***.**

2.1 Produção aquícola e geração de resíduos

O aumento da produção e do consumo de pescado[1] está diretamente relacionado à necessidade de se viabilizar tecnologias para o reaproveitamento dos resíduos gerados pela indústria aquícola (LIMA, 2013). Nessa perspectiva, a produção mundial de pescado passou de 90.836,477 milhões de toneladas (t) em 2006 para 91.336,230 milhões t em 2012. Destes, aproximadamente, 7.953,190 milhões t foram provenientes das águas continentais da América do Sul (FAO, 2014a).

Segundo a Organização das Nações Unidas para a Alimentação e a Agricultura (FAO), o consumo mundial *per capita* (população/renda) de pescado passou de 9,9 kg habitante/ano em 2005 para 18,9 kg habitante/ano em 2011, onde este grupo representou 16,7% do consumo de proteína animal total. Ainda em 2011, cerca de 86%, ou 136,2 milhões t da produção pesqueira total foi utilizada para consumo humano direto. Os 14% restantes (21,7 milhões t) foram destinados para outros produtos não-alimentares, principalmente para a fabricação de farinha e óleo de peixe (FAO, 2014b).

Com 12% da água doce disponível do planeta, um litoral de mais de oito mil quilômetros e uma faixa marítima, também conhecida como uma Zona Econômica Exclusiva (ZEE), equivalente ao tamanho da Amazônia, o Brasil possui enorme potencial para a aquicultura. É o quinto maior país do mundo, possuindo 1,7% do território do globo terrestre e representando 47% da América do Sul, ocupando uma área de 8.514.876,599 km², 7.367 km^2 de costa oceânica, 3,5 milhões de km^2 de Zona Econômica Exclusiva (faixa marítima sobre o qual o país detém os direitos de exploração, conservação e administração de todos os bens nela existentes). As dimensões continentais do Brasil favorecem sua relação com a produção pesqueira, entretanto, tem sido aproveitada apenas uma fração desta lâmina d'água. O país produz aproximadamente 2 milhões de t de pescado (levantamento preliminar de 2013), sendo 40% cultivados. Esta atividade gera um PIB pesqueiro de R$ 5 bilhões, mobilizando mais de 800 mil profissionais entre pescadores e aquicultores e proporciona 3,5 milhões de empregos diretos e indiretos. O

[1]Art. 438 - A denominação genérica, "PESCADO" compreende os peixes, crustáceos, moluscos, anfíbios, quelônios e mamíferos de água doce ou salgada, usados na alimentação humana (RIISPOA, Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal).

potencial brasileiro é enorme e o país pode se tornar um dos maiores produtores mundiais de pescado (MAPA, 2013).

A produção de pescado nacional para o ano de 2011 foi de 1.431.974,4 milhões de t, registrando-se um incremento de aproximadamente 13,2% em relação ao ano anterior. A pesca extrativa marinha continuou sendo a principal fonte de produção, responsável por 553.670,0 t (38,7% do total), seguida pela aquicultura continental (544.490,0 t; 38,0%), pesca extrativa continental (249.600,2 t; 17,4%) e aquicultura marinha (84.214,3 t; ~6%) (MAPA, 2013).

A região Nordeste registrou a maior produção de pescado do país, com 454.216,9 t, respondendo por 31,7% da produção nacional. Na análise da produção pesqueira marinha por espécie, observou-se que o grupo dos peixes representou 87% da produção total, seguido pelos crustáceos (10%) e moluscos (3%). A produção de peixes continentais foi de 243.820,7 t, representando 97,7% do total capturado. Entre as espécies que apresentaram os maiores volumes de captura (**Tabela 1**) em 2011, estão o curimatã (*Prochilodus spp*.) com 28.643,0 t, a piramutaba (*Brachyplatystoma vaillantii*) com 24.789,3 t, o jaraqui (*Semaprochilodus spp*.) com 16.556,8 t, a dourada (*Brachyplatystoma rousseauxii*) com 14.486,1 t, a pescada[2] (*Plagioscion spp*) com 13.150,3 t e o pacu (*Metynnis spp*) com 11.123,0 t. Essas seis espécies juntas representaram 44,6% da produção pesqueira continental do país. Na perspectiva da aquicultura marinha, a arabaiana[3] (*Seriola spp*.) apresentou volume total de 704,9 t, enquanto o tucunaré[4] (*Cichla spp.*) foi de 9.304,4 t, na pesca extrativista continental (MAPA, 2013). Ao mesmo tempo, também há um aumento gradual da produção de

[2]Capturada em regiões costeiras, desde lagoas salobras, estuários e mangues a baías abertas, em áreas de lodo, areia ou cascalho, entre 1-35 m de profundidade. Sua carne é considerada excelente. Formas de captura: com redes de cerco, espera, arrastos de praia. Características: corpo alongado, pouco comprimido; cabeça moderada; caudal romboidal em adultos; coloração cinza-prateada, com dorso mais escuro; ventre mais claro, com amplas áreas amarelas que incluem o flanco e nadadeiras inferior (LESSA e NOBREGA, 2000).

[3]Distribui-se em toda região do Nordeste do Brasil, em áreas de estuários a mar aberto, de 1 a 70 metros de profundidade. Importância comercial. São capturadas com artes semelhantes as outras Pescadas. Tamanho: comprimento máximo de 60 cm e médio de 40 cm de comprimento zoológico. Características: escamas ciclóides, coloração prateada (1), dorso acinzentado (2); nadadeiras claras, exceto a dorsal espinhosa, escura, bem como a margem da caudal (3).
(LESSA e NOBREGA, 2000).

[4]Peixe de água doce, salobra. Ocorre nas corredeiras, nas águas tranquilas, com profundidade média e substratos rochosos. Não é considerado ideal para a aquicultura, devido aos seus hábitos altamente predatórios. A reprodução ocorre durante todo o ano, com um pico no início da estação chuvosa. Cerca de 9.000 a 15.000 ovos por kg são liberados durante a desova. A desova acontece a cada dois meses, sobre uma pedra lisa em águas rasas (FISHBASE.ORG).

resíduos[5] gerados nas etapas de processamento do pescado. O termo "resíduo" refere-se às sobras e subprodutos do processamento, de baixo valor comercial e são constituídos principalmente por cabeça, escamas, espinhas, nadadeiras e vísceras (**Figura 1**).

Tabela 1: Produção de pescado (t) da pesca extrativa marinha em 2011, por espécie Neotropical.

Espécies Neotropicais		**Produção nacional de pescado (t)**		**Alimentação**
Nome Popular	***Nome Cientifico***	***2010***	***2011***	
Agulhão-branco	*Tetrapturus albidus*	35,0	59,7	Lulas, moluscos e peixes.
Albacora	*Thunnus alalunga*	589,9	595,4	Camarões e pequenos peixes.
Arabaiana	*Seriola dumerili*	697,8	704,9	Crustáceos, peixes e lulas.
Badejo	*Mycteroperca spp.*	1.934,6	1.604,0	Moluscos, crustáceos.
Baicu	*Lagocephalus laevigatus*	620,9	626,1	Camarões e peixes.
Beijupirá	*Rachycentron canadum*	922,9	930,4	Crustáceos, peixes e lulas.
Bonito-pintado	*Euthynnus alletteratus*	462,7	466,8	Crustáceos, peixes e lulas.
Camurupim	*Megalops atlanticus*	817,7	581,8	Peixes.
Corvina	*Micropogonias furnieri*	43.191,3	43.369,7	Crustáceos e peixes.
Dourado	*Salminus maxillosus*	7.999,3	4.379,2	Lulas e pequenos peixes.
Enchova	*Pomatomus saltatrix*	3.731,1	3.769,0	Peixes.
Merluza	*Merluccius hubbsi*	1.900,9	1.920,0	Invertebrados, peixes e lulas.
Namorado	*Pseudopercis numida*	635,1	641,5	Crustáceos, pequenos peixes.
Pescada-amarela	*Cynoscion acoupa*	20.878,6	21.074,2	Crustáceos, pequenos peixes.
Pescada-branca	*Cynoscion leiarchus*	948,1	956,3	Crustáceos, pequenos peixes.
Pescada-cambuçu	*Cynoscion virescens*	777,6	782,3	Crustáceos, pequenos peixes.
Pescada-olhuda	*Cynoscion guatucupa*	6.002,2	6.044,6	Crustáceos, pequenos peixes.
Robalo	*Centropomus spp.*	3.644,9	3.680,3	Crustáceos e peixes.
Tainha	*Mugil spp.*	17.866,1	18.045,9	Plâncton, plantas flutuantes.
Tucunaré	*Cichla ocellaris*	9.236,1	9.304,4	Camarões e pequenos peixes.
Xaréu	*Caranx hippos*	2.453,5	2.476,5	Camarões, invertebrados e peixes.
Outros	-	39.796,0	40.168,2	-

Fonte: Boletim Estatístico da Pesca e Aquicultura (MAPA, 2013). Adaptado.

*A produção pesqueira irá variar de acordo com o período do ano e a forma de captura.

**Alguns espécimes podem apresentar comportamento detritívoro em alguma fase do seu ciclo de vida (ou por completo). E ainda canibalismo devido à escassez de alimentos. Ex. Tucunaré.

***Algumas espécies citadas podem variar o nome popular de acordo com a região do país.

Somam-se a estes resíduos, as cabeças, cascas e caudas de crustáceos, produtos que são descartados, muitas vezes inadequadamente, no meio ambiente. Os resíduos da classe II da NBR 10.004 (não inertes, propriedades como: combustibilidade, biodegradabilidade ou solubilidade em água, como resíduos de pescado não-contaminados) da indústria pesqueira são os que apresentam maior potencial para implantação de tecnologias (LIMA, 2013), agregando valor, propiciando uma atividade economicamente viável e ecologicamente sustentável (BEZERRA et al., 2006).

[5]Os resíduos resultantes de manipulações de pescado, bem como o pescado condenado, devem ser destinados ao preparo de subprodutos não comestíveis (RIISPOA, Regulamento da Inspeção Industrial e Sanitária de Produtos de Origem Animal).

Figura 1: Espécies de peixes Neotropicais e produção de resíduos do processamento do Pescado. A- Tucunaré (*Cichla ocellaris*); B- Arabaiana (*Seriola dumerili*); C- Pescada-branca (*Cynoscion leiarchus*) e Tubo intestinal dissecado. Fonte: Fishbase (A,B), acervo pessoal (C).

2.2 Aproveitamento dos subprodutos da pesca

Vísceras digestivas de espécies marinhas e de água doce representam uma fonte rica e potencial de biomoléculas ativas, as quais podem ser aplicadas em diversos processos tecnológicos (BEZERRA et al., 2006; KIM e MENDS, 2006), tais como na extração de colágeno (MUYONGA et al., 2004; VIDOTTI e GONÇALVES, 2006), produção de óleo para a indústria farmacêutica e alimentícia humana (FELTES et al., 2010; LIMA, 2013), no processamento da carne, na produção de silagem (GODDARD e PERRET, 2005; ARRUDA et al., 2007; FELTES et al., 2010; GOOSEN et al., 2014), na produção de hidrolisado protéico (BEZERRA et al., 2006) e farinha de peixe (LIMA, 2013; OLIVEIRA et al., 2014). Neste sentido, várias pesquisas estão sendo realizadas visando encontrar fontes de outras espécies de peixes cultivados e/ou exóticos como novas possibilidades de aplicações para o aproveitamento biotecnológicos destes subprodutos (ALENCAR et al., 2003; BEZERRA et al., 2005; MARCHUSCHI et al., 2010; FREITAS JUNIOR et al., 2012; COSTA et al., 2013; SILVA et al., 2014).

Etapas tecnológicas para obtenção de enzimas visando o aproveitamento dos subprodutos da pesca têm sido reaizadas por grupos de pesquisadores na tentativa de demonstrar, por exemplo, seu aproveitamento como aditivos em processamento industrial (KTARI et al., 2012; YOUNES et al., 2014), e para que sua aplicação se torna viável, elas precisam atuar em faixa de temperatura e de pH, possuindo características físico-químicas compatíveis com a necessidade em cada etapa de processamento, como é o

caso da enzima extraída de vísceras digestivas de tambaqui (*Colossoma macropomum*), que apresentou pH ótimo entre 10,0 e 12,0 e temperatura ótima de 60°C (ESPOSITO et al., 2009), do extrato bruto de cecos pilóricos e intestinos de saramunete (*Pseudupeneus maculatus*), xaréu (*Caranx hippos*), budião (*Sparisoma sp*.) e traíra (*Hoplias malabaricus*) que apresentaram pH ótimo variando de 7,0 a 9,0, temperatura ótima de 55ºC e estabilidade térmica até 55ºC em xaréu e saramunete e até 45ºC em budião e traíra (ALENCAR et al., 2003). Em estudo com extrato purificado, Bezerra et al. (2005) observaram pH ótimo de 8,0, temperatura ótima de 50ºC e estabilidade térmica até 50ºC em intestinos de tilápia-do-Nilo (*Oreochromis niloticus*).

O processo de aproveitamento para obtenção de um produto finalizado a partir destes resíduos passa por uma sucessão de etapas, desde a obtenção das vísceras à identificação da biomolécula, passando processos sequenciais, tais como os de purificação, caracterização e sequenciamento para definir o perfil da enzima aplicável. Dentre estas, um grupo especial de enzimas vem recebendo especial atenção devido ao seu largo emprego comercial: as proteases (BEZERRA et al., 2005; SOUZA et al., 2007; ESPÓSITO et al., 2009; COSTA et al., 2013; SILVA et al., 2014). Estudos sobre purificação e caracterização de enzimas digestivas de peixes tropicais, neotropicais e subtropicais têm demonstrado tanto a presença, como o enorme potencial desses subprodutos como fontes de proteases (ALENCAR et al., 2003; BEZERRA et al., 2006; SOUZA et al., 2006). Dentre elas, merecem destaque a tripsina (SILVA et al., 2014), a quimotripsina (YANG et al., 2009) e a colagenase (DABOOR et al., 2012), devido às suas propriedades físico-químicas e aplicações industriais diversas, descritas no item a seguir desta fundamentação.

PARTE II: *Trato digestivo - Fonte alternativa de proteases*.

2.3 Proteases

No período de 1998 a 2005, o Brasil importou 25.982.968 kg de enzimas industriais e produtos relacionados, e exportou 18.441.529 kg. A organização das enzimas industriais importadas em 2005 por ordem decrescente de preço (US$/kg), forneceu a seguinte relação: bromelina (1,30 a 53,70); enzimas a base de transglutaminase (35,20); outras enzimas e seus concentrados (3,90 a 17,80); outras amilases (1,80 a 14,10); enzimas a base de celulase (4,20 a 14,00); outras proteases (4,37 a 13,40); outras

enzimas preparadas (5,00 a 10,50); α-amilase (6,00); enzimas para pré-curtimento (1,10 a 1,68). As enzimas classificadas como “outras”: NCM 3507.90.39 “Outras enzimas e seus concentrados” e NCM 3507.90.49 “Outras enzimas preparadas”, responderam, em 2005, a 73% das importações e a 33% das exportações (POLITZER e BON, 2006). As proteases que são utilizadas nas indústrias de alimentos e detergentes são preparadas em grandes quantidades e utilizadas como preparações brutas, enquanto que aquelas que são utilizadas em medicina são produzidas em pequenas quantidades mas requerem extensa purificação, antes de poderem ser utilizadas (RAO et al., 1998). A seguir, serão descritas definições e classificações para as proteases.

2.3.1 Definições e classificação

A diversidade biológica das espécies de peixes fornece uma ampla variedade de enzimas com propriedades únicas. As proteases (proteinases, peptidases ou enzimas proteolíticas, EC 3.4) são enzimas que clivam ligações peptídicas entre os aminoácidos das proteínas, cujo mecanismo é denominado de clivagem protéica, catalisando a hidrólise total das proteínas, comum em processos de ativação ou inativação de enzimas, envolvido principalmente na digestão (RAO et al., 1998), sendo derivadas de fontes animais, microbianas e de vetetais (BOUGATEF, 2013). Os avanços nas técnicas de análise demonstraram proteases que realizam modificações muito específicas e seletivas de proteínas, como a ativação de formas zimogênicas e enzimas por proteólise limitada, coagulação do sangue, lise de coágulos de fibrina, transformação e transporte de proteínas secretoras através das membranas (RAO et al., 1998).

De forma genérica, são divididas em dois grupos principais, dependendo do seu local de ação na molécula do substrato: endopeptidases e exopeptidases. As endopeptidases são caracterizadas por sua ação preferencial nas regiões internas da cadeia polipeptídica, entre as regiões N- (α-amino) e C- (α-carboxila) terminal. As endopeptidases são divididas em quatro subgrupos com base no seu mecanismo catalítico, (i) serino-proteases, (ii) aspartato-proteases, (iii) cisteíno-proteases, e (iv) metaloproteases. As exopeptidases atuam apenas nos finais das cadeias polipeptídicas na região N ou C terminal. Aquelas que atuam na região amino terminal livre liberam um único resíduo de aminoácido (aminopeptidases), um dipeptídeo (dipeptidil-peptidases) ou um tripeptídeo (tripeptidil-peptidases). As exopeptidases que atuam na região carboxi terminal livre liberam um único aminoácido (carboxipeptidases) ou um dipeptídeo (peptidil-

dipeptidases) (RAO et al., 1998; BOUGATEF, 2013). Exopeptidases, especialmente as aminopeptidases, são onipresentes, mas menos disponíveis como produtos comerciais, uma vez que muitas delas são intracelulares ou ligadas à membrana (BOUGATEF, 2013). As metaloproteases receberão uma descrição mais detalhada no item 2.3.2.3.

As proteases podem ser classificadas segundo o valor do pH no qual apresentam atividade máxima, diferenciando-se dessa forma em: proteases ácidas, neutras ou alcalinas (RAO et al., 1998). Tanto as ácidas quanto as básicas estão, comumente, presentes em peixes que apresentam estômago bem delimitado e funcional, enquanto espécies agástricas, geralmente, possuem apenas a básica, onde o esôfago está diretamente relacionado com o intestino (sendo aí que ocorrem os processos bioquímicos essenciais da digestão, consequência da atividade enzimática oriunda do pâncreas ou da sua própria mucosa) (SEIXAS FILHO, 2003). De acordo com a União Internacional de Bioquímica e Biologia Molecular (IUBMB), as proteases estão inseridas no subgrupo 4 do grupo 3 (hidrolases), pois clivam a proteína adicionando uma molécula de água à ligação peptídica (BERG et al., 2004), desempenhando um papel essencial no crescimento e sobrevivência de todos os organismos vivos. Para os animais aquáticos, proteases são produzidas predominantemente pelas glândulas digestivas, com destaque para pepsina, gastricsina, elastase, carboxipeptidase, esterase carboxila, tripsina, quimotripsina e colagenase (KLOMKLAO, 2008).

2.3.2 Tipos, Propriedades e Aplicações

As principais proteases tratadas neste trabalho são a tripsina, a quimotripsina e a colagenase, enzimas pancreáticas (zimogênicas) que atuam no intestino das espécies nos processos degradativos. Tais enzimas merecem especial atenção, devido às suas relações com a propriedade colagenolítica (decomposição das fibras de colágeno), função que será descrita, minuciosamente, adiante. Dessa forma, o texto a seguir trata das principais características de cada enzima, modo de atuação e aplicações biotecnológicas.

2.3.2.1 Tripsina (EC 3.4.21.4)

A tripsina (EC 3.4.21.4) é uma das mais importantes enzimas de peixes e invertebrados aquáticos (BOUGATEF, 2013; OLIVEIRA et al., 2014), sendo responsável pela hidrólise de proteínas oriundas da dieta (BARKIA et al., 2009; OLIVEIRA et al., 2014). É uma serinoprotease que hidrolisa ligações peptídicas constituídas por resíduos

de lisina e arginina (RAO et al., 1998; KLOMKLAO, 2008; OLIVEIRA et al., 2014). Os zimogênios secretados pelo pâncreas são ativados por clivagem proteolítica (KOOLMAN e ROEHM, 2005) no intestino pela enteroquinase, convertendo o zimogênio tripsinogênico pancreático em tripsina (KOLKOVSKI, 2001; KANNO et al., 2009; OLIVEIRA et al., 2014), pela remoção de um hexapeptídeo na porção N-terminal. A tripsina, subsequentemente, converte outras moléculas de tripsinogênio em tripsina. De tal modo, a enteroquinase desencadeia uma cascata de atividade proteolítica, pois a tripsina é o ativador comum de todos os zimogênios pancreáticos, como por exemplo o quimotripsinogênio (KOLKOVSKI, 2001; ROTTA, 2003; KLOMKLAO et al., 2008; OLIVEIRA et al., 2014).

As tripsinas de animais marinhos são muito semelhantes às tripsinas de mamíferos na sua massa molar, que pode variar entre 21 e 30 kDa (WANG et al., 2010; FREITAS-JUNIOR et al., 2012; BOUGATEF, 2013), composição de aminoácidos e sensibilidade a inibidores. Apresenta inibição ou instabilidade em faixas de pH abaixo de 5,0 e acima de 11,0, dependendo do substrato, e inibição por diisopropil-fluorfosfato (DFP) e pelo fluoreto de fenilmetilsulfonila (PMSF, inibidor de serinoprotease) (MARCUSCHI et al., 2010; CAI et al., 2011; KTARI et al., 2012; COSTA et al., 2013; CUENCA-SORIA et al., 2013; VILLALBA-VILLALBA et al., 2013; GUERRERO-ZÁRATE et al., 2014), inibidor de tripsina de soja (SBTI) e aprotonina. Esta enzima hidrolisa substratos sintéticos como N-α-benzoil-DL-arginina-p-nitroanilida (BApNA) e tosil-arginina-metil-éster (TAME) (WHITAKER, 1994; VILLALBA-VILLALBA et al., 2013). São enzimas sensíveis a diversos íons metálicos, tais como Ca^{2+} e Mg^{2+}, sendo inativadas pelos íons metálicos Fe^{2+}, Mn^{2+}, Cu^{2+}, Al^{3+}, Ba^{2+}, Co^{2+} e Zn^{2}, apresentando temperatura ótima em torno de 35-55°C (SOUZA et al., 2007; SILVA et al., 2011; FREITAS-JUNIOR et al., 2012; COSTA et al., 2013; CUENCA-SORIA et al., 2013; VILLALBA-VILLALBA et al., 2013; OLIVEIRA et al., 2014).

Algumas tripsinas de espécies aquáticas tropicais, neotropicais e subtropicais já foram isoladas e caracterizadas, dentre espécies com diferentes hábitos alimentares e habitats, tais como a trilha *Paretroplus maculatus* (SOUZA et al., 2007); tambaqui *C. macropomum* (MARCUSCHI et al., 2010); carapeba *Diaptereus rhombeus* (SILVA et al., 2011); pirarucu *Arapaima gigas* (FREITAS-JUNIOR et al., 2012); peixe-zebra *Salaria basilisca* (KTARI et al., 2012); Xaréu *C. hippos* (COSTA et al., 2013); castarrica *Cichlasoma urophthalmus* (CUENCA-SORIA et al., 2013); peixe-gato *Pterygoplichthys disjunctivus* (VILLALBA-VILLALBA et al., 2013); e o lagarto tropical *Atractosteus tropicus* (GUERRERO-ZÁRATE et al., 2014). As aplicações tecnológicas são diversas,

destacando-se suas utilizações nas formulações como aditivos para detergentes e sabão em pó (ESPOSITO et al., 2009; YOUNES et al., 2014) e na produção de hidrolisados protéicos, sobretudo para alimentação animal (BOUGATEF, 2013). Além do que, esta enzima apresenta o papel de ativador de colagenases e, em alguns casos, pode apresentar a propriedade colagenolítica frente aos tipos de colágeno, assunto que será discutido com detalhes na parte III desta fundamentação.

2.3.2.2 Quimotripsina (EC 3.4.21.1)

A quimotripsina (EC 3.4.21.1) é uma endopeptidase membro da família de serinoproteases, armazenada no pâncreas de vertebrados e invertebrados na forma de um precursor, o quimotripsinogênio, ativada através de um efeito cascata a partir da ativação da tripsina no duodeno (OLIVEIRA et al., 2014). Ao sofrer clivagem com a tripsina, o quimotripsinogênio é clivado em duas partes, que ainda ficam unidas por uma ponte dissulfeto (S-S). Quando o quimotripsinogênio clivado perde dois peptídeos pequenos, numa etapa chamada de trans-proteólise, obtém-se a quimotripsina. É uma enzima específica para a hidrólise de ligações peptídicas em que grupos carboxila são fornecidos por um dos três aminoácidos aromáticos, ou seja, fenilalanina, tirosina e triptofano. A enzima possui três estruturas diferentes (isoformas), variando ligeiramente na solubilidade, mobilidade eletroforética, ponto isoelétrico e especificidade de clivagem (RAO et al., 1998; APPLEBAUM e HOLT, 2003; KUZ'MINA e GOLOVANOVA, 2004; YANG et al., 2009; ZHOU et al., 2011). A tríade-catalítica desta enzima é formada por uma rede de pontes de hidrogênio entre a Ser^{195}, His^{57} e a Asp^{102} (POLGÁR, 2005; KOOLMAN e ROEHM, (2005). Tanto ela quanto a tripsina contêm a mesma "tríade" de resíduos cataliticamente ativos, como ilustrado na **Figura 2** (EISENMENGER e REYES-DE-CORCUERA, 2009).

Como uma das principais enzimas envolvidas na digestão de proteínas, quimotripsinas de peixes já foram extraídas, isoladas e caracterizadas a partir de vísceras digestivas de acará-disco-azul-marrom *Symphysodon aequifasciatus* (CHONG et al., 2002); sardinha *Sardinops sagax caerulea* (CASTILLO-YÁÑEZ et al., 2005; 2009); peixe-japonês *Carassius auratus* (YANG et al., 2009); castarrica *C. urophthalmus* (CUENCA-SORIA et al., 2013); e o lagarto tropical *A. tropicus* (GUERRERO-ZÁRATE et al., 2014).

De modo geral, estas enzimas apresentam massa molar variando entre 22 a 30 kDa, temperatura ótima oscilando entre 45 e 55°C, e pH ótimo de 7 a 9 (ALENCAR et al.,

2003; CASTILLO-YAÑEZ et al., 2009; YANG et al., 2009; CUENCA-SORIA et al., 2013; GUERRERO-ZÁRATE et al., 2014; OLIVEIRA et al., 2014), apresentando sensibilidade a determinados inibidores específicos, tais como tosil fenilalanina clorometil cetona (TPCK, inibidor de serinoproteases) (CUENCA-SORIA et al., 2013; GUERRERO-ZÁRATE et al., 2014), N-carbobenzoil-L-fenilalanina clorometil cetona (ZPCK, inibidor de quimotripsina-like) e inibidor de tripsina de soja (SBTI). São sensíveis a diversos íons metálicos, tais como Ca^{2+} e Mg^{2+}, enquanto são inativadas pelos íons de Fe^{2+}, Mn^{2+}, Cu^{2+} e Zn^{2} (YANG et al., 2009). As quimotripsinas de espécies aquáticas são instáveis a temperaturas acima de 55°C e em condições ácidas (DE VECCHI e COPPES, 1996), além de hidrolisar substratos sintéticos como o N-succinil-Ala-Ala-Pro-Phe-pNa (SApNA) e o Succinil fenilalanina ρ-nitroanilida (Suc-Phe-p-Nan) (CASTILLO-YAÑEZ et al., 2009; OLIVEIRA et al., 2014).

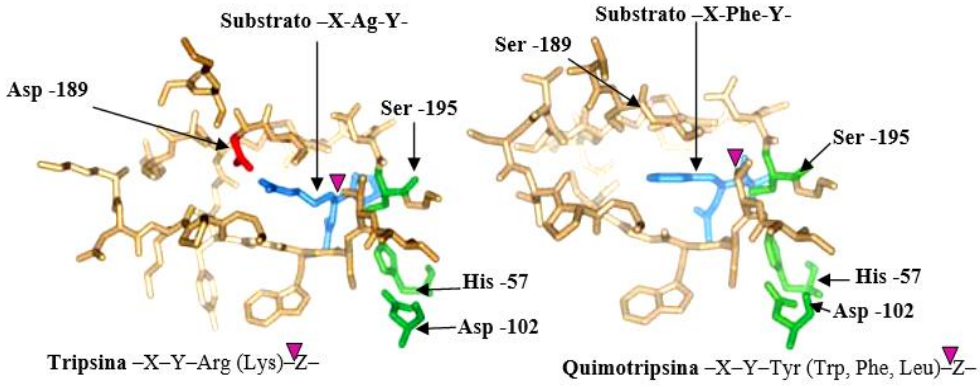

Figura 2: Ilustração da especificidade enzima-substrato. 1– Tripsina (esquerda); 2- Quimotripsina (direita); Asp: aspartato; His: histidina; Ser: serina. – ▼ local de clivagem. Fonte: Adaptado de Koolman e Roehm (2005).

A aplicabilidade industrial da quimotripsina é bastante difundida em diversos ramos, muito embora seja considerada uma enzima de alto custo. Sua concentração se dá em grande parte em aplicações analíticas e em fins diagnósticos, no ramo médico, sobretudo de doenças como catarata. Apesar do seu alto custo, ainda é comumente empregada na indústria alimentícia, principalmente no processamento da carne, em etapas de amaciação, além da produção e remoção de proteínas do osso, para aumentar o valor nutricional das proteínas dos alimentos; na indústria de processamento de couro; na indústria química, principalmente na de produção de detergentes (RAO et al., 1998; ZHOU et al., 2011). Esta enzima ainda é relacionada, assim como a tripsina, como

motivadoras da atividade colagenolíticas, como descrito por Teruel e Simpson (1995), temática que, assim como a tripsina, será discutida na parte III desta fundamentação.

PARTE III: ***Aplicação biotecnológica de colagenases na produção de peptídeos bioativos de colágeno*****.**

2.3.2.3 Colagenases e enzimas colagenolíticas

Colagenases verdadeiras e enzimas com propriedades colagenolíticas – muitas vezes atuando de forma auxiliar as primeiras –, fazem parte de um grupo de enzimas de importância vital, sobretudo, do ponto de vista fisiológico, atuando na manutenção e regeneração de órgãos e tecidos; além de sua extensa aplicabilidade, como será discutido adiante. Enzimas que apresentam a propriedade colagenolítica também são capazes de hidrolisar as ligações peptídicas de vários tipos de colágeno. São de considerável importância na medicina humana e veterinária (RAO et al., 1998; WATANABE, 2004; LIMA et al., 2014). Considerações gerais acerca das características, tipos, fontes e propriedades das colagenases serão descritas nos itens a seguir.

2.3.2.3.1 Definições e características gerais

As colagenases (EC 3.4.24.7) são um grupo de enzimas capazes de hidrolisar as ligações peptídicas de vários tipos de colágeno (descrito no item 2.3.2.3.4.1.2) – a proteína mais abundante nos mamíferos –, em condições fisiológicas de pH e temperatura, *in vivo* e *in vitro* (TERUEL, 1997; ZIMMER et al., 2009; DABOOR et al., 2010). De acordo com o sistema de classificação da comissão de enzimas, as colagenases são altamente específicas para o colágeno (nativo ou desnaturado), não apresentando atividade para qualquer outra proteína. Quando a molécula do colágeno é desnaturada, torna-se susceptível a hidrólise por outras proteases, como a tripsina e a quimotripsina, por exemplo (TERUEL, 1997), que tiveram suas características gerais e especificas de atuação descritas nos itens 2.3.2.1 e no 2.3.2.2.

O processo de ativação das colagenases é realizado através da destruição de um inibidor endógeno – ligado à enzima – ou por clivagem de um zimogênio, para convertê-la em sua forma ativa. Os ensaios empregados para a determinação de atividade das

colagenases podem ser agrupados em quatro tipos diferentes: (a) colorimétricos[6], (b) fluorescentes, (c) viscosimétricos e, por fim, por meio da (d) radioatividade (DABOOR et al., 2010).

A colagenase típica – metalocolagenases – é secretada como uma pró-enzima inativa (procolagenases), sendo necessário a atuação de ativadores, sendo os mais utilizados para esta conversão o 4-aminofenilmercúrico (DABOOR et al., 2010), a tripsina (SHINKAI et al., 1977) e o tiocianato de sódio (ou de potássio) (DABOOR et al., 2010; 2012). Woessner (1977) observou que a tripsina reforçou a atividade da colagenase em quase 30%. Vale salientar que, em virtude da tripsina ser considerada um ativador potente, supõe-se que, a ativação do seu zimogênio (tripsinogênio) em sua forma ativa, pode, indiretamente, ativar as colagenases presentes no meio. Nesse sentido, foi proposto por Bezerra et al. (2005) e seguido por Costa et al. (2013), Freitas-Junior et al. (2013) e por Silva et al. (2014) um processo de aquecimento do extrato bruto como forma de ativação do zimogênio e desnaturação de outras proteases de baixa resistência térmica, estabelecendo, assim, apenas as proteases resistentes a temperaturas de 45°C por tempo prolongado.

Nesse contexto, sabe-se que as colagenases e/ou as enzimas que apresentam esta propriedade, são tolerantes a essa temperatura, teoriza-se aqui que, esse aquecimento proposto pelos autores supracitados, sirva de ativação não apenas das formas tripsínicas, como da quimotripsina e das outras enzimas que apresentam a propriedade colagenolítica de forma auxiliar, além das colagenases verdadeiras (descritas no item 2.3.2.3.3). Os íons Ca^{2+} e Zn^{2+} também funcionam como ativador destas enzimas (BRINCKERHOFF e MATRISIAN, 2002; DABOOR et al., 2010; 2012), como será discutido nos itens 2.3.2.3.3.1 e no 2.3.2.3.3.2.

Os principais inibidores das colagenases são: mercaptoetanol e o ácido etilenodiamino tetra-acético (EDTA) (KRISTJÁMSSON et al., 1995; TERUEL e SIMPSON, 1995; ROY et al., 1996; KIM et al., 2002; PARK et al., 2002; DABOOR et al., 2010), além do inibidor de tripsina de soja (SBTI), especifico para tripsinas – quando estas são objeto

[6]O método básico para a determinação da atividade enzimática para metaloproteinases é o da ninhidrina. O ensaio de ninhidrina detecta aminoácidos e peptídeos liberados pela degradação do colágeno. O colágeno é incubado com a enzima e os peptídeos liberados são mensurados após incubação durante 5 horas a 37°C (DABOOR et al., 2010). Azocoll, um colágeno insolúvel que está ligado a um azo-corante vermelho brilhante, tem sido amplamente utilizado para a dosagem de enzimas serino proteolíticas (CHAVIRA et al., 1984). Este substrato tem sido empregado como forma alternativa na determinação da atividade de enzimas com propriedades colagenolíticas e/ou de colagenases verdadeiras (LIMA et al., 2009; ROSSO et al., 2013).

da atividade colagenolítica (DABOOR et al., 2012). Enzimas colagenolíticas podem ser extraídas a partir de uma variedade de técnicas que utilizam diferentes sistemas de tampão (tris-HCl, bicarbonato de sódio, cloreto de cálcio e cacodilato) (DABOOR et al., 2010; 2012). A eletroforese é uma forma utilizada para caracterizá-la, principalmente através da estimativa da massa molar (PARK et al., 2002; KIM et al, 2002; WU et al., 2010a), que pode variar, significativamente, em relação ao tipo de enzima e também a origem da mesma (microbiana ou proveniente de tecido animal) (BYUN et al., 2002; PARK et al., 2002; KIM et al., 2002; WU et al., 2010a; DABOOR et al., 2012; DUARTE et al., 2014). Uma provável explicação para esta variação é devida a proteólise de um precursor maior da colagenase. Para uso na indústria, tal variação será menos importante do que atividade colagenolítica no geral, mas o potencial para variação deve ser observado (DABOOR et al., 2010).

2.3.2.3.2 Fatores que afetam a atividade colagenolítica

Colagenases de várias fontes apresentam pequenas diferenças em suas características físico-químicas, quanto ao pH e temperatura, sobretudo, porque esses fatores são limitantes na retenção da atividade colagenolítica (TERUEL, 1997; DABOOR et al., 2010). Maiores detalhes serão descritos nos itens a seguir.

2.3.2.3.1 Efeito do pH

Enzimas colagenolíticas extraídas a partir de espécies de peixes e invertebrados exibem atividade ótima em pH fisiológico. Para a maioria das espécies, esse pH situa-se numa faixa entre 6,0 e 8,0 (DABOOR et al., 2010). Atividade ótima em pH 8,0 já foi reportada para o peixe filé, *Novoden modestrus* (KIM et al., 2002), para o sargo, *Pagrus major* (Wu et al., 2010a) e para a sardinha, *Sardinella aurita* (HAYET et al., 2011). Liu et al. (2010) e Lima et al. (2009) também observaram enzimas com propriedades colagenolíticas produzidas a partir de microrganismos e tendo atividade ótima em pH de 8,0 e 8,2, respectivamente, para *Bacillus cereus* e *Candida albicans*. Mukherjee et al. (2009) observaram para uma espécie de esponja (*Rhopaloeides odorabile*) pH ótimo de 5,0, enquanto Murado et al. (2009) encontraram pH de 6,0 para a espécie peixe-arraia (*Raja clavata*); e Kristjámsson et al. (1995) e Roy et al. (1996) encontraram pH de 7,0 para bacalhau do Atlântico (*Gadus morhua*) e para uma espécie de caranguejo (*Carcinus maenas*), respectivamente. Teruel e Simpson (1995), Byun et al. (2002), Park et al.

(2002), Souchet e Laplante (2011) e Daboor et al. (2012) relataram pH 7,5 para as espécies aquáticas solha (*Pseudopleuronectes americanus*), atum (*Thunnus thymus*), cavala (*Scomber japonicus*), camarão (*Chionoecetes opilio*) e em mistura de vísceras de diferentes espécies de peixes, respectivamente. A estabilidade enzimática às variações de pH foi relatada para espécies aquáticas (peixes, crustáceos, esponjas) e de microrganismos, variando entre 5,0 a 10,0, a escala de recuperação de atividade (KRISTJÁMSSON et al., 1995; TERUEL e SIMPSON, 1995; LIMA et al., 2009; MUKHERJEE et al., 2009; MURADO et al., 2009; WU et al., 2010; HAYET et al., 2011; SOUCHET e LAPLANTE, 2011).

2.3.2.3.2 Efeito da Temperatura

Colagenases isoladas a partir de subprodutos de peixes marinhos têm demonstrado atividade em uma variedade de temperaturas, dependendo do tecido e das espécies das quais elas foram isoladas (DABOOR et al., 2010), embora a faixa relatada até então esteja entre temperaturas > 20°C e < 60°C (KIM et al., 2002; PARK et al., 2002; DABOOR et al., 2010, 2012; WU et al., 2010; HAYET et al., 2011). Temperaturas de 55°C já foram relatadas para algumas espécies de peixes subtropicais, como descritos por Teruel e Simpson (1995) para a espécie solha (*P. americanus*), por Kim et al. (2002) para a espécie peixe filé (*N. modestrus*) e Park et al. (2002) para uma espécie de cavala (*S. japonicus*). Wu et al. (2010) encontraram para pargo (*P. major*) temperatura ótima de 40°C. Em contrapartida, Kristjámsson et al. (1995) observaram atividade enzimática ótima de 50°C para o bacalhau do Atlântico (*G. morhua*), com decréscimo na atividade enzimática de 50% a partir dos 55°C. Hayet et al. (2011) notaram atividade ótima aos 60°C, com diminuição de atividade a partir dos 70°C, para espécie sardinha (*S. aurita*); enquanto que Daboor et al. (2012) encontraram atividade ótima aos 35°C na mistura do processamento do pescado; e de 30°C como detectado por Roy et al. (1996) para uma espécie de caranguejo (*C. maenas*). Como teorizado anteriormente no item 2.3.2.3.1, a temperatura é um fator limitador para proteases menos sensíveis e funciona como ativador de proteases que convertem procolagenases, como descrito anteriormente.

2.3.2.3.3 Fontes e tipos de colagenases

As principais fontes de colagenases produzidas e disponíveis no mercado da atualidade são de origem microbiana (DUARTE et al., 2014), como a colagenase a partir

do *Clostridium histolyticum* (TERUEL e SIMPSON, 1995; KIM et al., 2007). Outras fontes microbianas já foram reportadas, como as obtidas a partir de *C. albicans* (LIMA et al., 2009), *Penicillium aurantiogriseum* (ROSSO et al., 2012; LIMA et al., 2013), *Bacillus pumilus* (WU et al., 2010b), *B. cereus* (LIU et al., 2010) e *B. licheniformis* (BAEHAKI et al., 2012). Uma das vantagens das colagenases de microrganismos é a afinidade por vários sítios ao longo da cadeia, como visualizado na **Figura 3**. Entretanto, uma grande desvantagem é a virulência de bactérias patogênicas produtoras deste tipo de enzima, uma vez que suas aplicações são em locais de mobilização imunológica (DUARTE et al., 2014). A degradação do colágeno e a formação dos peptídeos de colágeno serão descritos detalhadamente no item 2.3.2.3.4.1.3.

Colagenases de fontes vegetais também já foram reportadas na literatura, tal como a descrita por Kim et al. (2007). Entretanto, espécies de peixes e invertebrados tem sido objeto de investigação por possuírem fontes potenciais e disponíveis de colagenases e/ou de enzimas que apresentam a propriedade colagenolítica, como as descritas por Teruel e Simpson (1995), Park et al. (2002), Wu et al. (2010) e Roy et al. (1996).

