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RESÍDUOS DE PROCESSAMENTO DO PINTADO (*Pseudoplatystoma corruscans*) COMO FONTE DE PROTEASE ÁCIDA E COLÁGENO

Recife
2015

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco como pré-requisito para a obtenção do grau de doutor em Bioquímica e Fisiologia.

Orientador: Prof. Dr. Ranilson de Souza Bezerra

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A Deus, a Quem, durante todo o decurso de minha caminhada, encontrei forças e iluminação para prosseguir.

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“Quis mudar tudo. Mudei tudo. Agora, pós-tudo, ex-tudo mudo.”

Augusto de Campos

RESUMO

A presente tese reporta a purificação parcial e caracterização enzimática de uma protease ácida proveniente do estômago do *Pseudoplatystoma corruscans*, bem como o aproveitamento de resíduos deste peixe para aplicação na extração de colágeno. Neste âmbito, o primeiro capítulo tratou da caracterização enzimática de uma protease ácida do estômago do pintado e aplicação desta enzima na extração de colágeno da pele de *Oreochromis niloticus*. A caracterização com substratos e inibidores específicos sugere que a protease ácida trata-se de uma pepsina-símile. Esta enzima foi pouco sensível à exposição a íons metálicos como Cu^{2+}, Cd^{2+}, Hg^{2+}, Ca^{2+}, Al^{3+}, K^{+}, Mg^{2+} e Ba^{2+}. A protease demonstrou características interessantes como alta atividade, estabilidade em pH neutro e elevada temperatura ótima. Adicionalmente colágeno ácido e pepsino-solúvel (ASC e PSC) foram extraídos da pele de *O. niloticus*. Pepsina comercial e pepsina-símile proveniente do estômago do pintado foram utilizadas no processo de extração e favoreceram para o aumento do rendimento. No capítulo 2, foi realizada uma purificação parcial da protease ácida do estômago do pintado. Após as três etapas da purificação (tramento térmico, fracionamento salino e cromatografia de troca iônica) obteve-se um fator de purificação de 32 vezes. SDS-PAGE mostrou bandas proteicas entre 30,7 and 94 KDa, possivelmente isoformas da pepsina. O zimograma corroborou a presença da enzima. Além disso, o uso de inibidor específico produziu evidências de que a protease trata-se de uma pepsina-símile. A caracterização físico-química da protease ácida mostrou estabilidade da fração frente ao pH neutro, além de elevada temperatura ótima. Finalmente, o capítulo três objetivou a extração de colágeno (ácido e pepsino-solúvel) da pele do pintado, bem como a caracterização dessa proteína, sugerindo o seu uso como fonte alternativa de colágeno frente aos animais terrestres. ASC e PSC foram isolados da pele do pintado, evidenciando o aumento do rendimento da extração com a adição de pepsina. SDS-PAGE e espectro de absorção UV indicaram que o colágeno estudado foi do tipo I. ASC e PSC apresentaram alta solubilidade em pH ácido. Na presença de NaCl, PSC exibiu maior solubilidade em relação ao ASC. Diante dos resultados alcançados neste trabalho, pode-se dizer que os resíduos (pele e vísceras) do pintado apresentam um grande potencial para aplicações industriais, principalmente relacionadas à obtenção de colágeno.

Palavras-chave: Colágeno; Protease digestiva; Purificação; Víscera de peixes.

ABSTRACT

The present work reports on the partial purification and enzymatic characterization of a acid protease from *Pseudoplatystoma corruscans* stomach as well as the use of this waste for fish collagen extraction. In this context, chapter one treated the enzymatic characterization of an acidic protease from spotted sorubim stomach and application of this enzyme in collagen extraction from *Oreochromis niloticus* skin. The characterization with substrates and specific inhibitors suggests that it is the acid protease pepsin-like. This enzyme was not sensitive to the exposure to metal ions such as Cu^{2+}, Cd^{2+}, Hg^{2+}, Ca^{2+}, Al^{3+}, K^{+}, Mg^{2+} and Ba^{2+}. The protease showed interesting features like high activity, stability at neutral pH and high optimum temperature. Additionally, acid and pepsin soluble collagen (ASC and PSC) were extracted from the skin of *O. niloticus*. Commercial pepsin and pepsin-like from the spotted sorubim stomach were used in the extraction process and favored to increase the yield. In chapter two, it was performed a partial purification of acid protease from the spotted sorubim stomach. After the three steps purification it was obtained a 32 fold purification factor. SDS-PAGE revealed protein bands of 30.7 and 94 kDa, possibly pepsin isoforms. The zymography confirmed the presence of the enzyme. Furthermore, the use of specific inhibitor produced evidence that the protease is pepsin-like. The physicochemical characterization of acid protease showed stable fraction to neutral pH and high optimum temperature. Finally, chapter three brings the collagen extraction (acid and pepsin soluble) from the spotted sorubim skin as well the characterization this protein, suggesting its use as an alternative source of collagen front of land animals. ASC and PSC were isolated from the spotted sorubim skin showing increased extraction yield with the addition of pepsin. SDS-PAGE and UV absorption spectrum indicate that collagen studied is type I. ASC and PSC showed high solubility in acid pH. In the presence of NaCl, PSC exhibited higher solubility compared to ASC. With the results reported in the present thesis, it can be said that spotted sorubim wastes (skin and visceras) show great potential for industrial applications, mainly related to obtaining collagen as an alternative source.

Keywords: Collagen; Digestive protease; Purification; Fish viscera.

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ASC	Colágeno ácido solúvel
Asp	Ácido aspártico
BApNA	N-α-benzoil-L-arginina-p-nitoanilida
β-ME	β-Mercaptoetanol
BSE	Encefalopatia espongiforme bovina
DEAE-cellulose	Diethylaminoethyl cellulose
DMSO	Dimetilsulfóxido
D.N.O.C.S.	Departamento Nacional de Obras Contra a Seca
DNSA	Ácido 3,5 - dinitrossalicílico
E-64	L-3-carboxitrans- 2, 3-epoxi-propionil-L-leucin-4-guanidino-butilamida
EC	Comitê enzimático
EDTA	Etileno-diamina-tetra-acético
ES	Complexo Enzima-Substrato
FA	Febre aftosa
FAO	Organização das nações Unidas para Alimentação e Agricultura
FMD	Febre aftosa
Gly	Glicina
Hyp	Hidroxiprolina
IUBMB	União Internacional de Bioquímica e Biologia Molecular
k	Constante de velocidade
kDa	Quilo Daltons
mM	Milimolar
nm	Nanômetro
MPA	Ministério da Pesca e Aquicultura
PGs	Pepsinogênios
pH	Potencial Hidrogeniônico
pI	Ponto isoelétrico
PMSF	Fenilmetanossufonilfluoreto
Pro	Prolina
PSC	Colágeno pepsin solúvel
RNA	Ácido ribonucleico

SAPNA	Succinil-alanina-alanina-prolina-fenilalanina-*p*-nitroanilida
SDS	Dodecil sulfato de sódio
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
TAME	Tosil-arginina-metil-éster
TLCK	N-p-tosil-L-lisina clorometil cetona
TPCK	N-tosil-L-fenilalanina clorometil cetona
TSE	Encefalopatia espongiforme transmissível
UFPE	Universidade Federal de Pernambuco
U/mg	Unidades de atividade enzimática por miligrama
U/mL	Unidades por mililitro
UV-vis	Ultravioleta visível
v/v	Volume/volume
w/v	Peso/volume
µg	Micrograma
µL	Microlitro

SUMÁRIO

1. INTRODUÇÃO

A produção aquícola mundial tem passado por um constante crescimento devido a um aumento na demanda por produtos pesqueiros. Estima-se que as atividades de pesca extrativa e aquicultura em 2013 atingiram um novo recorde mundial, com 160 milhões de toneladas contra as 157 milhões de toneladas do ano anterior (FAO, 2014). No entanto, com a estagnação da quantidade de pescado proveniente da captura, a aquicultura vem assumindo, nos últimos anos, a responsabilidade de atender à demanda por produtos aquícolas, através do aumento da utilização de espécies e tecnologias adequadas. Segundo as projeções da FAO, os aquicultores produziram 70 milhões de toneladas de pescado no ano passado, 44% da produção total mundial (FAO, 2014).

A aquicultura brasileira, só há pouco tempo, vem dando ênfase para espécies de peixes nativos. Na procura por novidades para atender ao mercado, os produtores têm buscado espécies inovadoras, que tenham boa produtividade e aceitação para a pesca e o consumo. Dentre as espécies nativas, várias são aquelas com esse potencial, sem, contudo, terem sido estudadas suficientemente. Espécies carnívoras, como o pintado (*Pseudoplatystoma corruscans*), têm despertando interesse dos pesquisadores e produtores de peixes, principalmente devido ao valor comercial, à qualidade da carne e às características esportivas para a pesca que apresentam (LUZ et al., 2001).

A produção de pintado teve o primeiro registro nacional em 1998, com 329 toneladas, e em onze anos cresceu 546%, atingindo a marca de 2.126,7 toneladas em 2009. É importante ressaltar que o crescimento da produção no período de 2006 a 2009 foi de 94%, mostrando-se bastante consistente e expressivo, com média anual de 19,4% (IBAMA, 2010). Apesar de ter alcançado um crescimento vertiginoso, a criação desta espécie em cativeiro ainda apresenta limitações, sobretudo relacionadas à sua nutrição. A falta de dados concretos sobre o hábito alimentar e as exigências nutricionais nas diferentes fases de crescimento fazem com que as deficiências na alimentação e na nutrição desse peixe sejam responsáveis por altos índices de mortalidade (CREPALDI et al., 2006a).

Em contrapartida, o aumento da produção pesqueira é proporcional às concentrações de resíduos deixados através do processamento do pescado. Em geral os resíduos são descartados no ambiente e causam sérios problemas de poluição. Essa crescente poluição ambiental e o reconhecimento de que o uso dos recursos biológicos é limitado tem enfatizado a necessidade de utilização de subprodutos da indústria pesqueira (MONTE, 2013).

As vísceras dos peixes estão entre os resíduos que não são consumidos pelos humanos e correspondem por cerca de 10% do peso total do animal (SIMPSON e HAARD, 1987). De acordo com Bezerra et al. (2000), as vísceras dos peixes são conhecidas por serem ricas em proteases. Proteases ácidas como a pepsina representam uma alternativa para o uso em certos processos biotecnológicos: coagulação do leite (TAVARES et al., 1997), produção de silagem (GILDBERG et al., 2004) e extração de colágeno (BENJAKUL et al., 2010a). Devido a sua ampla aplicabilidade em processos biotecnológicos, pepsinas têm sido extraídas, purificadas e caracterizadas a partir de vísceras de peixes (WU et al., 2009; NALINANON et al., 2010; KHALED et al., 2011).

Em relação ao aproveitamento de resíduos pesqueiros, o número de trabalhos objetivando a extração de colágeno a partir de organismos aquáticos tem crescido consideravelmente. Colágeno da pele de várias espécies de peixes tem sido extraído e caracterizado, como *Ictalurus punctatus* (LIU et al., 2007), *Lutjanus lutjanus* (NALINANON et al., 2007), *Monoceros aluterus* (AHMAD et al., 2010), *Nemipterus hexodon* (NALINANON et al., 2011), *Diodon holocanthus* (HUANG et al., 2011), *Pangasianodon hypophthalmus* (SINGH et al., 2011), *Evenchelys macrura* (VEERURAJ et al., 2013), *Hypophthalmichthys molitrix* (SAFANDOWSKA et al., 2013) e *Acipensern schrenckii* (VEERURAJ et al., 2014).

Sendo assim, o presente trabalho aborda o estudo das vísceras do pintado com enfoque na obtenção de biomoléculas ativas e aplicação biotecnológica na extração de colágeno. Dessa forma, pode-se fornecer conhecimento da sua fisiologia digestiva, contribuindo para o entendimento da capacidade enzimática digestiva, bem como propiciar a utilização dos resíduos do seu processamento, agregando valor ao pescado.

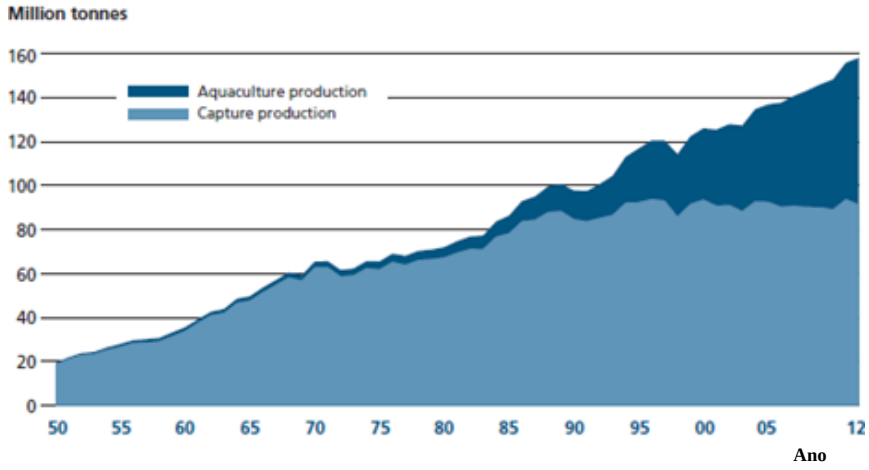
2. REVISÃO DA LITERATURA

2.1 Panorama da aquicultura e produção de pintado

A produção de pescado mundial tem crescido em ritmo constante nas últimas cinco décadas (Figura 1), com a oferta alimentar aumentando a uma taxa média anual de 3,2%, ultrapassando o crescimento da população mundial em 1,6%. O consumo mundial de peixe per capita aumentou de 9,9 kg em 1960 para 19,2 kg em 2012 (FAO, 2014).

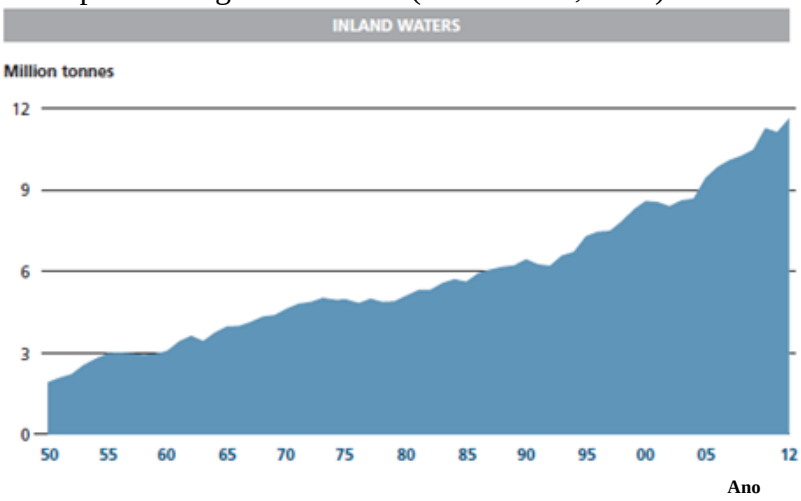
Este impressionante desenvolvimento tem sido impulsionado por uma combinação de fatores, como o crescimento populacional, aumento da renda e urbanização, além de ser facilitado pela forte expansão da produção de peixes e canais de distribuição mais eficientes (FAO, 2014).

Figura 1: Produção aquícola mundial (Fonte: FAO, 2014).



Por sua vez, a produção global advinda da captura em águas interiores atingiu 11,6 milhões de toneladas em 2012 (Figura 2). Embora esta tendência ascendente pareça contínua, sua participação na produção total de captura global não excedeu 13% (FAO, 2014).