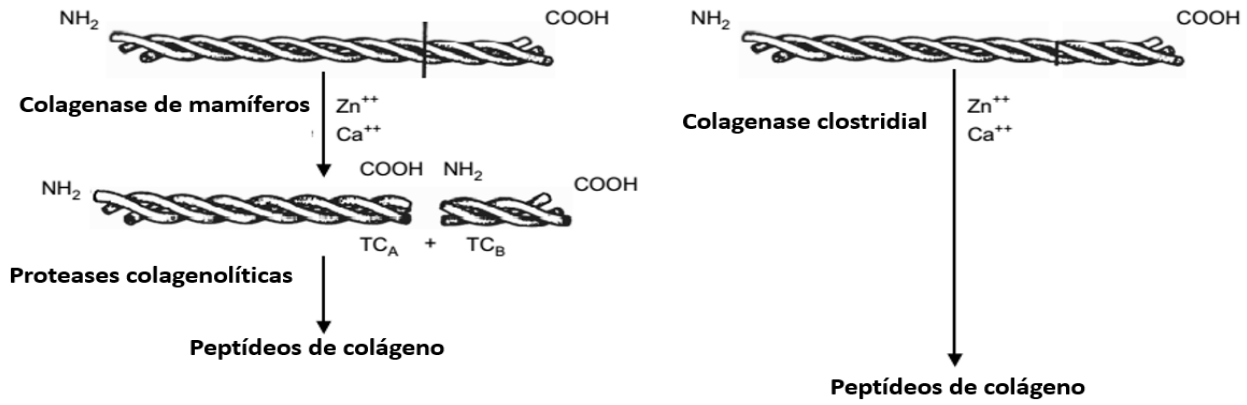

Figura 3: Ação das colagenases de mamíferos x colagenases clostridial. Colagenases bacterianas são metaloproteinases envolvidas na degradação da matriz extracelular de células animais, devido à sua capacidade para digerir colágeno nativo. Fonte: Jung e Winter (1998). Adaptado.

De modo geral, em espécies de peixes, a colagenase tem sido pesquisada em nível do fígado, do estômago – quando existe na espécie considerada, do intestino, eventualmente parte anterior e posterior, dos cecos pilóricos, quando existem, do pâncreas ou do hepatopâncreas e da gordura mesentérica (YOSHINAKA et al., 1976; 1977; 1978). Yoshinaka et al. (1978) investigaram qual seria o local de produção de colagenase em diferentes órgãos que compõem o sistema digestivo (fígado, estômago, intestino delgado, mesentério) de diferentes espécies de peixes subtropicais, tais como

nas sardinhas (*Sardinops melanosticta*), na truta arco-íris (*Salmo gairdnerii*), na carpa (*Cyprinus carpio*), no peixe-gato (*Parasilurus asotus*), na enguia (*Anguilla japonica*) e numa espécie de arabaiana (*Seriola quinquerdiota*). Ainda segundo os autores, dentre todas as vísceras analisadas, o estômago foi a que apresentou a menor atividade, quando comparado as demais vísceras digestivas, ou mesmo não demonstrou nenhuma atividade – dependendo da espécie.

Segundo Seixas-Filho (2003), a baixa atividade colagenolitica no estômago tem relação com a variabilidade morfológica do aparelho digestivo dos peixes, de acordo com a espécie. Diferentemente, as porções do intestino são sempre muito ricas destas proteases. Além disso, uma relação bastante satisfatória foi estabelecida entre a natureza do regime alimentar e a importância da atividade colagenolítica. De modo genérico, espécies herbívoras e onívoras possuem dieta pobre de colágeno ou simplesmente sem este, enquanto em espécies de peixes carnívoros é mais elevada. Dentre as diversas espécies carnívoras abundantes capturadas pela pesca brasileira (pescada, arabaiana, dourado, ariocó, por exemplo), o tucunaré (*C. ocellaris*), espécie em ascensão no mercado, que devido sua abundância da fase adulta pode causar competição intra-específica e justificando as altas taxas de canibalismo (GOMIERO e BRAGA, 2004), como visualizado na **Figura 4**, sendo necessária ação das colagenases e das proteases colagenolíticas para auxiliar nos processos de degradação do colágeno.

Figura 4: Espécie de Tucunaré (*C. Ocellaris*) ingerindo uma espécie de Pacu Prata (*Mylossoma duriventre*). Fonte: Revista Pescada e Companhia. Adaptado. Imagem meramente ilustrativa de uma hipótese de canibalismo.

De modo geral, as colagenases são subdivididas em 2 grupos bem definidos, as metalocolagenases – também conhecidas como colagenases verdadeiras ou de vertebrados –, e as serinocolagenases – conhecidas como falsas colagenases ou serinoproteases que apresentam a propriedade de decompor a parte não-helicoidal do colágeno. Essa divisão em dois grandes grupos toma por base funções fisiológicas distintas, tais como as descritas nos itens a seguir, para ambas as categorias.

2.3.2.3.3.1 Metalocolagenases

São endopeptidases integrantes da família das metaloproteinases (MMPs), que promovem a degradação da matriz extracelular, podendo também ser chamadas de matrixinas ou colagenases de vertebrados[7]. Todos os membros dessa família são secretados como pro-enzimas ligadas à membrana (CAWSTON, 1996; BRINCKERHOFF e MATRISIAN, 2002; GREENLEE et al., 2007; MURPHY e NAGASE, 2008; TALLANT et al., 2010). Essas pro-enzimas são liberadas por neutrófilos, monócitos, macrófagos, fibroblastos e, além disso, também podem ser secretadas pelas células tumorais em resposta a uma variedade de estímulos, tais como processos cicatriciais e tumorais, por exemplo. Apresentam massas molares entre 30.000 e 150.000 Da. Como todos os membros das MMPs, são enzimas Zn^{2+} dependentes, sendo inibidas por qualquer quelante que se ligue a esse íon, tendo a necessidade do Ca^{2+} para manutenção da sua estabilidade (JUNG e WINTER, 1998; DABOOR et al., 2010; 2012). Apenas as MMP 1, 8, 13, 14 e 18 possuem a atividade de decompor os tipos de colágeno nativo de cadeia tripla I, II, III, VII e X (DABOOR et al., 2010; TALLANT et al., 2010; ARAUJO et al., 2011). São comumente recuperadas de tecidos animais e de subprodutos do processamento do pescado, tais como ossos, barbatanas, peles e do hepatopâncreas de caranguejos marinhos (BRACHO e HAARD, 1995; BRINCKERHOFF e MATRISIAN, 2002; DABOOR et al., 2010).

MMPs podem ser divididas em: (I) colagenases verdadeiras[8], que clivam a tripla hélice do colágeno num único local ao longo das três cadeias, dando origem a produtos

[7]Foram descritas pela primeira vez em vertebrados por Gross e Lapierre (1962), incluindo os seres humanos, mas já foram encontrados em invertebrados e plantas. São distintas de outras endopeptidases por sua dependência aos íons metálicos como cofatores e sua capacidade de degradar outras proteínas da matriz extracelular (CAWSTON, 1996).

[8]Existem três tipos de colagenases: colagenase 1 (MMP-1), colagenase 2, também chamada de colagenase de neutrófilos (MMP-8) e colagenase 3 (MMP-13). Elas consistem de domínios pro-peptídeo, catalíticos e Hemopexina, desempenhando um papel importante na clivagem dos tipos de colágeno fibrilar I, II e III e

de 3/4 e 1/4 do comprimento da molécula original (MMP-1, 8 e 13); (II) gelatinases[9], que têm como alvo colágenos desnaturados e gelatinas (MMP-2 e 9); e (III) as estromelisinas[10], que têm uma ampla especificidade e podem degradar os proteoglicanos (MMP-3, 7 e 10), matrilisinas[11] (MMP-7 e 26), MMPs tipo membrana (MMP-14, 15, 16, 17 e 24) e outras MMPs 3, que são classificadas pela especificidade ao substrato e, principalmente, de acordo com sua estrutura (CAWSTON, 1996; GREENLEE et al., 2007; MURPHY e NAGASE, 2008; ARAUJO et al., 2011). Enzimas dessa matriz participam de processos fisiológicos que envolvem reparação tecidual durante a implantação do blastocisto, ovulação, pós-parto e involução pós-lactação, a reabsorção óssea, cicatrização de feridas, etc. Essa capacidade de processamento também é necessária durante a embriogênese e angiogênese (CHUNG et al., 2004; TALLANT et al., 2010).

A participação das MMPs em diversos eventos biológicos deve-se ao fato de que elas podem influenciar, potencialmente, o comportamento celular através de algumas ações, como clivagem de proteínas que fazem a adesão célula–célula, liberação de moléculas bioativas na superfície celular ou por clivagem de moléculas presentes na superfície celular, as quais transmitem sinais no ambiente extracelular (CAWSTON, 1996; ARAUJO et al., 2011). Para que ocorra a catálise (**Figura 5**), são necessários substratos que incluem outras (pró)-proteases, inibidores de protease, fatores de coagulação, peptídeos antimicrobianos, moléculas de adesão, fatores de crescimento, hormônios, citosinas, bem como os seus receptores e proteínas de ligação. Os substratos de MMPs clivam o montante de resíduos hidrofóbicos, tipicamente leucina (MURPHY e NAGASE, 2008; TALLANT et al., 2010). A desregulação[12] das MMPs pode dar origem à degradação e patologias decorrentes. Isto ocorre durante a inflamação, ulceração, artrite, periodontite,

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também apresentam atividade contra outras moléculas da ECM e proteínas solúveis. Os domínios catalíticos das colagenases podem clivar substratos não-colagenosos, mas eles são incapazes de clivar colágenos fibrilares nativas na ausência de seus domínios Hemopexina. A cooperação entre os dois domínios é considerada importante para a expressão da sua atividade colagenolítica (MURPHY e NAGASE, 2008).

[9]Ambas as enzimas possuem três repetições de fibronectina do tipo II inserida no domínio catalítico. Partilham a atividade proteolítica semelhante de decompor colágenos desnaturados, gelatinas e uma série de moléculas da ECM, incluindo colágenos do tipo nativo IV, V e XI (MURPHY e NAGASE, 2008).

[10]MMP-3, MMP-10 e MMP-11 são chamados estromelisinas 1, 2 e 3, respectivamente. Elas têm a mesma estrutura de domínio como as colagenases, mas não conseguem clivar colágenos intersticiais. MMP-3 e MMP-10 são semelhantes em estrutura e especificidade ao substrato, mas MMP-11. MMP-3 e MMP-10 podem digerir uma série de moléculas da MEC (MURPHY e NAGASE, 2008).

[11]A característica estrutural destas MMPs é que eles não têm o domínio hemopexina. MMP-7 é sintetizada pelas células epiteliais. Degradam componentes da matriz extracelular (MURPHY e NAGASE, 2008).

[12]A atividade dessas enzimas é controlada, principalmente, através de inibidores protéicos teciduais denominados TIMPs. As MMPs e seus inibidores determinam a arquitetura da MEC (ARAUJO et al., 2011).

doença cardiovascular, fibrose, e enfisema. Outras consequências da atividade MMP desregulamentada incluem processos associados ao câncer, como tumorigênese e neovascularização tumoral, invasão e metástase, assim como apoptose, ativação de defesa intestinal e patologias do sistema nervoso, tais como acidente vascular cerebral e doença de Alzheimer (MURPHY e NAGASE, 2008; TALLANT et al., 2010; ARAUJO et al., 2011).

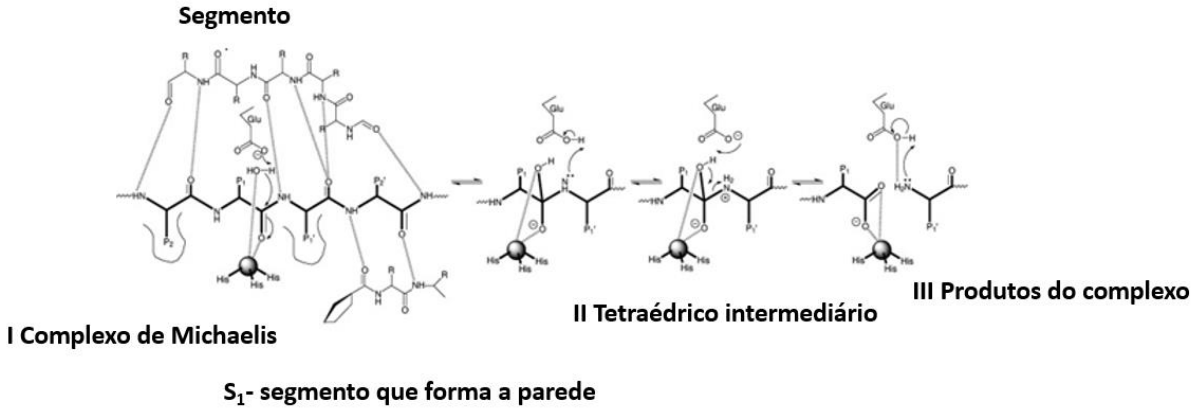

Figura 5: Mecanismo catalítico de MMPs. Esquema para o mecanismo de clivagem proposto para MMPs, com o íon Zn^{2+} como uma esfera e ligações de hidrogênio como linhas tracejadas. Os três ligantes de histidina são representados por traçados contínuos. Fonte: Tallant et al. (2010). Adaptado.

2.3.2.3.3.2 Serinocolagenases

As serinocolagenases ou serino proteases colagenolíticas (CE 3.4.21.32), como todas as serino proteases, contém um resíduo de serina no seu sítio catalítico. Estas enzimas são Ca^{2+} dependentes, apresentando massa molar variando entre 24.000 a 36.000 Da, estando muitas vezes associadas a órgãos digestivos, sobretudo, intestinos e cecos (YOSHINAKA et al., 1976; 1977; 1978; KRISTJÁMSSON et al., 1995; KIM et al., 2002; PARK et al., 2002, SEIXAS-FILHO, 2003; MURADO et al., 2009). Podem ser exopeptidases, endopeptidases, oligopeptidades e em grupos ɣ-peptidases. São geralmente ativas em pH alcalino e neutro, com um pH ótimo entre 7 e 11 (RAO et al., 1998; DABOOR et al., 2010).

Enzimas deste grupo são capazes de clivar a tripla hélice do colágeno dos tipos I, II e III, e muitas vezes estão envolvidas com a produção de hormônios, degradação de outras proteínas, coagulação sanguínea e fibrinólise (DABOOR et al., 2010). Serino proteases colagenolíticas já foram isoladas a partir de vísceras digestivas, músculo e de resíduos gerais do processamento do pescado (aglomerado com cabeça, barbatanas,

cauda, vísceras digestivas, reprodutivas, entre outras), tais como os relatados por Turkiewicz et al. (1991) para a espécie de crustáceo marinho Krill Antártico *Euphausia superba*, Kristjámsson et al. (1995) para o bacalhau do Atlântico *G. morhua*, Teruel e Simpson (1995) para o back preto *P. americanus*, Roy et al. (1996) para o caranguejo *C. maenas*, Byun et al. (2002) para o atum *T. thymus*, Kim et al. (2002) para o peixe filé *N. modestrus*, Park et al. (2002) para uma espécie de cavala *S. japonicus*, Mukherjee et al. (2009) para a esponja *R. odorabile*, Murado et al. (2009) para o peixe-arraia *R. clavata*, Wu et al. (2010) para o peixe pargo *P. major*, Hayet et al. (2011) para uma espécie de sardinha *S. aurita*, Souchet e Laplante (2011) para uma espécie de caranguejo *Chionoecetes opilio* e Sriket et al. (2011) para o camarão de água doce *Macrobrachium rosenbergii*. Órgãos digestivos podem servir tanto como fonte serinocolagenases quanto de metalocolagenases, mas a maioria dos estudos têm se concentrado em glândulas digestivas como fonte das colagenases portadoras do grupo serino. Assim, os resíduos de tecidos, além das glândulas digestivas, podem servir como um valioso e promissor manancial de moléculas bioativas (DABOOR et al., 2010).

2.3.2.3.4 Aplicações das colagenases

As colagenases demonstram aplicações em diversas áreas, podendo ser classificadas, segundo Watanabe (2004), dentro de duas categorias: (1) aquelas em que proteases colagenolíticas são utilizadas diretamente – como em soluções tópicas a partir da adição direta em pomadas, por exemplo; e (2) aquelas em que são utilizados os produtos resultantes da sua reação – como os peptídeos gerados a partir da degradação de colágenos extraídos da pele de espécies de peixes, por exemplo, empregando-os na produção e aplicação de moléculas bioativas para a indústria. Devido à sua capacidade de decompor o colágeno sem afetar as propriedades de outras proteínas, tornou-se uma ferramenta útil na indústria biomédica (TERUEL, 1997).

Colagenases e enzimas com propriedades colagenolíticas têm sido largamente utilizadas na medicina humana – e veterinária, também – com o propósito de limpar feridas necrosadas, escaras, cicatrizes pós-operatórias, no tratamento de psoríase e pediculoses, implantes e perdas ósseas, em lesões de mamilos de mulheres em aleitamento, no tratamento de cicatrizes hipertróficas, no tratamento de isquemias do coração e no debridamento de úlceras de pessoas diabéticas (TAKAHASHI et al., 1999; ÖZCAN et al., 2002; WATANABE, 2004; ARAKAWA et al., 2012; TALLIS et al., 2013).

Outra aplicação médica bastante recorrente (e recente) é o emprego da enzima produzida por *C. histolyticum* no tratamento das doenças de Peyronie[13] (LEVINE, 2013; LANGSTON e CARSON, 2014) e de Dupuytren[14] (contratura ou moléstia) (MARTÍN-FERRERO et al., 2013; PEIMER et al., 2013; HENTZ, 2014; LECLÈRE et al., 2014; MEALS e HENTZ, 2014), como pode ser visualizado na **Figura 6**.

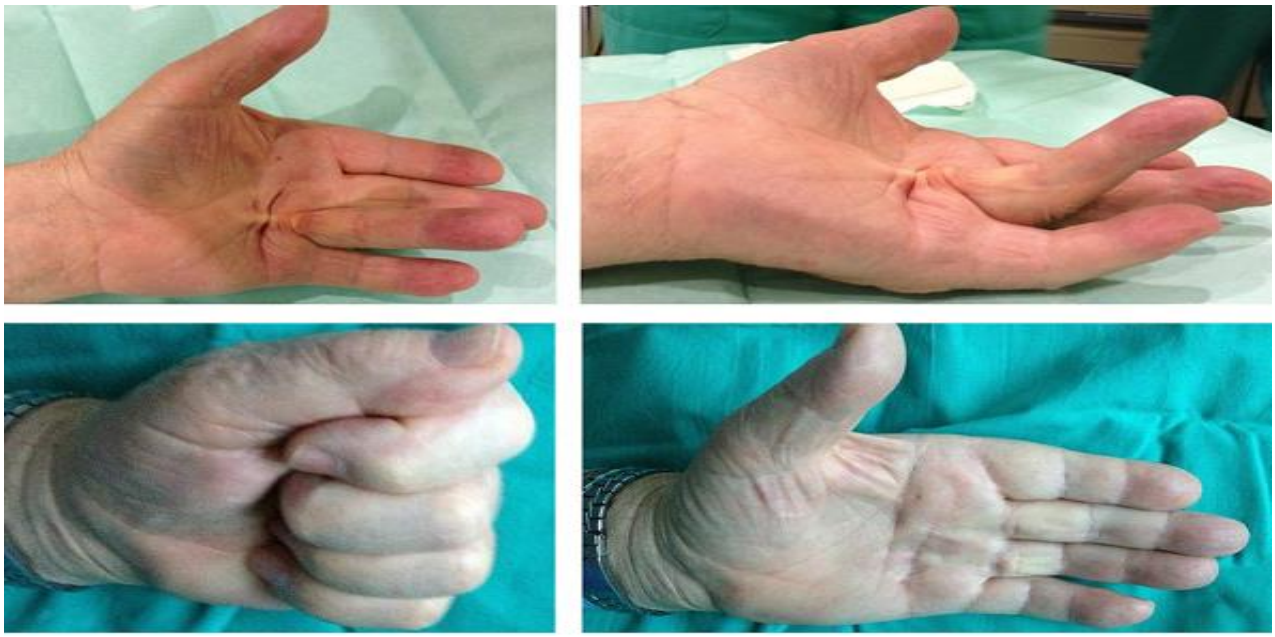

Figura 6: Aplicação da colagenase no tratamento da Doença de Dupuytren. Observar o quarto dedo antes a administração da colagenase e os avanços após 1 mês de administração. Fonte: Martín-Ferrero et al. (2013). Adaptado.

A cicatrização de feridas[15] da pele e remodelação do colágeno onde há degradação extensa é outra importante aplicação das colagenases atualmente, como visualizado na **Figura 7**.

[13]A doença de Peyronie é caracterizada pelo desenvolvimento de uma placa fibrótica ou de um nódulo que se instalam na túnica albugínea — estrutura que envolve os corpos cavernosos –, comprometendo sua elasticidade e impededindoque sua expansão (LEVINE, 2013; LANGSTON e CARSON, 2014).

[14]É uma contratura fixa da mão em flexão caracterizada pelo espessamento da fáscia palmar (tecido encontrado abaixo da pele da mão). Essa condição pode variar desde pequenos nódulos até faixas muito espessas, as quais podem tracionar dos dedos em direção à palma da mão (MARTÍN-FERRERO et al., 2013; PEIMER et al., 2013; HENTZ, 2014; LECLÈRE et al., 2014; MEALS e HENTZ, 2014).

[15]As colagenases, tal como com outras enzimas, são secretadas para o espaço extracelular pelo granulócitos e macrófagos, que participam do natural processo de desbridamento durante a fase inflamatória. Elas separam especificamente o colágeno em dois fragmentos, que são sujeitos a uma maior degradação por proteases inespecíficas (como as serino colagenases, por exemplo). Como as fibrilas de colágeno são os principais constituintes da pele – colágeno é responsável por 70 a 80% do peso seco –, sua degradação leva ao afrouxamento e facilita a sua remoção. Os peptídeos resultantes acabam por atrair as células necessárias para a subsequentes etapas de cicatrização (JUNG e WINTER, 1998).

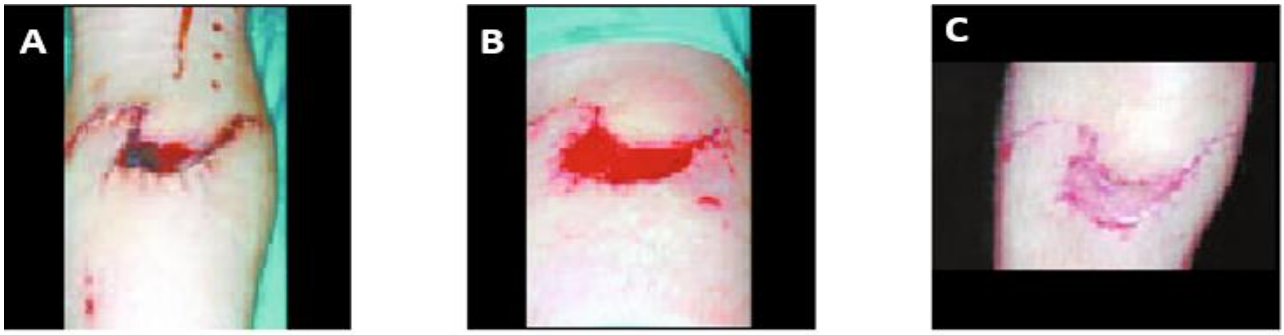

Figura 7: Caso clínico que demonstra o sucesso do uso de pomada a base de colagenase. Na primeira imagem (a esquerda, A), necrose parcial. Na imagem central, B, 2 semanas após e necrotomia, com subsequente aplicação da pomada a base de colagenase. Na figura a direita, C, após 4 semanas e cicatrização quase completa. Fonte: Jung e Winter (1998). Adaptado.

Uma vez que o colágeno é uma das principais proteínas da matriz, a má regulação dessas enzimas é prejudicial, sobretudo quando já há alguma injúria. Uma variedade de células produz colagenases, tais como neutrófilos, granulócitos, macrófagos, fibroblastos, queratinócitos e outros. Estas células desempenham um papel essencial nas diferentes fases de cicatrização de feridas. Os fibroblastos, por exemplo, são atraídos para a ferida por fatores segregados a partir de macrófagos. Eles produzem colágeno novo para a reconstrução da matriz extracelular. Existem inúmeras vantagens do uso de colagenases no tratamento de feridas: a) removem o tecido necrosado com maior eficiência por sua capacidade de hidrolisar vários tipos de colágeno; b) são indolores e não-hemorrágicos; c) podem ser usados por longos períodos e também em associação com outros medicamentos; d) atraem macrófagos e fibroblastos para o local da ferida; e) aumentam a formação de tecidos de granulação e estimulam o próprio organismo a promover a cicatrização (JUNG e WINTER, 1998).

Na indústria têxtil, tem sido aplicada no tratamento de couros e tingimento de tecidos, por funcionar como um biocatalisador não-tóxico, melhorando as características de tingimento, além de se tornar um processo ecologicamente viável (KANTH et al., 2008). Na alimentícia, são adicionadas nas etapas de beneficiamento de produtos, tais como no preparo de carnes e em processos de amaciamento – o colágeno insolúvel contribui para a resistência da carne e dessa forma a enzima atua no processo de repartição do tecido conectivo aumentando a textura e a qualidade de carnes e derivados (FOEGEDING e LARICK, 1986; KIM et al., 1993). Na indústria alimenticia, atua no processamento de carnes, atua na musculatura de peixes, auxiliando nas etapas para remoção da pele. Enzimas microbianas, como, por exemplo, bactérias e leveduras, têm sido utilizados como auxiliares no processamento de alimentos, no entanto, espécies de

microrganismos que produzem colagenases pertencem aos gêneros que contêm estirpes patogênicas. A presença de colagenase em algumas destas estirpes podem contribuir para a virulência destes microrganismos e contaminação do alimento. Assim, colagenases de fontes alimentares, por exemplo, oriundas de peixes, mariscos, camarões, entre outros, podem tornar-se uma alternativa potencial em substituição às colagenases microbianas (KIM et al., 1993; TERUEL, 1997).

Assim sendo, observa-se que a produção de enzimas que apresentem a propriedade colagenolítica pode ser muito vantajosa, sobretudo, oriundas de fontes alternativas, como a partir dos resíduos até aqui descritos, por exemplo. Todas as aplicações partem do princípio da degradação do colágeno (por colagenases verdadeiras e/ou proteases que apresentem as propriedades colagenolíticas – que atuam auxiliando o processo de fragmentação dessa proteína). Nesse contexto, enzimas com propriedades colagenolíticas isoladas de espécies de peixes neotropicais tornam-se uma fonte promissora e sustentável para futuras aplicações, especialmente, para aproveitamento destas enzimas na produção de peptídeos bioativos de colágeno, como os descritos no item a seguir.

2.3.2.3.4.1 Produção e aplicação de peptídeos de colágeno

Os avanços biotecnológicos buscam melhorar a qualidade e minimizar os custos para obtenção de fontes alternativas de enzimas com propriedades colagenolíticas. Como foco central desta pesquisa – as enzimas que apresentam esta propriedade e suas aplicações práticas na produção de moléculas bioativas –, a contextualização que se segue trata fundamentalmente dessa consequência – a obtenção destes fragmentos protéicos. Para tanto, faz-se necessário a promoção de um maior entendimento da proteína precursora – o colágeno –, quanto a sua função, estrutura e tipos encontrados nas diferentes vísceras e/ou sistemas orgânicos. Em seguida, trataremos da fase final deste capítulo relacionado à formação dos peptídeos bioativos de colágeno e alternativa de extração destas proteases por meio do método de separação em fases aquosas.

2.3.2.3.4.1.1 Colágeno, função e estrutura

O colágeno, uma proteína fibrosa, é o principal componente de tecidos conjuntivos em mamíferos e peixes, representando 30% do conteúdo de proteína total e 6% em peso do corpo humano (TONHI e PLEPIS, 2002; DABOOR et al., 2010; CHUNG e UITTO,

2010; JUNQUEIRA e CARNEIRO, 2011; FERREIRA et al., 2012; DUARTE et al., 2014). Constitui um tipo de proteínas selecionadas durante a evolução para exercer diferentes funções, principalmente estruturais. É o principal elemento estrutural dos ossos, cartilagens, pele, tendões, ligamentos, músculo liso, vasos sanguíneos, dentes, córneas, lamina basal e demais órgãos dos vertebrados (SENARATNE et al., 2006; UITO et al., 2008; CHUNG e UITTO, 2010; JUNQUEIRA e CARNEIRO, 2011).

A unidade básica do colágeno é o tropocolágeno (PRESTES, 2012). A molécula do colágeno consiste em três cadeias polipeptídicas ligadas entre si em tripla hélice (GELSE et al., 2003; FERREIRA et al., 2012), como visualizado na **Figura 8**.

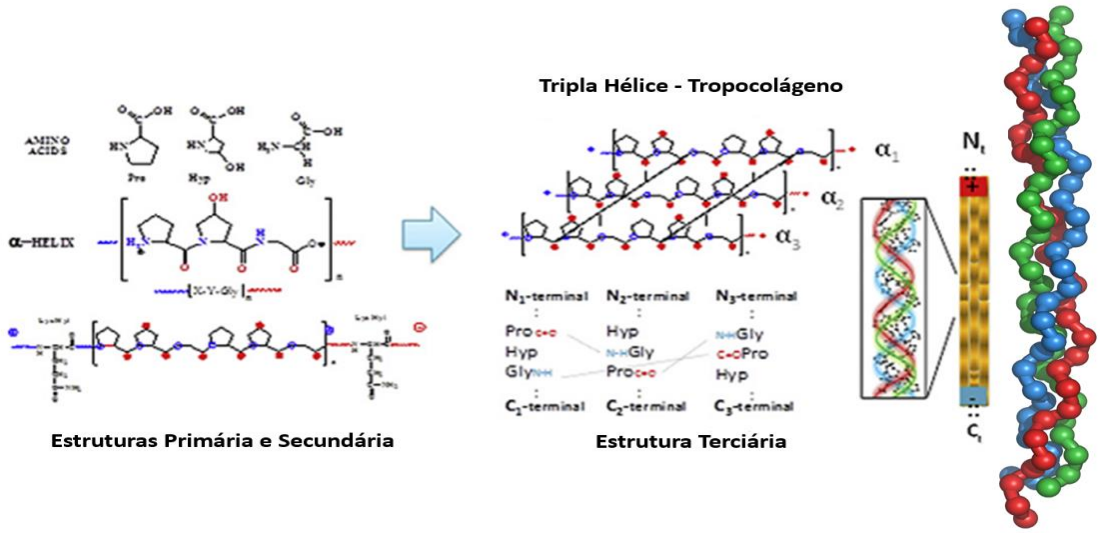

Figura 8: Desenho esquemático da estrutura hierárquica do colágeno. Montagem. Fonte: Ferreira et al. (2012). Adaptado.

As fibras se ligam por pontes de hidrogênio entre o grupo –NH da glicina e o grupo carbonila C=O de resíduos de outra cadeia polipeptídica, ou por pontes de hidrogênio com moléculas de água. A estrutura repetitiva de (Gli-X-Y)n é característica de todos os colágenos. O resíduo de glicina é pré-requisito estrutural para a tripla hélice. As posições X e Y são frequentemente ocupadas por prolina e hidroxiprolina (GELSE et al., 2003; DABOOR et al., 2010; FERREIRA et al., 2012). Além disso, a influência na estabilização da hélice de colágeno é também devido a pequenas interações, tais como: Van der Waals, hidrofóbica e eletrostática (PRESTES, 2012). Nas fibrilas de colágeno, ~25% dos aminoácidos na cadeia α são prolina e hidroxiprolina que bloqueiam efetivamente a rotação interna da cadeia de colágeno nestes sítios e estabilizam a estrutura em tripla hélice (WISNIEWSKI et al., 2007; FERREIRA et al., 2012).

2.3.2.3.4.1.2 Tipos de Colágeno

Atualmente, a família dos colágenos é composta por mais de 20 tipos geneticamente diferentes (**Tabela 2**). O colágeno do tipo I é o mais abundante, sendo amplamente distribuído no organismo, sobretudo no humano, espécie na qual é baseado a classificação. Ele ocorre como estruturas classicamente denominadas de fibrilas de colágeno e que formam ossos, dentina, tendões, cápsulas de órgãos, córnea, vasos sanguíneos e derme, desempenhando um papel importante na morfogênese e no metabolismo celular de novos tecidos, conferindo propriedades mecânicas e bioquímicas (MYLLYHARJU e KIVIRIKKO, 2004; SÖDERHÄLL et al., 2007; UITO et al., 2008; CHUNG e UITTO, 2010; JUNQUEIRA e CARNEIRO, 2011; FERREIRA et al., 2012; MAKAREEVA e LEIKIN, 2014).

Seu domínio central de cada uma das três cadeias, contém um segmento de repetição ininterrupta Gli-X-Y abrangendo cerca de 1000 aminoácidos (DABOOR et al., 2010; CHUNG e UITTO, 2010; JUNQUEIRA e CARNEIRO, 2011; MAKAREEVA e LEIKIN, 2014). Este colágeno é uma proteína macromolecular constituída de três cadeias polipeptídicas (duas α_1 e uma α_2) que estão sob a forma helicoidal em sua porção central e nas extremidades amínica e carboxílica permanecem na forma globular (PRESTES, 2012). É sintetizado como um precursor de procolágeno, o qual é composto de um pró-peptídeo N-terminal, domínio de colágeno central e de pro-peptídeo C-terminal (MAKAREEVA e LEIKIN, 2014). Em outros colágenos, como no tipo IV (o colágeno da membrana basal) e tipo VII (o colágeno de ancoragem das fibrilas), a sequência de repetições Gli-X-Y contém imperfeições que interrompem a conformação em tripla hélice (UITTO et al., 2008; CHUNG e UITTO, 2010).

Os colágenos tipos I, II, III, V e X alinham-se em grandes fibrilas extracelulares e são designados como colágenos formadores de fibrilas ou colágenos fibrilares. O colágeno tipo IV é organizado em um entrelaçamento dentro das membranas basais, enquanto o colágeno tipo VI forma microfibrilas distintas e o tipo VII forma fibrilas de ancoragem. Colágenos associados às fibrilas são estruturas curtas que ligam as fibrilas de colágeno umas às outras e os outros componentes da matriz extracelular, pertencendo a este grupo os colágenos do tipo IX, XII, XIV, XIX, XX e XXI (OLSEN, 1995; TERUEL, 1997; UITTO et al., 2008; CHUNG e UITTO, 2010). A abundância dos tipos V e XI é baixa, mas eles são encontrados associados com os tipos I e II, no osso e na cartilagem,

bem como em outros tecidos, com importante participação na função de resistência a tensão (DABOOR et al., 2010; JUNQUEIRA e CARNEIRO, 2011).

Tabela 2. Principais tipos de colágeno.

Tipo	Composição molecular	Localização genômica	Distribuição tecidual
	Colágeno formadores de fibrilas		
I	[α1(I)]2α2(I)	COL1A1 (17q21.31 – q22)	Osso, derme, tendão, ligamento, córnea.
II	$[\alpha1(II)]_3$	COL2A1 (12q13.11 – q13.2)	Cartilagem, corpo vítreo, núcleo pulposo.
III	$[\alpha1(III)]_3$	COL3A1 (2q31)	Pele, parede dos vasos, fibras reticulares da maioria dos tecidos (pulmões, fígado, baço, etc.).
V	α1(V), α2(V), α3(V)	COL5A1 (9q34.2 – q34.3)	Pulmão, córnea, ossos, tecidos fetais; juntamente com o colágeno tipo I.
XI	α1(XI) 2(XI)α3(XI)	COL11A1 (1p21)	Cartilagem, corpo vítreo.
	Colágeno da membrana basal		
IV	$[\alpha1(IV)]_2\alpha(IV)$; α1– α6	COL4A1 (13q34)	Membranas basais.
	Colágeno microfibrilar		
VI	α1(VI), α2(VI), α3(VI)	COL6A1 (21q22.3)	Derme, cartilagem, placenta, pulmões, da parede do vaso, disco intervertebral.
	Fibrilas de ancoragem		
VII	$[\alpha1(VII)]_3$	COL7A1 (3p21.3)	Pele, junções epidérmicas, cérvix.
	Colágenos formadores de rede hexagonal		
VIII	[α1(VIII)]2α2(VIII)	COL8A1 (3q12 – q13.1)	Células endoteliais.
X	$[\alpha3(X)]_3$	COL10A1 (6q21 – q22.3)	Cartilagem hipertrófica.
	Colágenos FACIT		
IX	α1(IX)α2(IX)α3(IX)	COL9A1 (6q13)	Cartilagem, humor vítreo, córnea.
XII	$[\alpha1(XII)]_3$	COL12A1 (6q12 – q13)	Pericôndrio, ligamentos, tendões.
XIV	$[\alpha1(XIV)]_3$	COL9A1 (8q23)	Derme, tendão, da parede do vaso, placenta, pulmões, fígado.
XIX	$[\alpha1(XIX)]_3$	COL19A1 (6q12 – q14)	Rabdomiossarcoma humano.
XX	$[\alpha1(XX)]_3$		Epitélio da córnea, pele embrionária, cartilagem esternal, tendão.
XXI	$[\alpha1(XXI)]_3$	COL21A1 (6p12.3 – 11.2)	Parede de vasos sanguíneos.
	Colágenos transmembranar		
XIII	$[\alpha1(XIII)]_3$	COL13A1 (10q22)	Epiderme, endomísio, intestino, condrócitos, pulmões, fígado.
XVII	$[\alpha1(XVII)]_3$	COL17A1 (10q24.3)	Junções derme-epidérmicas.
	Multiplexin		
XV	$[\alpha1(XV)]_3$	COL15A1 (9q21 – q22)	Fibroblastos, células musculares lisas, rim, pâncreas.
XVI	$[\alpha1(XVI)]_3$	COL16A1 (1p34)	Fibroblastos, queratinócitos.
XVIII	$[\alpha1(XVIII)]_3$	COL18A1 (21q22.3)	Pulmões, fígado.

Fonte: Gelse et al. (2003). Adaptado.

A derme humana é composta por colágenos do tipo I e III, associadas em fibras extracelulares, representando de 80% e 10% do volume total de colágeno, respectivamente. Outro colágeno presente na pele é o tipo IV, presente na junção dermo-epidérmica e na membrana basal vascular. Em adição a estes grandes colágenos, a pele humana contém vários colágenos menores que demonstram localização espacialmente restringida, contudo desempenham um papel crítico na estabilidade da pele. Um deles é colágeno VII, o principal, se não exclusivo, componente das fibrilas de ancoragem, que interage com o colágeno do tipo I (CHUNG e UITTO, 2010; JUNQUEIRA e CARNEIRO, 2011). O tipo V está presente na maioria dos tecidos conjuntivos, incluindo a derme onde representa menos de 5% do total de colágeno, localizando-se na superfície das fibras de colágeno formadas pelos tipos I e III, regulando o crescimento lateral dessas fibras (CHUNG e UITTO, 2010).

Devido suas características físico-químicas, o colágeno tem sido um dos biomateriais mais utilizados na atualidade, podendo ser facilmente modificados através de reação dos seus grupos funcionais, por introdução de ligações cruzadas ou de enxertos de moléculas biológicas, criando uma ampla variedade de materiais com propriedades mecânicas ou biológicas adaptadas. É fonte para engenharia de tecido ósseo devido a sua abundância, biocompatibilidade, alta porosidade, facilidade de combinação com outros materiais, processamento fácil, baixa antigenicidade e capacidade de absorção no corpo. Historicamente, os usos industriais de colágeno em forma de couro e gelatina são generalizados, incluindo aplicações de gelatina fotográfica, na indústria de cosméticos, alimentos e farmacêutica (PATI et al., 2010; FERREIRA et al., 2012).

Na medicina, suas aplicações estão relacionadas a suturas, agentes hemostáticos, substituição e/ou regeneração tecidual (vasos, ossos, cartilagens, pele, sangue, traquéia, esôfago), cirurgia plástica (lábios, pele), oxigenador de membrana, contraceptivos, matrizes biodegradáveis, implantes, bandagem da córnea, lentes de contato (FERREIRA et al., 2012).

Dentre várias espécies utilizadas para a obtenção de colágeno[16], os peixes merecem atenção, especialmente devido a sua grande disponibilidade, ausência de risco

[16]Em peixes, o colágeno total nos tecidos pode ser isolado pela extração direta com ácidos orgânicos (acético, cloroacético, cítrico e lático), ácidos inorgânicos (clorídrico) e extração enzimática. O rendimento da extração de colágeno dependerá da espécie de animal usada, idade e dos parâmetros de extração (temperatura, tempo, e pH), e seu pré-tratamento. O aumento da solubilidade do colágeno após o tratamento enzimático depende da espécie do peixe. O colágeno de algumas espécies de peixes é completamente solúvel em ácido acético após o tratamento enzimático (SENARATNE et al., 2006).

de transmissão de doenças, de barreias religiosas, alto rendimento no processo de extração e ausência de toxicidade (SENARATNE et al., 2006). Vários estudos têm focado a extração de diferentes colágenos a partir de várias espécies aquáticas, como a perca-do-Nilo *Lates niloticus* (MUYONGA et al., 2004) e do patim *Raja kenojei* (HWANG et al., 2007), por exemplo. Peles (**Figura 9**), ossos, nadadeiras e escamas são tecidos formados principalmente por colágeno e embora as propriedades físico-químicas do colágeno das espécies de peixes sejam diferentes dos mamíferos, não há associação destas proteínas a doenças, como no caso dos colágenos obtidos de outras fontes animais, tais como o bovino e a associação com a encefalopatia espongiforme bovina (BSE), encefalopatia espongiforme transmissível (TSE) e febre aftosa (FMD) devido à grande distância evolucionária entre peixes e humanos (GIRAUD-GUILLE et al., 2000; SONG et al., 2006).

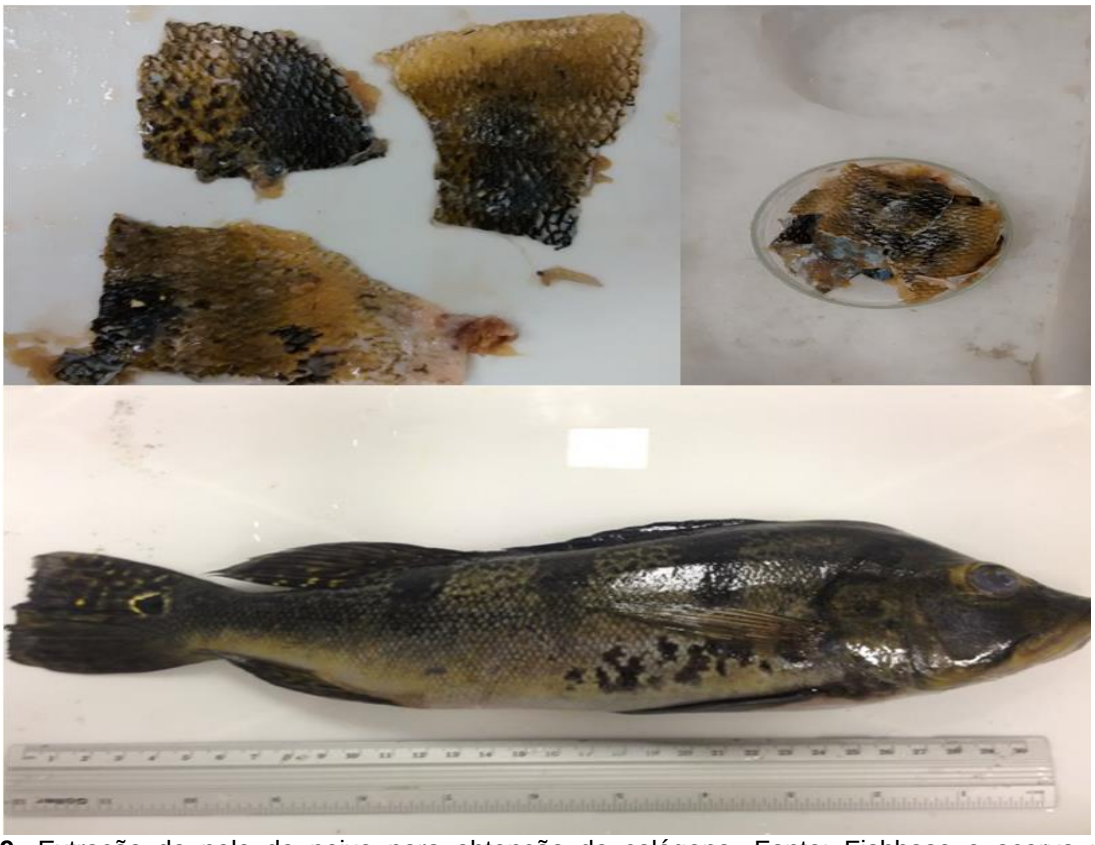

Figura 9: Extração da pele de peixe para obtenção de colágeno. Fonte: Fishbase e acervo pessoal. Adaptado.

Colágeno do tipo I já foi extraído da pele, ossos, barbatanas e escamas de peixes e de outros animais de água doce e marinhos, como lulas, águas-vivas e estrelas do mar. O papel das enzimas com propriedades colagenolíticas é fundamental na decomposição deste colágeno. Torna-se interessante e sustentável o emprego das próprias vísceras

oriundas do processamento do pescado na extração do colágeno da própria espécie, assim como na produção de peptídeos de colágeno a partir da fragmentação desse colágeno, dando um maior alcance ao processo produtivo, agregando valor ao produto final e reduzindo custos no tratamento/destino das peles. Além do que, essas moléculas bioativas são uma alternativa saudável para aplicação na indústria alimentícia e farmacêutica (PATI et al., 2010).

Grandes quantidades de resíduos são geradas a partir de fábricas de processamento de peixe, podendo servir como fontes do colágeno. Então, há um imenso espaço para utilização dos resíduos do processamento de peixe. Devido a essas vantagens, os resíduos de peixe têm o potencial para ser usado como uma fonte natural de colágeno novo (PATI et al., 2010). A fragmentação do colágeno e a produção destes peptídeos é tratado no item a seguir.

2.3.2.3.4.1.3 Formação de peptídeos de colágeno

Os produtos de reação produzidos por proteases colagenolíticas que agem nos diferentes tipos de colágeno são chamados de “peptídeos de colágeno” (**Figura 10**).

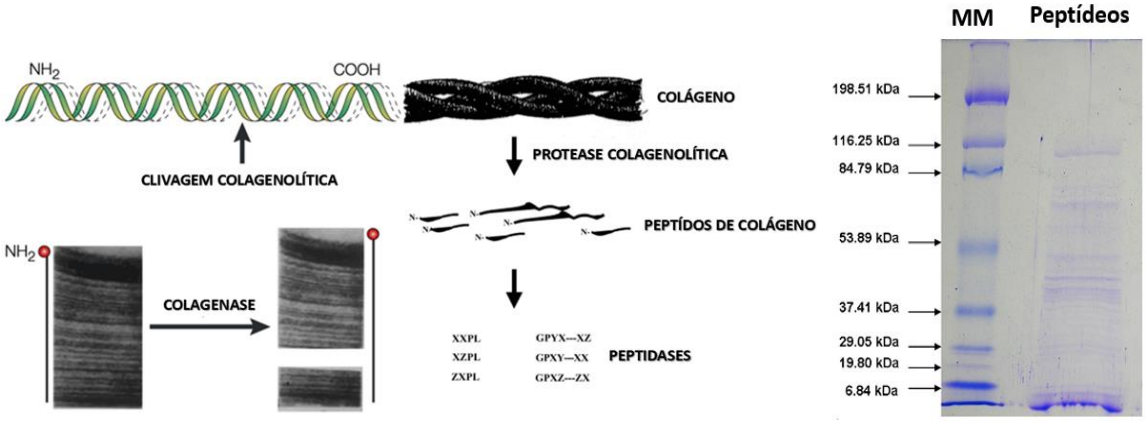

Figura 10: Digestão do colágeno intersticial por protease colagenolítica. Mapeamento de peptídeos de colágeno do tipo I do tendão de Aquiles bovino, após hidrólise pela colagenase extraído por APTS (17,5% de PEG 1500, 15% de concentração de fosfato e pH 6,0). MM: massa molecular. Fonte: Brinckerhoff e Matrisian (2002), Watanabe (2004) e Lima et al. (2013). Adaptado.

Peptídeos de colágeno têm sido referidos como sendo agentes eficazes no tratamento de várias doenças como, por exemplo, a supressão de artrite induzida por colágeno e de proteção contra bactérias patogênicas. Estes peptídeos têm sido administrados por via oral como um pré-tratamento contra a osteoporose (WATANABE, 2004). Para a preparação de peptídeos de colágeno, o método de clivagem química que

está atualmente em uso requer reações que utilizam brometo de cianogênio (CNBr) como catalisador. Portanto, as enzimas colagenolíticas – que apresentam elevada atividade, especificidade e são estáveis –, são as preferidas para a produção desses peptídeos visando aplicações industriais, com destaque para as áreas alimentícia e biomédica (TERUEL, 1997; WATANABE, 2004; UESUGI et al. 2008).

As fibrilas de colágeno são insolúveis na sua estrutura nativa, mas podem ser solubilizadas em solução aquosa se forem desnaturadas para procolágenos solúveis (FERREIRA et al., 2012). A tripla hélice de colágeno nativo é altamente resistente à ação da maioria das proteases. Entretanto, a ação da colagenase torna a molécula do colágeno um alvo fácil para outras enzimas proteolíticas, uma vez que, as células de tecido conjuntivo sintetizam uma série de proteases capazes de atuar sobre o colágeno em condições fisiológicas. Este grupo de proteases incluem metalo, aspártico, cisteíno e serino proteases, como a quimotripsina e a tripsina, por exemplo (TERUEL, 1997).

As metalocolagenases desempenham um papel crítico – dentre todas as colagenases – na produção destes peptídeos, clivando todo colágeno fibroso, de forma mais rápida nos tipos I e III, do que nos do tipo II. A ação deste tipo de colagenase é dependente da conformação, sob a tripla hélice de colágeno em um local definido, entre os resíduos de Gly-IIe ou Gly-Leu ou ligações em todas as três cadeias peptídicas. Entretanto, metalocolagenases apresentam pouca ação no mesmo local em cadeias α individuais. Estas colagenases não possuem efeito sobre a parte não-fibrosa dos colágenos. A degradação do colágeno requer a ação conjunta de colagenases verdadeiras e de pelo menos mais uma protease sobre os diferentes locais da molécula de colágeno (TERUEL, 1997). Vale salientar que, os processos de purificação empregados de forma rotineira acabam por tornar o produto final mais oneroso. Nessa perspectiva, a proposta desta pesquisa foi empregar um sistema formado pelo polietilenoglicol (PEG) e um sal, apresentando rápida separação das fases, baixo custo e elevada seletividade na separação de moléculas com base na solubilidade, como descrita no próximo item, a seguir.

2.3.2.3.4.1.4 Extração líquido-líquido/Sistema de Duas Fases Aquosas

A extração líquido-líquido utilizando o sistema de duas fases aquosas (SDFA) é um processo de bioseparação que permite um elevado grau de purificação e recuperação de materiais biológicos. Esta técnica pode ser utilizada nas etapas iniciais de um processo de

purificação (por exemplo, separando proteínas dos restos celulares), pois permite a remoção de contaminantes por um processo simples e econômico, e na substituição de técnicas complexas de separação sólido-líquido (SPELZINI et al., 2005; MALPIEDI et al., 2008). Sabe-se que a cromatografia é geralmente preferida na maioria dos processos de purificação. No entanto, algumas desvantagens do processo, como a baixa capacidade de ligação e os altos custos de resina (ROSA et al., 2011), aumentam a necessidade de técnicas alternativas para viabilizar as etapas de produção. Entre estas alternativas, o sistema de duas fases aquosas (SDFA) – uma estratégia de extração líquido-líquido (ELL) –, é utilizado como uma técnica potencial devido às suas múltiplas vantagens, incluindo: menor viscosidade, menor custo de produtos químicos, possibilidade de utilização de solventes com boa capacidade de extração ou seletivos; possibilita controle de pH, força iônica e temperatura, de forma a evitar a desnaturação de enzimas, biocompatibilidade e simplicidade de operação dos processos (MAZZOLA et al., 2008; MONTEIRO FILHO, 2010; COELHO et al., 2011; ESPITIA-SALOMA et al., 2014).

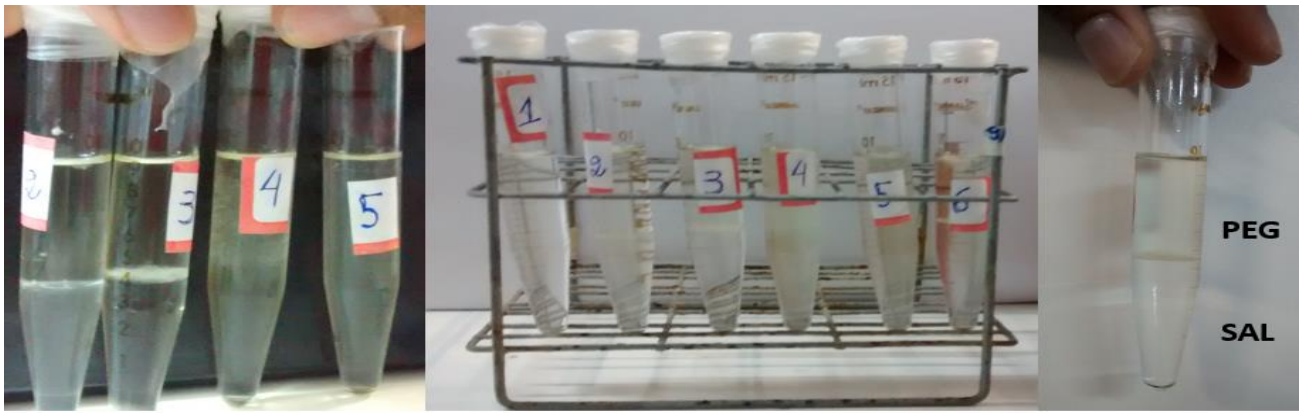

Figura 11: Sistema de duas fases aquosas. Ilustração das fases PEG (polímero) e SAL na extração de proteases com propriedades colagenolíticas a partir de vísceras intestinais da espécie de peixe tucunaré (*Cichla ocellaris*). Montagem. Fonte: Acervo pessoal.

Em 1956 foi mencionado pela primeira vez o uso do sistema de duas fases aquosas para a purificação de proteínas celulares e de partículas de células (ASENJO e ANDREWS, 2011). Desde essa época, a extração em SDFA tem sido aplicada a obtenção de produtos a partir de células animais (sobretudo, fragmentos da parede celular), de vegetais e microbianas, extração de vírus, organelas e ácidos nucleicos, devendo-se destacar a aplicação na purificação de enzimas, tais como as colagenases (PESSOA JÚNIOR e KILIKIAN, 2005; ROSSO et al., 2012; LIMA et al., 2013). Este sistema provou ser uma técnica eficaz na recuperação de biomoléculas (RITO-

PALOMARES, 2004; CHETHANA et al., 2007; MAZZOLA et al. 2008; PORTO et al., 2008; RUIZ-RUIZ et al., 2012; INGRAM et al., 2013; JARAMILLO et al., 2013; MEDEIROS E SILVA et al., 2013; MILOSEVIC et al., 2013; ESPITIA-SALOMA et al., 2014; WIBISONO et al., 2014).

A distribuição de proteínas entre as duas fases aquosas nos SDFA é caracterizada por um parâmetro denominado coeficiente de partição (K_p). A purificação é o resultado de uma partição seletiva da molécula-alvo e impurezas entre duas fases líquidas (**Figura 11**).

O elevado teor de água, 60 a 95% em massa (SILVA e LOH, 2006), garante a manutenção das propriedades biológicas das proteínas e a baixa tensão interfacial dos sistemas que protegem as proteínas. A separação entre a molécula a ser purificada e os contaminantes decorre das diferentes solubilidades apresentadas por esses solutos, em cada uma das fases aquosas (PESSOA JÚNIOR e KILIKIAN, 2005; ASENJO e ANDREWS, 2011).

De modo geral, os SDFA são formados por determinados polímeros, polieletrólitos, ou ainda, polímeros em combinação com solutos de baixa massa molar, em uma mesma solução, formando quatro grupos. No primeiro, podem ser considerados os sistemas formados por 2 polímeros não-iônicos: polietilenoglicol (PEG)/Ficoll; PEG/dextrana (Dx); PEG/polivinil álcool; polipropilenoglicol (PPG/Dextrana; metil celulose/hidroxipropildextrana e Ficoll/dextrana). No segundo, tem-se um polieletrólito e um polímero não-iônico: sulfato dextrana de sódio/polipropileno glicol e carboximetilcelulose de sódio/metil celulose (PESSOA JÚNIOR e KILIKIAN, 2005; SILVA e LOH, 2006).

Os SDFA formados por sulfato dextrana de sódio/carboximetildextrana de sódio e carboximetildextrana de sódio/carboxietilcelulose de sódio formam o terceiro grupo. Finalmente, no quarto grupo, tem-se um polímero não-iônico e um composto de baixa massa molar: PPG/Fosfato de potássio; metoxipolietilenoglicol/fosfato de potássio; PEG/glicose; PEG/sulfato de magnésio e PEG/citrato de sódio (PESSOA JÚNIOR e KILIKIAN, 2005). Para purificação de enzimas colagenolíticas, o mais empregado é o PEG/fosfato de potássio (ROSSO et al., 2012; LIMA et al., 2013). Segundo Asenjo e Andrews (2011), as propriedades de separação podem ser exploradas individualmente ou em conjunto para atingir uma separação eficaz de uma proteína particular, tais como: (i) a hidrofobicidade das próteínas, onde as propriedades hidrofóbicas de uma fase do sistema são utilizadas para a separação; (ii) eletroquímica, onde o potencial elétrico entre as fases

é usado para separar moléculas ou partículas de acordo com sua carga; (iii) tamanho-dependente, particionamento onde o tamanho ou a área das moléculas (proteínas) ou das partículas é o fator preponderante; e, por fim, (iv) afinidade-bioespecífica, em que a afinidade entre os sítios de ligação das proteínas e os ligantes de um dos polímeros é explorada para a separação. Enquanto que, os fatores que vão influenciar a separação são: (i) massa molar/tamanho dos polímeros; (ii) concentração do polímero; (iii) força iônica do sal; (iv) pH; e, por fim, (v) quantidade de sal adicional utilizado, que aumenta a hidrofobicidade do sistema.

O emprego dos sistemas PEG/Fosfato tem sido muito utilizado na extração de enzimas colagenolíticas, como descrito por Rosso et al. (2012) e Lima et al. (2013) devido ao fato do PEG possuir propriedades físicas favoráveis, especialmente no que se refere à viscosidade e à diferença de densidade entre as fases. Esta escolha é bastante influenciada por questões a que os processos de produção têm que obedecer, uma vez que o PEG é uma substância atóxica e não causa impacto ambiental (SARMENTO et al., 1997; PORTO, 2008; PADILHA et al., 2011). Em sistemas PEG/sal, a adição de outros sais interfere no coeficiente de partição, visto como, existe uma partição desigual entre eles, formando uma diferença de potencial elétrico. Além disso, íons mais hidrofóbicos se direcionam para a fase PEG, carregando proteínas igualmente hidrofóbicas e cátions induzem a transferência de proteínas para a fase PEG (PESSOA JÚNIOR e KILIKIAN, 2005). No caso em que a extração pelo SDFA não for suficiente, etapas subsequentes podem ter fases cromatográficas associadas (SILVA e LOH, 2006).

Por fim, sabe-se que a demanda por métodos de extração e purificação de biopartículas que sejam eficientes e economicamente viáveis é grande. Nessa linha, os sistemas aquosos bifásicos constituem um enorme potencial. Essa demanda por parte da indústria biotecnológica – com objetivo de trazer para o mercado consumidor produtos que possuam alto valor agregado, com ampla aplicação – certamente motivará a aplicação em escala industrial dos sistemas de duas fases aquosas (SILVA e LOH, 2006; MONTEIRO FILHO, 2010).

3. OBJETIVOS

3.1 Objetivo geral

Obter proteases com propriedades colagenolíticas a partir dos resíduos de três espécies de peixes Neotropicais (arabaiana *Seriola dumerili*; pescada-branca *Cynoscion leiarchus*; e tucunaré *Cichla* ocellaris) para aplicação na produção de peptídeos de colágeno.

3.2 Objetivos específicos

3.2.1 Extrair proteases a partir de resíduos viscerais obtidos de três espécies de peixes exóticas neotropicais (*S. dumerili*; *C. leiarchus* e *C. ocellaris*): tripsina, quimotripsina e colagenases.

3.2.2 Selecionar entre as espécies *S. dumerili*; *C. leiarchus* e *C. ocellaris* a que obtiver melhor atividade colagenolítica especifica.

3.2.3 Caracterizar físico-quimicamente a tripsina e quimotripsina do intestino das três espécies de peixes e investigar a especificidade dos extratos para com o colágeno bovino tipo I e do extraído de espécies exóticas neotropicais.

3.2.4 Caracterizar duas proteases com propriedades colagenolíticas, dentre as espécies pré-selecionadas, e investigar a capacidade de hidrólise do colágeno dos tipos I e do colágeno extraído da pele de espécies exóticas neotropicais.

3.2.5 Purificar uma protease com propriedades colagenolíticas da espécie que obtiver melhor resultado de atividade especifica para colagenase, através do método de extração Líquido-Liquido (ELL), por meio do Sistema de Duas Fases Aquosas (SDFA) e investigar a capacidade de hidrolisar o colágeno do tipo I e o extraído a partir da pele de espécies exóticas neotropicais.

3.2.6 Extrair colágeno da espécie pré-selecionada e caracterizar quanto a sua massa molar e rendimento, visando investigar a hidrólise do colágeno por meio de protease intestinal com propriedades colagenoliticas purificada por meio do SDFA.

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5. ARTIGO I - Use of fish byproducts: characterization of two serine proteases and determination of collagenolytic activity.

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Use of fish byproducts: characterization of two serine proteases and determination of collagenolytic activity.

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Abstract

Digestive viscera of fish are potential sources of biologically active molecules. Serine proteases were extracted and characterized from waste processing of greater amberjack (*Seriola dumerili*), hake (*Cynoscion leiarchus*) and peacock bass (*Cichla ocellaris*). Optimum temperature of trypsin was 60°C presenting stability between 25-60°C for the three species. For chymotrypsin, the same parameters were 40°C (*S. dumerili* and *C. leiarchus*) and 35°C (*C. ocellaris*) being stable at 25-45°C. The pH stability varied between 8.0-10.0 (trypsin) and 6.5-10.0 (chymotrypsin). The enzymes were sensitive to Fe^{3+}, Hg^{2+} and Cu^{2+}, TLCK and benzamidine. The kinetic parameters (Km and V_{max}) were within the range reported in the literature. Collagenolytic activity of freshwater species *C. ocellaris* (106.82 U/mg) was higher than in marine species *S. dumerili* (42.44 U/mg) and *C. leiarchus* (98.08 U/mg). The results suggest digestive viscera of *C. leiarchus* and *C. ocellaris* with collagenolytic activity as alternative source in the production of peptide collagen.

Keywords: Bioactive molecules, *Cichla ocellaris*, collagenolytic activity, *Cynoscion leiarchus*, Fish processing waste, *Seriola dumerili*.

Practical Applications

Digestive proteinases can be isolated as a value-added product from fish viscera and used as processing tools in industries to maximize the utilization of marine resources. Therefore, this study aimed to extract and characterize two serine proteases from the processing of waste from three species of Neotropical fish and investigate the collagenolytic properties present in their extract. Such collagenolytic enzymes are appreciated for their biotechnological use in food industry (meat softening), medicine (healing ointments), cosmetics (treatment of wrinkles and acnes), leather processing and production of bioactive collagen peptides (regeneration of cartilage tissue, among other features).

1. Introduction

Processing of byproducts of the fishing industry has been growing as a profitable alternative to the waste of fish. The use of proteases in stages of production of active biomolecules from digestive viscera and processing of wastes (carcasses, skin, bones, fins and heads) is an example of the potential use of enzymes in waste recovery, which could be improperly discarded, degrading the environment. In this context, fish enzymes can minimize production costs and function as an alternative and/or complementary profitable for industry and fishermen (Bezerra et al., 2006; Kim and Mends, 2006; Souza et al., 2007; Arvanitoyannis and Kassaveti, 2008; Marcuschi et al., 2010; Silva et al., 2011; Freitas Junior et al., 2012; Costa et al., 2013).

Proteases are degradative enzymes which catalyze the total hydrolysis of proteins. The serine proteases have been described as a group of endopeptidases (EC 3.4.21.X) with a serine residue in its catalytic center (Rao et al., 1998; Hedstrom, 2002). These enzymes play a key role in the digestion of dietary proteins and are responsible for the activation of trypsinogen, chymotrypsinogen and other zymogens. Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) are the major serine proteinases well characterized from the digestive glands of marine animals (Klomklao, 2008). Serine digestive proteinases have already been extracted and characterized from the guts of different fish species, as in the case of trypsin from Nile tilapia *Oreochromis niloticus* (Bezerra et al., 2005); spotted goatfish *Pseudupeneus maculatus* (Souza et al., 2007); masu salmon *Oncorhynchus masou* (Kanno et al., 2010); the silver mojarra *Diapterus rhombeus* (Silva et al., 2011); the pirarucu *Arapaima gigas* (Freitas Junior et al., 2012), crevalle jack *Caranx hippos* (Costa et al., 2013) and *Atractosteus tropicus* (Guerrero-Zárate et al., 2014); chymotrypsin of anchovy *Engraulis japonica* (Heu et al., 1995), Monterey sardine *Sardinops sagax*

caerulea (Castillo-Yañez et al., 2009) and tropical gar *Atractosteus tropicus* (Guerrero-Zárate et al., 2014).

The collagenolytic activity is a property also found in digestive glands of fish and involves the cleavage of the triple helix of collagen (types I, II and III) and induce protein degradation (Park et al., 2002; Kim et al., 2002; Daboor et al., 2012). These tasks are typical of an enzyme group called "collagenases". These are divided into two groups with distinct physiological functions: metalloproteases, involved in remodeling of the extracellular matrix, and serine proteases, involved in the digestion of food. The serine proteases with collagenolytic properties are more abundant than collagenolytic metalloproteinases and are of great importance for the development and survival of living organisms. Such proteolytic enzymes are appreciated for their biotechnological potential, for use in medicine, food industry, cosmetics, leather processing and obtainment of bioactive collagen peptides (Daboor et al., 2010; Lima et al., 2013). Enzymes with serine collagenolytic properties have been isolated and characterized from fishery waste, such as in winter flounder *Pseudopleuronectes americanus* (Teruel and Simpson, 1995), filefish *Novoden modestrus* (Kim et al., 2002), Mackerel *Scomber japonicas* (Park et al., 2002), Sea bream *Pagrus major* (Wu et al., 2010) and through the use of fish waste (Daboor et al., 2012).

Digestive proteinases can be isolated as a value-added product from fish viscera and used as processing tools in industries to maximize the utilization of marine resources (Bezerra et al., 2005; Souza et al., 2007; Freitas Junior et al., 2012; Daboor et al., 2012). Therefore, this study aimed to extract and characterize two serine proteases from the processing of waste from three species of Neotropical fish (greater amberjack *Seriola dumerili*, hake *Cynoscion leiarchus* and peacock bass *Cichla ocellaris*) and investigate the collagenolytic properties present in their extract.

2. Materials and methods

2.1 Materials

Azo dye-impregnated collagen (azocoll), azocasein, bovine serum albumin (BSA), Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), Succinyl-DL-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan), Type I collagen from calf skin, Tris (hydroxymethyl) aminomethane and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Glycine was acquired from Amersham Biosciences. HCl were obtained from Merck (Darmstadt, Germany). The spectrophotometer used was Bio-Rad Smartspec™ 3000. Microplate spectrophotometer used was Bio-Rad xMark™. The centrifuges were BioAgency Bio-Spin and Software MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

2.2. Fish Waste

Waste (intestine and muscle) of three species of Neotropical fish were used. Greater amberjack *Seriola dumerili* and hake *Cynoscion leiarchus* were obtained from the fishermen colony of Ponta Verde, Maceió, Alagoas, Brazil while a freshwater species, peacock bass *Cichla ocellaris* was obtained from São Francisco River, at Petrolândia, Pernambuco, Brazil. The viscera were kindly provided after evisceration process. Samples of 500 g intestine and muscle were collected separately, packaged in plastic containers, kept on ice and transported to the Laboratório de Enzimologia, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil, where they were stored at - 27°C for further processing.

2.3 Enzyme extraction

The processing of waste was in accordance with the methodology described by Teruel and Simpson (1995). The ratio of viscera to extraction buffer (0.05 M Tris-HCl pH 7.4, containing 5 mM $CaCl_2$) was 1:5 (w/v). The residues were separately homogenized by species and type (muscle and intestine) for 5 minutes at a speed adjustment homogenizer at 10,000-12,000 rpm (4°C) (IKA RW 20D S32, China). The homogenate was then centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 12,000 x *g* for 30 min at 4°C. The final supernatant (defined as the crude extract) was stored at - 25°C for further characterization steps and determination of enzyme activity.

2.4 Activity of serine proteases and protein determination

Protease activity was measured using BApNA and Suc-Phe-p-Nan (dissolved in DMSO) as specific substrate for trypsin and chymotrypsin, respectively. The final concentration used was 8 mM. The substrate (30 µl) was incubated in microplate wells with the enzyme (30 µl) and 140 µl of 0.05 M Tris-HCl pH 7.4, containing 5 mM $CaCl_2$. The release of p-nitroaniline was measured as an increase in absorbance at 405 nm in a microplate reader. Controls were performed without enzyme. One unit (U) of enzyme activity is considered as the amount of enzyme able to produce 1 µmol of p-nitroaniline per minute (Souza et al., 2007). The specific activity, calculated as the ratio between the protease activity (U/mL) and the total protein in the sample (mg/mL^{-1}), was expressed in U/mg. The protein concentration of all tissue extracts was determined according to Smith et al. (1985).

2.5 Michaelis–Menten kinetic assay (K_m and $V_{máx}$)

The substrates used in the kinetic tests were BApNA (final concentrations varying from 0 to 4.5 mM) and Suc-Phe-p-Nan (final concentrations varying from 0 to 4.5 mM) dissolved in DMSO. The reaction was performed in triplicate in microplates and consisted of a mixture of 30 µL crude extact, with 140µL of 0.5 M Tris–HCl buffer, pH 7.4 and 30 µL of substrate. The release of the product (p-nitroaniline) was monitored by a Microplate reader at 405 nm and the enzymatic activity was calculated as described in *section 2.4*. The activity values (U) obtained for each substrate concentration were plotted on a Michaelis–Menten graph using the MicroCal™ Origin® Version 8.0 (MicroCal, Northampton, MA, USA) (Souza et al., 2007).

2.6 Physicochemical properties of serine proteases

2.6.1 Optimum temperature and thermal stability

The effect of temperature on the enzyme activity and stability was evaluated at temperatures ranging from 25 to 80°C. For optimal temperatures, the assays were carried out by incubating the crude extract with the substrates, 8 mM BApNA or Suc-Phe-p-Nan, in a water bath. To test thermal stability, the enzyme was incubated in a water bath for 30 min and the remaining activity was then measured at 25°C. The activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%) (Silva et al., 2011).

2.6.2 Optimum pH and stability

These experiments were carried out in different pH ranges using the buffers: 0.5 M citrate–phosphate (pH 4.0-7.0), 0.1 M Tris–HCl (pH 7.5-8.5) and 0.1 M glycine-NaOH (pH 9.0-12.0), containing 5 mM $CaCl_2$, using 8 mM BApNA and Suc-Phe-p-Nan as substrate,

as previously described. Optimum pH was determined by mixing 30 µl of the crude extract with 140 µl of buffer solutions, then adding 30 µl of substrate for 10 min at 25°C. The influence of pH on enzyme stability was determined by incubating the enzyme with various buffer solutions, at a ratio of 1:1 for 30 min at 25°C. Then, 30 µl aliquots were withdrawn and used to assess the activity of the enzyme at the optimum pH presented using 8 mM substrate. The highest enzymatic activity observed for the enzyme in different buffers was defined as 100%. The activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run and that at the beginning, and expressed as percentage (%) (Freitas Junior et al., 2012).

2.7 Effect of metal ions

Samples of crude extract (30µL) were added to a 96-well microtitre plate with 1 mM (30 µL) of the ions Mn^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+}, Mg^{4+}, Al^{3+}, Fe^{3+} and Pb^{2+}. Deionized water was used to prepare the solutions of all metals. After 30 min of incubation, 110 µL of 0.05 M Tris-HCl pH 7.4, containing 5 mM $CaCl_2$, and 30 µL of 8 mM BApNA or Suc-Phe-p-Nan were added (Freitas Junior et al., 2012).

2.8 Effect of Inhibitors

Crude extract (30µl) was incubated for 30 min at 25°C with 30µl of protease inhibitors (8 mM): phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor; N-*p*-tosyl-*L*-lysin chloromethyl ketone (TLCK), a trypsin-specific inhibitor; benzamidine, a trypsin inhibitor; *N*-tosyl-*L*-phenylalaninechloromethyl ketone (TPCK), a chymotrypsin-specific inhibitor, diluted in DMSO; ethylenediamine tetra-acetic acid (EDTA), a chelating compound; and β-mercaptoethanol, a reducing agent; diluted in deionized water. After

incubation, 8 mM BApNA or Suc-Phe-p-Nan was added and the release of p-nitroaniline was measured as the increase in absorbance at 405 nm. The enzyme and substrate blanks were similarly assayed without enzyme and substrate solution, respectively. The 100% activity values were those established in the absence of the inhibitors. The activity was determined as the percentage of the proteolytic activity in an inhibitor-free control sample (Freitas Junior et al., 2012).

2.9 Determination of collagenolytic properties

The collagenolytic properties in the crude extract of intestine and muscle were determined according to the methodology modified by Adigüzel et al. (2009), using azocoll as substrate. A reaction mixture, which contained 5 mg of azocoll, 500 µl of 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$ and 500 µl of crude extract, was typically incubated at 55°C for 30 minutes, under stirring. Thereafter, was added 200 µl of trichloroacetic acid (TCA) and incubated to stop the reaction. After 10 minutes, the samples were centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 10,000 x *g* for 10 minutes at 4°C. The sample reading was performed using a spectrophotometer at a wavelength of 595 nm. One enzyme unit was defined as the amount of enzyme required to increase the absorbance in 0.01 at 595 nm.

2.10 Statistical analysis

All values are presented as means ± standard deviations. These data were statistically analyzed by ANOVA, followed by a post hoc (Tukey) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($p < 0.05$).

3. Results

The determination of the dosage of total protein and enzyme activity for trypsin and chymotrypsin of three Neotropical species tested in crude extract from intestinal viscera are described in Table 1. The specific activity of trypsin from crude extracts of greater amberjack *S. dumerili* was higher in comparison to hake *C. leiarchus* and peacock bass *C. ocellaris*. The specific activity of chymotrypsin was lower than trypsin in the three species under study.

Kinetic parameters of BApNA and Suc-Phe-p-Nan hydrolysis were examined in the present study (Table 2). The Michaelis-Menten (K_m) constant for the crude extract of *S. dumerili*, *C. leiarchus* and *C. ocellaris* was 0.35, 0.12 and 0.35 mM for trypsin and 0.34, 0.34 and 0.51 mM for chymotrypsin, respectively. The V_{max} was found to be 391.07, 705.99 and 148.15 U/mg for trypsin, and 108.08, 118.25 and 104.54 U/mg for chymotrypsin, of *S. dumerili*, *C. leiarchus* and *C. ocellaris*, respectively.

The effect of temperature on the activity of trypsin is shown in Fig. 1A. The optimum temperature of trypsin obtained from extracts of greater amberjack *S. dumerili*, hake *C. leiarchus* and peacock bass *C. ocellaris* was 60°C. The sharp decrease in enzyme activity was observed at temperatures above 65°C in all three analyzed species. The activity became insignificant at temperatures of 75°C in *S. dumerili* and *C. leiarchus* and 80°C for *C. ocellaris*. The optimum temperature of chymotrypsin was 35°C for *C. ocellaris* and 40°C for the species *S. dumerili* and *C. leiarchus* (Fig. 1B). In this work, abrupt reduction in activity was observed from 40°C for the species *C. ocellaris*, reaching complete inhibition at 70°C. For *S. dumerili* and *C. leiarchus* reducing activity occurred after 45°C decreasing steadily until 70°C and reaching complete inhibition at 75°C. With respect to thermal stability, the three fish trypsin analyzed were sensitive to temperatures above 60°C (Fig.

1C), while chymotrypsin was sensitive to temperatures of 40°C for *S. dumerili* and *C. leiarchus* and 45°C for *C. ocellaris* (Fig. 1D).

The determination of the optimum pH of trypsin from the three species under study showed higher enzyme activity from the neutral to alkaline pH range (7.0 to 11.0) (Fig. 2A). The extracted enzymes, when subjected to varying conditions of pH reached recovery of activity at a broad range of pH (6.0 to 12.0) for the three species (Fig. 2C). Tests to define the optimum pH of chymotrypsin indicated peak activity in the range of 8.0 to *S. dumerili* and *C. leiarchus*, and 8.5 for *C. ocellaris*, being unstable at pH below 5.0 (Fig. 2B). The optimum pH for other fish chymotrypsins ranged between pH 7.5 and 9.0 (Fig. 2D).

The effect of metallic ions on the activity of enzymes was evaluated and is presented in Table 3. The trypsin activity in the three species increased in relation to control (100%) when it was incubated in the presence of Mn^{2+}: 100.98%, 100.39% and 100.36% for *S. dumerili, C. leiarchus* and *C. ocellaris*, respectively, although there was no significant difference detected between the data (p <0.05). In this work, the protease in question was inhibited by the following ions, in decreasing order: *S. dumerili* - $Fe^{3+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Hg^{2+} > Pb^{2+} > Mg^{4+} > Al^{3+} > Mn^{2+}$, *C. leiarchus* - $Fe^{3+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Hg^{2+} > Pb^{2+} > Mg^{4+} > Al^{3+} > Mn^{2+}$, and finally, *C. ocellaris* - $Fe^{3+} > Pb^{2+} > Cu^{2+} > Cd^{2+} > Hg^{2+} > Zn^{2+} > Al^{3+} > Mg^{4+} > Mn^{2+}$. Chymotrypsin of the tested species proved to be more sensitive to metal ions than trypsin. The enzyme was inhibited by the following ions, in decreasing order: *S. dumerili* - $Hg^{2+} > Fe^{3+} > Zn^{2+} > Cd^{2+} > Mn^{2+} > Cu^{2+} > Al^{3+} > Pb^{2+} > Mg^{4+}$, *C. leiarchus*- $Hg^{2+} > Fe^{3+} > Zn^{2+} > Cd^{2+} > Mn_{2+} > Cu^{2+} > Al^{3+} > Pb^{2+} > Mg^{4+}$, and finally, *C. ocellaris* - $Pb^{2+} > Hg^{2+} > Fe^{3+} > Cu^{2+} > Cd^{2+} > Al^{3+} > Zn^{2+} > Mn^{2+} > Mg^{4+}$.

The trypsin inhibitors (TLCK and benzamidine) were those that brought a greater reduction of activity when using the specific substrate for trypsin of the three Neotropical species (Table 3).

315 The collagenolytic specific activity of freshwater species *C. ocellaris* (106.82 ±
316 0.0902 U/mg) was higher than in marine species *S. dumerili* (42.44 ± 0.0029 U/mg) and *C.*
317 *leiarchus* (98.08 ± 0.1519 U/mg). The *C. ocellaris* activity underwent the greater influence
318 by the inhibitors of serine proteases (PMSF) and metalloproteases (EDTA), 23.29 ±
319 0.00343 and 18.53 ± 0.00489 U/mg, respectively, when compared to *S. dumerili* (14.09 ±
320 0.02089 and 14.94 ± 0.00565 U/mg) and *C. leiarchus* (37.45 ± 0.00154 and 15.55 ±
321 0.00213 U/mg) (Fig. 3).
322
323 **4. Discussion**
324 The results for the trypsin from crude extract of the neotropical species tested were
325 higher than trypsin from crude extract of the species Japanese sea bass *Lateolabrax*
326 *japonicus* (0.30 U/mg) (Cai et al., 2011), zebra blenny *Salaria basilisca* (0.12 U/mg) (Ktari
327 et al., 2012), pirarucu *A. gigas* (0.37 U/mg) (Freitas Junior et al., 2012) and tropical gar *A.*
328 *tropicus* (0.000006 U/mg) (Guerrero-Zárate et al., 2014). Values higher than those
329 reported for *E. japonica* (0.05 U/mg) (Heu et al., 1995) and *A. tropicus* (0.0012 U/mg)
330 (Guerrero-Zárate et al., 2014) when compared with *C. leiarchus*, *S. dumerili* and *C.*
331 *ocellaris*, the present work. Cuenca-Soria et al. (2014) of 0.52 and 0.8 (U/mg) of the
332 Mayan cichlid *Cichlasoma urophthalmus*. Zhou et al. (2011) reported that chymotrypsin of
333 marine fish acclimated to cold regions have higher catalytic activities. Falcón-Hidalgo et al.
334 (2011) report for cuban limia *Limia vittata* and cuban gambusia *Gambusia punctate*
335 increased activity of trypsin and chymotrypsin during the development of the species.
336 Fish digestive enzymes, such as trypsin, for example, tend to have a higher binding
337 affinity for the specific substrate (lower Km) than bovine trypsin, for example, due to
338 differences in the region of substrate breakdown (Bougatef, 2013). The Km is used to
339 assess the affinity of the enzyme tested for the substrate and the results showed similar

values to that of alkaline trypsin from bigeye snapper *Pricanthus macracanthus* (Hau and Benjakul, 2006), mojarra *D. rhombeus* (Silva et al., 2011), pirarucu *A. gigas* (Freitas Junior et al., 2012) and crevalle jack *C. hippos* (Costa et al., 2013); while chymotrypsin species diverged from that reported by Castillo-Yañez et al. (2009).

The effect of temperature on the activity of trypsin in this work was similar to that of Chinook salmon *Oncorhynchus tshawytscha* (Kurtovic et al., 2006), Brownstripe red snapper *Lutjanus vitta* (Khantaphant and Benjakul, 2010) and Zebra blenny *Salaria basilisca* (Ktari et al., 2012). The loss of activity presumably was due to heat treatment. Another similar results are reported to Jacopever *Sebastes schlegelii* (Kishimura et al., 2007) and pirarucu *A. gigas* (Freitas Junior et al., 2012), both with optimal temperature at 65°C. Similar results for optimum temperature of chymotrypsin were found for rainbow trout *Oncorhynchus mykiss* (Kristjánsson and Nielsen, 1992), anchovy *E. japonica* (Heu et al., 1995) and Monterey sardine *Sardinops sagax caerulea* (Castillo-Yañez et al., 2009). Guerrero-Zárate et al. (2014) reported optimum temperature of 60°C for alkaline proteases (trypsin and chymotrypsin) in juveniles of tropical gar *A. tropicus*. Bougatef (2013) and Zhou et al. (2011) have reported that the temperature has a significant effect on the activity and stability of trypsin and chymotrypsin since the folding is lost during heating and one of the reasons why chymotrypsin is more sensitive to temperature is precisely the fact that chymotrypsin present less disulfide bonds compared with trypsin (Roach et al., 1997; Várallyay et al., 1997).