Figura 2: Produção mundial de pescados referente à captura em águas interiores (Fonte: FAO, 2014).



O Brasil possui 12% da água doce do planeta, condições climáticas favoráveis para o cultivo de peixes comerciais e uma diversidade de espécies aquáticas. Possui, portanto, um grande potencial de mercado tanto para a produção pesqueira quanto para os produtos advindos da aquicultura. Apesar deste potencial, historicamente, o país tem apresentado pequena participação no cenário mundial da atividade. Além do baixo investimento no setor, a pesca no Brasil tem concentrado esforços sobre poucas espécies (GEO BRASIL, 2002).

A escolha de uma espécie que atenda às exigências para sua produção comercial pressupõe, dentre outros fatores, existência de mercado, rusticidade e crescimento rápido, além de oferta contínua de alevinos. Estas características intrínsecas apontam o grande potencial de expansão do mercado e abrem grandes perspectivas para os empreendimentos voltados ao cultivo de peixes.

A produção do *Pseudoplatystoma corruscans* foi impulsionada na década de 1990, com o domínio em escala comercial da produção de alevinos, que se iniciou em algumas pisciculturas de Mato Grosso do Sul. Segundo dados do Ministério da Pesca e Aquicultura (BRASIL, 2010), a produção de surubim ou pintado alcançou 2.126,7 t em 2009. Considerando as primeiras informações de produção em 1998 (329 t) e os dados mais atuais, nota-se grande incremento na produção de pintado, que atende tanto o mercado interno quanto o externo.

Os mercados da região Sudeste, Sul e Centro-Oeste consomem a maior parte do pintado comercializado no país. Praticamente todos são oriundos da pesca extrativa na região do Pantanal do Mato Grosso e Mato Grosso do Sul, bem como na Amazônia brasileira. Os exemplares do rio São Francisco são geralmente comercializados nos

centros consumidores próximos aos locais de captura, visto que a quantidade desse peixe nessa bacia caiu drasticamente (KUBITZA et al.,1998).

2.2 Pintado (*Pseudoplatystoma corruscans*)

A espécie *P. corruscans* (AGASSIZ, 1829), é conhecida, popularmente, como surubim e, mais comumente, como pintado, devido à presença de pintas escuras espalhadas pelo corpo (Figura 3).

Figura 3: Exemplar de *Pseudoplatystoma corruscans*

Segundo Lauder & Liem (1983), a espécie ocupa a seguinte posição sistemática (Tabela 1):

Tabela 1: Sistemática Filogenética do pintado

Reino	Animalia
Filo	Chordata
Super Classe	Pisces
Classe	Osteichthyes
Subclasse	Actinopterygii
Ordem	Siluriformes
Subordem	Siluroidei
Familia	Pimelodidae
Gênero	*Pseudoplatystoma*
Espécie	*Pseudoplatystoma corruscans*

Fonte: LAUDER & LIEM (1983).

A ordem Siluriformes inclui os chamados peixes de couro, cuja principal característica externa é a ausência de escamas pelo corpo, que é revestido apenas de pele espessa ou coberto, parcial ou totalmente, por placas ósseas. Apresentam barbilhões e, frequentemente, o primeiro raio da nadadeira dorsal e das peitorais se

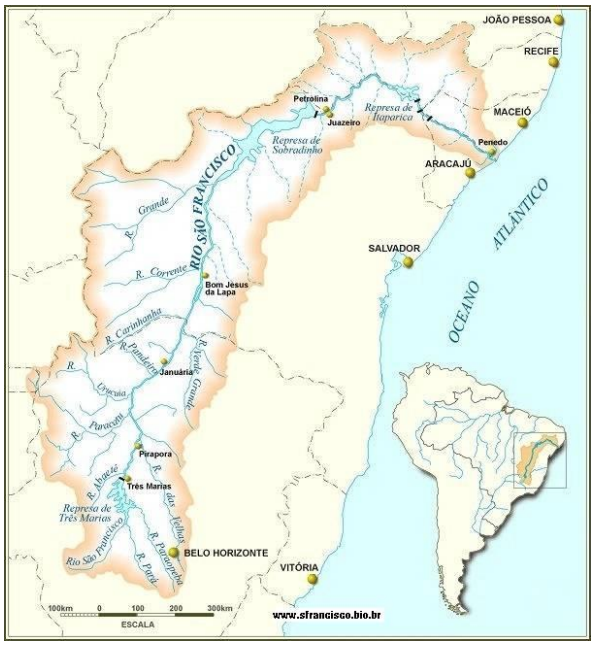
constitui de um acúleo forte e pungente (MIRANDA et al., 1997). Segundo Kubitza et al. (1998), a ordem Siluriformes engloba mais de 2.200 espécies de bagres espalhados por todos os continentes.

A subordem Siluroidei é composta por um significativo número de gêneros e espécies dulcícolas de hábitos noturnos e dieta variada. Compreende treze famílias na região neotropical, das quais sete ocorrem no rio São Francisco, dentre elas a Pimelodidae, que é a mais numerosa da subordem e tem como característica distinta a presença de três pares de barbilhões próximos à boca (MARINHO, 2007).

O gênero *Pseudoplatystoma* (BLEEKER, 1862), que inclui os maiores peixes da família Pimelodidae, é exclusivamente de água doce e pode ser encontrado nas principais bacias hidrográficas sul-americanas. Sua distribuição inclui os rios Amazonas, Orinoco, São Francisco, bem como no sistema do rio da Prata formado pelos rios Paraná, Paraguai e Uruguai (ROMAGOSA et al., 2003).

Este gênero é constituído por oito espécies. Três destas são muito semelhantes entre si, mudando apenas o formato das manchas negras sobre o fundo cinza/oliva. São elas: *P. corruscans* (surubim, pintado), *P. fasciatum* (cachara) e *P. tigrinum* (caparari, pirambucu). Na bacia amazônica, ocorrem as espécies *P. fasciatum* e *P. tigrinum*; na bacia do Paraná, *P. fasciatum* e *P. corruscans* e na bacia do São Francisco apenas *P.corruscans* (Figura 4) (BALDISSEROTO e GOMES, 2005).

Figura 4: Bacia do Rio São Francisco



(Fonte: http://www.espelhodagua.org/o-rio-sao-francsico.php).

Buitrago-Suárez & Burr (2007) descreveram o reconhecimento de cinco novas espécies: *P. magdaleniatum* (rio Magdalena, Colômbia); *P. metaense* (rio Orinoco, Colômbia e Venezuela); *P. orinocoense* (rio Oricono, Venezuela); *P. reticulatum* (rio Paraná) e *P. punctifer* (rio Amazonas).

Em relação ao aspecto morfológico, o pintado apresenta o corpo alongado e roliço, com cabeça deprimida e largura ao nível da boca ligeiramente menor que a largura do corpo. Possui mandíbula mais curta que a maxila superior e dentes viliformes no palato. Seu flanco e dorso apresentam máculas arredondadas e suas nadadeiras dorsal e caudal manchas menores (MIRANDA, 1997). Trata-se de um dos maiores peixes fluviais, podendo atingir 3 metros de comprimento corpóreo e chegando a pesar 100 a 120 kg (CREPALDI et al., 2006a).

2.2.1 Bioecologia do *P. corruscans*

De hábito alimentar carnívoro, principalmente piscívoro, essa espécie migratória parece percorrer grandes distâncias no período reprodutivo. Seu período reprodutivo é relativamente curto, compreendendo os meses de chuva (novembro a janeiro), quando os rios recebem um grande aporte de água (SATO e GODINHO, 2003).

A importância ecológica do *P. corruscans* pode ser evidenciada, pelo fato de ele ser, o segundo maior peixe predador da bacia do Paraná e o primeiro da Bacia do São Francisco (TAVARES, 1997). Outro aspecto é o fato, da espécie ocupar um habitat crescentemente alterado pelas ações antrópicas, com grandes riscos de desaparecimento. Segundo Mateus & Penha (2007), bagres pimelodídeos, dentre eles o pintado, são os principais alvos nas capturas de peixes de água doce da América do Sul.

A espécie vem sendo explorada de forma acelerada, sem que manejos de pesca sejam estabelecidos, principalmente pelo desconhecimento da sua dinâmica populacional. A pesca predatória tem contribuído perigosamente para a diminuição gradual dos estoques naturais, o que faz com que aumentem os esforços de captura e, consequentemente, o custo. Desde o final da década de 90, o pintado está incluído entre as espécies vulneráveis à extinção (FUNDAÇÃO BIODIVERSITAS, 2003).

Por ser um peixe reofílico, a degradação de seu ambiente nativo, levado pelas construções de represas, assoreamento dos rios, poluição e a intensificação da pesca predatória, são os principais fatores que contribuem para o declínio das populações naturais do pintado (MARINHO, 2007).

2.2.2 Importância econômica do *Pseudoplatystoma corruscans*

No Brasil, o pintado está entre as espécies de peixes de água doce com grande potencial de consumo, representativos na produção pesqueira de águas interiores, devido às suas grandes proporções e à qualidade de sua carne (BENÍTEZ, 2003). A grande aceitação pelos consumidores decorre do fato da espécie apresentar carne saborosa, com baixo teor de gordura e ausência de espinhos intramusculares, o que o torna adequado aos mais variados preparos. Estas características atendem as preferências de mercado e fazem da carne do pintado um produto com grandes possibilidades de exportação (INOUE et al., 2008).

A valorização econômica e social da espécie tem sido demonstrada não só pela representatividade das capturas, mas também devido à importância quanto à possibilidade de seu emprego na piscicultura empresarial. Além da sua grande estima popular, o pintado é também valioso e muito apreciado pelos pescadores esportivos. A agressividade e o grande porte que alcançam fizeram com que conquistassem rapidamente esse mercado. Nesse tipo de comércio, o quilo do peixe tende a alcançar maiores valores, tornando-se uma alternativa interessante para os piscicultores. Camargo e Petrere (2001) confirmaram a importância dessa espécie na pesca artesanal devido ao seu alto valor de mercado.

No Brasil existe grande número de espécies que têm potencial para o cultivo, pois preenchem os requisitos necessários para a escolha de uma espécie adequada para a piscicultura. Dentre elas, o *P. corruscans* tem se destacado por apresentar características comerciais e zootécnicas desejáveis, como rápido crescimento e eficiente conversão alimentar. As características e os rendimentos de carcaça (CREPALDI et al., 2008) e a capacidade de obtenção de gametas por meio da hipofisação (CREPALDI et al., 2006b) reforçam a qualificação da espécie para a piscicultura industrial. Por apresentar estas vantagens, a produção de pintado vem crescendo no Brasil (PILECCO et al., 2008).

Entretanto, apesar de possuir grande potencial zootécnico, o cultivo desta espécie apresenta limitações relacionadas ao manejo alimentar, por ser carnívora, podendo se alimentar de ração quando condicionada. Um exemplo dessa lacuna é a dificuldade para introduzir o alimento artificial na dieta das larvas e juvenis. Além disso, devido ao hábito noturno, a espécie só se alimenta durante o dia quando submetida a treinamento (BALDISSEROTO e GOMES, 2005).

No momento já há tecnologia adequada para a reprodução do pintado e obtenção de larvas, mas o maior desafio está na mudança da fase de larva para juvenil. Por

apresentar hábito alimentar piscívoro, aliado ao desconhecimento de técnicas de manejo alimentar adequadas, nessa fase ocorre um acentuado canibalismo, levando à redução da taxa de sobrevivência durante a criação inicial (ALVARADO et al., 2003).

No entanto, muito trabalho ainda tem que ser realizado para a elaboração de um pacote tecnológico, principalmente nas fases mais complicadas do cultivo: a larvicultura e o treinamento alimentar. Segundo Sato et al. (1997) o *P. corruscans* requer cuidados extremos, principalmente nas fases iniciais, das quais, embora seja grande o número de indivíduos produzidos numa única desova, os organismos são extremamente frágeis.

2.3 Utilização de resíduos da aquicultura

O aumento da produção pesqueira e, consequentemente, do volume de pescado processado mundialmente, tem gerado uma grande quantidade de resíduos e subprodutos. Por serem produtos altamente perecíveis, os peixes apresentam uma necessidade significativa de processamento, o que gera grande quantidade de resíduos líquidos (águas residuais) e sólidos (pele, ossos, vísceras, nadadeiras e cabeças) (DOODE, 1996).

Os resíduos da indústria pesqueira apresentam uma composição rica em compostos orgânicos e inorgânicos, o que gera preocupação relativa aos potenciais impactos ambientais negativos decorrentes da disposição deste material diretamente no ambiente (SEIBEL & SOARES, 2003). Tal fato representa um desafio para empresários e comunidade científica interessados no desenvolvimento sustentável da aquicultura. Como já é sabido, a redução do uso inconsciente da matéria prima além de evitar desperdícios e promover a reciclagem dos resíduos, garante processos mais econômicos e com menor impacto ambiental.

Assim, preocupados com problemas ambientais, pesquisadores em todo o mundo vêm desenvolvendo diversos esforços para obtenção de métodos que possibilitem a transformação desses materiais em co-produtos com valor agregado (ARVANITOYANNIS e KASSAVETI, 2008). A obtenção de biomoléculas com ampla aplicabilidade tecnológica a partir de resíduos de peixes tem sido uma necessidade por parte do setor industrial. Biomoléculas como enzimas, polissacarídeos e colágeno provenientes das sobras do processamento de peixes têm propiciado a criação de novos setores industriais, contribuindo também com a sustentabilidade econômica e ambiental da indústria pesqueira (COSTA, 2012).

Os resíduos da indústria pesqueira são passíveis de aplicação em diversos segmentos industriais, entre eles: obtenção de silagem de peixe (ARRUDA et al., 2006), com potencial para utilização como fonte proteica em rações (BORGHESI et al., 2007); produção de fertilizantes ou produtos químicos (CAVALCANTE JÚNIOR et al., 2005); iscas e artesanatos (BANCO do NORDESTE, 1999); na produção de biodiesel/biogás (KATO et al., 2004); produtos dietéticos (quitosana) (GILDBERG e STENBERG, 2001); pigmentos naturais (SACHINDRA et al., 2006); imobilização de cromo (OZAWA et al., 2003); embalagens de alimentos (quitosana, gelatina e colágeno) (GÓMEZ-ESTACA et al., 2009); obtenção de enzimas, principalmente proteases (ESPÓSITO et al., 2010; MARCUSCHI et al., 2010; SILVA et al., 2011; FREITAS-JÚNIOR et al., 2012; COSTA et al., 2013) e na indústria farmacêutica e de cosméticos (colágeno) (BENJAKUL et al., 2010b; LIU et al., 2012).

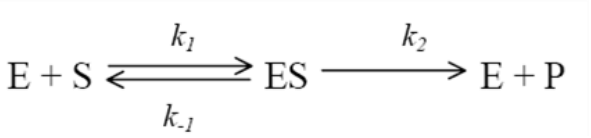
2.4 Enzimas

Enzimas são biomoléculas catalisadoras que atuam diminuindo o nível de energia de ativação, implicando no aumento da velocidade das reações bioquímicas. A eficiência das enzimas em catalisar reações é tal que a velocidade de uma reação pode ser aumentada em até 10^{20} vezes (HARVEY et al., 2009).

Todas as enzimas conhecidas, com exceção de certos RNAs catalíticos, são proteínas, e estão presente em todos os organismos vivos, sendo essenciais, tanto para a manutenção, como para o crescimento e a diferenciação celular. As enzimas digestórias agem em sequências organizadas e catalisam centenas de reações sucessivas, pelas quais as moléculas de nutrientes são degradadas. Essas biomoléculas catalisadoras não reagem quimicamente com as substâncias sobre as quais atuam, nem alteram o equilíbrio das reações (NELSON e COX, 2004).

De uma maneira geral, uma enzima liga-se ao seu substrato formando um complexo Enzima-Substrato (ES), de caráter transitório (Figura 5). Provavelmente, apenas uma fração da molécula, denominada sítio ativo, é a responsável pela ligação da enzima ao substrato, e essa fração determina a especificidade enzimática (NELSON e COX, 2004). Podem existir também outras regiões da cadeia polipeptídica que são sensíveis à presença de determinadas espécies químicas, modulando a atividade da enzima. Tais regiões são denominadas centros alostéricos (BERG et al., 2004).

Figura 5. Reação de catálise enzimática. Enzima (E), Substrato (S), Produto (P), constante de velocidade (*k*). (Fonte: BERG et al., 2004)



A atividade catalítica de uma enzima se constitui em um meio sensível e específico para sua determinação. Assim, para se medir a quantidade de uma enzima em uma amostra, mede-se a velocidade de reação catalisada pela enzima. Os resultados são geralmente expressos em unidades enzimáticas e as quantidades relativas de enzima em diferentes amostras podem ser então comparadas. Uma unidade de atividade enzimática (U) pode ser definida como a quantidade de substrato que reage ou do produto formado por um determinado tempo de reação (MURRAY et al., 2002).

Uma vez que a reação química catalisada por uma enzima é a propriedade específica que distingue uma enzima de outra, a IUBMB (União Internacional de Bioquímica e Biologia Molecular) organizou as enzimas em seis grandes classes (Tabela 2).

Tabela 2: Classificação das enzimas segundo a IUBMB.

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

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

Em relação à atividade enzimática, a manutenção da estrutura de uma enzima é de extrema importância. A presença da enzima em um meio em que fatores como o pH

ou a temperatura não favoreçam a estabilidade estrutural de sua cadeia polipeptídica pode provocar modificações ou até mesmo inibir a atividade (NELSON e COX, 2005).

Algumas enzimas necessitam também da presença de outras estruturas, genericamente denominadas cofatores, para efetuar a catálise. A natureza química dos cofatores é muito diversa. Estes podem ser íons metálicos, como o Mg^{2+}, o Zn^{+} e o Fe^{2+}, ou moléculas orgânicas, como o piridoxal fosfato ou a coenzima A. Outros grupos como moléculas orgânicas contendo metais, como o grupo heme (uma porfirina contendo ferro) ou a vitamina B_{12} (5'-desoxiadenosilcobalamina) também podem ser classificados como um cofator (NELSON e COX, 2005).

Além das modificações estruturais, as enzimas podem ainda ter sua atividade alterada pela presença de inibidores. Os principais mecanismos de inibição são classificados em reversíveis ou irreversíveis. Quanto à inibição reversível podemos classificá-la em competitiva e não competitiva. Na primeira, o inibidor apresenta semelhança estrutural com o substrato e se liga ao sítio ativo da enzima, formando o complexo enzima-inibidor e impedindo que a enzima se ligue ao substrato. Entretanto, diferentemente do inibidor irreversível, não há alteração na estrutura desta enzima. Quanto ao inibidor não competitivo, este se liga a outra região da enzima provocando uma alteração em sua estrutura principalmente em torno do sítio ativo, impedindo a reação de catálise mesmo quando o substrato está ligado a ela (VOET et al., 2006).

2.4.1 Carboidrases

O mecanismo do processo digestivo em peixes ainda não está totalmente elucidado, embora dados obtidos na literatura reportem que as enzimas digestivas de peixes são qualitativamente semelhantes às observadas em outros vertebrados. Algumas enzimas não proteolíticas, como amilase, lipase, esterase, quitinase e celulase, têm sido registradas no estômago de peixes (BALDISSEROTTO, 2009).

As espécies de peixes diferem em sua habilidade de digerir carboidratos. Esta variabilidade reflete as diferenças anatômicas e funcionais do trato gastrintestinal e órgãos associados (KROGDAHL et al., 2005), assim como a temperatura (KUZMINA et al., 1996). Estudos comparativos sobre a atividade de enzimas proteolíticas e carboidrases podem revelar a capacidade de diferentes espécies em utilizar proteínas e carboidratos como fonte de alimento (HIDALGO et al., 1999).

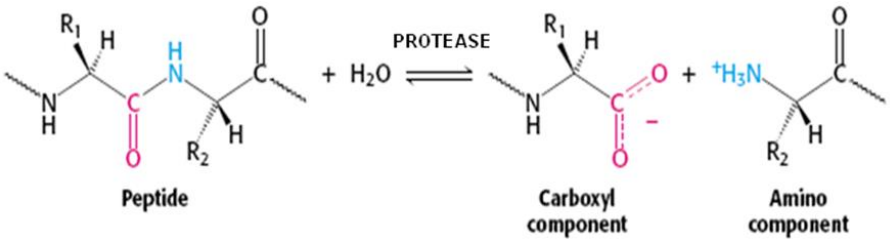
Para a realização da digestão do amido há a atuação de diversas enzimas. A α-amilase [EC 3.2.1.1] é uma endocarboidrase encontrada na saliva e no trato digestivo de

animais vertebrados (SALEH et al., 2005), responsável pela hidrólise de ligações glicosídicas α (1,4), no amido e glicogênio. Nesse processo são produzidos oligossacarídeos, α-dextrinas e maltose, que são hidrolisados à glicose pela ação complementar da α-glicosidase [EC 3.2.1.20], da sacarase-isomaltase [EC 3.2.1.48] e da α-dextrinase [EC 3.2.1.20]. Dentre essas, a α-glicosidase está diretamente relacionada à exo-hidrólise de ligações glicosídicas α (1,4) da maltose e demais oligossacarídeos formados após a atuação da α-amilase (ROSAS et al., 2000).

2.4.2 Proteases digestivas de peixes

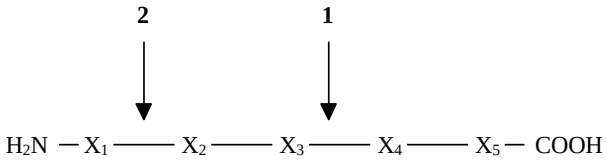
As proteases são enzimas que catalisam, *in vivo*, a hidrólise das ligações peptídicas entre os aminoácidos que constituem uma proteína. De acordo com a IUBMB, as proteases estão inseridas no subgrupo 4 do grupo 3 (Hidrolases), pois clivam a proteína adicionando uma molécula de água à ligação peptídica (Figura 6) (BERG et al., 2004).

Figura 6. Hidrólise enzimática de uma proteína hipotética (Fonte: BERG et al., 2004)



As enzimas proteolíticas são essenciais para a sobrevivência dos seres vivos, atuando na ativação de zimogênios, digestão de proteínas provenientes da dieta e do próprio organismo, coagulação sanguínea, etc. As proteases são subdivididas em dois grandes grupos, as exoproteases que clivam ligações peptídicas próximas às extremidades amino e carboxiterminais do substrato e as endoproteases que clivam as ligações peptídicas internas das cadeias polipeptídicas do substrato (Figura 7) (RAO et al, 1998).

Figura 7. Classificação das proteases: Endoproteases clivam ligações peptídicas dentro da proteína (1). Exoproteases, mais especificamente as aminopeptidases, clivam resíduos localizados na posição N-terminal da proteína (2). Figura modificada de Gonzales e Robert-Baudouy (1996).



As endopeptidases são divididas em subclasses, com base no mecanismo catalítico, e a especificidade é utilizada apenas para identificar enzimas individuais dentro dos grupos. As subsubclasses são: serina endopeptidases (serinoproteases) (EC 3.4.21), cisteína endopeptidases (cisteínoprotease) (EC 3.4.22), aspartato endopeptidases (aspartatoproteases) (EC 3.4.23), metaloendopeptidases (metaloproteases) (EC 3.4.24) e treonina endopeptidases (treoninoprotease) (EC 3.4.25).

Proteases estão entre as enzimas de peixes que recebem maior atenção, pois são responsáveis pela digestão de proteínas dos alimentos ingeridos, os componentes mais caros da alimentação de peixes. Levando-se em consideração o valor do pH no qual apresentam atividade máxima, estas enzimas podem ser classificadas em: proteases ácidas, neutras ou alcalinas (RAO et al., 1998).

Entre as proteases que atuam em pH alcalino, a tripsina apresenta grande importância, ocorrendo com abundância no sistema digestivo de organismos aquáticos. Ela faz parte da família das serinoproteases, caracterizadas por apresentar um mecanismo comum, envolvendo a presença de uma tríade catalítica composta de resíduos específicos: serina, histidina e ácido aspártico (Figura 8). Esta enzima cliva as ligações peptídicas no lado carboxila de resíduos de aminoácidos carregados positivamente como arginina e lisina (Figura 9) (KOMKLAO et al., 2007). Dentre os substratos sintéticos hidrolisados pela tripsina e usados em pesquisas científicas destacam-se: N-α-benzoil-L-arginina-p-nitoanilida (BApNA) e tosil-arginina-metil-éster (TAME) (SIMPSON, 2000).

Figura 8. Centro ativo de uma serinoprotease (Fonte: www.proteopedia.org).

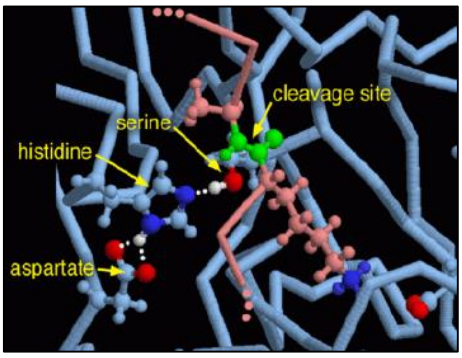
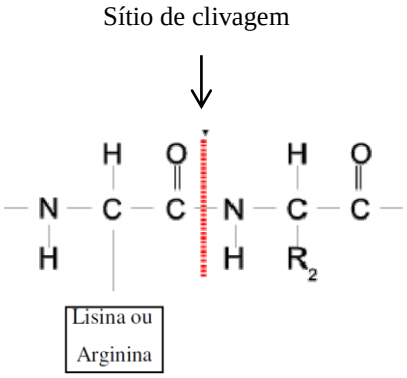
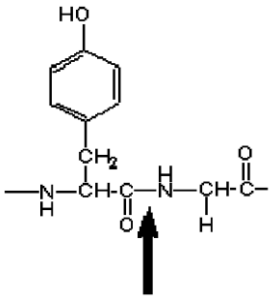


Figura 9. Sítio de hidrólise específico para tripsina (Fonte: BERG et al., 2004).



O suco pancreático dos peixes é rico em enzimas que atuam na digestão de proteínas. Além da tripsina, outras enzimas são armazenadas nas células pancreáticas na forma de zimogênios, como quimotripsina, carboxipeptidases e elastase (RUST, 2002). A quimotripsina é considerada a segunda enzima mais abundante no sistema digestório de organismos aquáticos (KOMKLAO et al., 2007). Esta enzima catalisa a hidrólise de ligações peptídicas de proteínas na porção carboxila de aminoácidos aromáticos como: fenilalanina, tirosina e triptofano e também substratos sintéticos, tais como Succinil-alanina-alanina-prolina-fenilalanina-*p*-nitroanilida - SAPNA (Figura 10) (CASTILLO-YAÑEZ et al., 2006).

Figura 10. Sítio de hidrólise específica para quimotripsina (Fonte: BERG et al., 2004).



No que diz respeito a biocatalisadores atuantes em pH ácido, destaca-se a pepsina, sendo considerada a principal protease ácida dos peixes, produzida na forma inativa de pepsinogênio (BALDISSEROTTO, 2009). A enzima é ativada inicialmente em condições ácidas, através da remoção de um peptídeo de baixo peso molecular pelo ácido clorídrico e continua o processo de transformação por autocatálise. O ácido clorídrico estimula ainda a liberação do hormônio secretina, responsável pela descarga do suco pancreático rico em íons bicarbonato que irão auxiliar na neutralização do pH no intestino (ROTTA, 2003).

2.5 Pepsina

A pepsina (EC, 3.4.23.1), incluída na categoria das endopeptidases, é a principal enzima digestiva estomacal de peixes. Pertencente à família das aspartatoproteases, possui especificidade preferencial por aminoácidos aromáticos, como fenilalanina, tirosina e triptofano. Esta enzima apresenta peso molecular em torno de 35 kDa, sendo de grande importância para a hidrólise das ligações peptídicas decorrente da degradação de proteínas sob condições ácidas (SIMPSON, 2000).

A atividade de pepsina medida pela sua capacidade de hidrolisar proteínas é influenciada pelo pH, temperatura e inibidores. Geralmente enzimas digestivas de peixes apresentam atividade ótima a temperaturas muito mais elevadas do que a temperatura do habitat dos peixes. Isto pode ser explicado pelo fato das temperaturas no interior do trato digestivo dos peixes serem mais altas em relação à temperatura ambiente (GILDBERG, 1988).

O pH ótimo e a estabilidade ao pH podem ter efeitos significativos sobre a atividade de pepsina dos peixes. Em geral, se o peixe apresenta mais de uma isoforma de pepsina, os pH ideais destas isoformas serão semelhantes. A temperatura ótima da

pepsina dos peixes costuma variar entre 30 a 55 ˚C. A temperatura ótima da pepsina de peixes depende muito dos tipos das espécies (água fria ou quente) (SHAHIDI et al., 2001).

Nem todos os inibidores de protease tem um efeito inibitório sobre a pepsina. Tem sido demonstrado que inibidores típicos, tais como fluoreto de fenil metil sulfonil (PMSF), inibidor de serino-protease; L-3-carboxytrans- 2, 3-epoxi-propionil-L-leucin-4-guanidino-butilamida (E-64), inibidor de proteinase cisteína e etileno-diamina-tetra-acético (EDTA) não tem qualquer efeito inibitório sobre pepsinas (ZHOU et al., 2008). Entretanto, pepstatina A (inibidor de proteinase aspártica) pode combinar-se com a pepsina de peixe, impedindo a ligação enzima – substrato, resultando assim numa completa inibição da atividade enzimática (COPELAND et al., 2005).

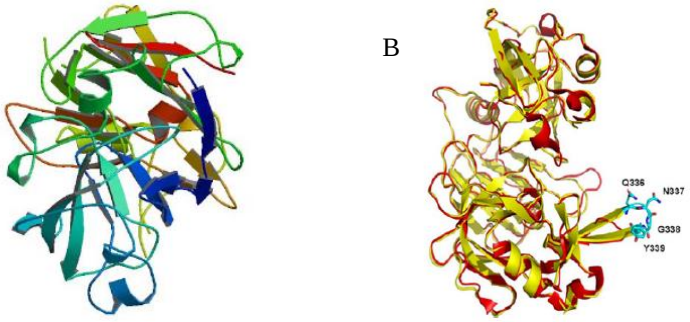
Pepsinas podem ser encontradas principalmente no suco gástrico do lúmen estomacal e podem ser isoladas a partir de uma variedade de espécies como mamíferos, aves e peixes. Há vários tipos de pepsinas estomacais ou isoformas, com estrutura proteica e propriedades enzimáticas distintas (SHAHIDI, 2001). Zimogênios de aspartatoproteases como pepsinogênios, também são secretados pelas glândulas da mucosa gástrica de vertebrados, incluindo peixes. Trata-se de uma forma antecedente inativa da enzima, a qual se torna funcional através da ação de cinases apropriadas ou outros ativadores (EFFRONT, 2007).