Optimum pH of trypsin from the tested species is consistent with that reported by Hau and Benjakul (2006) for the Bigeye snaper *Priacanthus macracanthus*, indicating an optimum pH range between 8.0 and 11. In this study, peak activity was found in the pH of 8.5, 9.0 and 10.0, respectively, for *C. ocellaris*, *S. dumerili* and *C. leiarchus*. Similar reports have been described for enzymatic crude extracts from Asian bony tongue *Scleropages*

formosus (Natalia et al., 2004), sardine *Sardina pilchardus* (Bougatef et al., 2007), Striped seabream *Lithognathus mormyrus* (Elhadj-Ali et al., 2009), Smooth hound *Mustelus mustelus* (Bougatef et al., 2010), Brownstripe red snapper *Lutjanus vitta* (Khantaphant and Benjakul, 2010), Amazonian fish tambaqui *Colossoma macropomum* (Marcuschi et al., 2010), Japanese sea bass *L. japonicas* (Cai et al., 2011) and pirarucu *A. gigas* (Freitas Junior et al., 2012) which showed optimum activity in the range from 8.5 to 10.0. These results are common for the activity of digestive enzymes of fish (Castillo-Yáñes et al., 2005).

Trypsin stability to pH variation was similar to the findings reported by Silva et al. (2011) for Silver mojarra *D. rhombeus* remaining stable in a range of alkaline pH (8.5-11.0) and showing unstable range below 8.5 and negligible activity at 4.5 while Cai et al. (2011) reported for Japanese sea bass *L. japonicus* recovery of activity being detected in the pH range from 7.0 to 11.0. Optimum pH of chymotrypsin was similar to the reports for Monterey sardine *S. sagax caeruleus* (Castillo-Yáñez et al., 2009), showing a relatively high activity in the interval of pH 8.0-10.0. Here, the optimum pH range (7.5-9.0) found for chymotrypsin indicated denaturation when subjected to extensive acidic range (below 5.0) or for long time at high alkaline ranges (12.0).

In the stability test, chymotrypsin was more sensitive to changes in pH than trypsin in the species *S. dumerili* and *C. leiarchus* (6.5 to 10.5), with a small range of recovery of activity when compared to *C. ocellaris* (6.0 to 12.0). Changes in pH of the medium will affect both substrate and enzyme, changing the charge distribution and conformation of molecules (Klomklao et al., 2006). Most enzymes undergo irreversible denaturation in a very acid or alkaline solution, resulting in the loss of activity (Silva et al., 2011).

The results obtained for optimal temperature and pH and for activity recovery when subjected to both heat stress and fluctuations of pH for both enzymes (trypsin and

chymotrypsin) are in accordance with parameters considered by the industry in various sectors, since it has been demonstrated that alkaline proteases derived from the waste of fish processing have biotechnological potential in the detergent industry (Esposito et al., 2009), biomedical and food applications (Zhou et al., 2011), by bearing the most stressful situations of physicochemical exposure (Bezerra et al., 2006).

The results of metallic ions for trypsin are in accordance with those obtained by Grey triggerfish *Balistes capriscus* (Jellouli et al., 2009), Striped seabream *L. mormyrus* (Elhadj-Ali et al., 2009) and Zebra blenny *Salaria basilisca* (Ktari et al., 2012). Costa et al. (2013) observed for crevalle jack *C. hippos* inhibition after incubation with Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Hg^{2+} at 1 mM, revealing high sensitivity of the enzyme to metal ions. Silva et al. (2011) reported reduction in trypsin activity of Silver mojarra *D. rhombeus* when it was incubated in the presence of Fe^{2+}, Cd^{2+}, Cu^{2+} and Al^{3+} in a proportion of 20 to 35%, while ions Hg^{2+} and Zn^{2+} inhibited about 53.11% and 71.23%, respectively. According to these authors, the Pb^{2+} ions completely inhibited the enzymatic action. Bougatef et al. (2007) reported for trypsin from the viscera of sardine *S. pilchardus* strong inhibition by Zn^{2+} and Cu^{2+}. Souza et al. (2007) reported for spotted goatfish *P. maculatus* the effects of Al^{3+}, Zn^{2+} and Hg^{2+} on the enzyme from the pyloric caeca (respectively, 2.51%, 25.06% and 29.12%) and intestine (respectively, 10.18%, 19.26% and 29.51%). The chymotrypsin was susceptible to various metal ions, such as Mg^{2+} and inactivated by the ions Fe^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+} as reported by Yang et al. (2009) for crucian carp *C. auratus*. In the present work, the ion Hg^{2+} completely inhibited the activity of chymotrypsin in the species *S. dumerili* and *C. leiarchus.*

The results of inhibition by benzamidine and TLCK are similar to those reported by Bezerra et al. (2005), Souza et al. (2007), Bougatef et al. (2010), Silva et al. (2011), Freitas Junior et al. (2012) and Costa et al. (2013). TLCK is a specific inhibitor of trypsin

and inactivate enzymes with similar activity. This inhibition occurs by a covalent bond with the histidine residue in the catalytic triad of the active site, thus blocking the binding of substrate to the enzyme (Jeong et al. 2000).

When subjected to a potent serine protease inhibitor (PMSF), were detected activities trypsin of 62.18 %, 71.06 % and 71.0 %, respectively, for *S. dumerili*, *C. ocellaris* and *C. leiarchus*. Natalia et al. (2004) reported that in the intestine of Asian bony tongue *S. formosus*, PMSF caused residual activity of 22.9%, a reduction of more than 50% of its activity. Bezerra et al. (2005) reported that trypsin of Nile tilapia *O. niloticus* was inhibited (approximately 55%) by PMSF. Cuenca-Soria et al. (2014) reported a high degree of inhibition in alkaline proteases (trypsin and chymotrypsin) from extracts of *C. urophthalmus* by specific inhibitors such as TPCK, TLCK, and EDTA (greater than 80%) and PMSF (60%). When the use of specific inhibitor of chymotrypsin (TPCK) was detected residual activity of 54.03% for the species *S. dumerili*, of 93.96% for *C. leiarchus* and 92.13% for *C. ocellaris*. Chymotrypsin has sensitivity to certain natural and synthetic specific inhibitors, which may result in partial decrease or no enzymatic activity. In this study, little chymotrypsin activity was influenced to when using their specific inhibitors, when compared with the other inhibitors tested, especially when subjected to trypsin inhibitors, TLCK and benzamidine.

The use of metalloprotease inhibitor reduced almost by half the enzymatic activity of this protease in the three species tested. According to the results of collagenolytic activity (Fig. 3), *S. dumerili* was more susceptible to PMSF and EDTA than *C. ocellaris*, and *C. leiarchus* was more susceptible to PMSF than *C. ocellaris*, suggesting a strong indication of the presence of serine proteases and metalloproteinases with collagenolytic properties in crude extracts of the three species, especially in *C. ocellaris*. The *C. ocellaris* has primarily piscivorous diet, occupying the upper levels of the food chain of rivers, lakes and

reservoirs. Due to the type of diet, its biochemical metabolism is geared for digestion of viscera from several prey species, including their own species (cannibalism) (Gomiero and Braga, 2006). This fact may have influenced the high ability to hydrolyze the substrate, providing high collagenolytic property to the material, due to the need for collagen hydrolysis of both skin and scales of their own species.

The high collagenolytic property was also reported for intestinal crude extract of filefish *N. modestrus* (114.15 U/mg) (Kim et al. 2002) and to a lesser extent for tuna *Thunnus thymus* (16.5 U/mg) (Byun et al., 2002) and for Mackerel *S. japonicus* (16.5 U/mg) (Park et al., 2002). Teruel and Simpson (1995) also found low collagenolytic activity (3.82 U/mg) in the crude extract of muscle winter flounder *P. americanus*, as well as Daboor et al. (2012) using a mixture of haddock, herring, flounder and ground fish (11.63 U/mg). Souchet and Laplante (2011) also detected the presence of collagenolytic activity (13.3 U/mg) in byproducts of Snow Crab *Chionoecetes opilio*. Unpublished results by our research group, as seen in Figure 4, also showed lower collagenolytic activity in crude extracts of other neotropical freshwater fish, such as the jaguar cichlid *Parachromis managuensis*, cobia *Rachycentron canadum*, tambaqui *C. macropomum*, Nile tilapia *O. niloticus*, catfish *Pseudoplatystoma corruscans*, lane snapper *Lutjanus synagris*, mackerel *Scomberomorus mackerel* compared to the peacock bass *C. ocellaris*, hake *C. leiarchus* and greater amberjack *Seriola dumerili*. Currently, much of the collagenase used in the market is of microbial origin (*Clostridium histolyticum*).

Wastes from the processing of fish, such as intestinal guts and leftover carcasses, are potential sources of enzymes and often still presenting high catalytic ability, at relatively low concentrations (Kim and Mends, 2006). Digestive enzymes of fish can be useful tools in industry, due to its remarkable properties: high optimum temperature and thermal resistance (45°C to 65ºC); optimum pH in alkaline medium, with high activity in a

broad pH range (7.0 -10.0). Some properties confer wide applicability to these proteins (Bezerra et al. 2006), such as the characteristics found in the tested enzymes. Therefore, the results of this study suggest the digestive guts of *S. dumerili*, C. *leiarchus* and *C. ocellaris* as potential sources of protein biomolecules for industrial and biotechnological processes and exploration of collagenolytic properties from extracts of *C. leiarchus* and *C. ocellaris* as bioactive molecules, aiming the production of collagen peptides for pharmaceutical and nutritional purposes.

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Figure Legends

Fig. 1. Effect of temperature and thermal stability on the activity of serine proteases extracted from greater amberjack (*Seriola dumerili*), hake (*Cynoscion leiarchus*) and peacock bass (*Cichla ocellaris*). (A) Optimum temperature for trypsin activity in a range of 25-80°C. (B) Optimum temperature for chymotrypsin activity in a range of 25-80°C. (C) Thermal stability of trypsin after 30 minutes incubation at a temperature in the range of 25-80°C. (D) Thermal stability of chymotrypsin after 30 minutes of incubation in the temperature range of 25-80°C.

Fig. 2. Effect of pH and stability at different pH on the activity of serine proteases extracted from greater amberjack (*Seriola dumerili*), hake (*Cynoscion leiarchus*) and peacock bass (*Cichla ocellaris*). (A) Optimal pH for the activity of trypsin, using different buffers in the pH range from 4.5 to 12.0, expressed as percentage of the maximum one obtained in 0.05 M Tris-HCl buffer. (B) The optimum pH for the activity of chymotrypsin using different buffers in the pH range from 4.5 to 12.0, expressed as percentage of the maximum one obtained in 0.05 M Tris-HCl buffer. (C) pH stability of trypsin after incubation for 30 minutes in the pH range 4.0 to 12.0. (D) pH stability of chymotrypsin after incubation for 30 minutes in the pH range 4.0 to 12.0.

Fig. 3. Collagenolytic activity of digestive proteases in intestine of greater amberjack (*Seriola dumerili*), hake (*Cynoscion leiarchus*) and peacock bass (*Cichla ocellaris*). Ethylenediamine tetraacetic acid (EDTA); Phenylmethylsulphonyl fluoride (PMSF).

Fig. 4. Intestinal collagenase activity in crude extracts Neotropical species and hydrolyzed shrimp. On results obtained in our laboratory. Protein hydrolyzate - from waste shrimp *Litopenaeus vannamei*. Species fish tested: jaguar cichlid (*Parachromis managuensis*, habitat: freshwater, feeding habits: carnivorous); cobia (*Rachycentron canadum*, marine, carnivorous); tambaqui (*Colossoma macropomum*, freshwater, herbivorous); Nile tilapia (*Oreochromis niloticus*, water sweet, omnivorous); surubim (*Pseudoplatystoma corruscans*, freshwater, carnivorous); lane snapper (*Lutjanus synagris*, marine, carnivorous), mackerel (*Scomberomorus cavala*, marine, carnivorous); peacock bass (*Cichla ocellaris*, freshwater, carnivorous); hake (*Cynoscion leiarchus*, marine, carnivorous); greater amberjack (*Seriola dumerili*, marine, carnivorous). Main fish feeding carnivorous cited: fish and shellfish. Activity measured at 55°C under agitation using azocoll as substrate for a period of 30 minutes, reading absorbance reading 595.

Figure 1

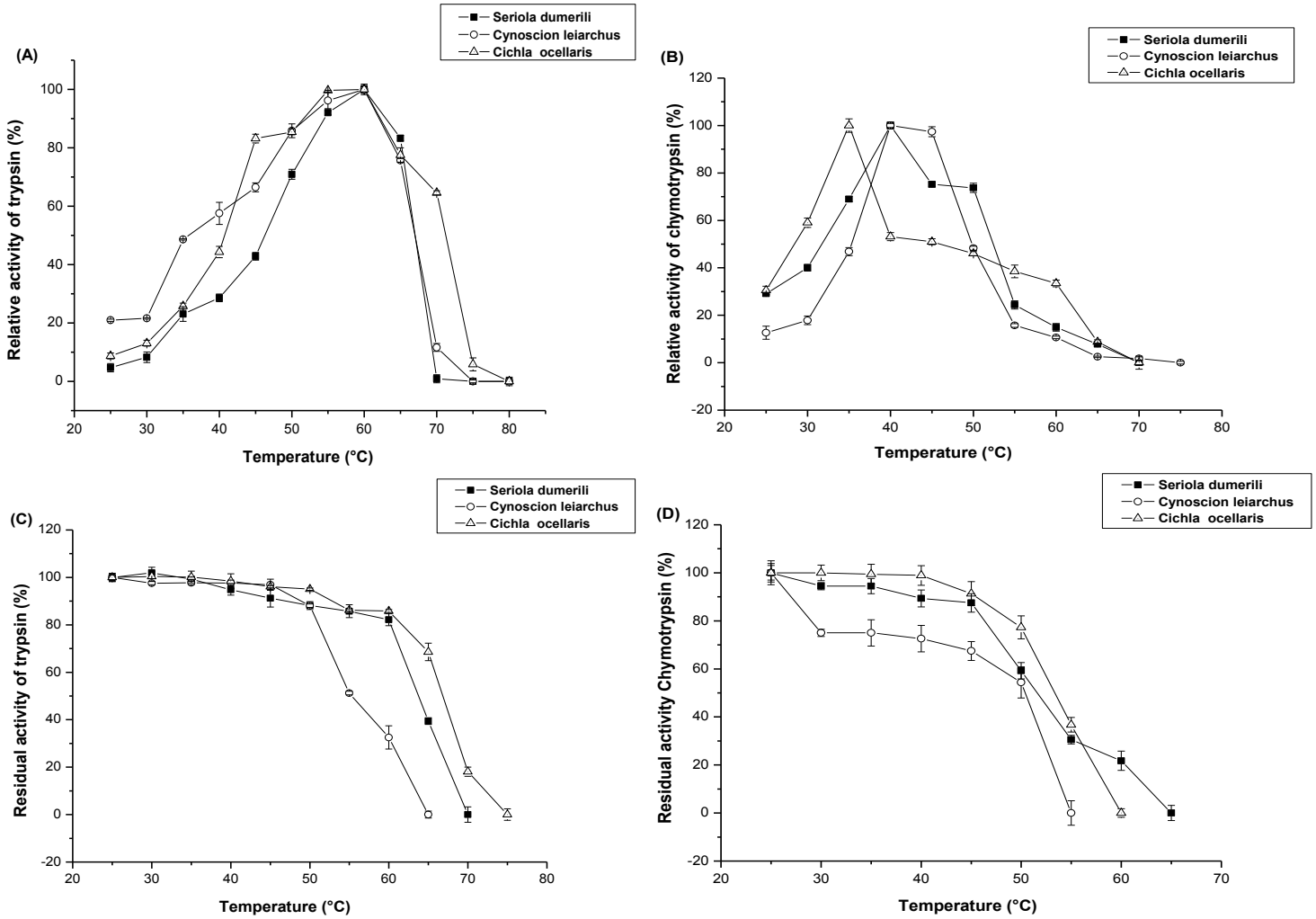

(A)
Seriola dumerili
Cynoscion leiarchus
Cichla ocellaris
Relative activity of trypsin (%)
Temperature (°C)
(B)
Seriola dumerili
Cynoscion leiarchus
Cichla ocellaris
Relative activity of chymotrypsin (%)
Temperature (°C)
(C)
Seriola dumerili
Cynoscion leiarchus
Cichla ocellaris
Residual activity of trypsin (%)
Temperature (°C)
(D)
Seriola dumerili
Cynoscion leiarchus
Cichla ocellaris
Residual activity Chymotrypsin (%)
Temperature (°C)

Figure 2

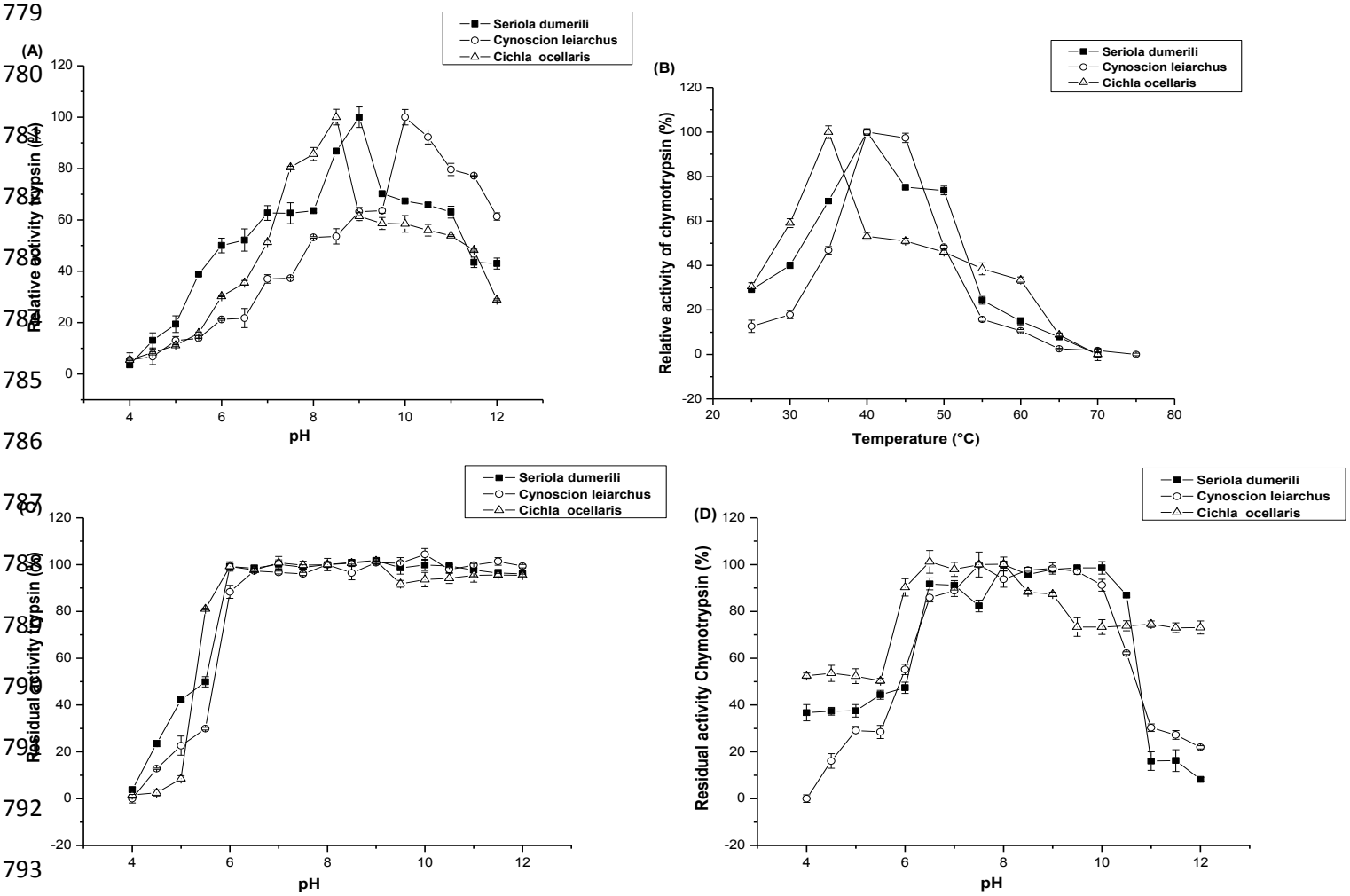
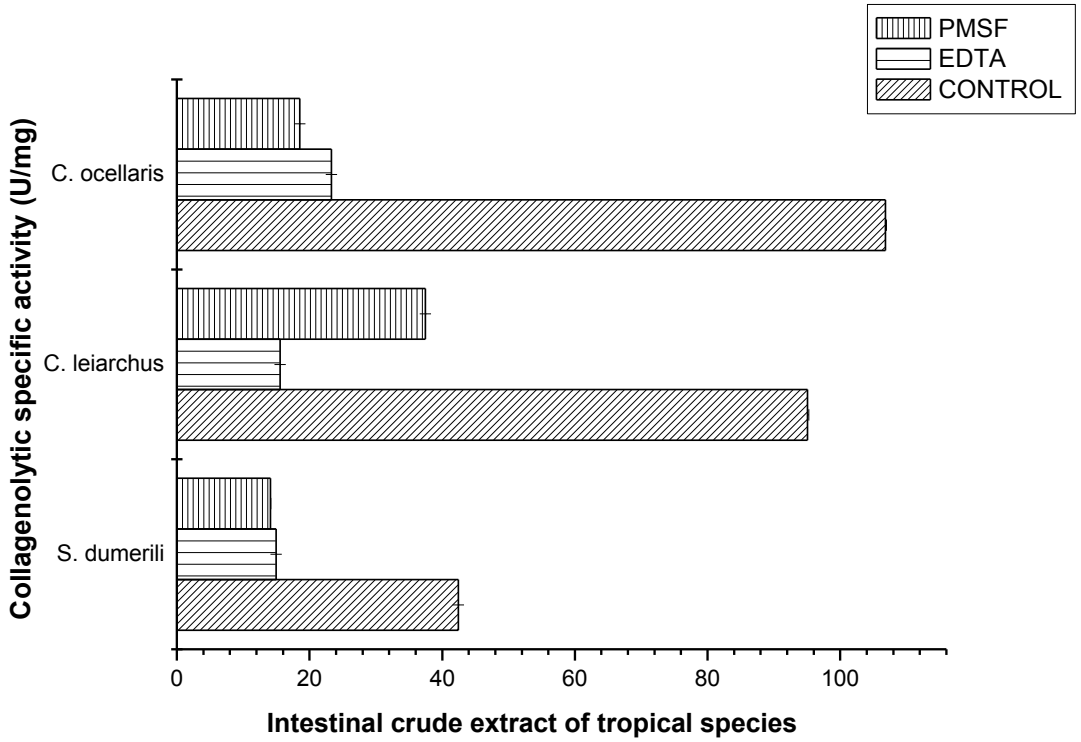

(A)
Seriola dumerili
Cynoscion leiarchus
Cichla ocellaris
Relative activity trypsin (%)
pH
(B)
Seriola dumerili
Cynoscion leiarchus
Cichla ocellaris
Relative activity of chymotrypsin (%)
Temperature (°C)
(C)
Seriola dumerili
Cynoscion leiarchus
Cichla ocellaris
Residual activity trypsin (%)
pH
(D)
Seriola dumerili
Cynoscion leiarchus
Cichla ocellaris
Residual activity Chymotrypsin (%)
pH

Figure 3


PMSF
EDTA
CONTROL
C. ocellaris
C. leiarchus
S. dumerili
Collagenolytic specific activity (U/mg)
0
20
40
60
80
100
Intestinal crude extract of tropical species

820 **Figure 4**
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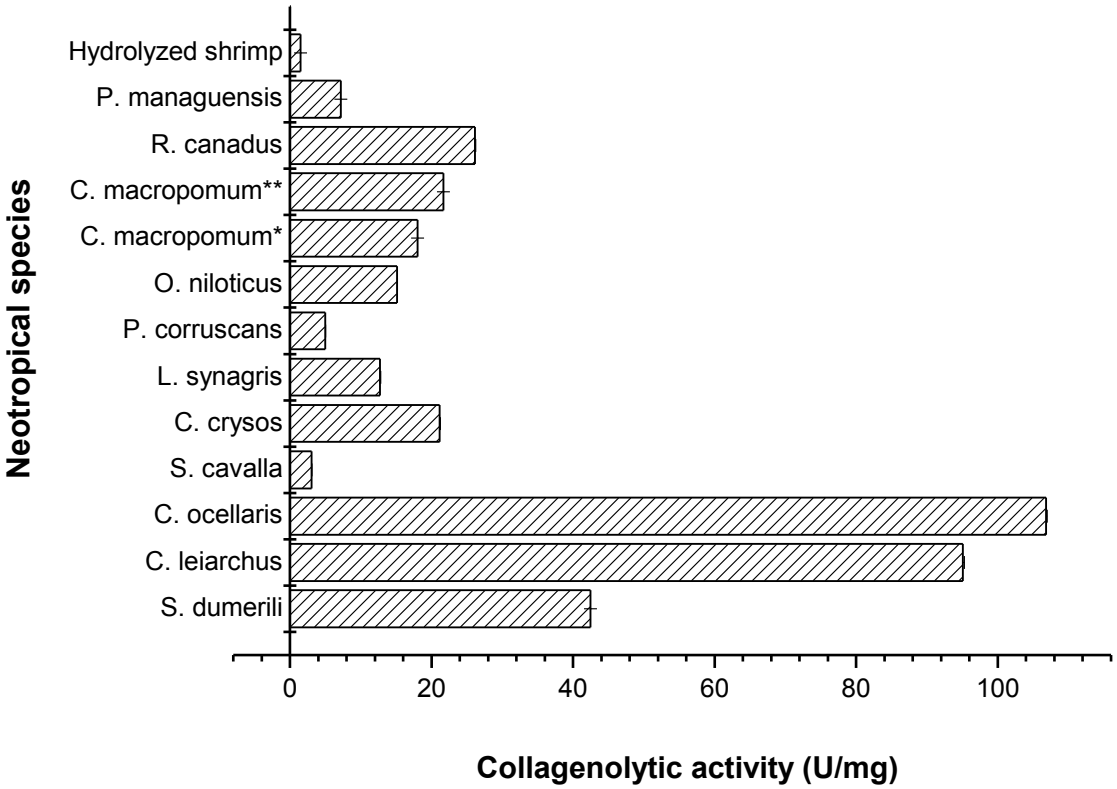

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Tables

Table 1. Characterization of digestive enzymes from intestinal waste of Neotropical species of fish (crude extract).

Species*	Enzyme	Total protein (mg/ml)	Enzyme activity (U)	Enzyme activity (U/mg protein^{-1})
	Trypsin			
S. dumerili		3.29 ± 0.01	4.82 ± 0.004	1.46 ± 0.0011
C. leiarchus		12.99 ± 0.10	6.92 ± 0.002	0.53 ± 0.0001
C. ocellaris		7.15 ± 0.004	8.04 ± 0.008	1.12 ± 0.001
	Chymotrypsin			
S. dumerili		3.29 ± 0.01	2.33 ± 0.003	0.71 ± 0.00079
C. leiarchus		12.99 ± 0.10	2.48 ± 0.002	0.19 ± 0.00013
C. ocellaris		7.15 ± 0.004	2.48 ± 0.002	0.35 ± 0.00024

*Common name/scientific name: greater amberjack *Seriola dumerili*, hake *Cynoscion leiarchus*, peacock bass *Cichla ocellaris*.

879 **Table 2.** Michaelis-Menten constant (K_m e $V_{máx}$) of serine proteases from Neotropical species
880 compared to other species.

Species	K_m (mM)		$V_{máx}$ (U/mg)		References
	BApNA	*Suc-Phe-p-Nan*	*BApNA*	*Suc-Phe-p-Nan*	
S. dumerili	0.35 ± 0 .09	0.34 ± 0.04	391.07 ± 12.80	108.08 ± 2.56	Present work
C. leiarchus	0.12 ± 0.03	0.51 ± 0.06	705.99 ± 20.90	118.25 ± 3.37	Present work
C. ocellaris	0.35 ± 0.14	0.34 ± 0.08	148.15 ± 7.50	104.54 ± 4.66	Present work
O. mykiss	-	0.035	-	-	Kristjansson and Nielson (1992)
S. sagax caerulea	-	0.074	-	-	Castillo-Yáñez et al. (2006)
P. macracanthus	0.312	-	-	-	Hau and Benjakul (2006)
*P. maculatus**	1.82 ± 0.19	-	-	-	Souza et al. (2007)
*P. maculatus***	1.94 ± 0.45	-	-	-	Souza et al. (2007)
S. sagax caeruleus	-	0.048	-	-	Castillo-Yáñez et al. (2009)
Bovine chymotrypsin	-	0.08	-	-	Yang et al. (2009)
Carassius auratus[a]	-	1.4	-	-	Yang et al. (2009)
Carassius auratus[b]	-	0.5	-	-	Yang et al. (2009)
M. mustelus	0.387 ± 0.02	-	-	-	Bougatef et al. (2010)
Suine trypsin	0.82	-	-	-	Freitas Junior et al. (2012)
C. hippos	0.69	-	-	-	Costa et al. (2013)

881 (−): data not reported. *Extracted from Intestine. **Extracted from pyloric caeca. BApNA: Nα-benzoyl-DL-
882 arginine-p-nitroanilide. Suc-Phe-p-Nan: Succinyl-DL-phenylalanine-p-nitroanilide. [a]Chymotrypsin A.
883 [b]Chymotrypsin B. Fish species: Common name/scientific name: greater amberjack (*Seriola dumerili*), hake
884 (*Cynoscion leiarchus*), peacock bass (*Cichla ocellaris*), pirarucu (*Arapaima gigas*), Monterey sardine
885 (*Sardinops sagax caerulea*), bigeye snapper (*Pricanthus macracanthus*), rainbow trout (*Oncorhychus*
886 *mykiss*), Nile tilapia (*Oreochromis niloticus*), crucian carp (*Carassius auratus*), spotted goatfish
887 (*Pseudupeneus maculatus*), Japanese sea bass (*Lateolabrax japonicus*), smooth hound (*Mustelus mustelus*)
888 and crevalle jack (*Caranx hippos*).
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Table 3. Effect of ions and inhibitors on the residual activity (%) of trypsin and chymotrypsin from Neotropical fish species.

Íons and Inhibitors	***S. dumerili***		***C. ocellaris***		***C. leiarchus***	
	BApNA	*Suc-Phe-p-Nan*	*BApNA*	*Suc-Phe-p-Nan*	*BApNA*	*Suc-Phe-p-Nan*
Metal íons (1 mM)*						
Mn^{2+}	100.98^{a}	21.49^{b}	100.39^{a}	65.70^{b}	100.86^{a}	80.86^{a}
Zn^{2+}	55.20^{b}	17.05^{b}	85.25^{a}	52.42^{b}	94.74^{a}	39.29^{b}
Fe^{3+}	41.71^{b}	2.14^{b}	58.71^{b}	33.76^{b}	30.20^{b}	35.09^{b}
Cu^{2+}	55.14^{a}	22.39^{b}	73.77^{a}	39.68^{b}	61.91^{b}	7.57^{b}
Hg^{2+}	65.61^{b}	0.0^{b}	82.86^{a}	27.76^{b}	79.98^{a}	0.0^{b}
Mg^{4+}	78.91^{a}	100.62^{a}	96.95^{a}	106.52^{a}	99.77^{a}	105.37^{a}
Cd^{2+}	59.20^{b}	19.41^{b}	81.45^{a}	41.19^{b}	62.30^{b}	3.17^{b}
Al^{3+}	79.69^{b}	25.45^{b}	85.29^{a}	43.55^{b}	60.37^{b}	63.11^{b}
Pb^{2+}	69.74^{b}	52.14^{b}	67.68^{b}	22.15^{b}	54.89^{b}	36.97^{b}
Inhibitor (8 mM)*						
PMSF	62.18^{b,ab}	68.04^{a,a}	71.00^{b,a}	75.61^{b,a}	72.06^{a,a}	70.84^{a,a}
TPCK	54.03^{b,a}	87.56^{a,a}	92.13^{a,b}	98.58^{a,b}	93.96^{a,a}	82.23^{a,a}
TLCK	26.71^{b,c}	26.28^{b,b}	18.18^{b,c}	9.34^{b,c}	8.90^{b,b}	15.90^{b,b}
Benzamidine	19.11^{b,c}	48.30^{b,b}	13.34^{b,c}	10.12^{b,c}	9.64^{b,bc}	16.18^{b,b}
β-Mercaptoethanol	43.40^{b,c}	66.84^{b,ab}	40.95^{b,d}	31.36^{b,d}	50.13^{b,b}	33.81^{b,c}
EDTA	45.02^{b,bc}	67.82^{b,ab}	42.99^{b,e}	57.87^{b,e}	55.16^{b,b}	37.60^{b,c}

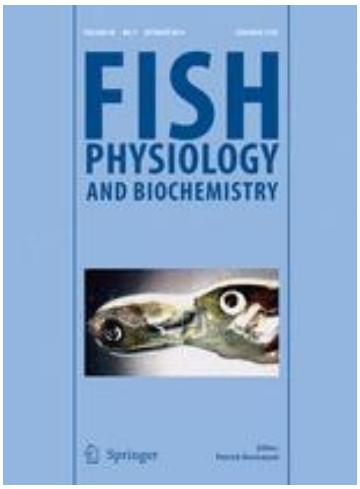
* In the controls, determinations were performed without ions or inhibitors. In the inhibitor values, the first superscript letter represents comparison with control group, that was considered as 100% (a). The second superscript letter is related to comparison between inhibitors. The initial concentration used for the assay with inhibitors was 8 mM. The final concentration used the assay with ions was 1 mM. Phenylmethylsulphonyl fluoride (PMSF); N-p-tosyl-L-lysin chloromethyl ketone (TLCK); *N-tosil-L-phenylalanine* chloromethyl ketone (TPCK); Ethylenediamine tetraacetic acid (EDTA). Different superscript letters represent statistical differences ($p < 0.05$).

6. ARTIGO II: I: Extraction and partial characterization of collagenolytic serine protease of hake *Cynoscion leiarchus* and specificity test on skin collagen from Neotropical fish species.

VAGNE DE MELO OLIVEIRA, DOUGLAS HENRIQUE DE HOLANDA ANDRADE, HELANE MARIA SILVA DA COSTA, CAIO RODRIGO DIAS ASSIS, CAROLINA DE ALBUQUERQUE LIMA, RANILSON DE SOUZA BEZERRA, ANA LÚCIA FIGUEIREDO PORTO

Este artigo será submetido para a revista:

Fish Physiology and Biochemistry



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Extraction and partial characterization of collagenolytic serine protease of hake *Cynoscion leiarchus* and specificity test on skin collagen from Neotropical fish species.

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Abstract

Enzymes with collagenolytic properties was extracted and physic-chemically characterized from digestive viscera of *C. leiarchus*. The optimum temperature of enzyme was 55°C and its activity showed thermostability between 25 - 60°C. The enzyme had optimal activity at pH 8.0 and remained stable between 6.5 and 11.5. The protease was inhibited by the metal ions, in decreasing order: Pb^{2+}> Al^{3+} > Cu^{2+}> Hg^{2+} > Cd^{2+} > Ba^{2+} > K^{+} > Zn^{2+} > Na^{+}. Ions Ca^{2+} and Mg^{2+} increased the activity; while were inhibited by order of activity, by Benzamidine > TLCK > EDTA > β-Mercaptoethanol > PMSF > TPCK. After 48 hours of incubation, enzyme was effective in hydrolysis the following types of collagen: *P. corruscans* skin collagen > *O. niloticus* skin collagen > bovine collagen type I. The characteristics displayed by the collagenolytic serine protease of hake *C. leiarchus* in this work are interesting for biotechnological applications, suggesting their employment in production of collagen peptides on an industrial scale, through their use in the softening of meat and fish processing, adding value to the fishery product and minimizing the environmental impact of disposal of these wastes.

KEYWORDS: By-products, collagenolytic protease, *Cynoscion leiarchus*, enzyme characterization, wastes.

Introduction

The collagenase (EC 3.4.24.7) is a group of enzymes that hydrolyze the peptide bonds of various types of collagen, the most abundant protein in mammals, under physiological conditions of pH and temperature both in *vivo* and in *vitro* (Teruel 1997; Daboor et al. 2010; Tallant et al. 2010; Hayet et al. 2011; Souchet and Laplante 2011). They are highly specific for native or denatured collagen, showing no activity to any other protein. If the molecule is denatured collagen, it becomes susceptible to hydrolysis by other proteases (Teruel 1997).

Generally, the collagenases are subdivided into two well defined groups, metalocollagenases, zinc-dependent endopeptidases that degrade and the helical part of collagen, commonly recovered from animal tissues and by-products from fish processing, such as bones, fins, skins and hepatopancreas of marine crabs (Bracho and Haard 1995; Roy et al. 1996; Brinckerhoff and Matrisian 2002; Daboor et al. 2012); and serinocollagenases, endo or exopeptidases, calcium-dependent enzymes containing a serine residue in its catalytic group and that have the property of decomposing the non-helical part of the collagen being often associated with the digestive organs, particularly the intestines and caeca (Kim et al. 2002; Park et al. 2002).

Collagenases have been widely used in human and veterinary medicine for the purpose of clear necrotic wounds, bedsores, scars and postoperative wounds, and psoriasis and in the pediculosis treatment , implants and bone loss, injuries in nursing women nipples, in the treatment of hypertrophic scars in the treatment of ischemic heart debridement of ulcers and diabetic individuals (Takahashi et al. 1999; Özcan et al. 2002; Watanabe 2004; Arakawa et al. 2012; Tallis et al. 2013). Another frequent medical application is the use of the enzyme produced by *C. histolyticum* in the treatment of Peyronie's disease (Levine 2013; Langston and Carson 2014) and Dupuytren (Martin-

1028 Ferrero et al. 2013; Peimer et al. 2013; Hentz 2014; Leclère et al. 2014; Meals and Hentz
1029 2014). In the textile industry, have been applied in the treatment of leather and fabric
1030 dyeing, by operating as a non-toxic biocatalyst, improving dyeing characteristics, and
1031 become an ecologically viable process (Kanth et al. 2008). In the food, they are added in
1032 steps of product processing, such as in the preparation of meat and softening processes
1033 (Foegeding and Larick 1986; Kim et al. 1993).

1034 Enzymes with collagenolytic properties can be extracted from microbial sources
1035 (Rosso et al. 2012; Lima et al. 2013), vegetables (Kim et al. 2007), and animals (Wu et al.
1036 2010a; Daboor et al. 2012). Today, the main sources produced and available in the market
1037 are of microbial origin (Duarte et al. 2014) as collagenase from *Clostridium histolyticum*
1038 (Teruel and Simpson 1995; Kim et al. 2007). Other microbial sources have been reported,
1039 such as those derived from *Candida albicans* (Lima et al. 2009), *Penicillium*
1040 *aurantiogriseum* (Rosso et al. 2012; Lima et al. 2013), *Bacillus pumilus* (Wu et al. 2010b),
1041 *Bacillus cereus* (Liu et al. 2010) and *Bacillus licheniformis* (Baehaki et al. 2012). However,
1042 species of microorganisms that produce collagenases belong to a genus that contains
1043 pathogenic strains (Duarte et al. 2014). Thus, collagenase food sources, for example,
1044 derived from fish, shellfish, shrimp, among others, may become a potential alternative to
1045 replace microbial collagenases (Kim et al. 1993; Roy et al. 1996; Teruel 1997; Kim et al.
1046 2002; Park et al. 2002; Hayet et al. 2011; Souchet and Laplante 2011; Daboor et al. 2012).

1047 Therefore, this study aimed to extract and characterize a protease with
1048 collagenolytic properties of hake (*Cynoscion leiarchus*), and evaluate their sensitivity to
1049 bovine achilles tendon collagen type I and collagen extracted from the skin of
1050 *Pseudoplatystoma corruscans* and *Oreochromis niloticus*, for possible enzyme application
1051 in industry.

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Materials and methods

Materials

Azo dye-impregnated collagen (azocoll), azocasein, bovine serum albumin (BSA), Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), Succinyl-DL-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan), bovine serum albumin (BSA), Tris (hydroxymethyl) aminomethane and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Glycine was acquired from Amersham Biosciences. HCl were obtained from Merck. The spectrophotometer used was Bio-Rad Smartspec™ 3000. Microplate spectrophotometer used was Bio-Rad xMark™. The centrifuges were BioAgency Bio-Spin and Software MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

Fish Waste

Viscera waste of *C. leiarchus* were obtained from the colony of fishermen from Ponta Verde, Maceió, Alagoas, Brazil. The viscera were kindly provided after evisceration process. Samples of 500 g intestine and 50 g muscle were collected separately, packaged in plastic containers and kept on ice and transported to the Laboratório de Enzimologia, Centro de Ciências Biológicas, Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil, where they were stored at - 27°C for further processing.