Comparado com a pepsina, o pepsinogênio contém 44 aminoácidos adicionais, sendo estável em ambientes neutro ou levemente alcalino. Na presença de ácido clorídrico proveniente do suco gástrico (pH 1,5–2,0), esses 44 aminoácidos são removidos proteoliticamente de uma forma autocatalítica resultando na ativação da pepsina (RAUFMAN, 2004). Pepsinogênios são sintetizados como precursores, contendo uma sequência sinal hidrofóbica de 15 -16 aminoácidos na região N-terminal servindo como função de transporte. A sequência sinal é perdida durante o processamento pós-transcricional. Esses pró-seguimentos são fundamentais na estabilização da forma inativa e prevenção contra a entrada do substrato na região do sítio ativo. Os pepsinogênios também diferem uns dos outros em relação às estruturas primárias e propriedades enzimáticas de suas formas ativas (KAGEYAMA, 2002).

As estruturas tridimensionais de pepsina e pepsinogênio a partir de espécies de peixes estão mostradas na Figura 11.

Figura 11: (A) Estrutura tridimensional da pepsina do bacalhau-do-Atlântico (*Gadus morhua*) (KARLSEN, 1998) e (B) pepsinogênio do peixe mandarim-ouro (*Sinipercascherzeri*) (DENG, 2010).

A B



Enquanto pepsinas de mamíferos têm sido completamente sequenciadas, pepsinas de peixes ainda não foram exploradas extensivamente. A primeira estrutura tridimensional proposta para pepsina do bacalhau foi idêntica à pepsina suína quanto à localização do domínio e sequência de aminoácidos (ANDREEVA et al., 2001). A estrutura da pepsina do bacalhau corresponde a uma cadeia proteica simples (um monômero) e dois domínios com dobramentos semelhantes separados por uma fenda (KARLSEN et al., 1998). Já o sítio catalítico da pepsina é formado pela junção de domínios e contém dois resíduos de ácido aspártico, Asp 32 e Asp 215 em cada domínio (WORTHINGTON, 2010).

Pepsinas e pepsinogênios têm sido isolados a partir da mucosa gástrica de várias espécies de peixes, incluindo *Latimeria chalumnae* (TANJI et al., 2007), *Sparus latus* Houttuyn (ZHOU et al., 2007), *Coryphaenoides pectoralis* (KLOMKLAO et al., 2007), *Mustelus mustelus* (BOUGATEF et al., 2008), *Siniperca chuatsi* **(**ZHOU et al., 2008), *Sardinella aurita* (KHALED et al., 2011), *Thunnus alalunga* (NALINANON et al., 2010) e *Anguilla anguilla* (WU et al., 2009).

2.5.1 Aplicabilidade industrial da pepsina

O emprego da pepsina no ramo industrial tem se tornado frequente nos últimos anos. Tecnologicamente utilizada na extração de colágeno e gelatina, a enzima também vem sendo vastamente aproveitada na terapêutica para fins de regulação da digestão, como antisséptico dental e no tratamento de algumas doenças, incluindo dispepsia, gastralgia, diarreia infantil e alguns tipos de câncer (GORGAS, 2009).

Combinados com HCl, comprimidos e cápsulas contendo pepsina têm sido desenvolvidos para melhorar a digestibilidade no trato gastrointestinal, bem como para aumentar o apetite dos pacientes (MURADO et al., 2009). Pepsina de suínos tem sido usada no tratamento de úlceras gástricas e na coagulação do leite para formação do coalho, importante processo na indústria alimentícia (ALTUN; CETINUS, 2007).

A pepsina é uma das enzimas utilizadas para análise de outras proteínas devido a sua eficiência em quebrar pontes envolvendo aminoácidos aromáticos, fenilalanina, triptofano e tirosina. Estudos têm reforçado o emprego desta enzima na purificação de soros antipeçonhentos aplicados à soroterapia de humanos picados por animais venenosos (BOUSHABA et al., 2003). A enzima ainda é aproveitada na produção de silagem de peixe, na indústria de processamento de pescado para produção de subprodutos e na indústria de ração animal em processos de digestibilidade proteica.

Assim, a pepsina é considerada uma protease promissora, com larga aplicação convencional e industrial, para tanto, técnicas eficazes para a sua recuperação e purificação devem ser desenvolvidas (NALINANON et al., 2010; ZENG et al., 2012).

2.6 Colágeno

Diversos tipos de proteínas estão presentes na matriz extracelular dos tecidos conjuntivos. As características biofísicas desta matriz são definidas pela disposição supramolecular de elementos fibrilares, redes microfibrilares, como também de proteínas, glicoproteínas e uma grande variedade de outras moléculas solúveis. Entre os diferentes tipos de tecido conjuntivo podemos encontrar variações na sua composição e estrutura (GELSE et al., 2003).

Em relação ao conteúdo proteico, o colágeno destaca-se como sendo a proteína mais abundante do tecido conjuntivo. Esta macromolécula é encontrada em várias partes da estrutura biológica animal, como por exemplo, na pele, nos ossos, nos tendões e nos dentes (SENARATNE et al., 2006). O termo colágeno é derivado de palavras gregas que significam "produzir cola" (COELHO et al., 2001), sendo essa, sua primeira aplicação industrial reportada na literatura. Esta proteína estrutural, comum nos vertebrados, constitui cerca de 30% do conteúdo proteico total e em humanos o colágeno representa 6% do peso corporal (MUYONGA et al., 2004).

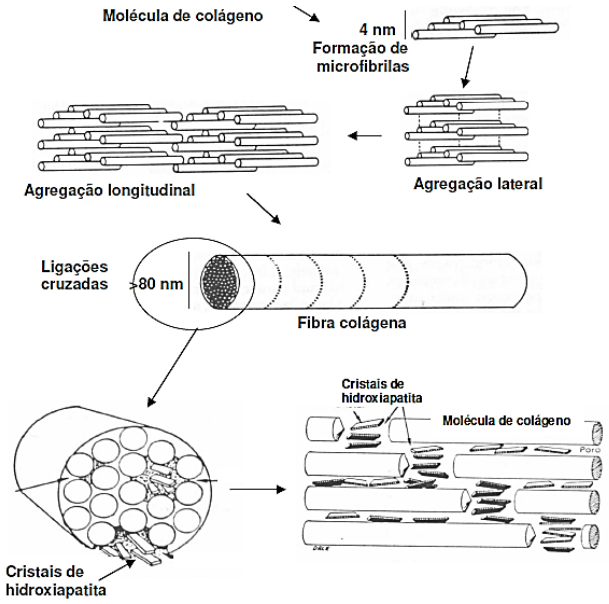
O colágeno apresenta como característica principal a capacidade de formação de fibras insolúveis e elásticas, que modulam forças externas e internas exercidas dentro do organismo. Apresenta também uma interessante capacidade de hidratação e

reabsorção e baixa antigenicidade. Outra importante função do colágeno é orientar tecidos em desenvolvimento. As fibras de colágeno começam a aparecer durante o desenvolvimento embrionário no processo inicial de diferenciação dos tecidos. Posteriormente, tornam-se, responsáveis pela integridade dos tecidos, dos ossos, cartilagens, pele e estrutura de vasos sanguíneos e outros órgãos (FRIESS, 1998).

2.6.1 Estrutura molecular do colágeno

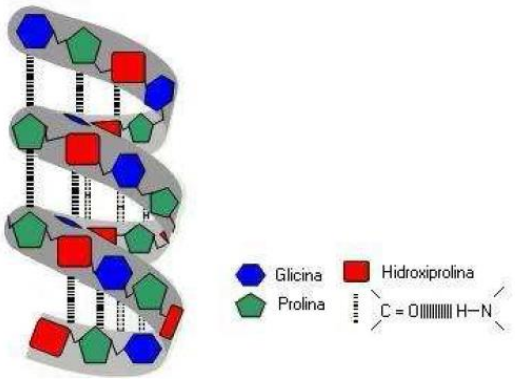
Ao microscópio óptico, o colágeno apresenta-se como fibras de espessura e orientação variadas, ocupando o compartimento extracelular, entre as células do tecido conjuntivo. Maiores detalhes estruturais só podem ser verificados através do microscópio eletrônico. A menor unidade é a fibrila com um padrão característico de estrias periódicas, que se repetem a cada 64 nm. Geralmente as fibrilas encontram-se agregadas, formando pequenos feixes, e tornam-se visíveis ao microscópio óptico quando excedem 0,2 μm, sendo chamadas de fibras colágenas. Os agregados de 4 a 8 moléculas de colágeno são formados durante o processo de fibrilogênese. Essas fibrilas possuem de 10 a 500 nm de diâmetro dependendo do tipo de tecido ou estágio do desenvolvimento. As fibrilas de colágeno se auto-organizam podendo formar moléculas ainda maiores, como as fibras (CAMPOS, 2008). A organização estrutural do colágeno durante a fibrilogênese, ou seja, durante o processo de agregação das microfibrilas, pode ser vista na Figura 12.

Figura 12: Desenho esquemático da organização do colágeno na sua estrutura supermolecular; adaptado de Ten Cate, 1994 e Yannas, 1996.



A proteína colágeno apresenta uma estrutura monomérica denominada tropocolágeno, constituído de três cadeias polipeptídicas que se apresentam na forma helicoidal e entrelaçam-se de modo a formar uma hélice tripla, sendo ancoradas umas nas outras por ligações de hidrogênio (MONTANHA et al., 2011). A manutenção da estrutura em tripla hélice é garantida devido a estas ligações de hidrogênio entre as fibras (grupos - NH de glicina e grupos carbonila C=O de resíduos localizados em outra cadeia polipeptídica) ou com moléculas de água (Figura 13).

Figura 13: Representação esquemática da estrutura da hélice tripla do colágeno adaptado por Brodsky e Ranshaw (1997).



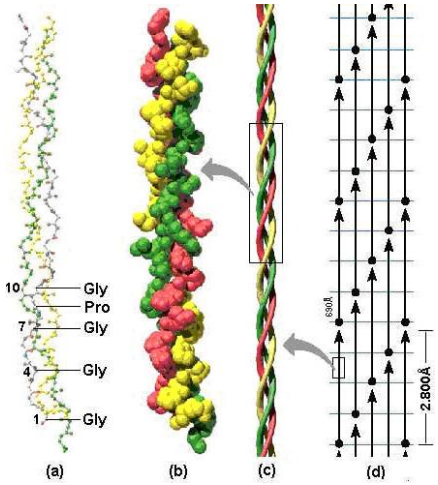
Estruturalmente, a tripla hélice, com orientação de sentido dextrógiro, apresenta um motivo, (Glicina-X-Y) n, comum a todos os vinte e nove tipos de colágeno e que se repete ao longo das cadeias. As posições X e Y são ocupadas com frequência por prolina e hidroxiprolina (MCCORMICK, 2009). A importância dessa estrutura está ligada a funções celulares como aderência e ativação da matriz extracelular (FIELDS, 1995).

O aminoácido glicina (Gly) possui o menor grupo radical e sua repetição ocorre a cada três posições na sequência das cadeias da hélice. Aproximadamente, 35% são posições de não-glicinas presentes na repetição de unidades Gly-X-Y. A posição X é usualmente ocupada pelo aminoácido prolina (Pro) e a posição Y, predominantemente, ocupada pela 4-hidroxiprolina. A molécula também contém um aminoácido não-usual chamado hidroxilisina, importante na formação da estrutura. Tanto a hidroxiprolina quanto a hidroxilisina alinham e estabilizam a tripla hélice. Por sua natureza alicíclica, elas formam ligações de hidrogênio, o que resulta na limitação da rotação da hélice.

Esse trio de aminoácidos, entrelaçados uns nos outros, formam uma estrutura semelhante a uma trança de forma helicoidal, formando uma cadeia extremamente resistente (CAMPOS, 2008).

A formação da estrutura hélice tripla estável (Figura 14) nos tecidos requer a modificação do aminoácido prolina (Pro), pela atividade da enzima prolil-hidroxilase (hidroxilação), para formar hidroxiprolina (Hyp) na cadeia do colágeno. Estudos mostram que a hidroxiprolina desempenha papel extremamente importante na estabilização da hélice tripla, pois defeitos na hidroxiprolina podem refletir na desorganização da hélice tripla e, portanto, de todo colágeno (SENA, 2004).

Figura 14: Estrutura do colágeno: (a) forma de tríplete presente nas matrizes colagênicas; (b) tropocolágeno; (c) hélice tripla; (d) modelo do quarto alternado pentafibrilar proposto por Smith (1968). (Fonte: SIONKOWSKA, 2006).



O arcabouço em tripla hélice do colágeno é uma estrutura altamente conservada que se encontra presente em todas as variações de colágeno. Em contrapartida, a presença de domínios não colagenosos entre os diferentes tipos de colágeno promove uma diversidade estrutural e funcional com diferentes variações desta proteína. Essa variedade contribui gerando estruturas que exigem a ação de enzimas específicas (colagenases) para a clivagem proteolítica. Na estrutura do colágeno, existem ainda regiões terminais amino e carboxi constituídas de 9-26 aminoácidos nas extremidades da molécula que não formam a estrutura da hélice. Essas regiões são denominadas telopeptídeos.

2.6.2 Tipos de colágeno

Diversos tipos de colágeno têm sido reportados na literatura, totalizando 29 estruturas, classificadas de I – XXIX. Esses subtipos proteicos exibem organização, sequência e função bem distintos, sendo caracterizados por possuírem considerável complexidade e variações na ocorrência de domínios não helicoidais. O colágeno pode ser dividido em três grandes grupos com base na sua estrutura macromolecular: a) colágeno fibroso estriado (I, II e III), b) colágeno não fibroso (IV), c) colágeno miofibrilar, o qual engloba os tipos VI e VII (matriz miofibrilar), V, IX e X (colágeno pericelular), e VIII e XI (XIONG, 1997).

Esses vários tipos de colágeno geralmente estão relacionados aos aspectos biomecânicos, entretanto além dessa função típica, essas proteínas realizam outras atribuições. Possuem importância fundamental no desenvolvimento de órgãos, envolvimento em processos de reparo de tecidos e cicatrização, podendo também atuar em eventos relacionados à sinalização celular, além de contribuir no armazenamento local de fatores de crescimento e citocinas. Essa capacidade de se vincular a fatores de crescimento e citocinas credencia estas moléculas como veículos de transporte com potencial para fins terapêuticos e farmacológicos (GELSE et al., 2003).

Dentre todos os tipos de colágenos existentes que já foram descobertos, o colágeno do tipo I tem despertado muita atenção por ser a proteína extracelular mais abundante, sendo também apontada como responsável pela manutenção da resistência mecânica nos ossos (SENA, 2004). Quanto à estrutura, o colágeno I trata-se de um heteropolímero formado por dois tipos de cadeias α, uma α_1 e uma α_2, em que a glicina constitui um terço do seu conteúdo de aminoácidos e possui baixos níveis de tirosina e histidina (MCCORMICK, 2009). Segundo Xiong (1997), o colágeno do tipo I é encontrado em todos tecidos conectivos, incluindo ossos e peles.