Extraction of collagenolytic enzymes and protein determination

The processing of waste was realized in accordance with the methodology described by Teruel and Simpson (1995). The ratio of viscera to extraction buffer (0.05 M Tris-HCl pH 7.5, containing 5 mM $CaC1_2$) was 1:3 (w/v). The extraction method followed systematic processes (Steps I, II, III and IV) for later analysis by which fractions were

obtained. In step I, the material was homogenized, homogenized and centrifuged. The resulting residual was again homogenized (Step II) and subsequently centrifuged through maceration new and homogenization (Step III). Then, the material was centrifuged and filtered in sterile syringe 0.22 µm and the resulting material was defined as stage IV. In each of maceration and homogenization step, all the viscera collected were homogenized separately for 5 minutes at a speed adjustment homogenizer at 10,000–12,000 rpm (4°C) (IKA RW 20D S32, China). The homogenate was then centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 12,000 x *g* for 30 min at 4°C. The supernatant fraction in relation to best dosage of total protein, specific and volumetric activity will be used as a crude extract (CE) for tests of physicochemical characterization of collagenolytic enzyme and sensitivity to collagen test. The material is stored at - 25°C for further processing. The protein concentration of all tissue extracts was determined according to Smith et al. (1985).

Activity of trypsin and chymotrypsin

Enzyme activity was measured using BApNA and Suc-Phe-p-Nan prepared with DMSO, as substrate specific for trypsin and chymotrypsin, respectively, with a final concentration of 8 mM. The substrate (30 µL) was incubated in microplate wells with the enzyme (30 µL) and 140 µL of 0.05 M Tris-HCl pH 7.4, containing 5 mM $CaCl_2$. The release of p-nitroaniline was measured as an increase in absorbance at 405 nm in a microplate reader, performed in quadruplicate. Controls were performed without enzyme. One unit of enzyme activity is considered as the amount of enzyme able to produce 1 µmol of p-nitroaniline per minute (Souza et al. 2013).

Determination of collagenolytic properties

The collagenolytic properties in the crude extract of intestine was determined according to the methodology modified by Adigüzel et al. (2009), using azocoll as substrate. A reaction mixture, which contained 5 mg of azocoll, 500 µL of 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$ and 500 µL of crude extract, was incubated at 55°C for 30 minutes, under stirring. Thereafter, was added 200 µL of trichloroacetic acid (TCA) and incubated to stop the reaction. After 10 minutes, the samples were centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 10,000 x *g* for 10 minutes at 4°C. The sample reading was performed using a spectrophotometer at a wavelength of 595 nm. One enzyme unit was defined as the amount of enzyme required to increase the absorbance in 0.01 at 595 nm. The assays were performed in quadruplicate.

Physicochemical properties of the collagenolytic enzyme

Optimum temperature and thermal stability

The effect of temperature on the enzyme activity and stability was evaluated at temperatures ranging from 25 to 90°C. For optimal temperatures, the assays were carried out by incubating the crude extract in a water bath. To test thermal stability, the enzyme was incubated in a water bath for 30 min and the activity was performed as described above (Kim et al. 2002; Park et al. 2002).

Optimum pH and stability

These experiments were carried out in different pH ranges using the buffers: 0.5 M citrate–phosphate (pH 4.0-7.0), 0.1 M Tris–HCl (pH 7.5-8.5) and 0.1 M glycine-NaOH (pH 9.0-12.0), containing 5 mM $CaC1_2$. The influence of pH on enzyme stability was determined by incubating the enzyme with various buffer solutions, at a ratio of 1:1 for 30 min. at 25°C. The activity was performed as described above. The highest enzymatic

activity observed for the enzyme in different buffers was defined as 100% (Kim et al. 2002; Park et al. 2002).

Effect of metal ions

The effect of metal ions on the enzyme activity was investigated by adding the monovalent (K^+ and Na^+), divalent metal ions (Hg^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+} and Ca^{2+}) and trivalent metal ion (Al^{3+}) to the reaction mixture. The final concentration of each metal ion was 1 mM. Each ion was incubated for 30 minutes at a ratio of 1:1, and then the activity was performed as described above blanks were performed with the absence of metal ions, being the results expressed as percentage activity (Park et al. 2002).

Effect of Inhibitors

The sensitivity of the enzymes with collagenolytic properties to the inhibitor was evaluated: phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor; N-*p*-tosyl-*L*-lysin chloromethyl ketone (TLCK), a trypsin-specific inhibitor; benzamidine, a trypsin inhibitor; *N*-tosyl-*L*-phenylalaninechloromethyl ketone (TPCK), a chymotrypsin-specific inhibitor; ethylenediamine tetra-acetic acid (EDTA), a chelating compound; and β-mercaptoethanol, a reducing agent. The final concentration of each inhibitor was 8 mM. Each ion was incubated for 30 minutes at a ratio of 1:1, and then the activity was performed as described above. The activity was compared with the reaction that is free of the corresponding inhibitors, being the results expressed as percentage activity (Park et al. 2002).

Assay specificity with collagen

The measure of the digestion of native collagen type I, skin collagen of *Pseudoplatystoma corruscans* and skin collagen of *Oreochromis niloticus* was performed according to the method of Moore and Stein (1954) and Park et al. (2002) with a slight modification. The skin collagen of *P. corruscans* and skin collagen of *O. niloticus* was extracted according Nagai et al. (2008). A reaction mixture, which contained 5 mg of collagen, 1 mL of 50 mM Tris-HCl (pH 7.5 with 5 mM $CaCl_2$) and 0.1 mL of the enzyme solution, was incubated at 37°C for 12, 24, 36 and 48 hours. The reaction was stopped by adding 0.2 mL of 50% trichloroacetic acid. After 10 min at room temperature, the solution was centrifuged at 1,800 × g for 20 min. The supernatant (0.2 mL) was mixed with 1.0 mL of a ninhydrin solution, incubated at 100°C for 20 min, and then cooled to room temperature. Subsequently, the mixture was diluted with 5 mL of 50% 1-propanol for an absorption measurement at 570 nm. A buffer (50 mM Tris-HCl, pH 7.5) that contained 5 mM $CaCl_2$ was used instead of an enzyme solution as the reference. The concentration of hydrolyzed-amino acids was determined using a standard curve of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme that is required for the hydrolysis of 1 mmol of substrate per h.

Statistical analysis

All values are presented as means ± standard deviations. These data were statistically analyzed by ANOVA, followed by a post hoc (Tukey) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($P < 0.05$).

Results

The fraction chosen for physicochemical characterization of collagenolytic enzyme was supernatant III to the process stage, taking into account the relative content of total proteins (mg/mL) and volumetric activity (U/mL) and specific (U/mg). Activity of serine proteases (trypsin and chymotrypsin) were determined as described in Table 1 for these two enzymes, the enzymatic activity benefit from the processing steps for reducing the material impurities, both in the gut extracts as muscle. The volumetric collagenolytic activity and specifies the pre-heated crude extract (step III) for 30 min at 45°C increased to 84.8 ± 0.02 (U/mL) and 25.74 ± 0.006 (U/mg), an increase of 16.96% and 17%, respectively, compared to the unheated crude extract.

The optimum temperature of the enzyme was 55°C (Figure 1A), showing thermostability in the range 25 to 60°C (Figure 1B), with a significant loss of activity at temperatures above 70°C and for stopping the complete 75°C. The enzyme had optimal activity at pH 8.0 (Figure 2A) and remained stable between 6.5 and 11.5 (Figure 2B).

The test with metal ions and natural and synthetic inhibitors are described in Table 2. Ions Zn^{2+}, Na^{+}, Ca^{2+} and Mg^{2+} showed no significant difference ($P < 0.05$), compared to the control group (100%), in contrast, all other ions showed a significant difference in the statistical test ($P > 0.05$). The protease was inhibited by metal ions tested, in decreasing order: $Pb^{2+} > Al^{3+} > Cu^{2+} > Hg^{2+} > Cd^{2+} > Ba^{2+} > K^{+} > Zn^{2+} > Na^{+}$. Ions Ca^{2+} and Mg^{2+} increased the collagenolytic activity. Regarding natural and synthetic inhibitors, was detected a significant difference ($P > 0.05$), compared to the control group in all exposures, in descending order of inhibition: Benzamidine > TLCK > EDTA > β-Mercaptoethanol > PMSF > TPCK. The degree of inhibition at TLCK and Benzamidine, protease inhibitors and EDTA, metalloprotease inhibitor.

The collagen was extracted from two Neotropical fish (*P. corruscans* and *O. niloticus*) with the unique purpose of application in the substrate affinity test comparing with the ability to hydrolyze insoluble collagen from bovine tendon type I. The specificity of the test collagen is illustrated in Figure 3. Cleavage of insoluble bovine collagen type I was detected from 24 h, reaching peak activity at 48 h. There was no record of activity below the 24 h period, using this type of substrate. When using collagen derived from the skin of *P. corruscans*, cleavage was recorded only after 48 h of incubation, there was no record of cleavage before this period. Regarding collagen obtained from skin of *O. niloticus*, cleavage was detected from 24 h and was not registered any activity in the previous period, and peak reaching 48 h of incubation. In general, for the tests with type I bovine tendon and skin *O. niloticus* from 24 hours, the activity showed a linear growth. The exception was the test with skin *P. corruscans*. However, when evaluated the activity achieved after 48 hours of testing, the skin of *P. corruscans* showed the highest enzymatic cleavage compared to the other two trials.

Discussion

Internal viscera, such as the intestine (or portions thereof) and pyloric caeca when present as a source of enzymes with collagenolytic properties have been reported from the crude extract for tuna *T. thymus* (16.5 U/mg) (Byun et al. 2002), for filefish *N. modestrus* (114.15 U/mg) (Kim et al. 2002) and mackerel *S. japonicus* (16.5 U/mg) (Park et al. (2002). Muscle of fish species as a research object of collagenases have been reported by Teruel and Simpson (1995) for winter flounder *P. americanus* (3.82 U/mg) and Wu et al. (2010a) for sea bream *P. major*. Notably that for the majority of the authors cited above, after performing technological treatments to obtain semi-pure enzyme detected significant

amounts of collagenolytic activity, such as described by Byun et al. (2002) and Park et al. (2002) with an increase of almost 40 times the activity of the crude extract.

Yoshinaka et al. (1978) investigated the site of production of collagenase in different organs that comprise the digestive system (liver, stomach, intestine) different species of fish, such as sardine *Sardinops melanosticta* (34.3 U/mg intestine), rainbow trout *Salmo gairdnerii* (2.8 and 36.8 U/mg liver and intestine, respectively), ginbuna *Carassius Carassius langsdorfii* (4.8 and 7.2 U/mg intestine anterior and posterior, respectively), carp *Cyprinus carpio* (10.4 U/mg intestine posterior), catfish *Parasilurus asotus* (1.4 and 3.1 U/mg liver and intestine, respectively), eel *Anguilla japônica* (1.1 and 33.9 U/mg stomach and intestine, respectively), yellow-tail *Seriola quinquerdiota* (0.7 U/mg pyloric caeca), bluefin tua *Thunnus thynnus*, umazurahagi *Novodon modestus* and shotted halibut *Eopsetta grigorjewi* (5.2, 66.3 and 82.8 U/mg stomach, intestine and pyloric caeca, respectively). The authors noted that portions of the intestine, pyloric cecum, mesentery, pancreas and adipose tissue are rich viscera collagenases. The activity often found in the intestine pancreatic collagenase was pre absorbed by the intestinal mucosa or were obtained from pancreatic tissue around the intestine, since it is impossible to completely separate the bowel contents and the surrounding tissue of the intestine may be caused.

The data go against what was described by Murado et al. (2009) for by-products (stomach, duodenum section including pancreas, final intestine) of rayfish (*R. clavata*), where the authors observed increased collagenolytic activity in the proximal portions of the intestine, rather than the stomach. According to the authors, in most species entered the carnivorous habits, demonstrated collagenolytic activity in his gut. The relation with the feeding habits due to the need to decompose the ingested during feeding and collagen degraded in the intestinal portion of the digestive tract or due to the acidic conditions of the

environment, since some fish species may exhibit protease collagenolytic property being stable under alkaline pH conditions (range 6 to 8), as described by Daboor et al. (2010).

The optimum temperature of the enzyme will vary according to the type of enzyme and the measured tissue and also evaluated depending on the species, the range of > 30°C and < 60°C has been reported that protease had the property of decomposing collagen, the collagenolytic (Kim et al. 2002; Park et al. 2002; Daboor et al. 2010, 2012; Wu et al. 2010a; Hayet et al. 2011; Suphatharaprateep et al. 2011; Baehaki et al. 2012; Lima et al. 2013). Identical values to this work were as described for winter flounder *P. americanus* (Teruel and Simpson 1995), filefish species *N. modestrus* (Kim et al. 2002), tuna *T. thymus* (Byun et al. 2002) and mackerel *S. japonicus* (Park et al. 2002).

Hayet et al. (2011) reported temperature of 60°C for sardinelle (*S. aurita*), with decreased activity at 70°C, while Kristjámsson et al. (1995) observed of 50°C for Atlantic cod (*G. morhua*), a decrease of enzyme activity of 50% from 55°C. Wu et al. (2010a) found for sea bream (*P. major*) optimum temperature of 40°C, similiar to that reportad by Murado et al. (2009) for rayfish (*Raja clavata*). Daboor et al. (2012) found optimal activity at 35°C in the mixture of haddock, herring, ground fish and flounder; and 30°C have been described by Roy et al. (1996) for greenshore crab (*C. maenas*). Sovik and Rustad (2006) reported decreased enzyme temperatures below 35°C in the guts of prey (*Brosme brosme*) and (*Molva molva*), while Mukherjee et al. (2009) for sponge (*Rhopaloeides odorabile*) observed activity optimum the 30°C. Enzymes with collagenolytic properties from microbial sources were also objects of investigation, having been reported by Wu et al. (2010b) who detected optimal activity at 45°C, when studying in bacterial strains of *Bacillus pumilus*, and showed that more than 50% of the original still remained activity after 5 min. of incubation at 70°C or 10 min at 60°C. Suphatharaprateep et al. (2011)

observed for collagenolytic protease of *Bacillus cereus* and *Klebsiella pneumoniae* optimum activity in 45°C and 40°C, respectively.

Optimum pH results presented in this study are identical to those reported for other fish species, such as *N. modestrus* filefish (Kim et al. 2002), sea bream *P. major* (Wu et al. 2010a) and sardinelle *S. aurita* (Hayet et al. 2011). Lima et al. (2009) and Liu et al. (2010) observed collagenolytic protease from *C. albicans* and *B. cereus* detected and optimum pH of 8.0 and 8.2, respectively. Teruel and Simpson (1995), Byun et al. (2002), Park et al. (2002), Laplante and Souchet (2011) and Daboor et al. (2012) reported pH of 7.5 to aquatic species winter flounder (*P. americanus*), tuna (*T. thymus*), Mackerel (*S. japonicus*), snow crab (*C. opilio*) and fish waste, respectively. Kristjámsson et al. (1995), Roy et al. (1996) and Baehaki et al. (2012) found a pH of 7.0 for Atlantic cod (*G. morhua*), greenshore crab (*C. maenas*) and *Bacillus licheniformis*, respectively. Suphatharaprateep et al. (2011) observed for collagenolytic protease of *K. pneumoniae* and *B. cereus* optimum pH of 7.0 and 6.0, respectively. Mukherjee et al. (2009) observed for a species of sponge (*R. odorabile*) optimum pH of 5.0. The stability of pH has also been reported for both aquatic species such as fish, crustaceans, and sponges, optimal values were found oscillating between < 5.0 and > 10.0 (Turkiewic et al. 1991; Kristjámsson et al. 1995; Teruel and Simpson 1995; Lima et al. 2009; Mukherjee et al. 2009; Murado et al. 2009; Wu et al. 2010a; Wu et al. 2010b; Hayet et al. 2011; Souchet and Laplante 2011; Suphatharaprateep et al. 2011; Baehaki et al. 2012).

The results described herein for temperature and pH conform to the needs required by industrial sectors for application collagenolytic enzyme properties in their segments, indicating enzyme with water prolonged heat resistance and a broad range of activity at different pH conditions. These factors are conditions for action of collagenase, as these enzymes are secreted as zymogens and require a change in its structure for its conversion

into active and practical activity (hydrolysis of collagen), and its activity retained by the control these physical and chemical parameters (Daboor et al. 2012).

Roy et al. (1996), Byun et al. (2002), Park et al. (2002), Kim et al. (2002), Liu et al. (2010) and Wu et al. (2010a) also observed increased activity of collagenolytic proteases greenshore crab (*C. maenas*), tuna (*T. thymus*), mackerel (*S. japonicus*), filefish (*N. modestrus*), *B. cereus* and sea bream *P. major*, respectively, when the presence of the ions Ca^{2+} and Mg^{2+}. Hayert et al. (2011) Baehaki et al. (2012) reported for sardinelle (*S. aurita*) and B*. licheniformis*, respectively, increased activity of Ca^{2+}, but with markedly reduced upon exposure to Mg^{2+}. Byun et al. (2002) reported further increases in activity when exposed to ions of Pb^{2+}, Ba^{2+} and Na^{+}, and decreased activity when the ions Cu^{2+}, Hg^{2+}, Cd^{2+}, K^{+}, and Zn^{2+}, while Wu et al. (2010a) detected increased for ions Zn^{2+}, Ba^{2+} and K^{+}, and marked reduction (55.2%) of the activity when exposed to Pb^{2+} ion. Hg^{2+} and Zn^{2+} were also objects of reduced activity in collagenolytic proteases. Roy et al. (1996), Park et al. (2002), Kim et al. (2002) and Hayert et al. (2011), while induced an increase in protease described by Liu et al. (2010).

Similar results for TLCK were reported by Kristjámsson et al. (1995), Kim et al. (2002), Byun et al. (2002), Park et al. (2002), Hayet et al. (2011) Sriket et al. (2011). Kristjámsson et al. (1995) reported strong inhibition of protease from Atlantic cod (*G. morhua*) exposed to the specific inhibitor of chymotrypsin, TPCK, differing from reports of Byun et al. (2002) for tuna (*T thymus*). Souchet and Laplante (2011) observed that proteases of snow crab (*C. opilio*), were inhibited by PMSF and TLCK but were insensitive to TPCK. In view of these properties, likely the proteases belong to the serine collagenase group. Inhibition by EDTA could be due to a mechanism other than Ca^{2+} chelation. The loss of activity upon exposure to EDTA has been observed in tuna species such as tuna *T. thymus* (Byun et al. 2002) and sardinelle *S. aurita* (Hayet et al. 2011), no inhibition was

observed for mackerel *S. japonicus* (Park et al. 2002). Kim et al. (2002), Wu et al. (2010b), Baehaki et al. (2012) and Daboor et al. (2012) also reported loss of activity upon exposure to EDTA. The reduction of enzyme activity when subjected to β-Mercaptoethanol, suggest that the structure of the enzyme has been tested disulfide, such as reported by enzyme linked Baehaki et al. (2012) in *B. licheniformis*.

The results of this study expressed opposite action activators and inhibitors are also inherent to those required by the industry, once determining the category type of collagenolytic enzyme is located (belonging to the serine group or the group of metalloproteinases) will define conditions of your application ideas. The inactivation of the enzyme, in whole or in part by inhibitors should, as suggested for Daboor et al. (2012), is related to interaction with other molecules (or metal ions) or with inhibitors of specific types of each category enzyme.

Hydrolysis of type I collagen has been detected through testing with collagenolytic proteases produced by various aquatic organisms, such as for the species of fish described by Kristjámsson et al. (1995) for Atlantic cod (*G. morhua*), Teruel and Simpson (1995) for winter flounder (*P. americanus*), Byun et al. (2002) for tuna (*T. thymus*), Park et al. (2002) for mackerel (*S. japonicus*), Kim et al. (2002) for filefish (*N. modestrus*), Herreiro-Hernandez et al. (2002) for iced cod (*G. morhua*), Hayet et al. (2011) for sardinelle (*S. aurita*); crab as described by Roy et al. (1996) for greenshore crab (*C. maenas*); and microorganisms such as described by Liu et al. (2010) fo*r B. cereus* and Wu et al. (2010b) for *B. pumilus*. Type I collagen has been extracted from the skin, bones, fins and scales of fish and other animals from freshwater and marine, such as squid, jellyfish and starfish. It is the most abundant type, being widely distributed in the body. It occurs as structures classically referred to as collagen fibrils that form bones, dentin, tendons, capsules bodies, corneal and dermal blood vessels, plays an important role in the

morphogenesis and cell metabolism of new tissue, providing mechanical and biochemical properties (Myllyharju and Kivirikko 2004; Söderhäll et al. 2007; Chung and Uitto 2010; Daboor et al. 2010; Ferreira et al. 2012; Junqueira and Carneiro 2013; Makareeva and Leikin 2014).

Turkiewic et al. (1991) reported serine proteinase isolated from *Euphausia superba* showed collagenolytic properties, it acting on collagens from Achilles tendon (type I and V) and reconstituted fibrils of calf skin collagen under conditions that do not denature the substrates. Kristjámsson et al. (1995) reported cleavage of native collagen types I, III, IV and V for marine species of fish in Atlantic cod (*G. morhua*). Byun et al. (2002) observed intestinal serine protease with collagenolytic properties with a preference for cleavage in the following decreasing order: collagen type V > collagen type II > type I collagen > type III collagen. For type V collagen, β-dimer was almost completely cleaved and the α1 chain was also partially degraded. The enzyme catalyzed hydrolysis of native collagen types II and III, and the release of peptides containing proline or hydroxyproline residues. The abundance of type V is low, but it is found associated with types I and II, bone and cartilage, and other tissues, with important participation in the function of tensile strength (Daboor et al. 2010; Junqueira and Carneiro 2013). Liu et al. (2010) observed various types of insoluble collagen and collagen detected efficiency in descending order type I > collagen type III > type II collagen. According Herreiro-Hernandez et al. (2002), since one collagenolytic enzyme works degrading collagen types, it is likely that other proteases may follow the process.

Associating all features, it is suggested that enzyme in question this is a protease being inhibited by the main protease inhibitors. Coupled to this, sum up their physical chemical characteristics, resistance to high temperatures and optimum activity at alkaline pH, as well as having been activated by Ca^{2+} ion and inactivated by Zn^{2+} ion, indicating

that the enzyme in question it is a serine protease capable of cleaving type I collagen and collagen extracted from fish skin Neotropical species, i.e. a collagenolytic serine protease with properties. All the features presented by the enzyme hake *C. leiarchus* are interesting for biotechnological applications, suggesting for the production of collagen peptides on an industrial scale.

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Figure legends

Fig. 1: Effect of temperature and thermal stability on the activity of collagenolytic serine protease of hake (*Cynoscion leiarchus*). (A) Optimum temperature in a range of 25–90°C. (B) Thermal stability after 30 minutes incubation at a temperature in the range of 25–90°C.

Fig. 2: Effect of pH and stability at different pH on the activity of collagenolytic serine protease of hake (*Cynoscion leiarchus*). (A) Optimal pH using different buffers in the pH range from 4.5 to 12.0. (B) pH stability after incubation for 30 minutes in the pH range 4.0 to 12.0.

Fig. 3: Activity of proteinase with collagenolytic properties extracted from digestive viscera of hake (*Cynoscion leiarchus*) against collagen type I and skin obtained from *P. corruscans* and *O. niloticus*, various incubation times (12-24-36-48 hours), at 37°C, mean of four replicates.

1598 **Figure 1**

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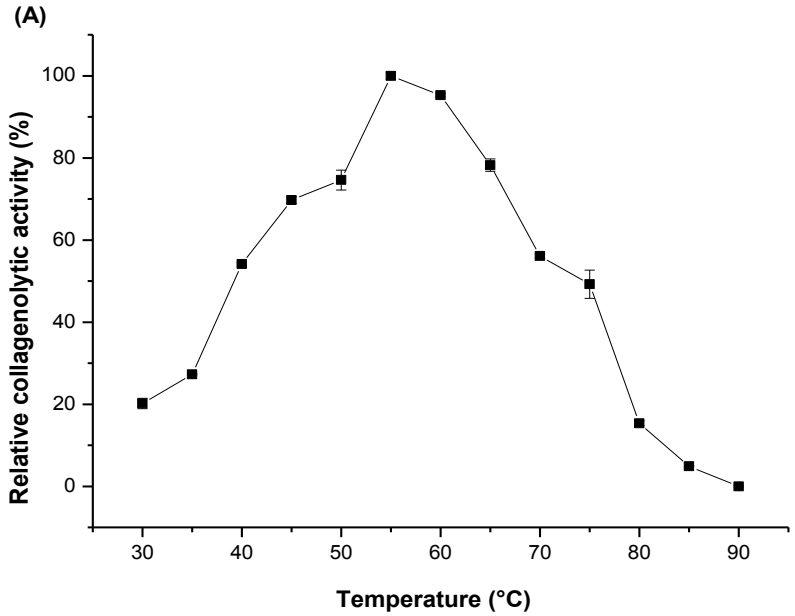

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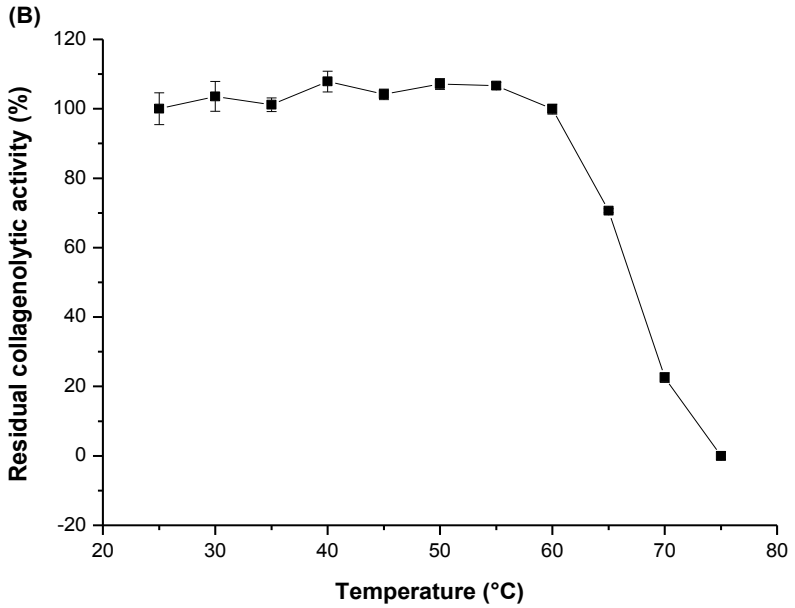

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1605 **Figure 2**

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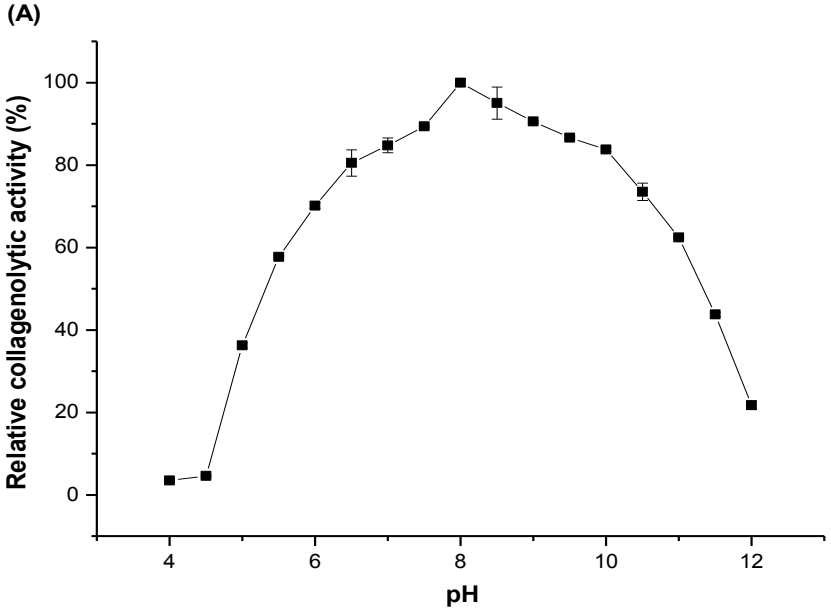

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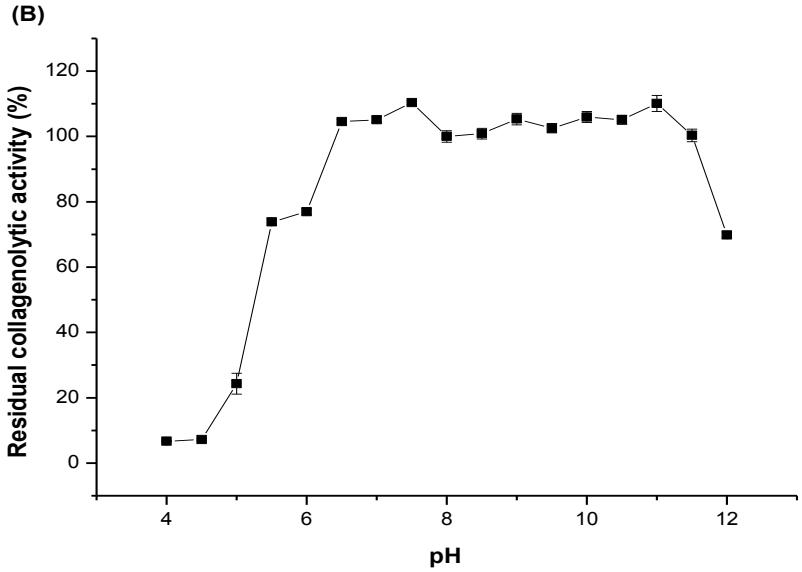

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Figure 3

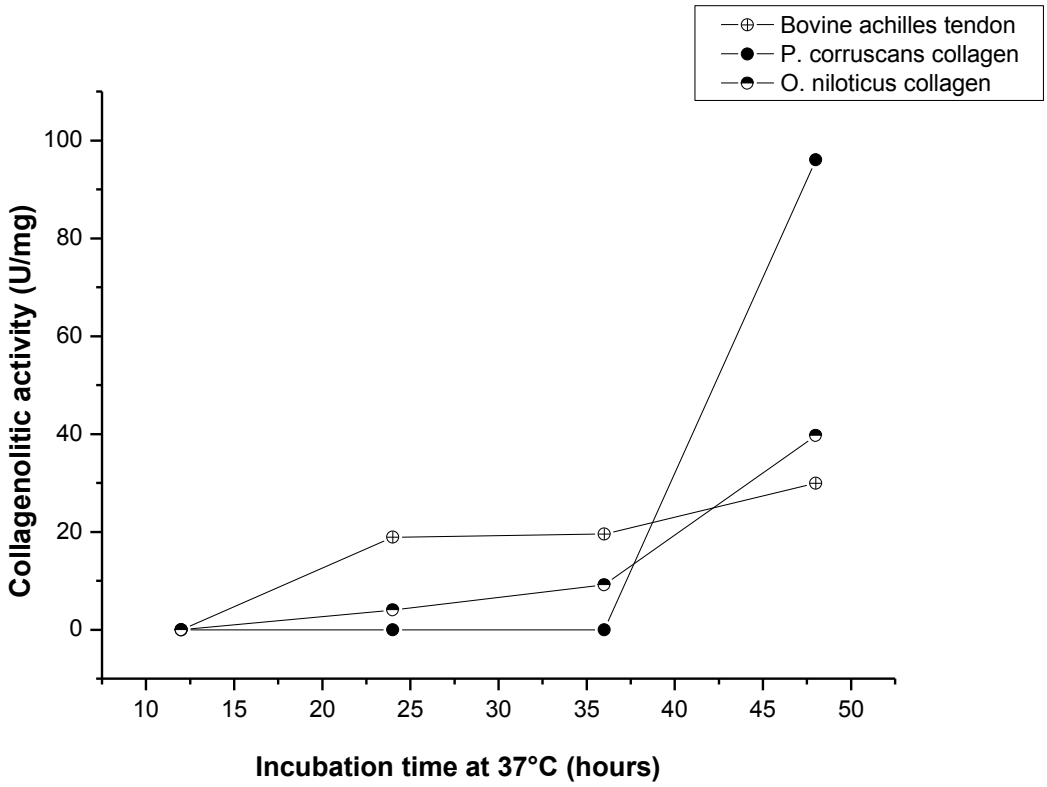
Bovine achilles tendon
P. corruscans collagen
O. niloticus collagen
Collagenolitic activity (U/mg)
0
20
40
60
80
100
10
15
20
25
30
35
40
45
50
Incubation time at 37°C (hours)

Table 1. Enzymatic activity of alkaline digestive proteases in enzymatic extracts of hake *Cynoscion leiarchus*.

Processing	Organ	Serino activity[1] (U/mg)	Serino activity[2] (U/mg)	Total protein (µg/ml)	Total protein (mg/ml)	*Collagenolytic Activity*[3]	
						Enzyme activity (U/mL)	Specific activity (U/mg)
Step I	*Intestine*	1.01 ± 0.07	0.48 ± 0.23	1931,37	1.93 ± 0.00	54.77 ± 0.00	28.36 ± 0.00
	Muscle	1.0 ± 0.13	0.82 ± 0.45	2754,90	2.75 ± 0.01	8.0 ± 0.01	2.90 ± 0.00
Step II	*Intestine*	0.83 ± 0.08	0.40 ± 0.24	3882,35	3.88 ± 0.00	56.02 ± 0.05	14.43 ± 0.01
	Muscle	1.17 ± 0.11	0.81 ± 0.16	3039,21	3.03 ± 0.00	16.5 ± 0.11	5.42 ± 0.03
Step III	*Intestine*	0.79 ± 0.19	0.61 ± 0.18	3294,11	3.29 ± 0.00	72.5 ± 0.08	22.0 ± 0.02
	Muscle	1.11 ± 0.10	1.04 ± 0.15	3970,58	3.97 ± 0.00	39.02 ± 0.58	9.82 ± 0.14
Step IV	*Intestine*	1.35 ± 0.02	0.63 ± 0.06	3372,54	3.37 ± 0.00	56.07 ± 0.03	16.62 ± 0.00
	Muscle	1.28 ± 0.08	1.51 ± 0.01	4147,05	4.14 ± 0.00	16.32 ± 0.01	3.93 ± 0.00

[1]Using Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), trypsin substrate.
[2]Using Succinyl-DL-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan), chymotrypsin substrate.
[3]Using Assaying proteinases with azocoll, collagenase substrate.

TABLE 2

Table 2. Effect of ions and inhibitors on the activity of collagenolytic enzyme from hake *Cynoscion leiarchus*.

Íons and Inhibitors	**Collagenolytic activity (%)**
Metal íons (1 mM)	
Control	100.0[a]
Cd^{2+}	66.23[b]
Cu^{2+}	64.49[b]
K^{+}	88.15[b]
Zn^{2+}	93.18[a]
Al^{3+}	63.21[b]
Hg^{2+}	64.92[b]
Na^{+}	93.91[a]
Pb^{2+}	60.57[b]
Ca^{2+}	105.52[a]
Ba^{2+}	75.68[b]
Mg^{2+}	100.39[a]
Inhibitors (8 mM)	
Control	100.0[a]
PMSF	61.67[b]
TPCK	89.58[b]
TLCK	26.84[b]
Benzamidine	23.73[b]
EDTA	38.11[b]
β-Mercaptoethanol	58.38[b]

*Mean value ± standart deviation. Values followed by different superscript letters are significantly different at $P < 0.05$

7. ARTIGO III: Collagenolytic proteinase from peacock bass (*Cichla ocellaris*): physicochemical characterization and specificity test of collagen.

VAGNE DE MELO OLIVEIRA, DOUGLAS HENRIQUE DE HOLANDA ANDRADE, RENATA CRISTINA DA PENHA FRANÇA, CAIO RODRIGO DIAS ASSIS, CAROLINA DE ALBUQUERQUE LIMA, LUIZ BEZERRA DE CARVALHO JUNIOR, RANILSON SOUZA BEZERRA, ANA LÚCIA FIGUEIREDO PORTO

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Collagenolytic proteinase from peacock bass (*Cichla ocellaris*): physicochemical characterization and specificity test of collagen

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Abstract

The physicochemical and kinetics of protease with collagenolytic properties from digestive viscera of peacock bass (*Cichla ocellaris*) and specificity collagen test were performed. The Michaelis-Menten constant (K_m) and $V_{máx}$ were 5.92 mM and 294.40 U/mg, respectively. Optimal pH and temperature of collagenolytic activity were 7.5 and 55°C, respectively, showing stability at pH between 6.5-11.5 and temperatures 25-60°C. The metal ions Hg^{2+}, Pb^{2+} and Cu^{2+} inhibited the enzyme activity, while K^{+}, Ca^{2+} and Mg^{2+} functioned as activators. The inhibition by Benzamidine and TLCK were stronger than the other test inhibitors. After 36 hours of incubation, the collagenolytic action was effective in all collagen types tested in the following order: bovine collagen type I > skin collagen of *P. corruscans* > skin collagen of *O. niloticus*. The physicochemical, kinetic and hydrolysis of collagen results provided data of the catalytic efficacy of enzyme, making it potentially enhanced in food processing.

Keywords: collagen, collagenolytic protease, digestive viscera, Neotropical species.

1. Introduction

According to FAO (2014), in 2011, global per capita consumption of fish was estimated at 18.9 kg, with fish accounting for 16.7% of the global population's intake of animal proteins and 6.5% of all proteins consumed. The increase in consumption is also linked to the need to increase production in order to meet the demand, resulting in increased production of waste, byproducts of processing, such as digestive viscera, fins, head and tail (Bezerra, Buarque, Amaral, Castro, Espósito & Carvalho Junior, 2006). Efficient utilization of byproducts has direct impact on the economy and environmental pollution of the country (Jayathilakan, Sultana, Radhakrishna & Bawa, 2012). Treated fish waste has found many applications and among them the most important are animal feed, biodiesel/biogas, dietary products and food-packaging applications (chitosan), protein hydrolyzate with fish and shrimp, natural pigments (after extraction), cosmetics (collagen) and enzyme isolation (Arvanitoyannis & Kassaveti, 2008). The use of proteases from the viscera of the fish brings an advantage over commercial enzymes related to production costs can be greatly reduced. To produce a protein hydrolyzate with pre-determined, for example, properties it is important first to determine the enzyme activity profile of the viscera used, since different activities can lead to the formation of peptides from different fractions with different physicochemical properties (Bezerra et al., 2006).

Fish internal organs represent rich sources of enzymes, and many of them exhibit high catalytic activity at relatively low concentrations. Considering the specific characteristics of these enzymes, fish processing byproducts are currently used for enzyme extraction, especially proteinases. Proteinases found in the digestive organs of fish include pepsin, gastricsin, trypsin, chymotrypsin, elastase, carboxypeptidase, carboxyl esterase and collagenase (Simpson, 2000; Byun, Park, Sung & Kim, 2002; Bezerra et al., 2006; Kim & Mends, 2006; Klomklao, 2008).

Enzymes which are capable of degrading the peptide bonds of various types of collagen (the most abundant protein in mammals and the major component of the extracellular matrix, constituent of skin, tendons, and cartilage as well as the organic component of bones, teeth, and the cornea) are generally known as collagenolytic enzymes (Harrington, 1996; Daboor, Budge, Ghaly, Brooks & Dave, 2010).

The real collagenolytic enzymes cleave helical regions of the fibrillar collagen molecule under physiological conditions (pH and temperature). These enzymes are divided into two groups: matrix metalloproteinases (MMPs) (zinc-dependent endopeptidases) and serine proteinase (exopeptidases, endopeptidases and oligopeptidades ɣ-peptidases groups). Due to the diversity of the structure of collagen, is extremely difficult to differentiate true collagenases from other proteinases with collagenolytic properties (Watanabe, 2004). However, it is known that numerous mammalian proteases including pepsin, trypsin, chymotrypsin, and other tissue enzymes can degrade gelatin and the nonhelical regions of collagen molecules (Harrington, 1996).

Digestive organs can serve as a source of both serinecollagenases and metallocollagenases but most studies have concentrated on digestive glands as a source of serine collagenase (Daboor et al., 2010). Proteinase with collagenolytic properties have been isolated and characterized from residues and byproducts from fish processing such as Atlantic cod (*Gadus morhua*) (Kristjámsson, Gudmundsdóttir, Fox & Bjarnason, 1995), tuna *Thunnus thymus* (Byun et al., 2002), filefish *Novoden modestrus* (Kim, Park, Kim & Shahidi, 2002), mackerel *Scomber japonicus* (Park, Lee, Byun, Kim & Kim, 2002), sea bream *Pagrus major* (Wu, Chen, Liu, Yoshida, Zhang, Su & Cao, 2010a), sardinelle *Sardinella aurita* (Hayet, Rym, Ali, Sofiane & Moncef, 2011) and a mixture of haddock, herring, ground fish and flounder (Daboor, Budge, Ghaly, Brooks & Dave, 2012).

The limited number of reports of fish byproducts, such as intestinal viscera of fish as a source of enzymes with collagenolytic properties highlights the need for further research to promote human health. In this context, this work aimed to extract and characterize physicochemically a protease with collagenolytic properties of peacock bass (*Cichla ocellaris*) and perform specificity test of collagen (bovine Achilles tendon insoluble collagen and collagen derived from the skin of two species of Neotropical fishes, pintado *Pseudoplatystoma corruscans* and Nile tilapia *Oreochromis niloticus*).

2. Materials and methods

2.1 Materials

Azo dye-impregnated collagen (azocoll), azocasein, bovine serum albumin (BSA), Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), Succinyl-DL-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan), Tris (hydroxymethyl) aminomethane and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Glycine was acquired from Amersham Biosciences. HCl were obtained from Merck. The spectrophotometer used was Bio-Rad Smartspec™ 3000. Microplate spectrophotometer used was Bio-Rad xMark™. The centrifuge was BioAgency Bio-Spin and Software MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

2.2 Fish Waste

Waste of digestive viscera and muscle of peacock bass *Cichla ocellaris* were obtained from the colony of fishermen from the town of Petrolândia, Pernambuco, Brazil. The material was kindly provided after evisceration process. Samples of intestine (500 g), of muscle, liver and stomach (50g) were collected separately, packaged in plastic

containers and kept on ice and transported to the Laboratório de Enzimologia (LABENZ), Centro de Ciências Biológicas, Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil, where they were stored at - 27°C for further processing.