2.6.3 Fontes de colágeno

As mais abundantes fontes de colágeno são mamíferos, especialmente bovinos e suínos, sendo a pele um dos subprodutos mais disponíveis para extração de colágeno (GÓMEZ-GUILLÉN et al., 2002). A pele de animais, por exemplo, contém 30 a 35% de proteínas e destas 90 a 95% são representadas pela fração de colágeno (COELHO et al., 1998). Os ossos constituem outra grande fonte de colágeno, no entanto sua composição varia consideravelmente de acordo com a espécie e o tipo do osso. Esta variação decorre, principalmente, do fato de que os ossos contêm várias estruturas

teciduais distintas, dentre elas: cartilagens, tecidos ósseos esponjosos, tecidos ósseos compactos, entre outras (COELHO et al., 1998).

O colágeno contido nos subprodutos de animais como bovinos, suínos e aves tem sido amplamente utilizado em processos industriais por apresentar propriedades como biocompatibilidade, biodegradabilidade e baixa antigenicidade (LIU et al., 2009). Porém, devido aos recorrentes casos de zoonoses como: encefalopatia espongiforme bovina (BSE), encefalopatia espongiforme transmissível (TSE), febre aftosa (FA) e gripe aviária, a busca por fontes alternativas e mais seguras desse composto tornou-se uma opção mais viável (ZHANG et al., 2007).

Organismos aquáticos, como os peixes, devido a grande disponibilidade, baixos riscos de transmissão de doenças, alto rendimento nos processos de extração e ausência de toxicidade, têm se destacado como uma alternativa frente ao colágeno de animais terrestres. Além disso, o uso de derivados de peixes não apresenta restrições religiosas para determinados grupos sociais (SENARATNE et al., 2006).

A indústria da pesca também tem gerado uma grande quantidade de resíduos ricos em colágeno, pois cerca de 30% do resíduo produzido consiste em pele e ossos com alto conteúdo de colágeno (GÓMEZ-GUILLÉN et al., 2002). Sendo assim, estudos envolvendo extração de colágeno proveniente de espécies aquáticas têm crescido continuamente.

Extrações bem sucedidas a partir de pele, ossos e escamas de peixes e outros organismos marinhos foram reportadas pela literatura nesses últimos anos, a exemplo das espécies *Sepia pharaonis* (AEWSIRI et al., 2009), *Priacanthus tayenus* e *Priacanthus macracanthus* (BENJAKUL et al., 2010a), *Pangasianodon hypophthalmus* (SINGH et al., 2011), *Hypophthalmichthys molitrix* (SAFANDOWSKA et al., 2013), *Evenchelys macrura* (VEERURAJ et al., 2013) e *Doryteuthis singhalensis* (VEERURAJ et al., 2014).

2.6.4 Aplicações industriais do colágeno

O colágeno, em sua forma purificada, possui várias aplicações industriais, principalmente nos setores farmacêutico, de cosméticos e alimentos. A qualidade e aplicação específica do colágeno extraído estão diretamente relacionadas com suas propriedades funcionais e pureza (RUSTAD, 2003).

No que tange ao setor farmacêutico, a biomolécula do colágeno é utilizada na fabricação de implantes vítreos, carreadores de drogas e produção de compostos

biologicamente ativos. Extensas pesquisas têm sido efetuadas a partir da utilização de colágeno como biomaterial em pacientes humanos. Esses dispositivos variam desde suturas reabsorvíveis, vasos sanguíneos sintéticos até proteção de córnea danificada, regeneração óssea, tratamento de queimaduras na pele e muitas outras utilizações (CAMPOS, 2008).

O uso do colágeno tem sido amplo no campo biomédico. Características importantes como: baixa antigenicidade, biodegradabilidade e propriedades mecânicas, hemostáticas e de matriz suporte para crescimento de células têm favorecido o emprego do colágeno nesta área (YANNAS, 1996). Dentre os biopolímeros, o colágeno é o material de origem animal mais abundante e fornecedor de uma ótima base para biomateriais. Depois de extraído, o colágeno pode ser processado para obtenção de filmes, membranas e fibras (KOKOSZKA et al., 2010).

Na área médica essa proteína pode ser aplicada no tratamento de doenças angiogênicas, hipertensão, incontinência urinária e osteoartrite (WOO et al., 2008). Além de todo este conjunto de opções para aplicação do colágeno, o biopolímero pode ser de grande utilidade na indústria fotográfica, de couro e tecidos (IKOMA et al., 2003; WOO et al., 2008; LIU et al., 2009).

Embora com diversas aplicações na indústria de alimentos, o colágeno é pouco aproveitado tecnologicamente, em relação às suas propriedades funcionais como extensor, umidificante, emulsionante, ligante e potencializador de textura. Diversas fontes de colágeno são utilizadas na fabricação de produtos cárneos emulsionados, dentre as quais destacam-se a pele suína, colágeno de ossos e do músculo esquelético. A grande vantagem é que o colágeno trata-se de uma proteína digestível de fácil obtenção, considerada pela maioria das legislações como um alimento e não como um aditivo (COELHO et al., 2001).

Recentemente tem se desenvolvido materiais biológicos que combinam diferentes biopolímeros e materiais inorgânicos ao colágeno e a outros compostos biológicos, como a quitosana, visando gerar compostos que cumpram requisitos funcionais específicos. Entre os produtos obtidos a partir do colágeno, a gelatina tem apresentado um grande poder de comercialização. Isso se deve à abundância dessa matéria prima que tem um baixo custo e possui excelentes propriedades funcionais (KOKOSZKA et al., 2010).

3. OBJETIVOS

3.1 Geral

- Extrair, semi-purificar e caracterizar uma protease digestiva ácida (pepsina símile) presente no estômago do pintado (*Pseudoplatystoma corruscans*), bem como avaliar o seu potencial biotecnológico para extração de colágeno, além de isolar e caracterizar colágeno da pele do pintado.

3.2 Específicos

- Caracterizar físico-quimicamente a atividade da protease ácida a partir do extrato bruto do estômago do pintado;
- Avaliar a sensibilidade da atividade proteolítica ácida presente no extrato bruto frente a íons metálicos e inibidores específicos e inespecíficos;
- Investigar o potencial uso da protease ácida para extração de colágeno da pele de *Oreochromus niloticus*;
- Purificar parcialmente uma protease ácida a partir do estômago do peixe *P. corruscans* através de cromatografia de troca-iônica;
- Avaliar a pureza e determinar a massa molecular aparente da enzima através de SDS-PAGE e zimograma;
- Caracterizar físico-quimicamente a enzima parcialmente purificada;
- Avaliar a sensibilidade enzimática da fração parcialmente purificada frente a íons metálicos e inibidores específicos e inespecíficos;
- Extrair colágeno ácido solúvel (ASC) e pepsino solúvel (PSC) a partir da pele do pintado (*P. corruscans*);
- Determinar o rendimento da extração do colágeno ASC e PSC;
- Caracterizar parâmetros de extração e solubilidade do colágeno ASC e PSC;
- Determinar o peso molecular aparente das amostras de colágeno através de SDS-PAGE.

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CAPÍTULO 1

"Spotted sorubim (*Pseudoplatystoma corruscans*) viscera as a source of acid protease for collagen extraction"

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Spotted sorubim (*Pseudoplatystoma corruscans*) viscera as a source of acid protease for collagen extraction

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ABSTRACT

The fact that the increase in fish production is proportional to the residue left by this industry, contributing to environmental pollution, has emphasized the need to found applications to these by-products. Therefore, the aim of this study was to characterize an acid protease from the *Pseudoplatystoma corruscans* stomach, as well as to evaluate its use for collagen extraction from the *Oreochromis niloticus* skin. The proteolytic activities were determined using hemoglobin (pepsin), BApNA (trypsin), Sapna (chymotrypsin) and Leu-p-Nan (leucine aminopeptidase) as substrates. Amylase activity was evaluated using starch as substrate. Effects of metal ions and protease inhibitors on proteolytic activity and its physical-chemical were determined. The enzyme activity was completely inhibited by pepstatin, whereas TPCK, PMSF and EDTA inhibited 16.9%, 9.3% and 7.0%, respectively. Maximum pepsin activity was observed at 55°C and pH 3.0. However, the protease was sensitive to temperatures above 55°C during 30 minutes incubation. The same remained stable over a wide pH range (1.5 to 10.0). By zymography, four proteolytic bands were observed. The protease activity was not sensitive to some metal ions like Ba^{2+}, K^{+}, Mg^{2+}, Al^{3+}, Hg^{2+}, Cd^{2+} and Ca^{2+}. The total yield of collagen extracted from the *Oreochromis niloticus* skin was 40.4% and 24% of dry weight, using commercial pepsin and pepsin from spotted sorubim, respectively. The enzymatic extract showed interesting features like high activity, stability at neutral pH and high optimum temperature. Specific substrate and inhibitors provided additional evidences that this protease is most likely a pepsin-like enzyme with potential industrial application for collagen extraction.

Key words: fish waste, digestive enzymes, pepsin, collagen.

1. Introduction

The spotted sorubim (*Pseudoplatystoma corruscans*) is a carnivorous freshwater fish distributed throughout the South American rivers (São Francisco, Amazonas and Paraná) (Crepaldi et al., 2006). This specie comprises the largest fish of the Pimelodidae family and may reach 100-120 kg. It is among the fish species that show great commercial value and potential for Brazilian aquaculture (Inoue et al., 2009).

Increased fish production and, consequently, the volume of fish processed worldwide, has generated a lot of waste and byproducts. This fact represents a great challenge for entrepreneurs and scientific community interested in pursuing strategies for the production of aquatic organisms, either coming from the extraction or aquaculture, become a sustainable activity (Costa et al., 2013).

In fish processing, it is generated a large amount of liquid (wastewater) and solid (skin, bones, guts and fins) wastes. These are usually discarded without treatment and have no commercial value, only causing environmental pollution. From an environmental and economic perspective, a better utilization of fish waste as a source of biomolecules is suggested; as an example it can be highlighted the use of viscera as sources of enzymes with potential industrial applications (Silva et al., 2011; Freitas-Júnior et al., 2012).

Pepsin is a major digestive enzyme present in the stomach of vertebrates and belongs to the class of aspartyl proteases. It is an endopeptidase that cleaves peptide bonds on the carboxyl side of aromatic amino acids residues: phenylalanine, tyrosine and tryptophan. Acid proteases such as pepsin have a particular importance due to their application in fish silage (Murado et al., 2009) and collagen extraction (Veeruraj et al., 2014).

The number of studies aiming to extract collagen from marine fish has grown continuously. Several studies have reported successful extraction from skin, bones and scales of fishes as striped catfish (Singh et al., 2011), marine eel-fish (Veeruraj et al., 2013), silver carp (Safandowska et al., 2013), amur sturgeon (Veeruraj et al., 2014), grass carp, silver carp, bighead carp and black carp (Liu et al., 2014), black rockfish, sea bass and red sea bream (Cho et al., 2014).

The use of commercial pepsin can be costly for collagen extraction, therefore, the use of pepsin from spotted sorubim stomach can be a promising alternative due to its abundance and unique properties. Thus the aims of this study were to characterize acid protease activity from the *P. corruscans* stomach, as well as to use spotted sorubim pepsin for collagen extraction from the *Oreochromis niloticus* skin.

2. Material and Methods

2.1. Materials

All reagents used in assays were of analytical grade and purchased from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) and Bio-Rad Laboratories (Ontario, Canada).

2.2. Obtaining samples and preparation of stomach extract

Juvenile specimens (medium length at first maturity =75 cm) of spotted sorubim (n=3) with total length of 76.3 ± 1.5 cm and weighing 3.3 ± 0.1 kg were obtained from the São Francisco River, in Pilão Arcado, Bahia, Brazil. Stomachs were removed, washed with distilled water and stored at 4°C for transport to the laboratory. Then the stomachs (25.0 ± 1.3 g) were homogenized in 0.9% NaCl (w/v) at 4°C in the ratio 1:5 (w/v) using an electric tissue homogenizer (IKA RW 20D S32, China). The material

obtained was centrifuged (Sorvall RC-6 Superspeed Centrifuge - North Carolina, USA) at 8,000 xg for 20 minutes at 4°C. The supernatant (crude extract) was stored at -20°C for later use.

2.3.Protein determination

The protein concentration in the crude extract was determined according to the methodology described by Bradford (1976), using bovine serum albumin as standard.

2.4. Specific proteolytic activities

Enzymatic activities of trypsin, chymotrypsin and leucine aminopeptidase were determined in microplates using Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA) succinyl proline alanine phenylalanine p-nitroanilide (SApNA) and leucine-p-nitroanilide (Leu-p-Nan) as specific substrates, respectively. The substrates were prepared in dimethyl sulfoxide (DMSO) at a final concentration of 4 mM. For the assays, 30 μL of the extract were mixed with 140 μL of 0.1 M Glycine-HCl buffer, pH 2.5 and 30 μL of specific substrate for 15 minutes at 25°C. The formation of p-nitroaniline (product) was followed at 405 nm with a microplate reader (Bio-Rad Model X-Mark^{TM} spectrophotometer, California, USA). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute (Bezerra et al., 2005).

Acid protease activity was performed according Pavlisko et al. (1997) using 2.0% (w/v) hemoglobin (prepared in 0.1 M Glycine-HCl, pH 2.5) as specific substrate. The enzymatic reaction was prepared in triplicate using microcentrifuge tubes to which were added 50 μL of sample, 350 μL of buffer 0.1 M Glycine-HCl, pH 2.5 and 100 μL of substrate. After 30 minutes at 37°C, 500 μL of 10% (w/v) trichloroacetic acid was

added to stop the reaction and the mixture was centrifuged at 10,000 xg for 15 minutes. The absorbance at 280 nm of the supernatant was measured with a microplate reader. One unit (U) of enzyme activity was defined as the amount of enzyme able to produce a change of 0,001 unit of absorbance per minute.

2.5. Amylase activity

Amylase activity was evaluated according to the methodology of Bernfeld (1955) using 2% (w/v) starch as substrate. For the assay, 60 μL of sample were mixed with 375 μL of 10 mM phosphate buffer, pH 7.5 and 375 μL of substrate. After 10 minute at 37ºC, 1 mL of DNSA (3,5 dinitrosalycilic acid) was added and the mixture was heated at 100°C for 10 minutes. The absorbance at 570 nm was measured with a microplate reader. A blank control was prepared, in which the sample was replaced by distilled water. The enzymatic activity was expressed as micrograms of maltose released per minute per milligram protein.

2.6. Physical-chemical properties

The influence of temperature on the proteolytic activity was measured by incubating the enzyme extract with 2% hemoglobin in a water bath at temperatures ranging from 25 to 85°C (Bezerra et al., 2005). For the assay of thermal stability, the enzyme extract was incubated for 30 minutes at different temperatures (25 – 85°C) and then evaluated for proteolytic activity as described in 2.3. The influence of pH on the proteolytic activity was assayed by incubating the extract at different 0.2 M buffer solutions (Glycine-HCl, pH 1.5-3.5; Citrate-Phosphate, pH 4.0-7.5; Tris-HCl, pH 8.0-9.0; and Glycine-NaOH, pH 9.5-10.0) for 30 minutes with 2% hemoglobin as substrate. The absorbance at 280 nm of the supernatant was measured with a microplate reader.

The pH stability of the enzyme activity of extract was determinated after pre-incubation for 30 min at the different buffers according to Klomklao (2007) modified.

2.7. Effect of protease inhibitors

This assay was performed using the synthetic protease inhibitors: phenylmethylsulphonyl fluoride (PMSF, serine protease inhibitor), N-p-tosyl-L-lysin chloromethyl ketone (TLCK, trypsin inhibitor), *N*-tosil-*l*-phenylalanine chloromethyl ketone (TPCK, chymotrypsin inhibitor), at 5 mM, dissolved in DMSO, as well as ethylenediamine tetraacetic acid (0.5 mM EDTA, metalloprotease inhibitor), 5 mM β-mercaptoethanol (reducing agent) prepared in water and 0.5 mM Pepstatin A , a pepsin inhibitor, prepared in 10% methanol. The inhibitors were incubated with enzyme extract at a proportion of 1:1 (v/v) for 30 minutes at 25°C. After incubation, the pepsin activity assay was performed using 2% hemoglobin as substrate. The residual activity of sample was estimated by comparing it to a positive control (100% activity), in which the extract was incubated with DMSO, water or 10% methanol, depending on the inhibitor's solvent (Bezerra et al., 2001).

2.8. Effect of metal ions

Samples of the enzyme extract (30 µL) were added to a 96-well microtiter plate with solutions 1.0 mM (30 µL) of $MgCl_2$, $BaCl_2$, KCl, $CuCl_2$, $CdCl_2$, $CaCl_2$, $HgCl_2$ or $AlCl_3$ for 30 minutes. After incubation, the pepsin activity assay was performed using 2% hemoglobin as substrate. The residual activity of sample was estimated by comparing it to a positive control (Wu et al., 2009).

2.9. Zymogram

Protease zymogram under native conditions was carried out following the procedure described by Garcia-Carreño et. al. (1993). Lyophilized extract (30 µg of protein) was mixed with a solution containing 10 mM Tris-HCl (pH 8.0), 2.5% SDS, 10% glycerol and 0.002% bromophenol blue and applied in the polyacrylamide gel (12.5% w/v). The electrophoresis proceeded at 4°C under constant electric current (11 A). After the migration of the proteins, the gel was incubated at 37°C and immersed in 2% hemoglobin at pH 2.5 for 60 minutes. Then the gel was stained with a solution composed of 0.01% Coomassie Brilliant Blue, 25% methanol and 10% acetic acid for 24 hours. The background of the gel was destained by washing in a solution containing 10% (v/v) acetic acid and 25% methanol (v/v).

2.10. Use of *P. corruscans* acid protease for collagen extraction from the *Oreochromis niloticus* skin

2.10.1. Obtaining and preparation of samples

Prior to collagen extraction, stomach extract from *P. corruscans* was subjected to precipitation by ammonium sulfate (80% saturation). The Nile tilapia skins were obtained from local fishing industry (Terra & Mar Pescados / Recife-PE, Brazil). After collecting, the material were washed with cold distilled water and cut into small pieces (0.5 – 0.5 cm^2).

2.10.2. Pretreatment of skin

The collagens were prepared by the method of Nagai and Suzuki (2000) with a slight modification. 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 3h 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. Then, it was added 10% butyl alcohol in the ratio of 1:10 (w/v) to remove fats. The mixture was continuously stirred for 6h at 4ºC and the skin was washed as previously described. Then, it was added 3% hydrogen peroxide at a ratio of 1:10 (w/v) for whitening the skin and was carried out in the wash.

2.10.3. Extraction of acid soluble collagen (ASC)

The residue was extracted with 0.5 M acetic acid at sample/acid ratio of 1:20 (w/v) for 3 days. The resulting viscous solution was centrifuged at 20,000g 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 centrifugation at 20,000g 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 (ASC).

2.10.4. Extraction of pepsin soluble collagen (PSC)

The pepsin soluble collagen (PSC) was obtained through the incubation of the insoluble material obtained in the previous steps with commercial porcine pepsin (EC 3.4.23.1; crystallized and lyophilized, Sigma, MO) or fraction 80% saturation fraction (ammonium sulfate) from stomach of *P. corruscans*. Porcine pepsin and fraction were tested at the same level (5000 U/mL) for the comparative study. Incubation occurred with constant homogenization for 24 hours at 4°C. The pepsin-solubilized collagen

(PSC) was obtained by the same method as the ASC. The yield of ASC or 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 lyophilized skin used (g).

2.11. Statistical analysis

Data of enzyme activity were analyzed using one-way analysis of variance (ANOVA) complemented with Tukey's test. Differences were reported as statistically significant when $P<0.05$, using the program MicrocalTM OriginTM version 8.0 (Software, Inc, U.S.).

3. Results and Discussion

3.1. Enzymatic assays

In vitro assays were performed to determine the action of enzymes present in the *P. corruscans* stomach extract. It was observed high activity of pepsin (23.24 ± 0.1 U/mg) by using 2% hemoglobin as substrate. The high proteolytic activity in the stomach is certainly associated with the carnivorous habit of the species and highlights the role of that structure in the digestive process. Acid proteolytic activities have been previously reported in carnivorous fish (Zhao et al., 2011).

Specific proteolytic activities showed discrete values (0.08 ± 0.0 U/mg and 0.15 ± 0.0 U/mg) with BApNA and Leu-p-Nan as substrates, respectively. The use of SApNA, specific chromogen substrate, was not effective to determine the chymotrypsin-like activity (Table 1). However, this is not the first report of such enzymes in fish stomach. Lundstedt et al. (2004) showed activity for trypsin and chymotrypsin in the stomach of *P. corruscans*. Deguara et al. (2003) discuss the presence of alkaline proteases in the stomach of *Sparus aurata* and refer to other

240 authors concerning this fact. The pattern observed in proteolytic activities is probably
241 explained as a function of the pH of the reaction medium, since this factor is critical for
242 stability of the polypeptide chain of the enzyme. The distribution of proteases varies
243 depending on the species and morphology of the digestive tract.

244 The total amylase activity present in *P. corruscans* stomach was detected using
245 the long chain substrate, 2% starch (1.26 ± 0.3 U/mg) (Table 1). Similar result was
246 reported by Lundstedt et al. (2004), where juveniles of *P. corruscans* underwent
247 experimental diets. Because it is a carnivorous fish, carbohydrate digestion in spotted
248 sorubim is not considered essential in the digestive process. However, digestive
249 carbohydrases such as amylase has been reported in gastric juice and *Clupea harengus*
250 and *Dorosoma cepidianun* (Fange et al., 1979). Compared with omnivorous species, the
251 amylase in carnivorous fish has been less active (Horn et al., 2006; Al-Tameemi et al.,
252 2010).

253 Studies by Seixa Filho et al. (1999) reported the amylolytic activity of
254 omnivorous freshwater fish, as piracanjuba (*Brycon orbignyanus*) and piau (*Leporinus*.
255 *friderici*) compared with spotted sorubim. These authors found higher amylolytic
256 activity in spotted sorubim. Regarding amylase, several factors may influence its
257 activity in fish, among them, eating habits and the structural complexity of carbohydrate
258 (Al-Tameemi et al., 2010). The influence of hormones, metabolites and temperature on
259 amylase activity in aquatic organisms have been reported (Van Wormhoudt, 1980), a
260 fact that makes it even more difficult to compare quantitative results in the literature.

261

262 3.2. Effect of temperature on acid protease activity and stability

263 Physico-chemical parameters were analyzed for acid protease activity present in
264 the stomach extract of *P. corruscans*. The effect of temperature on the activity of acid

protease is illustrated in Figure 1A. Enzymatic assays have shown that protease exhibited maximum activity at 55°C. The activity of the enzyme at 25 – 35°C corresponded to more than 50% of the activity at optimum temperature. In the temperature range between 40 and 50°C, acid protease showed more than 90% of its activity (Fig. 1A).

Results on optimum temperature of pepsin in some species of fish have been reported in the literature, such as 37°C for *Parona signata* (Pavlisko et al., 1997), 45 to 50°C for *S. Houttuyn latus* (Zhou et al., 2007), 40°C for *Mustelus mustelus* (Bougatef et al., 2008), 40 to 45 °C for *Siniperca chuatsi* (Zhou et al., 2008), 35 to 40°C for *Anguilla anguilla* (Wu et al., 2009). Komklao et al. (2007) obtained optimum temperature of 45°C for pepsin in the stomach of *Coryphaenoides pectoralis*. The optimal temperature of *Thunnus alalunga* pepsin was 50°C (Nalinanon et al., 2010) and 40°C for *Sardinella aurita* aspartic protease (Khaled et al., 2011).

It is not known why the digestive enzymes of fish and other aquatic organisms present high activity at temperatures above the physiological temperature. Probably, the answer to this question is in the adaptations of their ancestors to climate change through evolution (Freitas-Júnior et al., 2012). The decrease of acid protease activity from *P. corruscans* at 60°C is presumed to be due to enzyme denaturation. Determination of the optimum temperature for maximum activity of an enzyme is interesting for future comparative studies, providing information on their expected activity under various physiological conditions (Fernandez et al., 2001). Furthermore, pepsins may possibly be used in industrial applications that require low processing temperatures, as collagen extraction.

The resistance of acid protease from *P. corruscans* to heating treatment is shown in Figure 1B. The enzyme remained practically stable at temperatures 25 - 45°C for 30

290 minutes, losing more than half of its activity after heating above 55°C. According to the
291 literature, the acid protease of *Oreochromis niloticus* retained more than 50% of its
292 activity after heating between 50 and 60°C for 30 minutes (El-Belgaty et al., 2004).
293 Pepsins from *Pectoralis coryphaenoides* were stable up to 40°C (Klomklao et al., 2007).
294 Acid proteins of *Sardinops sagax caerulea* were not stable above 45°C (Castillo-Yanez
295 et al., 2004). *Thunus alalunga* pepsin was stable up to 50°C (Nalinanon et al., 2010)
296 whereas the enzymatic activity from the viscera of *Sardinella aurita* was stable up to
297 40°C (Khaled et al., 2011). The enzymatic characteristics relating to thermal stability
298 tests can be useful for possible application in industrial processes (gelatin extraction,
299 milk clotting). The acid protease of *P. corruscans* fits well in the requirements for
300 industrial application of the enzyme, being an interesting alternative.
301
302 3.3. Effect of pH on acid protease activity and stability
303 The effect of pH on the activity of acid protease from *P. corruscans* was
304 determined over the pH range (1.5 to 6.5). Protease was active between pH 1.5 to 3.5,
305 with the optimum pH in the range of 3.0 (Figure 1C). Above pH 4.0, over 50% of the
306 activity of the acid protease was lost and enzymatic activity was not detected in pH
307 greater than 5.0. Optimum pH values between 2.0 and 4.0 have been reported for pepsin
308 of *S. latus houttuyn* (Zhou et al., 2007). Pepsins from the stomach of *Coryphaenoides*
309 *pectoralis* exhibited maximal activity at pH 3.0 and 3.5 (Klomklao et al., 2007). Similar
310 result was found for pepsins from mandarin fish (Zhou et al., 2008). The optimal pH for
311 hydrolysis of hemoglobin by pepsin was found at pH 2.0 for *Mustelus mustelus*
312 (Bougatef et al., 2008) and *Thunnus alalunga* (Nalinanon et al., 2010). Aspartic
313 proteinase isolated from the stomach of *Anguilla anguilla* showed optimal pH values of

2.5 and 3.5 (Wu et al., 2009). Khaled et al. (2011) reported maximum activity of pepsin from the viscera of sardinelle around pH 3.0.

To investigate the pH stability, the enzyme was incubated in different buffers in the pH range from 2.0 to 10.5 and enzymatic activity is shown in Figure 1D. The acid protease retained more than 80% of its original activity in all the pH range tested. At pH 2.0 the enzyme retained 100% of its activity. The stability of acid protease from the *P. corruscans* stomach at neutral pH suggests some interesting information about the storage of the enzyme. According Klomklao et al., (2006), stability of pepsins to particular pH can be determined by the net charge of the enzyme. Castillo-Yanez et al., (2004) also reported that acidic enzymes from *Sardinops sagax caerulea* were stable between pH 3.0 to 6.0. *Thunus alalunga* pepsin was stable in the pH range of 2 – 5 (Nalinanon et al., 2010). Pepsins were stable in the pH range of 2.0 – 6.0 (Klomklao et al., 2007) and 2.0 – 5.0 (Khaled et al., 2011). At pH 1.0 and 4.0, the acid protease retained 85.3% and 61.5% of its initial activity, respectively (Bougatef et al., 2008). Differences related to pH stability between various species of fish indicate that different molecular properties may be involved, such as enzyme conformation and stability of structural links (Klomklao et al., 2007).

3.4. Effect of protease inhibitors

In order to better understand the class of proteases in the stomach from spotted sorubim, its sensitivity to various inhibitors was tested. The proteolytic activity (with the substrate hemoglobin) was completely inhibited by pepstatin A (100%), classic inhibitor of aspartate proteases. The enzymatic activity was weakly affected by serine protease inhibitors: TPCK (17.0%), PMSF (9.4%) and TLCK (2.7%) (Table 2). The low inhibition in the presence of EDTA (7.1%) (metalloenzyme inhibitor) indicates that

these proteases activities are not fully ion-dependent. Likewise, β-Mercaptoethanol (3.6%) did not show high inhibitory effect on the proteases, suggesting that the reduction of disulfide bonds little affect their activity. These results show that the sample is composed mainly of aspartate proteases, especially pepsin.

Khaled et al. (2011) reported that protease derived from the stomach of *Sardinella aurita* was almost completely inhibited by pepstatin A (2 mM). Activity of pepsin from the stomach of albacore tuna was strongly inhibited by pepstatin A with the concentration range of 0.1– 10 μM (Nalinanon et al., 2010). Pepsins from the stomach of *Anguilla anguilla* and *Siniperca chuatsi* also were almost completely inhibited by pepstatin A, but specific inhibitors of serine proteinases (PMSF) and metalloproteinases (EDTA) had no inhibitory effect on the activity of pepsin (Wu et al., 2009; Zhou et al., 2008, Bougatef et al., 2008). However, information regarding the inhibition of proteases *in vitro* does not adequately reflect what occurs *in vivo*, where other factors may modify the physiological response of fish (El-Sayed et al., 2000).

3.5. Effect of metal ions

Some enzymes require additional chemical component (cofactor) such as inorganic ions for its activity. On the other hand, due to their bio-accumulative and non-biodegradable properties, some metals constitute one of the main groups of aquatic pollutants (Ramesh, 2006). The effect of metallic ions on the activity of acid digestive protease from spotted sorubim stomach was evaluated and is presented in Table 3. Compared with the control, highest inhibition of the pepsin activity was found in the presence of Ba^{2+} (23.7%), Mg^{2+} (20.7%) and K^{+} (16.9%) at 1 mM. Ions Al^{3+}, Ca^{2+}, Hg^{2+}, Cd^{2+} and Cu^{2+} inhibited 10.9%, 9.2%, 7.4%, 6.6% and 5.0%, respectively.