2.3 Extraction of collagenolytic enzymes and protein determination

The processing of waste was realized in accordance with the methodology described by Teruel and Simpson (1995). The ratio of viscera to extraction buffer (0.05 M Tris-HCl pH 7.5, containing 5 mM $CaC1_2$) was 1:3 (w/v). The extraction method followed systematic processes (Steps I, II, III and IV) for later analysis by which fractions were obtained. In step I, the material was homogenized, homogenized and centrifuged. The resulting waste was again homogenized (Step II) and subsequently centrifuged through maceration new and homogenization (Step III). Then, the material was centrifuged and filtered in sterile syringe 0.22 µm and the resulting material was defined as stage IV. In each of maceration and homogenization step, all the viscera collected were homogenized separately for 5 minutes at a speed adjustment homogenizer at 10,000–12,000 rpm (4°C) (IKA RW 20D S32, China). The homogenate was then centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 12,000 x *g* for 30 min at 4°C. The supernatant fraction in relation to best dosage of total protein, specific and volumetric activity will be used as a crude extract (CE) for tests of physicochemical characterization of collagenolytic enzyme and sensitivity to collagen test. The material is stored at - 25°C for further processing. The protein concentration of all tissue extracts was determined according to Smith et al. (1985).

2.4 Activity of serine proteases

Protease activity was measured using BApNA and Suc-Phe-p-Nan prepared with DMSO, as specific substrate for trypsin and chymotrypsin, respectively, with a final concentration of 8 mM. The substrate (30 µL) was incubated in microplate wells with the enzyme (30 µL) and 0.05 M Tris-HCl pH 7.4, containing 5 mM $CaC1_2$ (140 µL), performed in quadruplicate. The release of p-nitroaniline was measured as an increase in absorbance at 405 nm in a microplate reader. Controls were performed without enzyme. One unit of enzyme activity is considered as the amount of enzyme able to produce 1 µmol of p-nitroaniline per minute. The specific activity, calculated as the ratio between the protease activity (U/mL) and the total protein in the sample (mg/mL^{-1}), was expressed in U/mg (Souza et al., 2007).

2.5 Determination of collagenolytic properties

The collagenolytic properties in the crude extract of intestine was determined according to the methodology modified by Adigüzel, Bitlisli, Yasa & Eriksen (2009), using azocoll as substrate. A reaction mixture (in quadruplicate), which contained 5 mg of azocoll, 500 µL of 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ and 500 µL of crude extract, was incubated at 55°C for 30 minutes, under stirring. Thereafter, was added 200 µL of trichloroacetic acid (TCA) and incubated to stop the reaction (room temperature). After 10 minutes, the samples were centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 10,000 x *g* for 10 minutes at 4°C. The reaction was read at 595 nm. One enzyme unit was defined as the amount of enzyme required to increase the absorbance in 0.01 at 595 nm.

2.6 Michaelis–Menten kinetic assay (K_m and V_{max})

The substrates used in the kinetic tests were azocoll (final concentrations ring from 1 to 20.0 mg/mL). The reaction was performed in quadruplicate. The sample reading was performed as described in section 2.5. One enzyme unit was defined as the amount of enzyme required to increase the absorbance in 0.01 at 595 nm. The activity values (U) obtained for each substrate concentration were plotted on a Michaelis–Menten graph using the MicroCal™ Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

2.7 Biochemical properties of collagenolytic protease

2.7.1 Optimum temperature and thermal stability

The effect of temperature on the enzyme activity and stability was evaluated at temperatures ranging from 25 to 90°C. For optimal temperatures, the assays were carried out by incubating the crude extract in a water bath. To assay thermal stability, the enzyme was incubated in a water bath for 30 min. and the remaining activity was then measured as described in item 2.5. The activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%) (Kim et al., 2002; Park et al., 2002).

2.7.2 Optimum pH and stability

These assays were carried out in different pH ranges using the buffers: 0.5 M citrate–phosphate (pH 4.0-7.0), 0.1 M Tris–HCl (pH 7.5-8.5) and 0.1 M glycine-NaOH (pH 9.0-12.0), containing 5 mM $CaC1_2$. The influence of pH on enzyme stability was determined by incubating the enzyme with various buffer solutions, at a ratio of 1:1 for 30 min. at 25°C. The activity was measured as described in item 2.5. The highest enzymatic activity observed for the enzyme in different buffers was defined as 100%. The activity was

calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%) (Kim et al., 2002; Park et al., 2002).

2.7.3 Effect of metal ions

The effect of metal ions on enzyme activity was investigated by adding the monovalent (K^+ and Na^+), divalent metal ions (Hg^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+} and Ca^{2+}) and trivalent metal ion (Al^{3+}) to the reaction mixture. The final concentration of each metal ion was 1 mM. Each ion was incubated for 30 minutes at a ratio of 1:1, and then the activity was determined as described under item 2.5. The activity was compared with the reaction that is free of the corresponding metal ions. The activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%) (Park et al., 2002).

2.7.4 Effect of Inhibitors

The sensitivity of enzymes with collagenolytic properties to some inhibitors was tested: phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor; N-*p*-tosyl-*L*-lysin chloromethyl ketone (TLCK), a trypsin-specific inhibitor; benzamidine, a trypsin inhibitor; *N*-tosyl-*L*-phenylalaninechloromethyl ketone (TPCK), a chymotrypsin-specific inhibitor, diluted in DMSO; ethylenediamine tetra-acetic acid (EDTA), a chelating compound; and β-mercaptoethanol, a reducing agent, diluted in deionized water. The final concentration of each inhibitors was 8 mM. Each ion was incubated for 30 minutes at a ratio of 1:1, and then the activity was determined as described under item 2.5. The activity was compared to the reaction with absence of the corresponding inhibitors. The activity

1941 was determined as the percentage of the proteolytic activity in an inhibitor-free control
1942 sample (Park et al., 2002).
1943
1944 2.8 Assay for substrate specificity
1945 The measure of the digestion of native collagen from bovine achilles tendon type I,
1946 type I, skin collagen of *P. corruscans* and skin collagen of *O. niloticus* was performed
1947 according to the method described by Moore & Stein (1954) and Park et al. (2002) with a
1948 slight modification. The skin collagen of *P. corruscans* and skin collagen of *O. niloticus*
1949 was extracted according Nagai, Suzuki & Nagashima (2008). A reaction mixture, which
1950 contained 5 mg of collagen, 1 mL of 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$
1951 and 0.1 mL of the enzyme solution, was typically incubated at 37°C for 12, 24, 36 and 48
1952 hours; and 55°C for 1 hour. The reaction was stopped by adding 0.2 mL of 50%
1953 trichloroacetic acid. After 10 min at room temperature, the solution was centrifuged at
1954 1,800 × g for 20 min. The supernatant (0.2 mL) was mixed with 1.0 mL of a ninhydrin
1955 solution, incubated at 100°C for 20 min, and then cooled to room temperature.
1956 Subsequently, the mixture was diluted with 5 ml of 50% 1-propanol for an absorption
1957 measurement at 570 nm. A buffer (50 mM Tris-HCl, pH 7.5) that contained 5 mM $CaCl_2$
1958 was used instead of an enzyme solution as the reference. The concentration of
1959 hydrolyzed-amino acids was determined by a standard curve that was based on a solution
1960 of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme that is
1961 required for the hydrolysis of 1mmol of substrate per h.
1962
1963 2.9 Statistical analysis

All values are presented as means ± standard deviations. These data were statistically analyzed by ANOVA, followed by a post hoc (Tukey) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($p < 0.05$).

3. Results and Discussion

The collagenolytic activity is present in several teleost fish species, having, in this study, the enzyme was extracted from the digestive viscera *C. ocellaris* through IV processing steps, have been determined at all stages of trypsin activity, chymotrypsin and collagenolytic, as has already been reported collagenolytic proteases that had been fit with properties and physicochemical characteristics similar to the grouping of serine proteinases such as those described by collagenolytic enzymes Kim et al. (2002) and Park et al. (2002).

In the present study, activity of trypsin-like and chymotrypsin-like were measured throughout the process for all viscera described observing a reduction in activity of 15% and 52%, respectively, along the steps to the bowel. Teruel & Simpson (1995) observed high activity of chymotrypsin-like in the muscle of winter flounder (*P. americanus*) decreased activity was observed throughout the processing for isolation of collagenolytic enzyme. Concerning collagenolytic activity increased 35% compared to stage I processing. The specific activity of the initial enzyme (after step IV) was 94.35 ± 0.002 (U/mg) as described in Table 1, while the activity of the enzyme was pre-heated to 127.44 ± 009 (U/mg), bringing about 35% of enzyme activity. The extract of heart showed the second highest collagenolytic activity and the highest activity with substrates for trypsin-like and chymotrypsin-like, in the IV stage of processing.

Kinetic parameters of azocoll hydrolysis were examined in the present study (Table 2), in comparison with other biological organisms. The Michaelis-Menten constant (*K*m) for the crude extract of *C. ocellaris* was 5.92 mM, while V*max* was 294.40 U/mg. No results were found of studies that employ the azocoll substrate in fish species with the purpose of kinetic parameters. Collagenase enzymes, as specific enzymes for the collagen substrate, have been isolated and characterized from both microbial cells and animal tissues (Daboor et al., 2010). As a result, the data presented in this study are compared with other substrates already employed with some subtropical fish species and strains of microorganisms. The kinetic parameters will provide efficacy data of the enzyme, making it potentially enhanced in food processing (Park et al., 2002).

The optimum temperature of the enzyme activity was 55°C as shown in Figure 1A, with marked reduction above 65°C, and total loss of activity at 75°C, remaining stable between 25°C and 60°C as illustrated in Figure 1B. Similar results have been reported for some subtropical fish species, as described by Teruel & Simpson (1995) for the species winter flounder (*P. americanus*), Kim et al. (2002) for filefish species (*N. modestrus*), Byun et al. (2002) for *T. thymus* and Park et al. (2002) for mackerel species (*S. japonicus*). Wu et al. (2010a) found for sea bream (*P. major*) optimum temperature of 40°C. In contrast, Kristjámsson et al. (1995) observed optimal enzymatic activity at 50°C for Atlantic cod (*G. morhua*) and a decrease of enzyme activity of 50% from 55°C. Hayet et al. (2011) found optimal activity at 60°C, with decreased activity at 70°C for sardinelle species (*S. aurita*); while Daboor et al. (2012) found optimal activity at 35°C in the mixture of haddock, herring, ground fish and flounder; and 30°C as detected by Roy, Bernard & Patrick (1996) for the species greenshore crab (*C. maenas*). Sovik & Rustad (2006) reported decreased of enzyme activity at temperatures below 35°C in the guts of prey (*Brosme brosme*) and (*Molva molva*). The optimum temperature of the collagenolytic enzyme has been explored

in other species of organisms as described by Wu, Li, Li, Chen & Shuliang (2010b) who detected optimal activity at 45°C, when studying in bacterial strains of *Bacillus pumilus*, and showed that more than 50% of the original activity still remained after 5 min of incubation at 70°C for 10 min at 60°C. The optimum temperature of the enzymes with collagenolytic properties will depend on the type of tissue and the extracted species in question, although hitherto reported range in temperatures between 30 and 60°C (Kim et al., 2002;. Park et al., 2002; Daboor et al., 2010, 2012; Wu et al., 2010a; Hayet et al., 2011).

The optimum pH of the enzyme was 7.5 (Figure 2A). The results of optimum pH are similar to the ones reported by Teruel & Simpson (1995), Byun et al. (2002), Park et al. (2002), Souchet & Laplante (2011) and Daboor et al. (2012) for the aquatic species winter flounder (*P. americanus*), tuna (*T. thymus*), Mackerel (*S. japonicus*), snow crab (*C. opilio*) and mixed viscera of different fish species, respectively, differing from Kim et al. (2002) for filefish (*N. modestrus*), Wu et al. (2010a) for sea bream (*P. major*) and Hayet et al. (2011) for sardinelle (*S. aurita*) reported pH of 8.0. Liu, Ma, Ca, Yang & Wang (2010) and Lima et al. (2009) also observed in enzymes with collagenolytic properties produced from microorganisms and having optimum activity at pH 8.0 and 8.2, respectively, in *B. cereus* and *C. albicans*. Mukherjee, Webster & Llewellyn (2009) observed for a species of Sponge (*R. odorabile*) optimum pH of 5.0, while Murado, González & Vázquez (2009) found a pH of 6.0 to rayfish species (*R. clavata*); and Kristjámsson et al. (1995) and Roy et al. (1996) found a pH of 7.0 for Atlantic cod (*G. morhua*) and greenshore crab (*C. maenas*), respectively. The stability of the collagenolytic enzyme properties in different pH ranges, has also been reported by various authors for different aquatic species (fish, crustaceans, sponges), and microorganisms, varying in the range of 5.0 to 10.0 (Kristjámsson et al., 1995; Teruel & Simpson, 1995; Lima et al., 2009; Mukherjee et al., 2009; Murado et al.,

2009; Daboor et al., 2010; Wu et al., 2010a; Hayet et al., 2011; Souchet & Laplante, 2011), have been detected in the present work enzymatic activity in a wide range of pH conditions, remained stable between 6.5 and 11.5, as seen in Figure 2B. The collagenolytic enzyme properties of this study showed high resistance to high temperatures and prolonged heat exposure, as well as variations in the pH conditions tested, suggesting becoming up for application on an industrial scale after successive processes to obtain the enzyme in its pure state. The ideal tacit knowledge of temperature and pH of the enzyme is essential, especially because they are contributing factors to the proper functioning and performance in the breakdown of collagen fibers, which if not controlled, can retain enzyme activity, as described by Daboor et al. (2012).

Collagenases factors that may need to convert their zymogen form of active molecules, as latent enzymes require a change in structure to achieve the desired collagenolytic activity. Inactive forms of collagenase may also is present by interactions with other molecules that act as inhibitors, cofactors are needed for conversion (Daboor et al., 2012). In this sense, the use of activators and inhibitors is essential for such action happen and controlled, especially the industrial processing point of view in the food industry. The test with metal ions and natural and synthetic inhibitors are described in Table 3. Ions K^{+}, Ca^{2+}, Ba^{2+} and Mg^{2+} showed no significant difference ($p < 0.05$), compared to the control group (100%), differing from the other metal ions with statistically significant data ($p > 0.05$). There was inhibition, in descending order, by the following ions: $Hg^{2+} > Pb^{2+} > Cu^{2+} > Cd^{2+} > Na^{+} > Al^{3+} > Zn^{2+} > Ba^{2+}$. While the ions K^{+}, Ca^{2+} and Mg^{2+} induced an increase in activity, although not significantly. Byun et al. (2002) observed a marked reduction in the activity of a serine protease extracted from tuna (*T. thymus*) when subjected to Cd^{2+}, Hg^{2+} and Zn^{2+} and exposed to increasing Ca^{2+} and Mg^{2+}. Kim et al. (2002) observed for filefish (*N. modestrus*) reduced activity of a serine protease caused by

the ions Cu^{2+}, Cd^{2+} and Zn^{2+}, and it was activated by Ba^{2+}, K^{+}, Ca^{2+} and Mg^{2+}; while Park et al. (2002) reported for the species mackerel (*S. japonicus*) decreased activity when subject to the ions Zn^{2+}, Hg^{2+}, Pb^{2+} and Cu^{2+}, and activation in the presence of Cd^{2+}, K^{+}, Ca^{2+} and Mg^{2+}. In tests with collagenolytic protease from *B. cereus*, Liu et al. (2010) observed reduced activity in the presence of Al^{3+} and Cu^{2+} while an increase in Ca^{2+} and Mg^{2+}. This increase is identical to that reported by Wu et al. (2010b) for the enzyme from *B. pumilus* and also observed reduction when exposed to Pb^{2+}. Analyzing protease from sea bream (*P. major*), Wu et al. (2010a) observed a complete inhibition when in presence of Cu^{2+}, Cd^{2+} and Zn^{2+}. Baehaki, Suhartono, Sukarno, Syah, Sitanggang, Setyahadi & Meinhardt (2012) reported for *B. licheniformis* marked reduction of activity by Mg^{2+} and increase by Ca^{2+}.

The degree of inhibition for Benzamidine and TLCK, protease inhibitors were stronger in relation to the other inhibitors tested, although all presented significant difference ($p > 0.05$), compared to control (100%). Similar results with TLCK and PMSF were reported by Kristjámsson et al. (1995), Kim et al. (2002), Byun et al. (2002), Park et al. (2002), Hayet et al. (2011), Souchet and Laplante (2011) and Sriket et al. (2011). Kristjámsson et al. (1995) reported strong inhibition of protease from Atlantic cod (*G. morhua*) exposed to the specific inhibitor of chymotrypsin, TPCK, differing from reports of Byun et al. (2002) for tuna (*T. thymus*). The assay with inhibitors of metalloproteinases (EDTA) detected reduced enzyme activity, although it can be considered low if compared to inhibitors of serine proteases tested (PMSF and TLCK). Marked loss of activity when subjected to EDTA has been reported for tuna *T. thymus* (Byun et al., 2002) and sardinelle *S. aurita* (Hayet et al., 2011) and no inhibition for mackerel *S. japonicus* (Park et al. 2002). However, other proteases showed marked reduction of collagenolytic activity, such as those described by Kim et al. (2002) for filefish (*N. modestrus*), Souchet & Laplante (2011)

for snow crab (*C. opilio*), Wu et al. (2010b) for *B. pumilus*, Baehaki et al. (2012) for *B. licheniformis* and Daboor et al. (2012) for fish waste. There was also reduced activity when subjected the enzyme to β-Mercaptoethanol, indicating that the structure of the enzyme presents disulfide bonds, such as the report by Baehaki et al. (2012) for *B. licheniformis*.

Enzymes belonging to the class of metalloproteases require, in general ions Zn^{2+} to maintain their optimum activity and stability, and have their activities significantly reduced upon exposure to EDTA, a chelating agent for ions Ca^{2+}. Considering that in this study, there was a reduction of enzyme activity upon exposure to ion Zn^{2+} and a minor reduction of activity by EDTA, could be evidence which this protease does not specifically belong to the group of metalloproteases. However, the enzymes belonging to the group of serine proteases that have collagenolytic properties require Ca^{2+} and enzyme cofactor, which in most cases lead to an increase of the enzymatic activity, as can be observed in this study. Here, the data evidenced that the enzymes under study are calcium-dependent, as well as they present disulfide bonds and can be inhibited by potent and specific inhibitors of serine proteases, such as Benzamidine, PMSF and TLCK. Such features, in turn, suggests that the enzymes under study are serine proteases that have collagenolytic properties, such as those already described by Byun et al. (2002), Kim et al. (2002), Park et al. (2002) and Wu et al. (2010a).

The collagen was extracted from two neotropical fish (*P. corruscans* and *O. niloticus*) with the unique purpose of application in the substrate affinity test comparing with the ability to hydrolyze insoluble collagen from bovine tendon type I, since the species *P. corruscans* is present in the diet of *C. ocellaris*, and *O. niloticus* biotechnological potential has been highlighted as well as a species of easy cultivation. The specificity of the test collagen is illustrated in Figure 3. The collagenolytic enzyme properties of *C. ocellaris* cleaved the native collagen types I, skin *P. corruscans* and *O. niloticus* which can

be seen in Figure 3A. The cleavage rate by the proteinase for the substrate was indicated as follows: type I > *O. niloticus* > *P. corruscans*, demonstrating affinity for the three substrates at 55°C for 1 hour. The proteinase has been identified by its collagenolytic property because of its ability to cleave the three tested types of collagen (Park et al., 2002). The assay for detecting cleavage over 48 hours can be seen in Figure 3B. Cleavage of collagen type I was detected from 24 hours, reaching peak activity at 48 hours. There was no record of activity below the 24 hours period, using this type of substrate. When using collagen derived from the skin of *P. corruscans*, cleavage was recorded after 36 hours of incubation and activity peaks were recorded at 48 hours. Regarding collagen obtained from skin of *O. niloticus*, cleavage was detected from 24 hours and was not registered any activity in the previous period, and peak reaching 48 hours of incubation. In general, after 36 hours, the activity showed a linear growth. After 36 hours, all types of collagen were effective in a proportion of insoluble bovine type I collagen > collagen extracted from the skin of *P. corruscans* > collagen extracted from the skin of *O. niloticus*. Bovine collagen showed the higher cleavage considering at 48 hours of incubation, but the collagen obtained from skin of *O. niloticus* promoted the highest initial activity at 24 hours of incubation.

Collagen type I is the most abundant protein in all vertebrates, being found in all connective tissues, including bone, tendon and skin. It is also the main component of the extracellular matrix and has the function to provide mechanical strength to tissues and organs and assist in regulation of cell medium (Liu et al., 2010; Makareeva and Leikin, 2014). This type of collagen has been used as a substrate for enzymes with collagenolytic properties produced by species of fish such as those described by Kristjámsson et al. (1995) for Atlantic cod (*G. morhua*), Teruel & Simpson (1995) for winter flounder (*P. americanus*), Byun et al. (2002) for tuna (*T. thymus*), Park et al. (2002) for mackerel (*S.*

japonicus), Kim et al. (2002) for filefish (*N. modestrus*), Herreiro-Hernandez, Duflos, Malle & Bouquelet (2003) for iced cod (*G. morhua*), Hayet et al. (2011) for sardinelle (*S. aurita*); crab as described by Roy et al. (1996) for greenshore crab (*C. maenas*); and microorganisms such as described by Liu et al. (2010) for *B. cereus* and Wu et al. (2010b) for *B. pumilus*. Liu et al. (2010) observed various types of insoluble collagen and detected collagenolytic efficiency in descending order type I > collagen type III > type II collagen. In contrast, Byun et al. (2002) observed intestinal serine protease with collagenolytic properties with a preference for cleavage in the following descending order: collagen type V > collagen type II > type I collagen > type III collagen. Once one collagenolytic enzyme acts degrading collagen types, it is likely that other proteases may carry on the process (Herreiro-Hernandez et al., 2003).

4. Conclusions

The proteinase extracted from digestive viscera of peacock bass (*C. ocellaris*) showed interesting characteristics for biotechnological applications, such as: collagenolytic property (the enzyme was able to cleave type I collagen, the collagen of the skin of *O. niloticus* and skin collagen of *P. corruscans*), high specific activity, stability over wide range of alkaline pH and thermostability. *C. ocellaris* is a freshwater species that is of increasing interest in Brazilian fish farming. However, reports on the functionality of this digestive enzymes targeted to human health are still scarce. Notably, the characterized proteinase shows potential for application in food industry and therapeutic and investigations are in progress in our research group.

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Legends

Fig. 1. Effect of temperature and thermal stability on the activity of proteinase with collagenolytic properties extracted from digestive viscera of peacock bass (*C. ocellaris*). (A) Optimum temperature in a range of 25-90°C. (B) Thermal stability after 30 minutes of incubation in the temperature range of 25-90°C.

Fig. 2. Effect of pH and stability on the activity of proteinase with collagenolytic properties extracted from digestive viscera of peacock bass (*C. ocellaris*). (A) Optimal pH, using different buffers in the pH range from 4.5 to 12.0. (B) pH stability after incubation for 30 minutes in the pH range 4.5 to 12.0.

Fig. 3. Substrate specificity and activity of proteinase with collagenolytic properties. (A) Substrate specificity of the proteinase with collagenolytic properties of peacock bass (*C. ocellaris*) on various collagens (bovine Achilles tendon insoluble collagen type I; collagen extracted from skin of pintado, *P. corruscans*; collagen extracted from the skin of the Nile tilapia, *O. niloticus*. The collagens were incubated with the enzyme: substrate ratio (1:200) for 1 h at 55°C. (B) Activity of proteinase with collagenolytic properties extracted from digestive viscera of peacock bass (*C. ocellaris*) against collagen type I and skin obtained from *P. corruscans* and *O. niloticus*, various incubation times (12-24-36-48 hours), at 37°C, mean of four replicates.

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Figure 1

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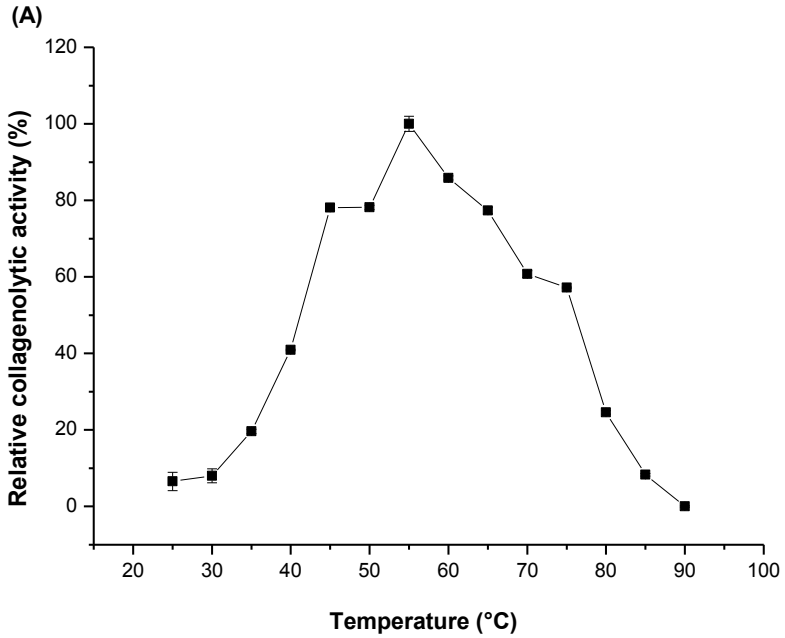

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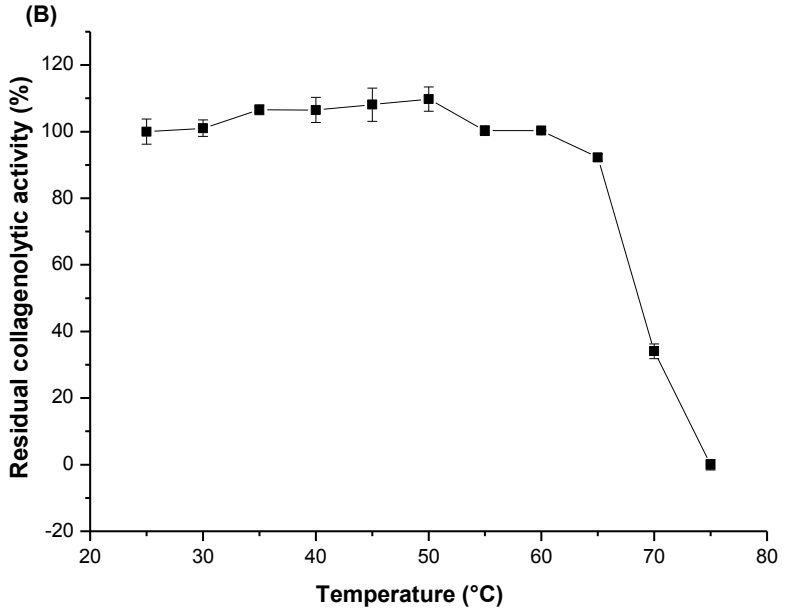

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2344 **Figure 2**

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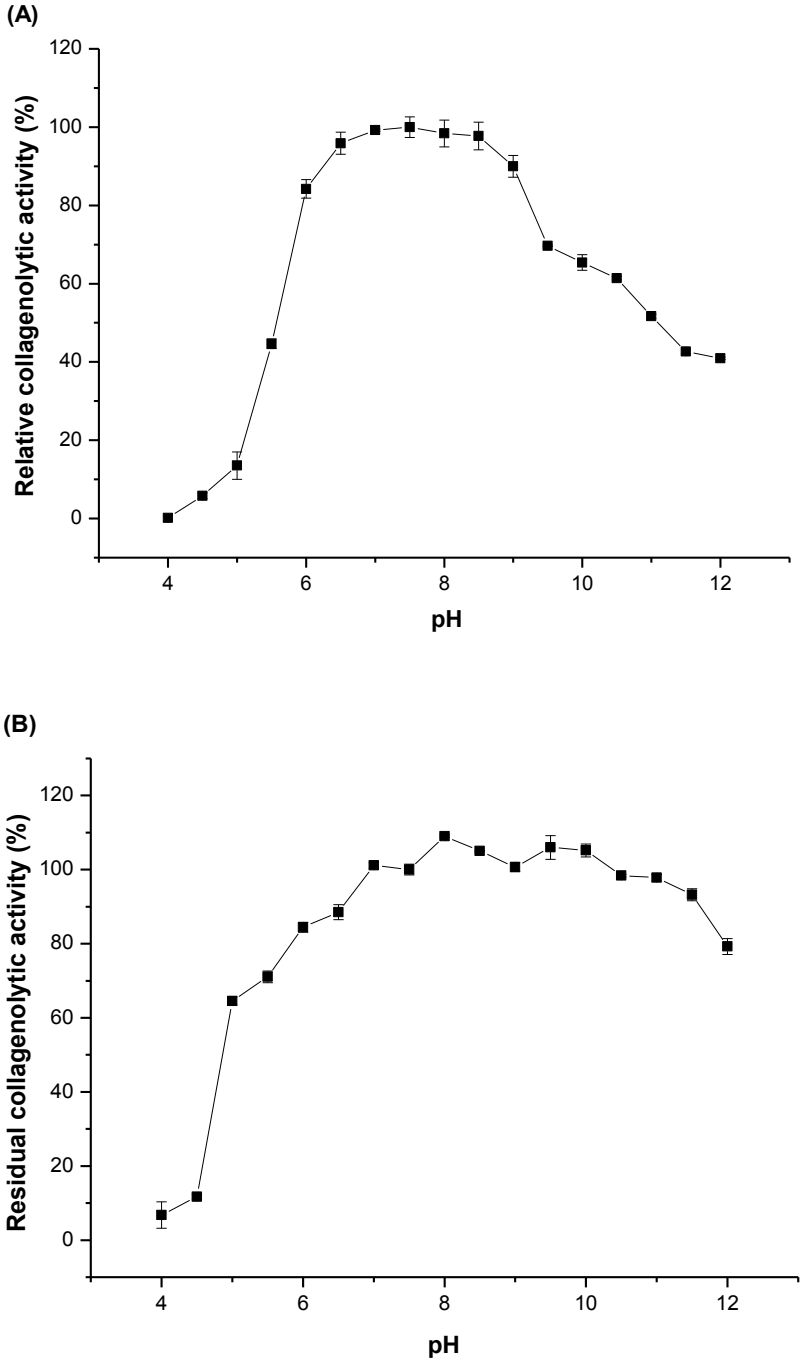

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2351 **Figure 3**

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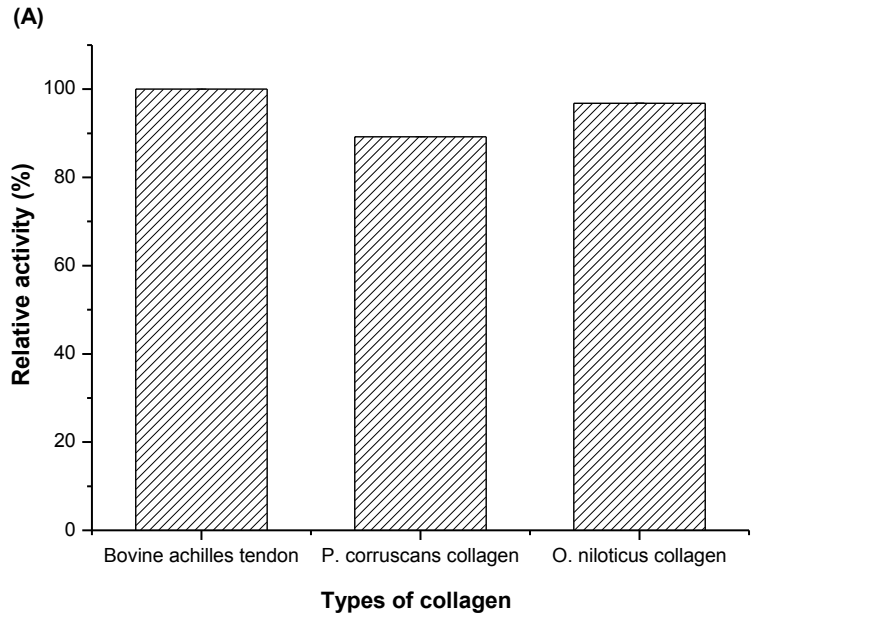

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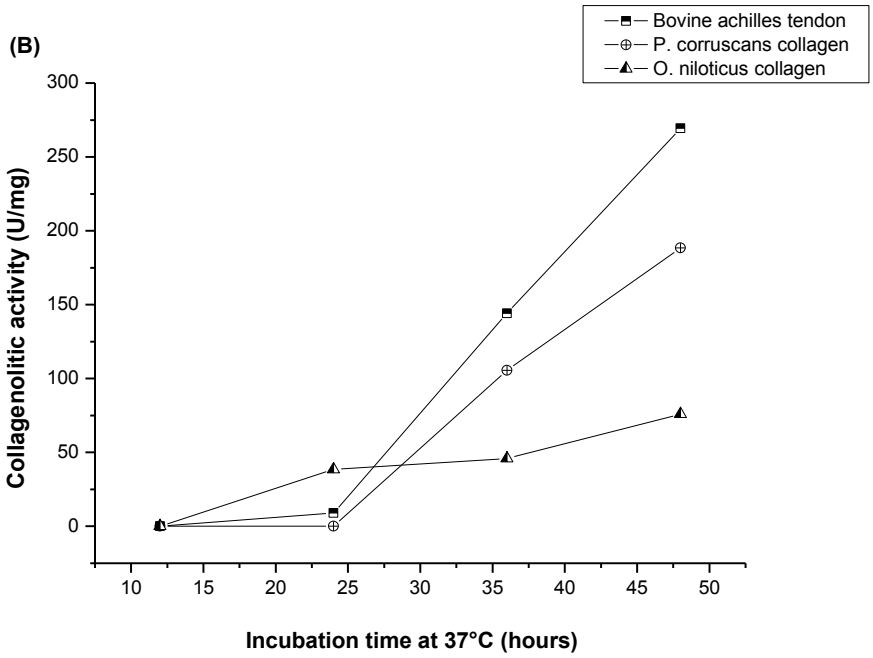

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2359 **Table 1.** Activity of alkaline digestive proteases in enzymatic extracts of peacock bass *C. ocellaris*.

Processing	Organ	Serino[1] activity (U/mg)	Serino[2] activity (U/mg)	Total protein (µg/ml)	Total protein (mg/ml)	Collagenolytic Activity	
						Enzyme activity (U/mL)	Specific activity (U/mg)
Step I	*Intestine*	9.89 ± 0.080	1.94 ± 0.123	1009.803	1.00 ± 0.012	87.87 ± 0.046	45.49 ± 0.024
	Liver	2.26 ± 0.044	1.75 ± 0.021	4019.607	4.01 ± 0.002	51.4 ± 0.022	12.81 ± 0.005
	Stomach	0.86 ± 0.070	0.59 ± 0.032	3509.803	3.50 ± 0.006	7.12 ± 0.022	2.03 ± 0.006
	Heart	1.78 ± 0.028	0.66 ± 0.020	9647.058	9.64 ± 0.001	51.4 ± 0.022	12.81 ± 0.005
	Muscle	0.59 ± 0.043	0.34 ± 0.137	2401.960	2.40 ± 0.010	11.35 ± 0.053	4.72 ± 0.022
Step II	*Intestine*	5.55 ± 0.025	0.87 ± 0.039	1794.117	1.79 ± 0.016	68.17 ± 0.021	37.99 ± 0.011
	Liver	2.20 ± 0.025	1.71 ± 0.150	4529.411	4.52 ± 0.001	77.95 ± 0.036	17.24 ± 0.008
	Stomach	0.56 ± 0.082	0.50 ± 0.137	3794.117	3.79 ± 0.008	7.02 ± 0.005	1.85 ± 0.001
	Heart	9.49 ± 0.025	9.98 ± 0.015	1147.058	1.14 ± 0.040	7.17 ± 0.045	6.25 ± 0.039
	Muscle	2.47 ± 0.072	2.48 ± 0.063	1598.039	1.59 ± 0.002	15.57 ± 0.051	9.79 ± 0.03
Step III	*Intestine*	7.88 ± 0.031	0.75 ± 0.218	1049.019	1.04 ± 0.022	76.07 ± 0.071	72.52 ± 0.068
	Liver	2.04 ± 0.026	1.74 ± 0.026	4911.764	4.91 ± 0.000	77.5 ± 0.022	15.78 ± 0.004
	Stomach	0.80 ± 0.137	0.85 ± 0.053	3666.666	3.66 ± 0.003	9.57 ± 0.020	2.61 ± 0.005
	Heart	17.4 ± 0.041	22.48 ± 0.032	450.980	0.45 ± 0.042	5.05 ± 0.013	11.19 ± 0.030
	Muscle	0.91 ± 0.063	0.77 ± 0.057	4950.980	4.95 ± 0.001	9.15 ± 0.010	1.84 ± 0.002
Step IV	*Intestine*	7.45 ± 0.01	0.93 ± 0.141	990.196	0.99 ± 0.012	93.42 ± 0.027	94.35 ± 0.028
	Liver	2.18 ± 0.028	1.50 ± 0.035	5872.549	5.87 ± 0.001	90.65 ± 0.075	15.44 ± 0.004
	Stomach	0.80 ± 0.097	0.54 ± 0.128	4519.607	4.51 ± 0.001	17.27 ± 0.014	3.83 ± 0.003
	Heart	21.53 ± 0.033	25.85 ± 0.027	431.372	0.43 ± 0.021	26.4 ± 0.003	61.2 ± 0.007
	Muscle	1.15 ± 0.041	1.20 ± 0.065	2882.352	2.88 ± 0.006	36.25 ± 0.020	12.58 ± 0.007

2360 [1]Using Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), trypsin substrate.
2361 [2]Using Succinyl-DL-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan), chymotrypsin substrate.
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Table 2. Michaelis-Menten constant (K_m and V_{max}) of collagenolytic enzyme from digestive viscera of peacock bass, *C. ocellaris*, compared to other species.

Scientific name	Species	Source extraction	Substrate*	K_m (mM)	V_{max} (U/mg)	References
Chicla ocellaris	Fish	Intestine	Azocoll	5.92	294.40	Present work
Bacillus licheniformis F11.4	Bacterium	Production**	Collagen from fish skin	0.26	0.27	Baehaki et al. (2012)
Sardinella aurita	Fish	Internal organs	SAAPNA	0.033	-	Hayet et al. (2011)
Pagrus major	Fish	Skeletal muscle	BocLeu-Lys-Arg-MCA	3.58	-	Wu et al. (2010a)
Bacillus pumilus Col-J	Bacterium	Production**	Collagen from calf skin	0.79	129.5	Wu et al. (2010b)
Bacillus cereus MBL13	Bacterium	Production**	Collagen Type I	1.31	12.54	Liu et al. (2010)
Thunnus thymus	Fish	Pylonc caeca	Collagen Type I	3.82	851.5	Byun et al. (2002)
Scomber japonicus	Fish	Internal organs	Collagen Type I	1.1	2.343	Park et al. (2002)

(−): data not reported.
*****The different types of substrate are related to the different proteinases showed the property to degrade collagen, according to the authors.
**Production - through fermentation processes with the use of microorganisms.

Table 3. Effect of ions and inhibitors on the activity of collagenolytic enzyme from Peacock bass *C. ocellaris*.

Íons and Inhibitors	**Collagenolytic activity (%)**
Metal íons (1 mM)	
Control	100.0[a]
Cd^{2+}	67.29[b]
Cu^{2+}	52.12[b]
K^{+}	101.54[a]
Zn^{2+}	72.18[b]
Al^{3+}	71.06[b]
Hg^{2+}	40.53[b]
Na^{+}	68.07[b]
Pb^{2+}	46.28[b]
Ca^{2+}	100.31[a]
Ba^{2+}	89.13[a]
Mg^{2+}	107.53[a]
Inhibitors (8 mM)	
Control	100.0[a]
PMSF	38.23[b]
TPCK	74.60[b]
TLCK	18.14[b]
Benzamidine	22.90[b]
EDTA	48.79[b]
β-Mercaptoethanol	84.89[b]

*Mean value ± standart deviation. Values followed by different superscript letters are significantly different at $P < 0.05$

8. ARTIGO IV: Aqueous two-phase partitioning and characterization of collagenolytic protease from digestive waste peacock bass (*Cichla ocellaris*).

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Aqueous two-phase partitioning and characterization of collagenolytic protease from digestive waste peacock bass (*Cichla ocellaris*)

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Abstract

Collagenolytic protease from digestive viscera of *C. ocellaris* was extracted by aqueous two-phase system (ATPS), using the PEG/phosphate. Experiments were carried out according to a 2^4-full factorial design using the PEG molar mass (MPEG), PEG concentration (CPEG), phosphate concentration (CPHOS) and pH as the independent variables, and the purification factor (PF), partition coefficient (K) and activity yield (Y) as the responses. The ATPS was composed of PEG (molar mass of 1500, 4000 and 8000 g/mol) at concentrations of 12.5, 15.0 and 17.5% (w/w) and phosphate at concentrations of 10.0, 12.50 and 15.00% (w/w). The best extraction results (K: 3.57, Y: 119.0%, PF: 8.24) were obtained at pH 8.0 using 17.5% (w/w) PEG 8000 and 15.0% (w/w) phosphate. The optimum temperature of the protease was 55°C, with optimal activity at pH 7.5. The enzyme was activated by Ca^{2+} and inhibited by Zn^{2+}, and have reduced activity when exposed to PMSF and TLCK. In the substrate specificity test, cleavage of type I collagen was detected after 24 hours of incubation with PEG-collagenolytic protease, reaching peak 48 hours. The results of this preliminary study demonstrate that the selected ATPS is satisfactorily selective for the extraction of such a collagenolytic protease. The enzyme recovered by ATPS system was able to cleave collagen type I and had physicochemical characteristics and compatible with the desired hydrolysis for industrial applications with emphasis on production of bioactive collagen peptides, meeting the needs of the food, cosmetic and pharmacologic industry.