363 The aspartic protease activity from *S. aurita* was slightly affected by Mn^{2+}, Ba^{2+},
364 and Zn^{2+}. Ions Ca^{2+}, Na^{+} and K^{+} no affected on the activity. However, the addition of
365 Mg^{2+}, Cu^{2+} and Hg^{2+} decreased the activity by 50%, 50% and 60%, respectively
366 (Khaled et al., 2011). According Klomklao et al., (2007), the activity of pepsins was
367 increased in the presence of divalent cations ($CaCl_2$, $MgCl_2$ and $CoCl_2$), especially
368 when the concentration used increased. The influence of metal ions on the activity of
369 pepsin was also verified by Krejpcio et al. (2002), where there was an increase of
370 enzyme activity with increasing concentrations of Al^{3+}. The different effects of trace
371 elements on the activity of pepsin could perhaps be explained in terms of the stability
372 constants for the complexes of the various ions with pepsin. It is known that sub-lethal
373 concentrations of heavy metals can disturb the digestive enzymatic activity of the
374 exposed species (De Coen et al., 1998).
375
376 3.6. Zymogram
377 The acid proteolytic activity of the stomach from *P. corruscans* was evaluated
378 by zymogram. It was observed the presence of four protein bands, possibly isoforms of
379 pepsin (Figure 2). Several authors have reported the use of zymogram for confirmation
380 of activity of acid protease (Nalinanon et al., 2010; Khaled et al., 2011). Zymography
381 analysis indicated that pepsins of mandarin fish revealed degrading activity against
382 acid-denatured bovine hemoglobin (Zhou et al., 2008). Through the zymogram can
383 identify the number of active forms of certain enzyme in different species, thereby
384 providing a source of data for the knowledge and comparison of their digestive
385 physiology.
386
387 3.7. Collagen extraction

In relation to the collagen extraction, the total yield of collagen obtained from the skin of Nile tilapia was 40.4% and 24% of dry weight, using commercial pepsin and pepsin from spotted sorubim, respectively. ASC, PSC (commercial pepsin) and PSC (stomach from spotted sorubim) showed yields of 19.3%, 21.1% and 4.7%, respectively (Table 4). This result shows that the collagen present in the skin Nile tilapia is not completely soluble in acetic acid 0.5 M. The fact that the PSC (commercial pepsin) showed a greater extraction yield when compared to ASC, can be due to possibility of the collagen molecules in the *O. niloticus* skin are linked through crossed covalent bonds. These crosslinks happen through the condensation of groups aldehydes in the telopeptides areas and intermolecular of the collagen fact that causes a decrease in solubility of the protein in acetic acid (Foegeding et al., 1996, Zhang et al., 2007). The low yield of PSC (stomach from spotted sorubim) can be explained by the purification step used, resulting in an extract with various interfering substances.

By submitting the residue extraction with acetic acid to the action of pepsin, is obtained as a result of the cleavage telopeptide region and hydrolysis of crosslinks (Balian et al., 1977). Thus pepsin could be used as an aid for increasing the extraction yield of collagen from the skin of Nile tilapia. The yield of ASC from the skin of *O. niloticus* was higher than that from Brownstripe red snapper skin (9%) and lower than that from the skins of Japanese sea-bass (51.4 %), chub mackerel (49.8%), and bullhead shark (50.1%) (Nagai et al., 2000).

Others works reported in the literature showed the following yield for the extraction of collagen from skin: *Aluterus monóceros* (7.6%) (Ahmad et al., 2010), *Priacanthus tayenus* e *Priancanthus macracanthus* (7.7% e 7.1%) (Benjakul et al., 2010). These variations in yields are related both to different biological conditions, which each species is subject, as well as the conditions and methods of extraction

(McCormick, 2009). Also worth pointing out that structural differences in collagen is directly related to the performance of the extraction, because if the molecules in telopeptide region are highly cross-linked the collagen solubility in acid tends to decrease (Foegeding et al., 1996).

4. Conclusion

The characterization with specific substrate and inhibitors generates evidence that this protease is probably a pepsin-like enzyme. It was also observed that this protease showed low sensitivity to various metal ions. The enzyme showed interesting features like high activity, stability at neutral pH and high optimum temperature. Acid soluble and pepsin soluble collagen were extracted from the skin of *O. niloticus*. Thus, *P. corruscans* pepsin could be used as an aid for increasing the extraction yield of collagen from the skin of Nile tilapia. These features indicate that the stomach of *P. corruscans* can be used as a source of acid proteases with a potential for industrial application (collagen extraction).

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627 **Tables**

628

629 **Table 1:** Specific proteolytic and amylolytic activities from the *Pseudoplatystoma*
630 *corruscans* stomach. The results are represented by mean ± standart deviation (n=3).

Enzymes	**Specific activities (U.mg^{-1})**
Pepsin (2% hemoglobin)*	23.24 ± 0.1
Trypsin (8 mM BApNA)	0.08 ± 0.0
Chymotrypsin (8 mM SApNA)	ND
Leucine aminopeptidase (8 mM Leu-p-Nan)	0.15 ± 0.0
Amylase (2% starch)*	1.26 ± 0.3

631
632 Specific substrates: BApNA, benzoyl-DL-arginine-p-nitroanilide; SApNA, succynil-
633 alanine-alanine-proline-phenylalanine-p-nitroanilide; Leu-p-Nan, leucine-p-nitroanilide.
634 *(w.v^{-1})
635 ND = not detectable
636

637 **Table 2.** Inhibitory effect on the activity of proteases from *P.*
638 *corruscans* stomach using 2% hemoglobin ($w.v^{-1}$) as
639 substrate at 37ºC for 30 minutes. The results are represented
640 by mean ± standart deviation (n=3).

Inhibitors	**Residual activity (%)**
Control*	100^{a} ± 1.7
TLCK (5 mM)	97.3^{a} ± 3.1
β-Mercaptoethanol (5 mM)	96.4^{a} ± 0.4
EDTA (0.5 mM)	92.9^{a} ± 0.4
PMSF (5 mM)	90.6^{ab} ± 2.8
TPCK (5 mM)	83.0^{b} ± 5.9
Pepstatin A (0.5 mM)	0^{c} ± 0.0

641 *Activity without presence of inhibitors.

642

643 **Table 3.** Effects of metal ions on the activity of acid
644 digestive protease from *P. corruscans* stomach.
645
646

	Residual Activity (%)
Control*	100.00[a]
Ions (1 mM)	
Cu^{2+}	95.0[b] ± 2.6
Cd^{2+}	93.4[b] ± 4.9
Hg^{2+}	92.6[b] ± 1.0
Ca^{2+}	90.8[b] ± 2.3
Al^{3+}	89.1[b] ± 0.7
K^{+}	83.1[b] ± 0.5
Mg^{2+}	79.3[b] ± 0.1
Ba^{2+}	76.3[b] ± 0.5

647 *Activity without presence of ions.
648 Deionized water was used in the preparation of metal
649 ions. After incubation, the activity of pepsin assay
650 was performed using 2% hemoglobin ($w.v^{-1}$) as
651 substrate at 37ºC for 30 minutes. The results are
652 represented by mean ± standart deviation (n=3).
653

654 **Table 4.** Yield of collagen from the skin of *Oreochromus niloticus*.

Collagen extraction	**Yield [*] (%)**
ASC	$19.3^{a} \pm 1.5$
PSC (commercial pepsin)	$21.1^{a} \pm 2.3$
PSC (stomach from *P. corruscans*)	$4.7^{b} \pm 0.6$

655 [*] Mean value ± standart deviation from three separate samples. Values
656 followed by different superscript letters are significantly different at $P < 0.05$.

657

Figures

Figure 1. Effect of temperature and pH on the acid digestive protease from *P. corruscans*. (A) Optimum temperature for activity of enzyme. (B) Thermal stability of enzyme, after 30 min of incubation in the temperature range 25 – 80°C. (C) pH optimum for activity of enzyme, utilizing various buffers in the pH range 1.5 - 6.5. (D) pH stability of enzyme, after incubation of 30 min in the pH range 2.0 - 10.5.

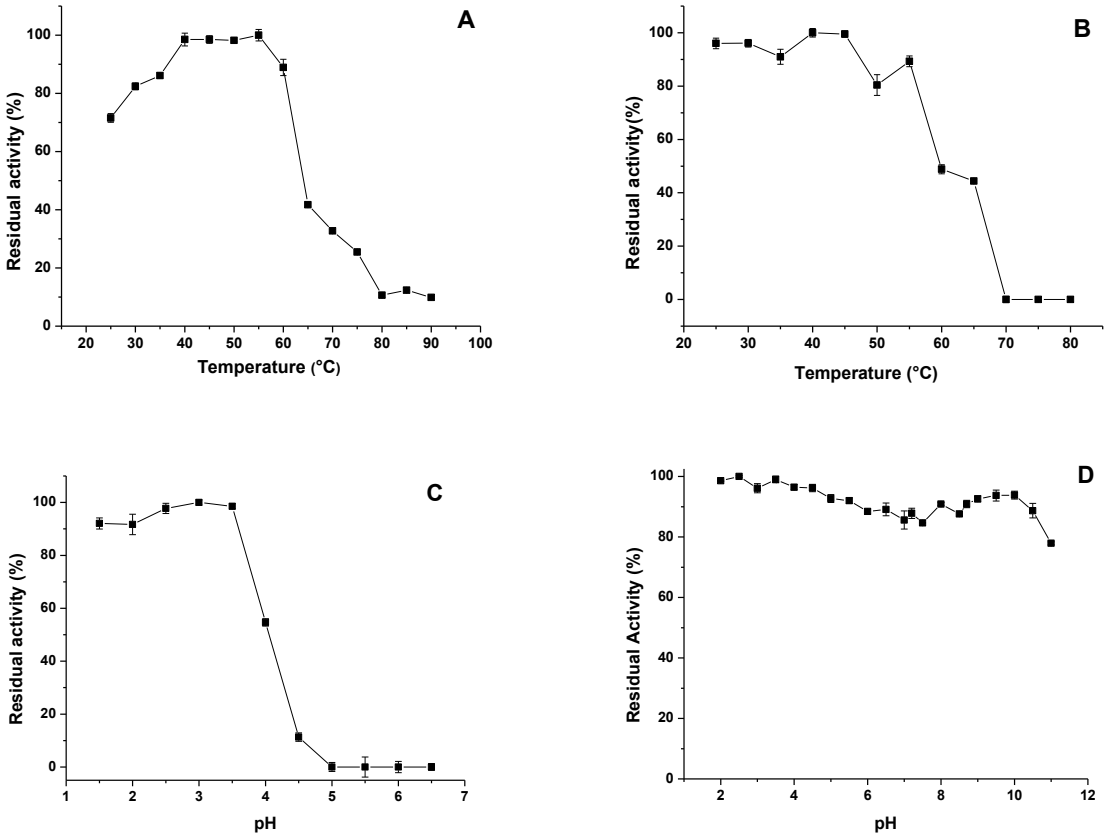
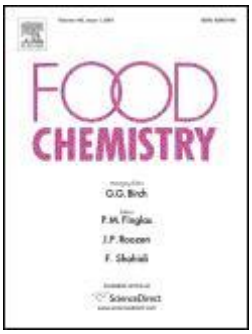


Figure 2. Zymogram of protease activity from *P. corruscans* stomach extract. The enzyme activity occurred in the presence of 2% hemoglobin as substrate. The zymogram was performed in the absence of SDS and 12.5% (w/v) separating gel. The gel was stained for protein overnight in a solution containing 0.25% (w/v) Coomassie Brilliant Blue, 10% (v/v) acetic acid and 25% methanol. The background of the gel was destained by washing in a solution containing 10% (v/v) acetic acid and 25% methanol (v/v).

CAPÍTULO 2

“Extraction, partial purification and characterization of acid protease from viscera of spotted sorubim (*Pseudoplatystoma corruscans*)”



A ser submetido ao periódico

FOOD CHEMISTRY

Extraction, partial purification and characterization of acid protease from viscera of spotted sorubim (*Pseudoplatystoma corruscans*)

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ABSTRACT

This work aimed to extract and partially purify pepsin-like present in the spotted sorubim stomach and determine the physical and chemical parameters of this protease. The crude extract was subjected to three purification steps: heat treatment, fractionation with ammonium sulfate and ion exchange chromatography on DEAE-cellulose column. The fractions were checked for the presence of protein and pepsin activity. All steps were monitored by SDS-PAGE. Effects of metal ions and protease inhibitors on its activity and its physical-chemical were determined. A purification factor of 32 times was obtained after application of fraction 0-60% in chromatography column. The SDS-PAGE showed polypeptide bands between 30.7 and 94 kDa. Maximum acidic protease activity was observed at 55°C and pH 3.0. The enzyme was stable at 25 and 30°C for 30 minutes The acidic protease remained stable at acid and neutral pH. The proteolytic activity was strongly inhibited by pepstatin A (87.3%), classic inhibitor of aspartate proteases. These features indicate that the stomach of *P. corruscans* can be used as a source of acidic proteases with potential for industrial application.

Key words: acidic protease, fish waste, partial purification.

1. Introduction

The global aquaculture production has experienced a steady growth due to an increase in demand for fish products. It is estimated that fishing and aquaculture activities in 2013 reached a new world record with 160 million tons. However, with the stagnation of the amount of fish from capture, aquaculture has taken in recent years, the responsibility to meet the demand for aquaculture products, by increasing the use of appropriate species and technologies (FAO, 2014).

Increased fish production and, consequently, the volume of fish processed worldwide, has generated a lot of waste and byproducts. These are usually discarded without treatment and have no commercial value causing environmental pollution. From environmental and economic perspective, a better utilization of fish waste as a source of biomolecules is suggested; as an example can highlight the use of visceras as sources of enzymes with potential industrial applications (Silva et al., 2011; Freitas-Jr et al., 2012; Costa et al., 2013).

Protease enzymes are responsible for the hydrolysis of peptide bonds present in proteins or peptides and correspond to 60% of all marketed worldwide enzyme. These biomolecules have different applications in a wide variety of industries such as detergents, foods, agrochemicals and pharmaceuticals (Gupta et al., 2002). Among the digestive protease, pepsin is the major enzyme present in the stomach of vertebrates. It is an endopeptidase (aspartyl protease) that cleaves peptide bonds on the carboxyl side of residues of aromatic amino acids: phenylalanine, tyrosine and tryptophan (Pavlisko et al., 1997).

Acid proteases such as pepsin, have a particular importance due to their application in cheese making (Aehle, 2007), fish silage (Murado et al., 2009) and collagen extraction (Veeruraj et al., 2014). Pepsins have been extracted, purified and

characterized from fish viscera as *Sparus latus* Houttuyn (Zhou et al., 2007); *Coryphaenoides pectoralis* (Klomklao et al., 2007); *Mustelus mustelus* (Bougatef et al., 2008); *Anguilla anguilla* (Wu et al., 2009); *Thunnus alalunga* (Nalinanon et al., 2010); *Sardinella aurita* (Khaled et al., 2011).

The spotted sorubim (*Pseudoplatystoma corruscans*) is a carnivorous freshwater fish distributed throughout the South American rivers (São Francisco, Amazonas and Paraná) (Crepaldi et al., 2006). This specie comprises the largest fish of the Pimelodidae family and may reach 100-120 kg. It is among the fish species that show great commercial value and potential for Brazilian aquaculture (Inoue et al., 2009). Hence, this paper aims to extract and partially purify pepsin-like present in the spotted sorubim stomach and determine the physical and chemical parameters of this protease for use in biotechnological applications.

2. Material and Methods

2.1. Materials

All reagents used in assays were of analytical grade and purchased from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) and Bio-Rad Laboratories (Ontario, Canada).

2.2. Obtaining, collecting samples and preparation of stomach extract

Juvenile specimens (medium length at first maturity =75 cm) of spotted sorubim (n=3) with total length of 76.3 ± 1.5 cm and weighing 3.3 ± 0.1 kg were obtained from the São Francisco River, in Pilão Arcado, Bahia, Brazil. Stomachs were removed, washed with distilled water and stored at 4°C for transport to the laboratory. Then the

stomachs (25.0 ± 1.3g) were homogenized in 0.9% NaCl (w/v) at 4°C in the ratio 1:5 (w/v) using an electric tissue homogenizer (IKA RW 20D S32, China). The material obtained was centrifuged (Sorvall RC-6 Superspeed Centrifuge - North Carolina, USA) at 8,000 xg for 20 minutes at 4°C. The supernatant (crude extract) was stored at -20°C for later use.