Keywords: aqueous two-phase system, fish waste, digestive protease, collagen hydrolysis.

1. Introduction

Partitioning in aqueous two-phase systems (ATPS) is a suitable method for separating and purifying mixtures of biomolecules. Using an ATPS it is possible to generate two immiscible aqueous phases [1-3], which can be applied to the recovery and purification of biological products [4], such as proteins, enzymes, nucleic acids, viruses, antibodies and cell organelles [5,6]. The partitioning between both phases is dependent on the surface properties of the biomolecules and on the properties of the two-phase system [7].

The use of PEG/phosphate system has been widely used due to the fact that PEG have favorable physical properties providing viscosity and density differences between the phases as well as the low cost [6], viscosity and density difference between the phases. This choice question is greatly influenced by manufacturing processes, since PEG is a non-toxic substance and does not cause environmental impact [7-9].

PEG/phosphate ions are directed more hydrophobic PEG phase systems for carrying hydrophobic proteins and cations also induce the transfer of proteins to PEG phase [10]. The high water content of 60 to 95% by weight [11], guarantees the maintenance of the biological properties of proteins and low interfacial tension within the system which protect proteins [7,10]. The observed partition coefficient (Kp) is a result of van der Waals, hydrophobic, hydrogen bonding, ionic interactions of biomolecules with the surrounding phase. Partitioning of biomolecules occurrence factors are concentration, size and molecular weight of the employed polymer, pH, presence of neutral salts and the surface properties of the targeted molecule [6,12].

The method of extraction by ATPS has been employed for the recovery of digestive proteases of biomedical and industrial interest, such as pepsin [13], trypsin [14] and chymotrypsin [15]. However, the need for new alternative sources, lead us to explore the

extraction from waste of fish processing, a rich source of proteins that present biotechnological potential compatible with market needs. Among them, there are the collagenases, a group of enzymes that hydrolyze the peptide bonds of various types of collagen. These enzymes are classified into two subgroups: metalocollagenases, an endopeptidase Zn^{2+}-dependent, and serinocollagenases, which are exopeptidases, endopeptidases, oligopeptidades and ɣ peptidases groups, Ca^{2+}-dependent, being able to cleave the triple collagen helix of types I, II and III especially the extracted residues in fish processing [16].

After the collagen hydrolysis by collagenase, the so-called collagen peptides are formed [16], bioactive molecules that act as hypotensive agents (ACE inhibitory), mineral-binding, antimicrobial, immunomodulatory, cytomodulatory, antithrombotic, antioxidant and hypocholesterolemic [17,18]. Digestive viscera of fish, such as intestines and caeca are rich sources of enzymes that have the property of decomposing collagen, particularly enzymes from the group of serinocollagenases [19,20], becoming a potential source of industrial and laboratory supplies, and a new product to market enzymes.

Research efforts aim to discover new peptides that provide health benefits. Concomitantly, there is a need for new viable sources of these biomolecules that can compete in the market. Under these assumptions, this study aimed to extract/concentrate a protease with collagenolytic properties of a Neotropical fish peacock bass (*Cichla ocellaris*). For this purpose, a 2^4-full factorial design was applied to the process to identify the optimal levels of PEG molar mass, pH, phosphate and PEG concentrations for pre purification or extraction of collagenolytic protease. Finally, the main biochemical properties of the extracted collagenolytic protease were determined and its application in the hydrolysis of collagen.

2. Materials and methods

2.1. Reagents

Azo dye-impregnated collagen (azocoll), azocasein, bovine serum albumin (BSA), collagen Type I from bovine Achilles tendon (Sigma Chemical Co., St. Louis, MO), Tris (hydroxymethyl) aminomethane and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Glycine was acquired from Amersham Biosciences. HCl were obtained from Merck. The spectrophotometer used was Bio-Rad Smartspec™ 3000. Microplate spectrophotometer used was Bio-Rad xMark™. The centrifuge was BioAgency Bio-Spin and Software MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

2.2 Fish Waste

Waste of digestive viscera of peacock bass *Cichla ocellaris* were obtained from the colony of fishermen colony of Petrolândia, Pernambuco, Brazil. The material was kindly provided after evisceration process. Samples of intestine (500 g) were collected separately, packaged in plastic containers and kept on ice and transported to the Laboratório de Tecnologia de Produtos Bioativos, Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco – UFRPE, Recife, Pernambuco, Brazil, where they were stored at - 27°C for further processing.

2.3 Extraction and enzymatic steps to recovery

2.3.1 Extraction of collagenolytic protease

The processing of waste was realized in accordance with the methodology described by Teruel and Simpson [21]. The ratio of viscera to extraction buffer (0.05 M Tris-HCl pH 7.5, containing 5 mM $CaC1_2$) was 1:3 (w/v). The extraction method followed

systematic processes (Steps I, II, III and IV) in which fractions were obtained for later analysis. In step I, the material was homogenized and centrifuged. The resulting waste was again homogenized (Step II), subsequently centrifuged and performed a new maceration and homogenization (Step III). Then, the material was centrifuged, filtered in sterile syringe 0.22 µm and the resulting material was defined as stage IV. In each of maceration and homogenization step, all the viscera collected were homogenized separately for 5 minutes at a speed adjustment of 10,000–12,000 rpm (4°C) (IKA RW 20D S32, China). The homogenate was then centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 12,000 x *g* for 30 min at 4°C. The best fraction of the supernatant passed through a recovery process in aqueous two-phase system (ATPS).

2.3.2 Preparation of the aqueous two-phase systems

Aqueous two phase systems were prepared in 15 mL graduated tubes with 40% (w/w) of phosphate salts mixture and 50% (w/w) PEG solutions at different pH values (6.0, 7.0, 8.0) at 25 ± 1 (ºC) according to statistical design described in item 2.4.3. Monobasic sodium phosphate (11 - 53.1%) and dibasic potassium phosphate (45-90%) were used in different concentrations allowing for obtention of different pH values. K_2HPO_4 and NaH_2PO_4 display greater solubility than their respective monobasic and dibasic salts [22]. Water was added to a final amount of 10g. After vortex shaking for 1.0 min, the two phases were separated by settling for 60 minutes. Then, the top and bottom phases were measured and analyzed separately. The both phases were assayed for protein concentration and collagenolytic activity.

2.4 Analytical techniques

2.4.1 Azocoll assay for collagenase activity

The collagenolytic properties in the crude extract of intestine was determined according to the methodology modified by Adigüzel et al. [23], using azocoll as substrate. A reaction mixture (in quadruplicate), which contained 5 mg of azocoll, 500 µL of 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ and 500 µL of crude extract, was incubated at 55°C for 30 minutes, under stirring. Thereafter, was added 200 µL of trichloroacetic acid (TCA) and incubated to stop the reaction (room temperature). After 10 minutes, the samples were centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 10,000 x *g* for 10 minutes at 4°C. The reaction was read at 595 nm. One enzyme unit was defined as the amount of enzyme required to increase the absorbance in 0.01 at 595 nm.

2.4.2 Protein determination

The protein concentration of all tissue extracts was determined according to Smith et al. [24], using bovine serum albumin as the standard.

2.4.3 Experimental design

A 2^4-full factorial design was utilized to evaluate the influence of the four independent variables, namely, PEG molar mass (x_1), PEG concentration (x_2), phosphate salt concentration (x_3) and pH (x_4) on the selected responses: partition coefficient, purification factor and yield of the protease partial purification. The experimental design was composed of 16 runs and 3 repetitions at the central point, needed to calculate the pure error (Table 1). A linear regression model was employed to predict the response according to eq. (1):

$$R = b_0 + \sum b_i x_i + \sum b_j x_j + \sum b_{ij} x_i x_j \quad (1)$$

where b_0 is the interception coefficient, b_i and b_j are the linear coefficients, b_{ij} are the interaction coefficients and the independent variables are x_i and x_j. The goodness of the model fit was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA); the first-order model equation was determined by Fischer's test. The experimental and predicted values were compared and the developed model validated with *Statistica 8.0* [25].

Table1. Experimental design 2^4 for collagenolytic protease partition using PEG-phospate ATPS.

Variables	Levels		
	Low (-1)	Central (0)	High (+1)
[a]$M_{PEG\ (g/mol)}$	1500	4000	8000
[b]$C_{PEG\ (\%\ w/w)}$	12.5	15	17.5
[c]$C_{PHO\ (\%\ w/w)}$	10	12.5	15
pH	6.0	7.0	8.0

[a] PEG molar mass. [b] PEG concentration. [c] Phosphate concentration.

2.5.4 Determination of partition coefficient, yield and purification factor

The partition coefficient, *K* (dimensionless), for collagenolytic activity in the aqueous two-phase system was defined as the ratio of protease activity (U/mL) in the top phase (A_t) to that in the bottom phase (A_b) (Eq. 2):

$$K = \frac{A_t}{A_b} \quad (2)$$

The purification factor (*P*, dimensionless parameter) was calculated as the ratio of the specific activity of protease in the top phase and initial specific activity of crude extract. Specific activity (U/mg) is given by the ratio between volumetric activity (U/mL) and protein concentration (mg/mL) (Eq. 3):

$$P = \frac{A_t / C_t}{A_i / C_i} \quad (3)$$

A_i is protease activity in the initial crude extract; C_t and C_i are total protein concentrations, expressed as mg/mL, in the top phase and crude extract, respectively.

The yield (Y %) was determined as the ratio of protease activity (A_t) (U/mL) in the top phase and in the crude extract (A_i) (U/mL) expressed as percentage (eq. 4):

$$Y = \left(\frac{A_t}{A_i} \right) \cdot 100 \quad (4)$$

2.6 Characterization of the extracted collagenolytic protease

2.6.1 Effects of temperature and pH

2.6.1.1 Optimum temperature

The effect of temperature on the enzyme activity and stability was evaluated at temperatures ranging from 25 to 90°C. For optimal temperatures, the assays were carried out by incubating the crude extract in a water bath. The activity was calculated as the ratio between the enzymatic activity [19,20].

2.6.1.2 Optimum pH

These assays were carried out in different pH ranges using the buffers: 0.5 M citrate–phosphate (pH 4.0-7.0), 0.1 M Tris–HCl (pH 7.5-8.5) and 0.1 M glycine-NaOH (pH 9.0-12.0), containing 5 mM $CaC1_2$. The highest enzymatic activity observed for the enzyme in different buffers was defined as 100%. The activity was calculated as the ratio between the enzymatic activity [19,20].

2.6.2 Effect of metal ions

The effect of metal ions on enzyme activity was investigated by adding the monovalent (K^+ and Na^+), divalent metal ions (Hg^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+} and Ca^{2+}) and trivalent metal ion (Al^{3+}) to the reaction mixture. The final concentration of each metal ion was 1 mM. Each ion was incubated for 30 minutes at a ratio of 1:1. The activity of the incubated samples was compared with that in absence of the corresponding metal ions. The activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%) [20].

2.6.3 Effect of Inhibitors

The sensitivity of enzymes under study to some inhibitors was tested: phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor; N-*p*-tosyl-*L*-lysin chloromethyl ketone (TLCK), a trypsin-specific inhibitor; benzamidine, a trypsin inhibitor; *N*-tosyl-*L*-phenylalaninechloromethyl ketone (TPCK), a chymotrypsin-specific inhibitor, all of them diluted in DMSO; ethylenediamine tetra-acetic acid (EDTA), a chelating compound; and β-mercaptoethanol, a reducing agent, diluted in deionized water. The final concentration of each inhibitor was 8 mM. Each ion was incubated for 30 minutes at a ratio

of 1:1. The activity was compared to the reaction with absence of the corresponding inhibitors. The activity was determined as the percentage of the proteolytic activity in an inhibitor-free control sample [20].

2.6.4 Statistical analysis

All values are presented as means ± standard deviations. These data were statistically analyzed by ANOVA, followed by a post hoc (Tukey) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($p < 0.05$).

2.7 Assay for substrate specificity

The measure of the digestion of native collagen from bovine Achilles tendon type I was performed according to the method described by Park et al. [20] and Moore and Stein [26] with a slight modification. A reaction mixture, which contained 5 mg of collagen, 1 mL of 50 mM Tris-HCl (pH 7.5) that containing 5 mM $CaCl_2$ and 0.1 mL of the enzyme solution, was typically incubated at 37°C for 12, 24, 36 and 48 hours. The reaction was stopped by adding 0.2 mL of 50% trichloroacetic acid. After 10 min at room temperature, the solution was centrifuged at 1,800 × g for 20 min. The supernatant (0.2 mL) was mixed with 1.0 mL of a ninhydrin solution, incubated at 100°C for 20 min., and then cooled to room temperature. Subsequently, the mixture was diluted with 5 mL of 50% 1-propanol for an absorption measurement at 570 nm. A buffer (50 mM Tris-HCl, pH 7.5) that contained 5 mM $CaCl_2$ was used instead of an enzyme solution as the control. The concentration of hydrolyzed-amino acids was determined by a standard curve that was based on a solution of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme that is required for the hydrolysis of 1mmol of substrate per h.

3. Results and discussion

3.1 Effect of independents variables on the partition coefficient of collagenolytic protease extraction using ATPS

Experimental design results for partition coefficient (K) of collagenolytic enzyme extraction using ATPS PEG/phosphate are presented in Table 2. According to binodal curve obtained with PEG 1500g/mol [28], no phase separation was observed for runs (1 and 5) as salt and PEG concentrations were below the curve. The influence of independent variables, PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}) phosphate salt concentration (C_{PHO}) and pH on the protease partition coefficient is described by equation (5) (x_1, x_2, x_3, and x_4 are the coded values for the independent variables)

$$K = \mathbf{2.36} + \mathbf{0.79}x_1 + 0.06x_2 + 0.35x_3 + \mathbf{1.43}x_4 - \mathbf{0.87}x_1x_2 - 0.28x_1x_3 - 0.06x_1x_4 - 0.26x_2x_3 - 0.03x_2x_4 - 0.15x_3x_4 \quad (5)$$

The independents variables PEG molar mass, phosphate salt concentration and the PEG concentration and PEG molar mass interaction (bold numbers) were statistically significant at 95% confidence level. In spite of this, all others terms were maintained in the model to minimize the error determination. In conformity with the results presented in Table 2, in the majority of runs, protease partition occurred preferentially on the top phase rich in PEG.

According to equation 2, the interaction of C_{PEG} and M_{PEG} displayed negative effects, indicating that the increase on C_{PEG} and a decrease on M_{PEG} or the opposite causes an improvement on protease partition to top PEG rich phase. In this work, the results obtained indicate that the polymer size not creates a repulsive effect on protease partition, in the meantime, the interaction of both variables is more significant in the coefficient partition (runs 6 and 15).

2758 **Table 2.** Matrix of the full factorial design (2^4) with conditions and results of the
2759 collagenolytic protease partition**.**

Run	M_{PEG}[a] (g/mol)	C_{PEG}[b] (%)	C_{PHO}[c] (%)	pH	K[d]	*Y*[e] (%)	*P*[f]	FA[g] (U/ml)
1[h]	1500	12.5	10	6.0	0	0	0	0
2	8000	12.5	10	6.0	0.69	40	3.47	25.62
3	1500	17.5	10	6.0	0.44	37	3.99	23.73
4	8000	17.5	10	6.0	1.53	142	2.20	89.57
5[h]	1500	12.5	10	8.0	0	0	0	0
6	**8000**	**12.5**	**10**	**8.0**	**3.57**	**119**	**8.24**	**75.31**
7	1500	17.5	10	8.0	0.80	103	3.43	65.30
8	8000	17.5	10	8.0	2.29	123	2.43	78.09
9	1500	12.5	15	6.0	1.64	121	2.97	76.49
10	8000	12.5	15	6.0	5.36	121	1.36	76.44
11	1500	17.5	15	6.0	2.59	100	1.50	63.01
12	8000	17.5	15	6.0	5.76	150	1.78	94.58
13	1500	12.5	15	8.0	1.80	122	1.48	77.47
14	8000	12.5	15	8.0	7.19	153	2.79	96.79
15	1500	17.5	15	8.0	7.12	169	1.91	106.82
16	8000	17.5	15	8.0	0.76	76	0.54	48.03
17	4000	15	12.5	7.0	0.76	124	3.67	78.69
18	4000	15	12.5	7.0	1.04	116	3.06	73.42
19	4000	15	12.5	7.0	1.46	117	3.15	73.95

2760 [a]PEG molar mass. [b]PEG concentration. [c] Phosphate concentration. [d] Partition coefficient.
2761 [e]Yield. [f] Purification factor. [g]Collagenolytic activity in the top phase. [h] No phase formation after
2762 addition of extract.

The lower molecular weight PEGs may interact strongly with proteins, while higher molecular weight PEGs have the ability to form intramolecular bonds. Systems with PEGs of higher molecular mass give the highest resolution to exploit hydrophobicity in partitioning [29].

The transfer of the protein into one of the phases requires the breaking of the phases of the components interact to create a cavity in which the protein is added. Therefore, the energy balance can be positive or negative depending on whether the protein/polymer interactions are attractive or repulsive, and it depends on the PEG molecular weight [6,30]. The higher the molecular weight of the polymer, the lower the concentration of polymer required for phase separation. The concentration of PEG provides a similar effect, since a high polymer concentration provides a greater number of polymer units involved in the separation of the protein and thus a larger amount of the target molecule partitions into the PEG phase due to the increased number of hydrophobic interaction can be formed between the protein and the polymer molecules [6,31].

3.2 Protease partition, yield and purification factor

Protease partition using PEG/phosphate salts ATPS showed different yield values (from 37 to 169 %) (Table 2). Yields above and near 100% are frequently reported for enzyme extraction using aqueous two-phase systems. These results are probably explained by the elimination of inhibitors during the purification process and by the composition of the systems, which PEG can modifies the structure of enzyme active site and favors the enzymatic activity [32,33].

The means variables that influenced in the yield response were PEG molar mass and phosphate salts concentration. The effect of the C_{PHO} and M_{PEG} interaction was negative meaning that high phosphate concentration makes protease migrate to the PEG

rich phase. This can be explained by a salting out effect, where the biomolecule is directed to the other phase because of the great amount of salt in the bottom phase [34]. The addition of salts in an aqueous solution of PEG leads to an orderly arrangement of water molecules around the PEG molecules due to the ability of the salts to destabilize the structure of water. The formation of a layer of water around cations results in a more compact structure, with a smaller volume occupied by the PEG molecule [13]. The volume occupied by the polymer increases with both the polymer concentration and chain length (or molar mass), which results in reduced space for biomolecules in the top phase. Consequently, the biomolecules tend to partition to the bottom phase, which is inferred as volume exclusion effect [35-38].

Phosphate ions can influence the protein partition by electrostatic interactions between biomolecules and the components of aqueous two-phase system. With an increase in C_{PHO}, negatively charged proteins prefer the PEG-rich phase, because of the repulsion force caused by salt anions [39]. The behavior observed in the statistical analysis for purification factor (*P*) obtained in this study is also in agreement with yield response, i.e. the negative effect of the C_{PHO} and M_{PEG} interaction influenced negatively in the *P*.

According to statistical analysis, pH (x_4) increase has a positive effect in the yield and volumetric protease activity, i.e. at alkaline pH, protein partition preferentially to top PEG rich phase. The pH of the system influences the ionizable groups of a protein and alters the protein surface charges. At high pH values, the protein is more negatively charged than at low pH, and therefore, the partition coefficient of the protein increases with increasing the pH [31], which may be due to the electrostatic interactions between the protein and the PEG units [40].

Through the study including the statistical analysis for the three variables responses (*K*, *Y* and *P*), the best results was obtained in run 6 (pH 8.0, M_{PEG} 8000 g/mol, C_{PEG} 12.5% w/w and C_{PHO} 10% w/w). As suggested that hydrogenionic forces favour the purification in the top phase where as major purification occurred in alkaline pH. The validity of the model was verified by analysis of variance and all determination coefficient-R^2 were around 0.80, a value close to 1 indicated agreement between the experimental and model predicted. The estimated effects and the corresponding *p*-values indicate that independent variables have a significant effect on the response studied ($p < 0.05$).

The highest purification factor (PF= 8.24) was close to that (M_{PEG} 1500 g/mol, K: 1.52, PF= 5.23, pH: 6.0, y: 61.68%) found by Lima et al. [41] who utilized the same ATPS to remove collagenase from a broth fermented by *Penicillium aurantiogriseum*. The use of high molecular weight PEG (M_{PEG} 10,000 g/mol, K: 0.3, PF: 4.8, pH: 8.5, y: 131%) in ATPS system has also been successfully described for Porto et al. [37] to recover an proteases from a *Clostridium perfringens* fermentation broth. When compared to other purification techniques already employed to isolate collagenase species of fish (Table 3), the ATPS system showed PF close to extraction by DEAE Sephadex A-50, crossing the crude extract the first column purification, highlighting the advantages of using ATPS system over other enzymes of recovery from the group of collagenase, as disclosed by Rosso et al. [38] were able to recover high collagenase extracted (M_{PEG} 550 g/mol, PF: 23.5, pH 6.0, K: 1.01, y: 242%).

2837 **Table 3.** Comparison of the developed and the previously reported processes for the
2838 purification of collagenase from different sources.

Scientific name	Species	Purification method	PF	Y (%)	Reference
Cichla ocellaris	Fish	ATPS	8.24	119.0	This work
Muscles, fins and bones of mixed fish samples of haddock, herring, ground fish and flounder	Fish	After fractionation with ammonium sulfate (40 - 80%)	1.42	80.63	[42]
		Ammonium sulfate free solution	1.66	68.24	
		After purification with Sephadex G-100	15.70	22.77	
Scomber japonicus	Fish	DEAE Sephadex A-50	11.5	17.9	[20]
		Sephadex G-100	23.3	0.7	
		DEAE Sephacel	26.4	0.3	
		Sephadex G-75	39.5	0.1	
Novoden modestrus	Fish	1st DEAE-Sephadex A50	8.60	38.16	[19]
		2nd DEAE-Sephadex A50	89.39	28.77	
		Sephadex G-150	92.40	10.90	
Pseudopleuronectes americanus	Fish	Ammonium sulfate fraction (40-80%)	1.54	76.85	[21]
		IEX fraction	5.81	17.18	
Penicillium aurantiogriseum	Microorganism	ATPS	5.3	48.1	[41]
		Q-Sepharose 1st	2.8	58.0	
Vibrio vulnificus	Microorganism	Q-Sepharose 2nd	3.3	19.8	[43]
		Superdex 200 1st	7	14.4	
		Superdex 200 2nd	13.2	11.4	
Bacillus subtilis	Microorganism	Ultrafiltration	4.8	34.4	[44]
		DEAE Sepharose	6.4	17.3	
		CM cellulose	6.9	5.2	
		Butyl-Toyopearl	13.7	3.4	

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3.3 Effect of temperature and pH on collagenolytic activity

The effect of temperature and pH on the relative collagenolytic protease activity was investigated and the results of these tests are shown in Figure 1. The maximum activity was observed at 55°C (Fig. 2A), according to the described collagenolytic enzymes purified extracted from fish species that have gone through recovery and purification processes, such as those described for the winter flounder (*P. americanus*) [21], filefish species (*N. modestrus*) [19], tuna *T. thymus* [45] and mackerel species (*S. japonicus*) [20]. The enzyme reduced more than 50% of its activity after reaching the temperature of 65°C. In this temperature range, the molecular kinetic energy becomes large enough to start the denaturation of the enzyme molecule itself [46], leading to a consequent decrease in enzyme activity.

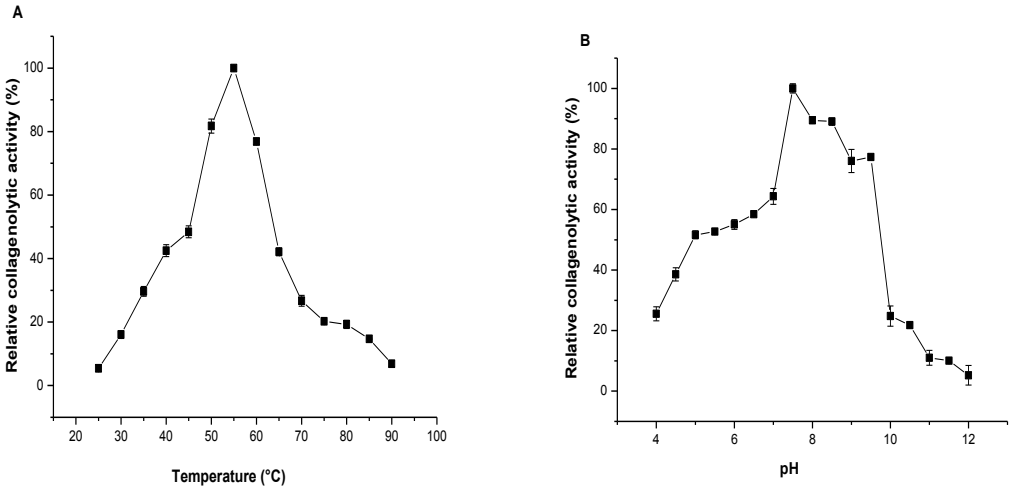

Figure 1. Effects of temperature (A) and pH (B) on the relative activities of collagenolytic-PEG protease from peacock bass (*Cichla ocellaris*) partitioning with 12.5% PEG 8000, 17.5% phosphate concentration and pH 8.0. The relative activities are expressed as percentages of the maximum ones obtained (A) in 0.05 M Tris–HCl buffer (pH 7.5) and (B) at 55°C, respectively. Each value is the mean of results of three experiments, and the error bars show the standard deviations.

The effect of pH was investigated in the range of 4-12, with the results shown in Figure 2B. Optimal activity was observed at pH 7.5, maintaining more than 60% relative activity in the range of 6.5 to 9.5. The optimum pH of results are similar to the ones Reported for winter flounder (*P. americanus*) [21], tuna (*T. thymus*) [46], mackerel species (*S. japonicus*) [20], snow crab (*C. opilio*) [47] and mixed viscera of different fish species [42]. Parameters such as temperature and pH are limiting factors for a good performance of enzymes that have collagenolytic properties producing peptides [16], which can be exploited in the food, cosmetic and pharmacologic industry. The results described in this work suggest PEG-collagenolytic extracted as alternative method for obtainment of this protease.

3.4 Metal ions and inhibitors effect on collagenolytic activity

Tests with metal ions and natural and synthetic inhibitors are described in Table 4. Only Ca^{2+} and Mg^{2+} showed no significant difference ($p < 0.05$) compared with the control group. There was inhibition, in descending order, by the following ions: Al^{3+}, Cu^{2+}, Hg^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+} and Mg^{2+}. These results are in agreement with those described for purified collagenolytic enzymes from filefish (*N. modestrus*) [19] and mackerel (*S. japonicus*) [20] for Cu^{2+}, Cd^{2+} e Mg^{2+}. Analyzing collagenolytic protease from sea bream (*Pagrus major*), Wu et al. [48] Observed the complete inhibition when in presence of Cu^{2+}, Cd^{2+} and Zn^{2+}.

Regarding the inhibitors tested, there was a significant difference between all treatments compared to the control group. The highest degree of inhibition was specific for trypsin inhibitors (Benzamidine and TLCK), but it was also detected high degree of inhibition (67.03%) for the serine protease inhibitor (PMSF). The test with metalloprotease

inhibitors (EDTA) found reduced activity, but in a lower degree in relation to that for serine protease inhibitors.

Table 4. Effect of ions and inhibitors on the activity of collagenolytic protease of *C. ocellaris* extracted ATPS system

Íons and Inhibitors	**Collagenolytic activity (%)**
Metal íons (1 mM)	
Control	100.0[a]
Cd^{2+}	54.29[b]
Cu^{2+}	41.98[b]
Zn^{2+}	74.26[b]
Al^{3+}	37.067[b]
Hg^{2+}	53.40[b]
Pb^{2+}	55.08[b]
Ca^{2+}	102.86[a]
Mg^{2+}	95.45[a]
Inhibitors (8 mM)	
Control	100.0[a]
PMSF	32.97[b]
TLCK	32.94[b]
TPCK	78.20[b]
Benzamidine	31.83[b]
EDTA	63.53[b]
β-Mercaptoethanol	40.63[b]

*Mean value ± standart deviation. Values followed by different superscript letters are significantly different at $P < 0.05$

Enzymes belonging to the class of metalloproteases generally require Zn^{2+} to maintain optimum activity and stability while have their activities significantly reduced after exposure to EDTA, a chelating agent for the Ca^{2+}. Thus, the increase in activity induced by Ca^{+} and the inhibition brought about by classical inhibitors of serine proteases besides the capacity cleave collagen type I, suggest that the enzyme in question belongs to the group

of serine collagenolytic proteases, such as those described for the species of tuna fish *T. thymus* [46], filefish *N. modestrus* [19], Mackerel *S. japonicus* [20] and for sea bream *P. major* [48].

3.5 Use of bovine Achilles tendon insoluble collagen

The assay using type I collagen demonstrating specificity for the substrate is shown in Figure 2. The PEG-collagenolytic extracted protease was able to cleave collagen starting from 24 hours of incubation and reaching maximum activity after 48 hours of incubation. There is no record in less than 24 hours, taking as standard the intervals between readings taken (12 hours). The results are similar to those reported for collagenase purified from the waste fish processing, such as those described for winter flounder (*P. americanus*) [21], Mackerel (*S. japonicus*) [20] and iced cod (*Gadus morhua*) [49].

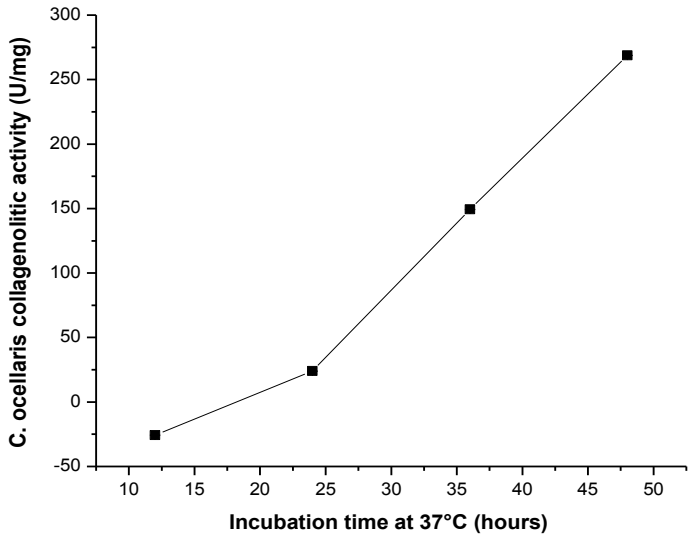

Figure 2. Activity of fish PEG-collagenolytic protease on extracted from digestive viscera of peacock bass (*C. ocellaris*) against collagen type I, various incubation times (12-24-36-

48 hours), at 37°C, mean of four replicates. The collagen was incubated with the purified enzyme: substrate ratio (1:200).

4. Conclusions

There is a growing need for methods of protein extraction and purification that are fast, efficient and economically viable. In this line, the aqueous two-phase systems (ATPS) are a potential alternative. In this study, we successfully recover a protease with collagenolytic properties from peacock bass (*C. ocellaris*) through ATPS system (PEG/phosphate) with the same physical and chemical characteristics (temperature, pH, sensitivity to metal ions) desired for the industrial market. The partition coefficient (K), the activity yield in the top phase (Y^t) and the purification factor (PF) of the collagenolytic protease were determined. The highest value of PF (8.24) Obtained using 20.0% (w/w) PEG 8000 and 12.5% (w/w) phosphate at pH 8.0, was better than those reported in the literature for similar ATPS. This technique can be used in the initial stages of a purification process allowing the removal of contaminants by a fast, simple and economical process, or even as a primary purification step, where it meets the need for isolation and production of collagen peptides, as reported here. Thus, given its simplicity and high performance by adding lower investments make ATPS a promising option for collagenase recovery from fish processing waste on an industrial scale, targeting the food, pharmaceutical and cosmetics industry.

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9. ARTIGO V. Isolation and characterization of skin collagen of peacock bass (*Cichla ocellaris*)

VAGNE DE MELO OLIVEIRA, THIAGO PAJEÚ NASCIMENTO, ROBSON COELHO DE ARAÚJO NERI, FLÁVIA THUANE DUARTE DO MONTE, CAIO RODRIGO DIAS ASSIS, RANILSON SOUZA BEZERRA, ANA LÚCIA FIGUEIREDO PORTO

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ISOLATION AND CHARACTERIZATION OF SKIN COLLAGEN OF PEACOCK BASS (*CICHLA OCELLARIS*)

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Running title: Characterization of fish skin collagen and peptide production.

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Abstract

Soluble collagen extracted with pepsin (PSC) was isolated and characterized from the skin of Neotropical fish peacock bass *Cichla ocellaris*. The yield on the dry weight was 3.9%. Its electrophoretic profile had consisted of two different α chains (α 1 and α 2) and their dimers (β chain), featured as collagen type I. The activity with ultraviolet (UV) showed maximum absorption at 211 nm and the solubility of NaCl up to 3% (w/v). Specificity test was applied to collagen. Cleavage have been detected after 24 hours incubation with collagenase pre purified using aqueous two-phase system (ATPS) obtaining, thereafter, native bioactive collagen peptides. The results suggest possible employment of *C. ocellaris* collagen as alternative source of biomolecules, particularly for applications in the food, biomedical and nutraceutical industry through the generated peptides.

Keywords: ATPS, collagen, pepsin soluble collagen, fisheries by-products.

1. Introduction

The collagen, a fibrous protein is the main component of connective tissues in mammals, representing 30% of the total protein content and 6% by weight of the human body (Chung & Uitto, 2010; Daboor, Budge, Ghaly, Brooks & Dave, 2010; Ferreira, Gentile, Chiono & Ciardelli, 2012). It is a type of selected proteins during evolution to perform different functions, primarily structural. It is the main structural component of bones, cartilages, skin, tendons, ligaments, smooth muscle, blood vessels, teeth, corneas, basal lamina and other organs of vertebrates (Chung & Uitto, 2010).

Currently, the family of collagens comprises more than 28 genetically distinct types (He & Theato 2013). The type I collagen is the most abundant, widely distributed in the body. It occurs as classically termed collagen fibril structures that form bones, dentin, tendons, capsule bodies, corneal and dermal blood vessels, plays an important role in the morphogenesis and cell metabolism of new tissue, providing mechanical and biochemical properties (Söderhäll, Marenholz & Kerscher, 2007; Chung & Uitto, 2010; Ferreira et al., 2012; Makareeva & Leikin, 2014).

Because of its physico-chemical characteristics, the collagen has been one of the biomaterials most currently used and can be easily modified through reactions of their functional groups by introducing crosslinks or grafts of biological molecules, creating a wide variety of materials mechanical or biological properties adapted. It is a source for bone tissue engineering because of their abundance, biocompatibility, high porosity and ease of combination with other materials, easy processing, low antigenicity and absorption in the body. Historically, industrial uses in the form of collagen leather and gelatin are widespread, including gelatin photographic applications in cosmetics, food and pharmaceuticals (Ferreira et al, 2012). In medicine, the collagen peptides have many applications such as in ulcerative processes (Azuma, Osaki, Tsuka, Imagawa, Okamoto &

Minami, 2014) in sutures, hemostatic agents, replacement and/or regeneration of tissue (vessels, bone, cartilage, skin, blood, trachea, esophagus), plastic surgery (lips, skin), membrane oxygenator, contraceptives, biodegradable matrices, implants, corneal bandage contact lenses, osteodystrophy (Elsaid & Chichester, 2006; Ferreira et al, 2012).

Among several species used for obtaining collagen, fish deserve attention, especially due to: 1) wide availability; 2) absence of risk of disease transmission, such as bovine collagen and the possible association with bovine spongiform encephalopathy (BSE), spongiform encephalopathy transmitted (TSE) and mouth disease (FMD) due to the large evolutionary distance between fish and humans; 3) religious obstacles; 4) high-yield in the process of extraction and 5) lack of toxicity (Veeruraj, Arumugam & Balasubramanian, 2013; Veeruraj, Arumugam, Ajithkumar & Balasubramanian, 2015).

Skin collagen has been extracted and characterized from several fish species such as brownbanded bamboo shark *Chiloscyllium punctatum* and blacktip shark *Carcharhinus limbatus* (Kittiphattanabawon, Benjakul, Visessanguan & Shahidi, 2010), ornate threadfin bream *Nemipterus hexodon* (Nalinanon, Benjakul, Kishimura & Osako, 2011), balloon fish *Diodon holocanthus* (Huang, Shiau, Chen & Huang, 2011), striped catfish *Pangasianodon hypophthalmus* (Singh, Benjakul, Maqsood & Kishimura, 2011), marine eel-fish *Evenchelys macrura* (Veerurajet al., 2013), olive flounder *Paralichthys olivaceus*, black rockfish *Sebastes schlegeli*, sea bass *Lateolabrax maculatus* and red sea bream *Pagrus major* (Cho, Jin, Rha, Kim & Hwang, 2014) and squid *Doryteuthis singhalensis* (Veeruraj et al., 2015).

According to MPA (Brazilian Fisheries and Aquaculture Ministry) (2011), Brazil produced approximately 1.4 millions tons (t), being among the twenty larger fish producers of the world. Of these, there were 9.304,400 t of peacock bass *Cichla ocellaris* (Schneider, 1801). The *C. ocellaris* is a common species in the Amazon basin, carnivorous habits,

which was widespread and adapted along the São Francisco River basin. This species is not cultured and the capture is focused on food and reports on the nutritional quality of his flesh are scarce. Their waste (skins and scales) are rich sources of collagen and were poorly exploited. In this perspective, the objective of this study was to isolate and extract the collagen from the skin of peacock bass *Cichla ocellaris*, as well as characterize and conduct a substrate specificity test with different species of Neotropical fish.

2. Materials and methods

2.1 Materials

Type I collagen from calf skin was purchased from Sigma-Aldrich (St. Louis, MO, USA), Glycine was acquired from Amersham Biosciences (Piscataway, NJ, USA). HCl were obtained from Merck. The spectrophotometer used was Bio-Rad Smartspec™ 3000. Microplate spectrophotometer used was Bio-Rad xMark™. The centrifuges were BioAgency Bio-Spin and Software MicroCal® Origin® Version 8.0 (Northampton, MA, USA).

2.2 Extraction of collagen from *C. ocellaris*

2.2.1 Collection and storage of samples

Adult specimens of peacock bass (*C. ocellaris*) with total length of 70.5 ± 1.5 cm and weighing 4.3 ± 0.5 kg were obtained from the fishermen colony of the town of Petrolândia, Pernambuco, Brazil. The specimens were transported to the Laboratório de Enzimologia (UFPE). Upon arrival, fish were washed using distilled water and after that were deskinned. The skin was washed with cold water (5 – 8ºC) and cut into small pieces

(0.5 – 0.5 cm^2). The prepared skin samples were packed in polyethylene bags and kept at - 20 ºC for further assays.

2.2.2 Pretreatment of skin

To remove non-collagenous proteins, the prepared fish skin was mixed with 0.2 M NaOH at a skin/alkali solution ratio of 1:10 (w/v). The mixture was continuously stirred for 3 h at 4ºC and the alkali solution was changed every 30 minutes. The treated skin was then washed with cold distilled water until a neutral or faintly basic pH of wash water was reached. The pH of wash water was determined using a digital pH meter (Sartorious North America, Edgewood, NY, USA). Then was added 10% butyl alcohol in the ratio of 1:10 (w/v) to remove fats. The mixture was continuously stirred for 6 h at 4ºC and the skin was washed as previously described. Then was added 3 % hydrogen peroxide at a ratio of 1:10 (w/v) for whitening the skin and was carried out in the wash (Singh et al., 2011).

2.2.3 Extraction of pepsin soluble collagen (PSC)

PSC was prepared by the method of Nagai & Suzuki (2000) with a slight modification. The pepsin soluble collagen was obtained through the incubation of the insoluble material obtained in the previous steps with commercial pepsin solution (EC 3.4.23.1; Sigma, MO) at enzyme/skin ratio of 1:20 (w/w) for 3 days. The resulting viscous solution was centrifuged at 20,000 x *g* for 30 min at 4°C. The supernatants of the extract were combined and salted-out by adding NaCl to give a final concentration of 0.9 M, followed by precipitation of the collagen by the addition of NaCl to the final concentration of 2.5 M in 1.5 M Tris–HCl (pH 8.8). After standing overnight, the resulting precipitate was collected by centrifuging at 20,000 x *g* for 60 min and then dissolved in 10 volumes of 0.5 M acetic acid. The solution obtained was dialyzed against 0.1 M acetic acid and

subsequently against distilled water. The dialysate was freeze-dried and referred to as acid soluble collagen (PSC). The yield of PSC was calculated as: Yield (%) = (M/M_0) x 100, where M is the weight of lyophilized collagen (g), and M_0 is the weight of drought scale used (g).

2.3 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed following the method of Laemmli (1970). Solubilized samples (25 µg of protein) were mixed at a ratio 1:1 (v/v) with the sample buffer (containing 0.5 M Tris HCl, pH 6.8, 4 % SDS, 20 % glycerol and 10 % b-ME), heated in a bath (IKA® Works Inc., China) at 85 °C for 5 minutes and were loaded on to polyacrylamide gels comprising a 7.5 % running gel and a 4 % stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel for 1 h and 30 min. using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.05 % (w/v) Coomassie blue R-250 in 15 % (v/v) methanol and 5 % (v/v) acetic acid and destained with 30 % (v/v) methanol and 10 % (v/v) acetic acid. Type I collagen from calf skin was also prepared following similar procedure and 10 µl were loaded as standard collagen. High-molecular-weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins.

2.5 UV absorption spectrum

UV absorption spectra from spotted peacock bass (*C. ocellaris*) skin was carried out according to Nalinanon et al. (2011), using a GeneQuant 1300. The collagen samples were dissolved in 0.5 M acetic acid solution with a sample/solution ratio of 1:1000 (w/v). The solutions were then placed into a quartz cuvette with a path length of 1 mm. UV spectra were measured at wavelength 200 – 280 nm.

2.5 Solubility

The solubility was determined by the method of Montero, Jimennez-Colmenero & Borderias (1991) with a slight modification. Collagens were dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/mL and the mixture was stirred at 4°C for 24 h. Thereafter, the mixture was centrifuged at 20,000 x *g* for 60 min at 4°C. The supernatant was used for solubility study.

2.5.2 Effect of NaCl on solubility

Collagen solution (0.5 mL) was mixed with 0.5 mL of NaCl in 0.5 M acetic acid at various concentrations to give the final concentrations of 0 %, 1 %, 2 %, 3 %, 4 %, 5 % and 6 % (w/v). The mixture was stirred continuously at 4°C for 60 min., followed by centrifuging at 20,000 x g for 60 min at 4°C. Protein content in the supernatant was measured and the relative solubility was calculated as previously described. All values are presented as means ± standard deviations. These data were statistically analyzed by ANOVA, followed by a post hoc (Tukey) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($p < 0.05$).

2.6 Extraction and enzymatic steps to recovery

2.6.1 Fish Waste

Intestinal waste of peacock bass (*C. ocellaris*) were obtained from the fishermen colony of the town of Petrolândia, Pernambuco, Brazil. The viscera were kindly provided after evisceration process. Samples of 300 g intestine were collected separately, packaged in plastic containers and kept on ice and transported to the Laboratório de Enzimologia, Centro de Ciências Biológicas, Departamento de Bioquímica, Universidade

Federal de Pernambuco, Recife, Pernambuco, Brazil, where they were stored at - 27°C for further processing.

2.6.2 PEG-Collagenolytic protease extraction steps and protein determination

The processing of waste was realized in accordance with the methodology described by Teruel & Simpson (1995). The ratio of viscera to extraction buffer (0.05 M Tris-HCl pH 7.5, containing 5 mM $CaC1_2$) was 1:3 (w/v). The extraction method followed systematic processes (Steps I, II, III and IV) for later analysis by which fractions were obtained. In step I, the material was homogenized and centrifuged. The resulting waste was again homogenized (Step II) and subsequently centrifuged through new maceration and homogenization (Step III). Then, the material was centrifuged and filtered in sterile syringe 0.22 µm and the resulting material was defined as step IV. In each maceration and homogenization step, all the viscera collected were homogenized separately for 5 minutes at an adjusted speed of 10,000 – 12,000 rpm (4°C) (homogenizer IKA RW 20D S32, China). The homogenate was then centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 12,000 x *g* for 30 min at 4°C. The best fraction of the supernatant passed through a recovered process in aqueous two-phase system (ATPS), using pH 8.0, 17.5% (w/w) PEG 8000 and 15.0% (w/w) phosphate salt. The protein concentration was determined according to Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano, Fujimoto, Goeke, Olson & Klenk (1985).

2.7 Hydrolysis of collagen by PEG-collagenolytic protease

The digestion measure of peacock bass (*C. ocellaris*) native collagen was according to the method of Moore & Stein (1954) and Park, Lee, Byun, Kim & Kim (2002) with a

slight modification. A reaction mixture, which contained 25 mg of *C. ocellaris* skin collagen, 5 mL of 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$ and 0.5 mL the PEG-collagenolytic protease, was typically incubated at 37°C for 12, 24, 36 and 48 hours. The reaction was stopped by adding 0.2 mL of 50% trichloroacetic acid. After 10 min (at room temperature), the solution was centrifuged at 1,800 x g for 20 min. The supernatant (0.2 mL) was mixed with 1.0 mL of a ninhydrin solution, incubated at 100°C for 20 min and then, cooled to room temperature. Subsequently, the mixture was diluted with 5 ml of 50% 1-propanol for an absorption measurement at 570 nm. A buffer (50 mM Tris-HCl, pH 7.5) that contained 5 mM $CaCl_2$ was used instead of an enzyme solution as the reference. The concentration of hydrolyzed-amino acids was determined by a standard curve that was based on a solution of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme that is required for the hydrolysis of 1mmole of substrate per h.

3. Results and Discussion

3.1 Yield of PSC from the skin of peacock bass (*C. ocellaris*)

The yield of collagen isolated from *C. ocellaris* was 2.9%, based on dry weight of skin. The high yield of peacock bass may be due to the possibility of its skin collagen molecules being connected via covalent cross-links through condensation of aldehyde groups in the telopeptides of collagen and intermolecular areas, a fact that causes a decrease in the solubility of the protein in acetic acid (Foegeding, Lanier & Hultin, 1996; Zhang, Liu, Li, Shi, Miao & Wu, 2007). Pepsin was able to cleave specifically the telopeptide region of the collagen at the species tested. Thus, pepsin can be used as an auxiliary tool for increasing the yield of the extraction of collagen from the skin of peacock bass.

Other works reported in the literature showed the following yield for the extraction of collagen from skin of horse mackerel *Trachurus japonicus* (1.51%), lizardfish *Saurida spp.* (0.79%), flying fish *Cypselurus melanurus* (0.72%), yellowback seabream *Dentex tumifrons* (0.90%) and grey mullet *Mugil cephalis* (0.43%) (Thuy, Okazaki & Osako, 2014), skipjack tuna *Katsuwonus pelamis* (5.62%) (Di, Chang-Feng, Bin, Guo-Fang & Zhong-Rui, 2014), bigeye snapper *Priacanthus tayenus* and *Priancanthus macracanthus* (7.7% and 7.1%) (Benjakul, Thiansilakul, Visessanguan, Roytrakul, Kishimura, Prodprand & Meesane, 2010), brownbanded bamboo shark *Chiloscyllium punctatum* (8.86%) (Kittiphattanabawon, Benjakul, Visessanguan, Kishimura & Shahidi, 2010) and balloon fish *Diodon holocanthus* (19.5%) (Huang, Shiau, Chen & Huang, 2011).

These variations in yields are related both to different biological conditions, in which each species is subjected, as well as the conditions and methods of extraction (Regenstein & Zhou, 2007). Also, is noteworthy that the structural differences in collagen molecules are directly related to the performance of the extraction, because if the molecules in telopeptide region are highly cross-linked, collagen solubility in acid tends to decrease (Foegeding et al., 1996). The difference in efficacy of pepsin in extracting collagen might be determined by fish species, collagen composition and configuration or amount of pepsin used.

3.2 SDS–PAGE patterns of collagen

The skin collagen of peacock bass (*C. ocellaris*) was solubilized by pepsin and examined by SDS-PAGE, using a 7.5% polyacrylamide gel (Fig. 1). The composition of collagen subunits showed a distribution of the bands similar to that observed in type I collagen derived from bovine skin, used as standard, and collagen derived from fish skin and scales and described currently (Singh et al., 2011; Li et al., 2013; Veeruraj, et al.,

2013). The estimated molecular weight of α chain, using globular protein standards, was approximately 120 kDa, which might be overestimated because of the difference between globular protein and collagenous protein with high content of relatively small amino acid residues (Gly and Ala) (Muyonga, Cole & Duodu, 2004).

All collagens comprised at least two different α chains (α1 and α2) and their dimers (β chain) and trimmers (γ chain), and the intensity of α1 chain was about double than that of α2 chain. One of the characteristics observed in the electrophoretic profile of type I collagen is a 2:1 ratio in the intensity of bands α1 and α2, respectively (Singh et al., 2011). The β and γ subunits present molecular mass above 200 kDa (Foegeding et al., 1996). According to the results of the electrophoresis, the data suggest that the extracted collagen is type I, which was already described for balloon fish *D. holocanthus* (Huang et al., 2011), ornate threadfin bream *Nemipterus hexodon* (Nalinanon et al., 2011) and marine eel-fish *E. macrura* (Veeruraj et al., 2013).

3.3 UV-vis spectra

The UV absorption spectrum of collagen was obtained for the skin of peacock bass (*C. ocellaris*) at the wavelength range of 200 - 280 nm (Fig. 2). Most proteins have a maximum ultraviolet absorption at 280 nm (Huang et al., 2011). The assay for *C. ocellaris* showed a higher rate of absorption of ultraviolet rays at 211 nm. Other works exhibited a higher absorption rate of ultraviolet rays at 230 nm for collagen from skin of ornate threadfin bream *N. hexodon* (Nalinanon et al., 2011), collagen of frog skin, 236 nm (Li, Liu, Gao & Chen, 2004), balloon fish *Diodon holocanthus*, 240 nm (Huang et al., 2011), eel-fish *Evenchelys macrura*, 228 nm (Veeruraj et al., 2013), skipjack tuna *K. pelamis*, 220 nm (Di et al., 2014) and squid *Doryteuthis singhalensis*, 222 nm (Veeruraj et al., 2014).

The absorption spectrum of ultraviolet rays can measure the amount of tyrosine and phenylalanine, in addition to being able to measure the integrity of the non-helical telopeptides (Na, 1988). The phenylalanine and tyrosine are sensitive chromophores and absorb ultraviolet rays in a range between 251 and 253 nm (Liu & Liu, 2006). Most works on processes of extraction and characterization of collagen reports a small amount of these amino acids in this protein (Liu & Liu, 2006; Huang et al., 2011; Singh et al., 2011).

Furthermore, according to Liu & Liu (2006) due to the characteristics of collagen, it can be observed the integrity of the non-helical telopeptides regions and to verify the presence of protein contaminants. Based on this information it may be suggested that collagenous material extracted in this work in both cases is type I collagen and has no large amount of contaminating proteins. A similar result was described for cellate puffer fish *Takifugu rubripes* (Nagai, Araki & Suzuki, 2002), skate *Raja kenojei* (Hwang, Mizuta, Yokoyama & Yoshinaka, 2007) and brownbanded bamboo shark *C. punctatum* (Kittiphattanabawon et al., 2010) in which the type I collagen was the main type isolated.

3.4 Effect of NaCl concentration on collagen solubility

The effect of NaCl on the solubility of extracted skin of peacock bass (*C. ocellaris*) is shown in Fig. 3. The PSC remained more than 90% soluble under most of the variations in NaCl concentration (0 - 3 %), having precipitation of most of the its content when the salt concentration achieves 4 % in the solution. Similar results were reported for the species skipjack tuna K. *pelamis* in which collagen lost its solubility when the NaCl concentration was above 2% (w/v). The decrease in solubility of collagens could be described as being due to a “salting out” effect, which occurred at relatively high NaCl concentrations (Li, Wang, Chi, Zhang, Gong, Tang, Luo & Ding, 2013; Di et al., 2014)

An increase in ionic strength causes a reduction in protein solubility by enhancing hydrophobic–hydrophobic interactions between protein chains, and increasing the competition for water with the ionic salts, leading to the induced protein precipitation (Bae, Osatomi, Yoshida, Osako, Yamaguchi & Hara, 2008; Huang et al., 2011; Di et al., 2014). The greater solubility of PSC could be due to the partial hydrolysis of high MW cross-linked molecules by pepsin (Singh et al., 2011). Considerable decrease also was noticeable when the concentration was increased to more than 4% for pepsin-soluble collagens from the skin of balloon fish *Diodon holocanthus* (Huang, Shiau, Chen & Huang, 2011). Expressive decrease in PSC solubility was observed with 3% NaCl or above for collagen extracted from the skin of *Evenchelys macrura* (Veeruraj et al., 2013).

3.5 Assay for substrate specificity: pre-purified enzyme by ATPS

The specificity test of the collagen extracted from the skin of *C. ocellaris* is illustrated in Figure 4. To this aim, the collagenolytic enzyme was extracted and pre-purified through the aqueous two-phase system (ATPS), as described above. The isolated collagenolytic protease was able to cleave native collagen from 24 hours of incubation. Lower cleavage time has not been detected, but reaching its peak at 48 hours of incubation. The collagen molecule consists of three polypeptide chains linked together in a triple helix (Ferreira et al., 2012). Enzymatic specificity test using collagenolytic protease have been described by Teruel & Simpson (1995) for winter flounder (*P. americanus*), Park et al. (2002) for mackerel (*S. japonicus*) and Herreiro-Hernandez, Duflos, Malle & Bouquelet (2003) for iced cod (*G. morhua*). Once one collagenolytic enzyme acts degrading collagen types, it is likely that other proteases may carry on the process (Herreiro-Hernandez et al., 2003).

4. Conclusions

Collagen was isolated from the skin of Neotropical fish peacock bass *Cichla ocellaris*, through employment with pepsin (PSC), obtaining income compatible with the extractions previously described in the literature for collagen from the skin of fish with employment of the same method. Based on SDS-PAGE patterns and UV absorption spectrum of collagen it may be suggested that collagenous materials extracted in this work in both cases is type I, the most commercially important. The extracted skin showed decreased solubility in the presence of NaCl at concentrations equal to or greater than 4%. The collagen was further cleaved by a prepurified collagenolytic enzyme through ATPS system, indicating specificity of the group of collagenase enzymes. Therefore, the skin collagen extracted from *C. ocellaris* may be an alternative, especially as a source of collagen and bioactive collagen peptides for industrial purposes, particularly in the food, biomedical and nutraceutical industry.

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Figure Captions

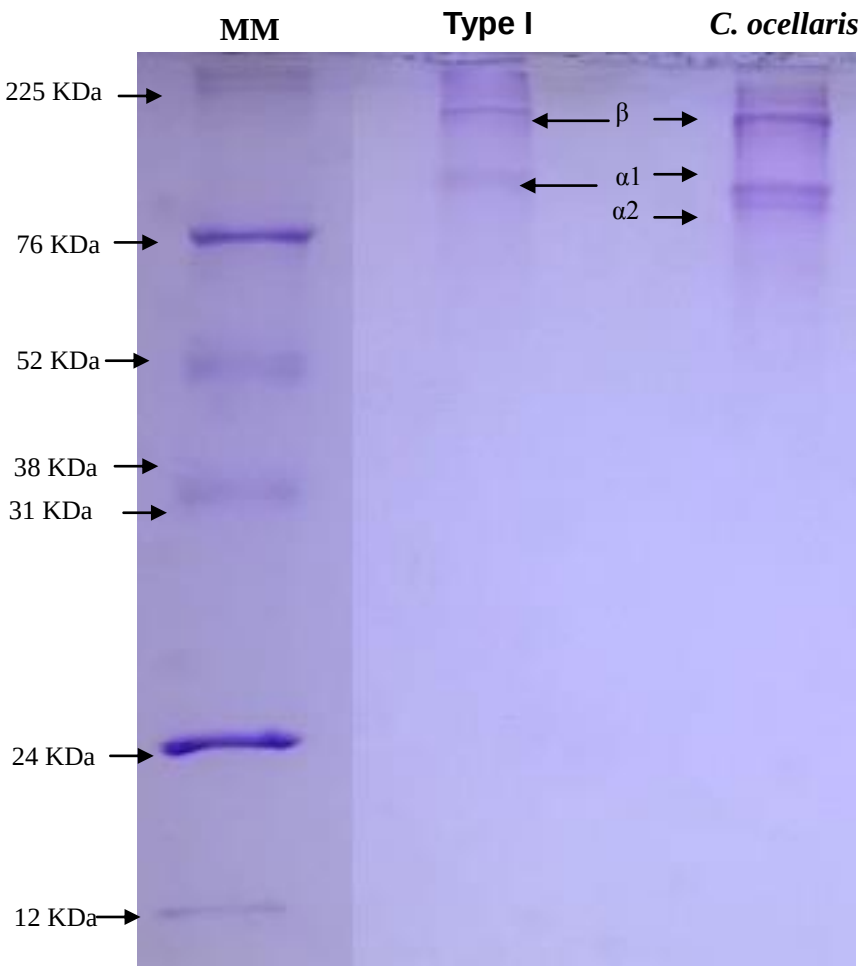
Fig. 1. SDS-PAGE patterns of pepsin soluble collagen (PSC) from the peacock bass *C. ocellaris* skin. M, high-molecular weight markers; I, type I collagen from calf skin.

Fig. 2. UV-Vis spectra of collagen isolated from the skin of peacock bass *C. ocellaris* in 0.5 M acetic acid (1:1).

Fig. 3. Relative solubility (%) of PSC from peacock bass *C. ocellaris* skin in 0.5 M acetic acid with different NaCl concentrations.

Fig. 4. Substrate specificity and activity of proteinase with collagenolytic properties. The collagen was incubated with the enzyme: substrate ratio (1:200) for 1 h at 55°C. Activity of proteinase with collagenolytic properties extracted from digestive viscera of peacock bass *C. ocellaris*, through aqueous two-phase system (ATPS), various incubation times (12-24-36-48 hours), at 37°C, mean of four replicates.

Figure 1


MM
Type I
C. ocellaris
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76 KDa
52 KDa
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24 KDa
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β
α1
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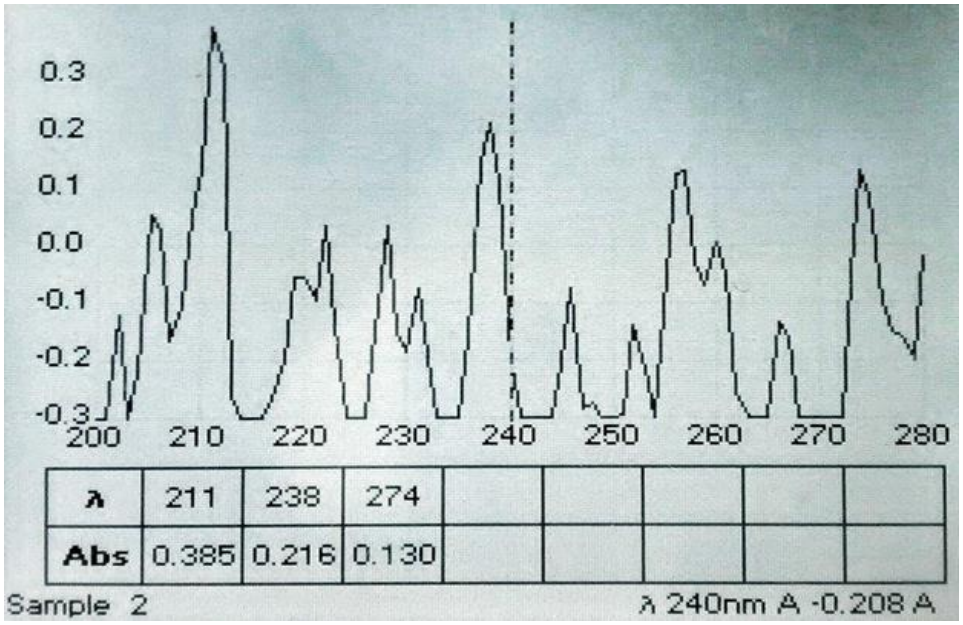
Figure 2


0.3
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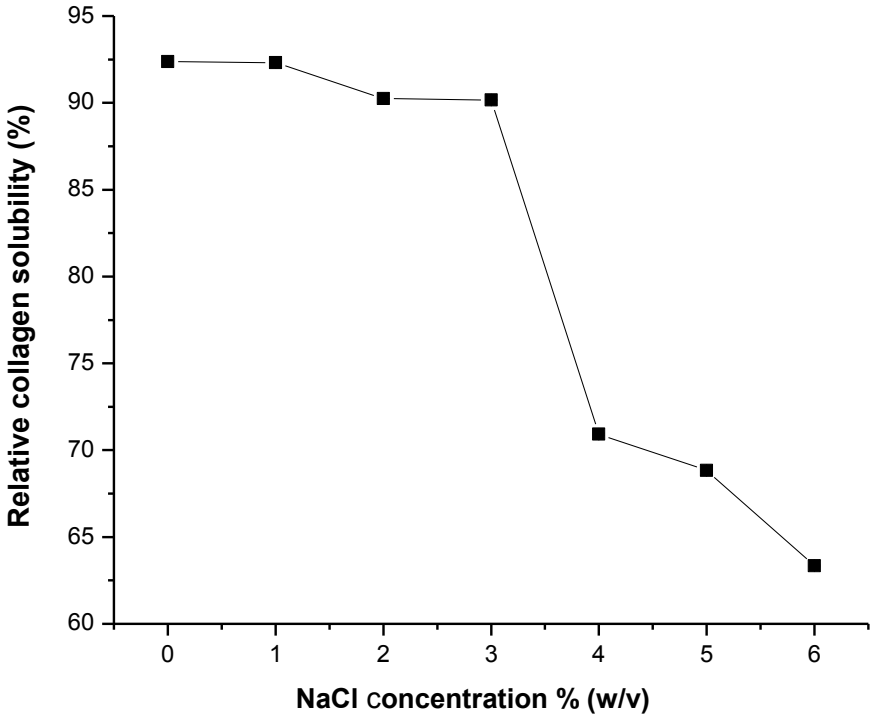
-0.1
-0.2
-0.3
200 210 220 230 240 250 260 270 280
λ 211 238 274
Abs 0.385 0.216 0.130
Sample 2
λ 240nm A -0.208 A

3598 **Figure 3**



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3612 **Figure 4**

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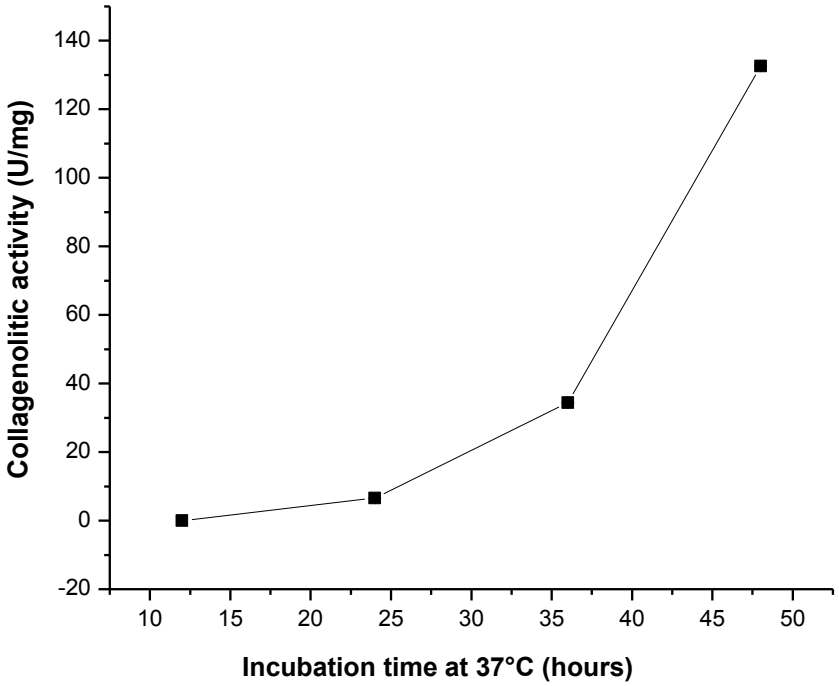

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10. CONSIDERAÇÕES FINAIS

Os ensaios desenvolvidos no presente trabalho demonstraram que,

i. Todas as três espécies de peixes Neotropicais pré-selecionadas (arabaiana *Seriola dumerili*; pescada-branca *Cynoscion leiarchus*; e tucunaré *Cichla ocellaris*) apresentaram potencial para fornecimento de moléculas biologicamente ativas;

ii. As tripsinas de *S. dumerili*, *C. leiarchus* e de *C. ocellaris* apresentaram propriedades físico-químicas e cinéticas similares a enzimas de outras espécies de peixes após processos de isolamento, sugerindo a potencialidade destas, tendo em vista que o objeto do presente estudo se deu a partir do extrato bruto, após sucessivas etapas de extração, destacando-se a tripsina de *S. dumerili* (1.46 U/mg atividade especifica, temperatura ótima de 60°C, mantendo-se estável de 25-60°C, K_m 0.35 mM e $V_{máx}$ 391.07 U/mg, respectivamente);

iii. As quimotripsinas extraídas também apresentaram propriedades físico-quimicas de interesse para aplicação industrial, simalares as enzimas de outros organismos marinhos, destacando-se a enzima de *S. dumerili* (0.71 U/mg atividade especifica, temperatura ótima de 40°C, mantendo-se estável de 25-45°C, K_m 0.34 mM e $V_{máx}$ 108.08 U/mg, respectivamente);

iv. As três espécies de peixes demonstraram potencial para fornecimento de enzima com propriedades colagenolíticas (colagenases), em ordem crescente: *S. dumerili* (42.44 U/mg), *C. leiarchus* (98.08 U/mg) e de *C. ocellaris* (106.82 U/mg). Partindo desta análise, permaneceu-se com as duas últimas espécies para prosseguimento nos processos laboratoriais, embora, de acordo com a literatura cientifica, as três espécies detacam-se quando comparadas a colagenases determinadas a partir do extrato bruto de espécies tropicais e subtropicais;

v. A enzima extraída de *C. leiarchus* apresentou propriedades físico-químicas compatíveis com o desejado pelo mercado industrial, resistindo a temperaturas de até 70°C, com atividade ótima de 55°C, em condições de pH de 8,0, mantendo-se estável numa larga faixa, de 6,5 a 11,5. A enzima ainda foi sensível aos íons de Ca^{2+} e de Mg^{2+}, funcionando como ativadores, e aos íons de Zn^{+} e Ba^{2+}, atuando como inibidores potenciais, sendo inibida ainda por inibidores específicos de serino colagenases (TLCK, benzamidina e PMSF). Ainda, a enzima foi capaz de clivar os

diferentes tipos de colágeno testados, após 48 horas de incubação. Somados, a enzima em questão trata-se de uma serinoprotease que apresenta propriedades colagenolíticas. Todas essas características são fundamentais para a resitência deste tipo de enzima em processos biotecnológicos industriais, tais como, para sua aplicação na produção de peptídeos de colágeno.

vi. A enzima extraída de *C. ocellaris* foi a que melhor apresentou potencial para clivagem dos diferentes tipos de colágeno testados, na relação tempo de exposição *x* clivagem, clivando na seguinte ordem: colágeno bovino tipo I > colágeno da pele de *P. corruscans* > colágeno da pele de *O. niloticus*.

vii. Ainda, apresentou propriedades físico-químicas de acordo com o requerido para enzima desta categoria, tais como: atividade ótima a 55°C e em pH de 7,5, mantendo-se estável na faixa de 25-60°C e de 6,5-11, respectivamente. Além do que, a enzima em questão foi ativada por inibidores de serinocolagenases, o íon de Ca^{2+}, e inbidida por ativadores de metalocolagenases, o Zn^{+}, além de ter sua atividade reduzida quando exposta aos inibidores de serino proteases, tais como benzamidina, PMSF e TLCK. Outra característica positiva da enzima foram seus parâmetros cinéticos, com K_m e $V_{máx}$ de 5.92 mM e 294.40 U/mg, respectivamente, indicando a especificidade desta enzima ao substrato e sua eficiência para aplicação nos processos produtivos. Com todas essas propriedades, a enzima em questão trata-se de uma serino protease que apresenta propriedades colagenolíticas, pertencente ao grupo das colagenases, sendo indicada para aplicações na produção de peptídeos d ecolágeno em escala industrial para os mais diversos fins.

viii. A protease colagenolitica de *C. ocellaris* foi recuperada através de um sistema de extração líquido-líquido (ELL), por meio do sistema de duas fases aquosas (ATPS) usando PEG/fosfato, através de análises dos seguintes parametros: O coeficiente de partição (K), seu rendimento nas fases (YT) e o fator de purificação (PF). O valor mais elevado de PF (8,24), obtido usando 20,0% (w/w) de PEG 8000 e 12,5% (w/w) sal de fosfato a pH 8,0, foi melhor do que os relatados na literatura para ATPS semelhante.

ix. A protease PEG-colagenolítica ainda apresentou as mesmas características físico-químicas (temperatura, pH, sensibilidade a íons metálicos e a inidibores naturais e

sintéticos) desejadas pelo mercado industrial (resistência a altas temperaturas, por exemplo).

x. O colágeno da pele de *C. ocellaris* foi extraído com sucesso, obtendo-se 2.9% de rendimento (peso seco). O colágeno extraído consistiu de duas cadeias diferentes α (α 1 e α 2), seus dímeros (cadeia β) e aparadores (cadeia γ), e foi caracterizado como sendo colágeno do tipo I. No ensaio ultravioleta (UV) do espectro de absorção, o colágeno mostrou uma máxima absorção de 211 nm. Os resultados obtidos neste estudo indicam a possibilidade de utilização de pele Tucunaré como uma fonte de biomoléculas com grande potencial de aplicação industrial e biotecnológica.

xi. O colágeno extraído da pele do tucunaré ainda serviu de parâmetro para teste de especificidade e hidrólise a partir da protease PEG-colagenolítica, indicando potencial de clivagem para a produção de peptídeos bioativos de colágeno.

xii. Enfim, é notório o potencial de *C. ocellaris* para o fornecimento de biomoléculas, sobretudo de proteases com propriedades colagenolíticas (colagenases), levando-se em consideração também a redução dos resíduos despejados no meio ambiente e a agregação de valor ao produto, além de se ter uma enzima extraída de fonte alternatica para competir no mercado mundial, além do que, a técnica de ATPS pode ser utilizada nas etapas iniciais de um processo de purificação, permitindo a remoção de contaminantes por um processo rápido, simples e econômico, ou mesmo como etapa de principal de purificação, quando satisfizer a necessidade de isolamento, como na produção de peptídeos de colágeno, como descritos no presente trabalho. Assim sendo, considerando sua simplicidade e alto rendimento somando investimentos mais baixos tornam ATPS uma opção promissora de recuperação de colagenases a partir de resíduos do processamento do pescado em escala industrial, visando a indústria alimentícia, farmacêutica e de cosméticos.

11. ANEXOS

11.1 Produção cientifica (2011-2015)

11.1.1 Premiações

Prêmio Jovem Pesquisador/Categoria Pós-Graduação.
2° Lugar: Caracterização parcial de tripsina de *Cichla ocellaris* por íons metálicos.
Concedente: I Congresso Internacional de Ciências Biológicas (2013).

Prêmio Jovem Pesquisador/Categoria Pós-Graduação.
3° Lugar: Proteases de *Ctenosciaena leiarchus*como biomarcadores marinhos.
Concedente: I Congresso Internacional de Ciências Biológicas (2013).

Menção Honrosa para o trabalho Characterization of Brain Acetylcholinesterase from Peacock Bass, *Cichla Ocellaris* (Bloch & Schneider, 1801) and in vitro Effect of Ions and Pesticides, XI Reunião Regional Nordeste da SBBq e IV International Symposium in Biochemistry of Macromolecules (2012).

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Renata Maria do Nascimento. Caracterização parcial de colagenase de cinco espécies de peixes Neotropicais. Início: 2014. Orientação de outra natureza. Universidade Federal de Pernambuco.

Deborah Cibelle da Silva Lacerda. Estudo histopatológico do fígado de alevinos de tilápia-do-Nilo *Oreochromis niloticus* LINNAEUS, 1758 (Perciformes: Cichlidae) exposto a metal pesado. 2013. Iniciação Científica. (Graduanda em Bacharelado em Ciências Biológicas), Universidade Federal de Pernambuco.

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Nathalia Albuquerque. Purificação e caracterização de enzimas digestivas de Tucunaré (*Cichla ocellaris*). 2013. Iniciação Científica. (Graduando em Bacharelado em Ciências Biológicas), Universidade Federal de Pernambuco.

Raphael Luiz de Oliveira Neves. Atividade butirilcolinesterásica muscular de *Oreochromis niloticus* expostos a metal pesado. 2012. Iniciação Científica. (Graduando em Licenciatura Plena em Ciências Biológicas), Universidade Federal Rural de Pernambuco.

Raquel Pereira Freitas da Silva. Aplicação biotecnológica de proteases digestivas de tilápias como biomarcadores de exposição ao $CuCl_2$ e ao $FeCl_2$. 2011. Iniciação Científica. (Graduando em Biomedicina), Universidade Federal de Pernambuco.

11.1.3 Orientações e co-orientações em andamento

Nathalia Albuquerque. Caracterização parcial de colagenase de Tucunaré (*Cichla ocellaris*). 2014. Iniciação científica e Monografia de conclusão de curso (Graduanda em Bacharelado em Ciências Biológicas), Universidade Federal de Pernambuco, Conselho Nacional de Desenvolvimento Científico e Tecnológico.

Luiz Henrique Svintiska Lino. Purificação e caracterização de colagenase e atividade fibrinolítica de espécies de peixes Neotropicais. 2014. Iniciação Científica (Graduando em Bacharelado em Ciências Biológicas), Faculdade Frassinetti do Recife, Conselho Nacional de Desenvolvimento Científico e Tecnológico.

11.1.3 Trabalhos completos publicados (como primeiro autor)

OLIVEIRA, V.M.; BEZERRA, R.S.; PORTO, A.L.F. Caracterização parcial de tripsina de Cicha ocellaris por íons metálicos. In: I Congresso Internacional de Ciências Biológicas, II Congresso Nacional de Ciências Biológicas, VI Simpósio de Ciências Biológicas, 2013, Recife. Anais de Biodiversidade e água: desafios e cooperação, v.1. p.603-612, 2013.

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OLIVEIRA, V.M.; ASSIS, C.R.D.; FRANCA, R. C. P.; VILA NOVA, M. X.; CARVALHO JUNIOR, L. B.; BEZERRA, R.S.; PORTO, A.L.F. Study of genes from beijupirá (Rachycentron canadum) through bioinformatics. In: XIV Congresso Latino-Americano de Ciências do Mar COLACMAR, 2011, Balneário Camboriú, SC. XIV Congresso Latino-Americano de Ciências do Mar COLACMAR, 2011. p. 1-3.

11.1.4 Textos de jornais e revistas (como primeiro autor)

OLIVEIRA, V.M.; SOARES, K.L.S.; SANTOS, B.A C.; BEZERRA, R.S.; PORTO, A.L. F. Sensibilidade da tripsina de cinco espécies de peixes tropicais ao calcio e ao ferro. Revista Higiene Alimentar (ISSN 0101-9171), CBMVHA, Gramado, p. 1282 - 1286, 23 abr. 2013.

OLIVEIRA, V. M.; SOARES, K.S.; SANTOS, B.A.C.; BEZERRA, R.S.; PORTO, A.L. F. Sensibilidade de tripsina extraída de vísceras digestivas de Seríola dumerili (RUSSO, 1810) a íons metálicos. Revista Higiene Alimentar (ISSN 0101-9171), CBMVHA, Gramado, p. 1287 - 1290, 23 abr. 2013.

11.2 Normas para submissão

11.2.1 Journal of Food Biochemistry

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RIZVI, S.S.H. 1986. Thermodynamic properties of foods in dehydration. In *Engineering Properties of Foods*, (M.A. Rao and S.S.H. Rizvi, eds.) pp. 133-214, Marcel Dekker, New York.

MICHAELS, S.L. 1989. Crossflow microfilters ins and outs. Chem. Eng. 96, 84-91.

LABUZA, T.P. 1982. *Shelf-Life Dating of Foods*, pp. 66-120, Food & Nutrition Press, Trumbull, CT.
References in Articles
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11.2.2 Fish Physiology and Biochemistry

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References

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- Negotiation research spans many disciplines (Thompson 1990).

- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
Reference list entries should be alphabetized by the last names of the first author of each work.

- Journal article
  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8
  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:
  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329
- Article by DOI
  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086
- Book
  South J, Blass B (2001) The future of modern genomics. Blackwell, London
- Book chapter
  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257
- Online document
  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
- Dissertation
  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations, see

- ISSN.org LTWA

If you are unsure, please use the full journal title.
For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

- EndNote style (zip, 2 kB)

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

11.2.3 LWT - Food Science and Technology

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic

artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

All lines must be consecutively numbered throughout the manuscript.

Article structure
Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**
• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract
A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Abstracts should not exceed 200 words for research papers and research notes, or 300 words for reviews.

Highlights
Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings

of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).
See http://www.elsevier.com/highlights for examples.

Keywords
Immediately after the abstract, provide a maximum of 5 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.
If possible the Food Science and Technology Abstracts (FSTA) Thesaurus should be used (IFIS Publ., Shinfield, Reading RG2 9BB, UK http://www.foodScienceCentral.com).

Chemical compounds
You can enrich your article by providing a list of chemical compounds studied in the article. The list of compounds will be used to extract relevant information from the NCBI PubChem Compound database and display it next to the online version of the article on ScienceDirect. You can include up to 10 names of chemical compounds in the article. For each compound, please provide the PubChem CID of the most relevant record as in the following example: Glutamic acid (PubChem CID:611). The PubChem CIDs can be found viahttp://www.ncbi.nlm.nih.gov/pccompound. Please position the list of compounds immediately below the 'Keywords' section. It is strongly recommended to follow the exact text formatting as in the example below:
Chemical compounds studied in this article
Ethylene glycol (PubChem CID: 174); Plitidepsin (PubChem CID: 44152164); Benzalkonium chloride (PubChem CID: 15865)
More information is available at: http://www.elsevier.com/PubChem.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Units
Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Do not use %, ppm, M, N, etc. as units for concentrations. If analytical data are reported, replicate analyses must have been carried out and the number of replications must be stated.

Database linking
Elsevier encourages authors to connect articles with external databases, giving their readers one-click access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Math formulae
Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).
Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Artwork
Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the printed version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
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TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
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Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
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Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
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Figure captions
Figures must be comprehensible without reference to the text. Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used in the caption. If analytical data are reported, replicate analyses must have been carries out. State the number of replications and provide standard error or other evidence of reliability of the data.

Tables
Number tables consecutively in accordance with their appearance in the text. Include a short but informative title. Provide the experimental conditions, as far as they are necessary for understanding. The reader should not have to refer to the text in order to understand the tables.
Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.
If analytical data are reported, replicate analyses must have been carried out. State the number of replications and give standard error or other evidence of reliability of data.
Probabilities may be indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

References
Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.
Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can

be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference style

Text: Citations in the text should follow the referencing style used by the American Psychological Association. You are referred to the Publication Manual of the American Psychological Association, Sixth Edition, ISBN 978-1-4338-0561-5, copies of which may be ordered from http://books.apa.org/books.cfm?id=4200067 or APA Order Dept., P.O.B. 2710, Hyattsville, MD 20784, USA or APA, 3 Henrietta Street, London, WC3E 8LU, UK.

List: references should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2010). The art of writing a scientific article. *Journal of Scientific Communications, 163*, 51–59.

Reference to a book:

Strunk, W., Jr., & White, E. B. (2000). *The elements of style.* (4th ed.). New York: Longman, (Chapter 4).

Reference to a chapter in an edited book:

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11.2.4 Fluid Phase Equilibria

NEW SUBMISSIONS

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process. As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay-out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.

If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.

Divide the article into clearly defined sections.

Figures and tables embedded in text

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

REVISED SUBMISSIONS

Use of word processing software
Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

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You are recommended to use the Elsevier article class *elsarticle.cls* (http://www.ctan.org/tex-archive/macros/latex/contrib/elsarticle) to prepare your manuscript and BibTeX (http://www.bibtex.org) to generate your bibliography.
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Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.
Materials and Methods
Provide sufficient detail to allow the work to be reproduced. In the case of experimental papers the numerical purity (mass fraction or mole fraction) of the investigated substances should be indicated, as well as the method of purity determination, if known. Any subsequent purification of the sample, such as distillation, crystallization, drying, etc., should be described.
Methods already published should be indicated by a reference: only relevant modifications should be described.
Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.
Results
Results should be clear and concise.
Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.
Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.
Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.
Nomenclature
Authors must provide a Nomenclature, to be published between the text of the paper and the list of references. The Nomenclature should be a list of all mathematical symbols in one column and their definitions with units, preferably including the equation number of first use, in an adjacent column. The symbols should follow the notation of the IUPAC, “Quantities, Units, and Symbols in Physical Chemistry, 2nd Ed.”,http://old.iupac.org/publications/books/gbook/green_book_2ed.pdf. In addition, all unusual abbreviations and acronyms used in the paper should be included in the Nomenclature. Authors should also consider defining symbols and acronyms when first used within the paper.

Essential title page information
• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding**

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• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract
A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract
Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images and in accordance with all technical requirements: Illustration Service.

Highlights
Highlights are a short collection of bullet points that convey the core findings of the article. Highlights are optional and should be submitted in a separate file in the online submission system. Please include 3 to 5 bullet points (max. 85 characters per bullet point including spaces). See http://www.elsevier.com/researchhighlights for examples.
Note: for Asian authors, interpreting a character as a word, max 85 characters per bullet point corresponds with approx. 20 words max per bullet point.
Keywords
Immediately after the abstract, provide a maximum of 5 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature and units
Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI. You are urged to consult IUPAC: Nomenclature of Inorganic Chemistry:http://www.iupac.org/ for further information.

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Math formulae
Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork
Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.

• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files.
A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions.
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

Tables
Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.
References
Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links
Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.
Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.
References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.
Reference management software
This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:
Reference to a journal publication:
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Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

11.2.5 Food Chemistry

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