2.3. Enzyme partial purification

For the partial purification of the enzymes, the stomach crude extract was first submitted to a heat treatment at 30°C for 30 minutes and centrifuged at 8,000 xg for 20 minutes at 4°C. The supernatant was collected and fractioned with ammonium sulfate at a final salt concentration of 60%. After two hours a 4°C the sample was centrifuged at 8,000 xg for 20 minutes at 4 °C. The precipitate (fraction 0-60%) was resuspended in 10 mL of 0.9% NaCl (w/v) and dialyzed against this same buffer for 24 hours at 4°C. Then, the fraction was subjected to ion exchange chromatography on DEAE-cellulose column at a flow rate of 0.3 $mL.min^{-1}$. The chromatography fractions were checked for the presence of protein (absorbance at 280 nm) and pepsin activity. Fractions with higher pepsin activity were pooled and stored at -20°C for subsequent experiments. All steps were monitored by SDS-PAGE.

2.4. Pepsin activity

Pepsin activity was performed according Pavlisko et al. (1997) using 2.0% (w/v) hemoglobin (prepared in 0.1 M Glycine-HCl, pH 2.5) as specific substrate. The enzymatic reaction was prepared in triplicate using microcentrifuge tubes to which were added 50 µL of sample, 350 µL of buffer and 100 µL of substrate. After 30 minutes at 37°C, 500 µL of 10% (w/v) trichloroacetic acid was added to stop the reaction and the

mixture was centrifuged at 10,000 xg for 15 minutes. The absorbance of the supernatant was measured with a microplate read (Bio-Rad Model X-Mark^{TM} spectrophotometer, California, USA) at 280 nm. One unit (U) of enzyme activity was defined as the amount of enzyme able to hydrolyze hemoglobin to produce a change of 0,001 unit of absorbance per minute.

2.5. Protein determination

The protein concentration in the crude extract was determined according to the methodology described by Bradford et al. (1976), using bovine serum albumin as standard.

2.6. SDS-PAGE and zymogram

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the procedure described by Laemmli (1970), using a 4% ($w.v^{-1}$) stacking gel and a 12.5% ($w.v^{-1}$) separation gel. Protease zymogram under native conditions was carried out following the procedure described by Garcia-Carreño et al. (1993). Liofilizated sample (30 µg of protein) was mixed with a solution containing 10 mM Tris-HCl (pH 8.0), 2.5% SDS, 10% glycerol and 0.002% bromophenol blue and applied in the electrophoresis gel (14% w/v). The electrophoresis proceded at 4°C under constant electric current (11A). After the migration of the protein bands, the gel was incubated at 37°C, immersed in 2% hemoglobin at pH 2.5 for 60 minutes. Then the gel was treated with a solution composed of 0.01% Coomassie Brilliant Blue, 25% methanol and 10% acetic acid for 24 hours. The background of the gel was destained by washing in a solution containing 10% (v/v) acetic acid and 25% methanol (v/v).

134 2.7. Physical-chemical properties

135 The assays of temperature were performed in a water bath at temperatures
136 ranging from 25°C to 85°C. The influence of temperature on the proteolytic activity was
137 measured by incubating the enzyme extract with 2% hemoglobin for 30 minutes at each
138 temperature (Bezerra et al., 2005). For the assay of thermal stability, the enzyme extract
139 was incubated for 30 minutes at different temperatures and then incubated with 2%
140 hemoglobin for 30 minutes at 37°C. The influence of pH on the proteolytic activity was
141 assayed with different 0.2 M buffer solutions (Glycine-HCl, pH 1.5-3.5; Citrate-
142 Phosphate, pH 4.0-7.5; Tris-HCl, pH 8.0-9.0) for 30 minutes to each buffer using 2%
143 hemoglobin as substrate. The pH stability of the enzyme extract was determinate after
144 pre-incubation for 30 min at different buffers according to Klomklao (2007) modified.

145

146 2.8. Effect of protease inhibitors

147 This assay was performed using the synthetic protease inhibitors:
148 ethylenediamine tetraacetic acid (4 mM EDTA, metalloprotease inhibitor), 4 mM β-
149 mercaptoethanol (reducing agent) prepared in water and 0.5 mM Pepstatin A , a pepsin
150 inhibitor, prepared in 10% methanol. The inhibitors were incubated with enzyme extract
151 at a proportion of 1:1 (v/v) for 30 minutes at 25°C. After incubation, the pepsin activity
152 assay was performed according Pavlisko et al. (1997) using 2% hemoglobin as
153 substrate. The residual activity of sample was estimated by comparing it to a positive
154 control (100% activity), in which the sample was incubated with water or methanol,
155 depending on the inhibitor's solvent (Bezerra et al., 2001).

156

157 2.9. Statistical analysis

158 Data of enzyme activity were analyzed using one-way analysis of variance
159 (ANOVA) complemented with Tukey's test. Differences were reported as statistically
160 significant when $P<0.05$, using the program MicrocalTM OriginTM version 8.0
161 (Software, Inc, U.S.).
162
163 **3. Results and Discussion**
164 The three steps partial purification of the acid protease from the spotted sorubim
165 stomach are summarized in Table 1. After the first step (heat treatment) the specific
166 activity was higher than that in initial crude extract activity and the yield was 92.6%.
167 The second (ammonium sulfate fractionation) and third (ion exchange chromatography
168 on DEAE-cellulose column) steps presented yields of 41.1 and 30%, respectively. The
169 final supernatant (after chromatography) showed no enzymatic activity, demonstrating
170 efficiency in this stage of the partial purification process. A purification factor of 32
171 fold was obtained after application of fraction 0-60% in chromatography column.
172 According to the literature, usually, two saturations of $(NH_4)_2SO_4$ solution are applied
173 to achieve purification. Wu et al. (2009) used 20-60% saturation to achieve high
174 purification of European eel pepsinogens (PGs) obtaining purification factor of 28.3,
175 36.3 and 64.2 fold. Bougatef et al. (2008) used 20-70% saturation to purification smooth
176 hound PGs (38.3% recovery after chromatography). Klomklao et al. (2007) used 30-
177 70% saturation to purify pepsins from pectoral rattail (purification fator of 7.1 and 13.0
178 fold after chromatography).
179 The chromatogram of the elution of proteins (ion exchange chromatography on
180 DEAE-cellulose column) is shown in Figure 1. In this step, proteins (including pepsin)
181 are absorbed to stationary phase with oppositely charged molecules with different
182 absorption abilities. As the concentration gradient of eluant is gradually increased,

different types of proteins can be eluted and separated (Roe et al., 2001). Higher enzyme activity was found in the second peak. Tanji et al. (2007) used a DEAE–cellulose column eluted by a gradient of 0-0.5 M NaCl in a sodium phosphate buffer at 0.5 ml/min of a flow rate for the purification of different types of PGs from African coelacanth and North Pacific bluefin tuna. Bougatef et al. (2008) used a DEAE–cellulose column equilibrated by a Tris–HCl buffer with 0-0.5M NaCl to elute the column at a flow rate of 1.3 ml/ min for the purification of one type of PG from smooth hound.

After to the chromatography, the poll of the peak (higher enzyme activity) was applied to a polyacrylamide gel for performing an SDS-PAGE. The polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2A) shows proteins between 30.7 and 94 KDa. According to the literature, pepsins from fish have molecular masses between 28 and 42 kDa. Bougatef et al. (2008) found the molecular weight of one type of PG and its corresponding pepsin from smooth hound to be 40 and 35 kDa, respectively. Zhou et al. (2008) used a similar method for molecular weight determination and obtained 36, 35, 38 and 35 kDa for four purified PGs and 31, 30, 32 and 30 kDa for the four corresponding pepsins from mandarin fish, respectively.

The acid proteolytic activity of sample after chromatography process was evaluated by zymogram and it was observed the presence of one protein band only (Figure 2B). Several authors have reported the use of zymogram for confirmation of activity of acid protease (Nalinanon et al., 2010; Khaled et al., 2011). Zymography analysis indicated that pepsins of mandarin fish revealed degrading activity against acid-denatured bovine hemoglobin (Zhou et al., 2008). Through the zymogram can identify the number of active forms of certain enzyme in different species, thereby

207 providing a source of data for the knowledge and comparison of their digestive
208 physiology.

209 Physico-chemical parameters were analyzed for acid protease activity of partial
210 purified enzyme. The effect of pH on the activity of pepsin is illustrated in Figure 3A.
211 Enzymatic assays have shown that acid protease was active between pH 1.5 to 4.0 and
212 exhibited maximum activity at pH 3.0. Pepsin activity usually decreases at different pH
213 from optimum pH, presumably due to conformational changes of the enzyme because of
214 the influence of pH on the amino acid residues that compose it.

215 Optimum pH values between 2.0 and 4.0 have been reported for pepsin of *S.*
216 *latus Houttuyn* (Zhou et al., 2007). Similar results to the optimum pH of purified
217 pepsins from the stomach of other fish species can be found in the literature, such as
218 *Anguilla anguilla* (pH 3.5, 2.5 and 2.5 for three pepsins separate this specie) (Wu et al.,
219 2009), *Coryphaenoides pectoralis* (pH 3.0 to 3.5 for two pepsins isolated this specie)
220 (Klomklao et al., 2007). Khaled et al. (2011) reported maximum activity of pepsin from
221 the viscera of sardinelle around pH 3.0. Different results were found for *Thunnus*
222 *alalunga* (Nalinanon et al., 2010) and *Mustelus mustelus* (Bougatef et al., 2008), where
223 the purified pepsin had optimum pH 2.0.

224 To investigate the pH stability, the enzyme was incubated in different buffers in
225 the pH range from 1.5 to 9.0 and residual activity shown in Figure 3B. The acid
226 protease remained stable at acid and neutral pH. Castillo-Yanez et al. (2004) also
227 reported that acid enzymes from *Sardinops sagax caerulea* were stable between pH 3.0
228 to 6.0. Albacore tuna pepsin was stable in the pH range of 2-5 (Nalinanon et al., 2010).
229 Pepsins were stable in the pH range of 2.0-6.0 (Klomklao et al., 2007) and 2.0-5.0
230 (Khaled et al., 2011).

231 According Klomklao et al. (2006) the stability of pepsins to particular pH
232 appears to be determined by the net charge of the enzyme. Pepsins are irreversibly
233 inactivated at alkaline pH (Gildberg and Raa, 1983), therefore, the irreversible
234 inactivation in alkaline condition indicates that the acid protease obtained from *P*.
235 *corruscans* may be a pepsin. Furthermore, this characteristic of being stable at neutral
236 pH is very interesting with regard to the storage of the material, since the inactive
237 enzyme can be stored at a neutral pH solution, and again be subjected to an acid pH, it
238 returns the its enzymatic activity without major losses. Differences related to pH
239 stability between various species of fish indicate that different molecular properties may
240 be involved, such as enzyme conformation, stability of structural links, in addition to
241 anatomical location (Klomklao et al., 2007).

242 The effect of temperature (25-80ºC) on the activity of partial purified enzyme
243 was evaluated and is presented in Figure 4A. The acid protease showed maximal
244 activity at 55°C, but remained more than 50% of its maximum activity in the
245 temperature range from 25 to 65ºC. Results on optimum temperature of pepsin in some
246 species of fish have been reported in the literature, such as 37°C for *Parona signata*
247 (Pavlisko et al., 1997), 45 to 50°C for *S. Houttuyn latus* (Zhou et al., 2007), 40°C for
248 *Mustelus mustelus* (Bougatef et al., 2008), 40 to 45°C for *Siniperca chuatsi* (Zhou et al.,
249 2008), 35 to 40°C for *Anguilla Anguilla* (Wu et al., 2009). Komklao et al. (2007)
250 obtained optimum temperature of 45°C for pepsin in the stomach *Coryphaenoides*
251 *pectoralis*. The optimal temperature of *Thunnus alalunga* pepsin was 50°C (Nalinanon
252 et al., 2010) and 40°C for *Sardinella aurita* aspartic protease (Khaled et al., 2011).

253 The resistance of acid protease from *P. corruscans* to heat treatment is shown in
254 Figure 4B. The enzyme was stable at 25 and 30°C for 30 minutes, losing about 37 to
255 55% of its activity at temperatures of 35 to 40°C, respectively. According Klomklao et

al. (2005) digestive proteases of many aquatic organisms are active in adverse conditions, as temperatures above the physiological. Pepsins from *Pectoralis coryphaenoides* were stable up to 40°C (Komklao et al., 2007). Acid proteins of *Sardinops sagax caerulea* were not stable above 45°C (Castillo-Yanez et al., 2004). Albacore tuna pepsin was stable up to 50°C (Nalinanon et al., 2010) whereas the enzymatic activity from the viscera of sardinelle was stable up to 40°C (Khaled et al., 2011).

Assays of thermal stability of the enzyme are important because provides data from its relationship with possible industrial processes. Most processes where pepsins can be used is realized at low temperatures or ambient temperature due to the degradability of the material (collagen extraction, milk coagulation) or decrease the economic burden caused by the energy consumption for heating which indicates the possibility of using pepsin obtained from *P. corruscans* for industrial applications.

In order to better understand the class of proteases in the stomach from spotted sorubim, its sensitivity to various inhibitors was tested. The effect of inhibitors on the activity of partial purified protease are shown in Table 2. The proteolytic activity (with the susbstrate hemoglobin) was strongly inhibited by pepstatin A (87.3%), classic inhibitor of aspartate proteases. Can be seen in the results after 30 minutes of incubation, that the partial purified enzyme retained 100% of its activity in the presence of EDTA, metalloenzyme inhibitor, and about 76.8% of its activity in the presence of β-Mercaptoethanol, inhibitor of cysteine proteases. These results lead one to believe the sample is composed mainly of aspartate proteases, especially pepsin.

Pepsins from the stomach of *Anguilla anguilla* and *Siniperca chuatsi* also were almost completely inhibited by pepstatin A, but specific inhibitors of serine proteinases (PMSF) and metalloproteinases (EDTA) had no inhibitory effect on the activity of

pepsin (Wu et al., 2009; Zhou et al., 2008, Bougatef et al., 2008). Activity of pepsin from the stomach of albacore tuna was strongly inhibited by pepstatin A with the concentration range of 0.1-10 µM (Nalinanon et al., 2010). Khaled et al. (2011) reported that protease derived from the stomach of *Sardinella aurita* was almost completely inhibited by pepstatin A (2 mM).

4. Conclusion

The study of enzymes from visceras of spotted sorubim (*P. corruscans*) can contribute to sustainable development through the use of waste fish. This study, an acid protease from spotted sorubim stomach was partial purified. Physical chemical parameters were characterized and showed interesting features like high activity, stability at neutral pH and high temperature optimum. The use of a specific inhibitor produced evidence that this partial purified fraction is probably a pepsin-like enzyme. These features indicate that the stomach of *P. corruscans* can be used as a source of acid proteases with potential for industrial application.

Acknowledgements

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416 Chem., 56, 5401 – 5406.

417 **Tables**

418

419 **Table 1.** A summary of the purification of acid digestive protease from *P. corruscans* stomach.

Purification steps	Total protein amount (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (folds)
Crude extract	218.08	1.47	0.007	100.0	1.0
Heath crude extract	101.01	1.36	0.013	92.61	2.0
Ammonium sulphate (0-60%)	24.15	0.60	0.025	41.15	3.71
Ion exchange chromatography	2.05	0.44	0.215	30.09	31.96

420 *Excep for the heated crude extract (30°C for 30 min), all steps were performed at 4°C. The

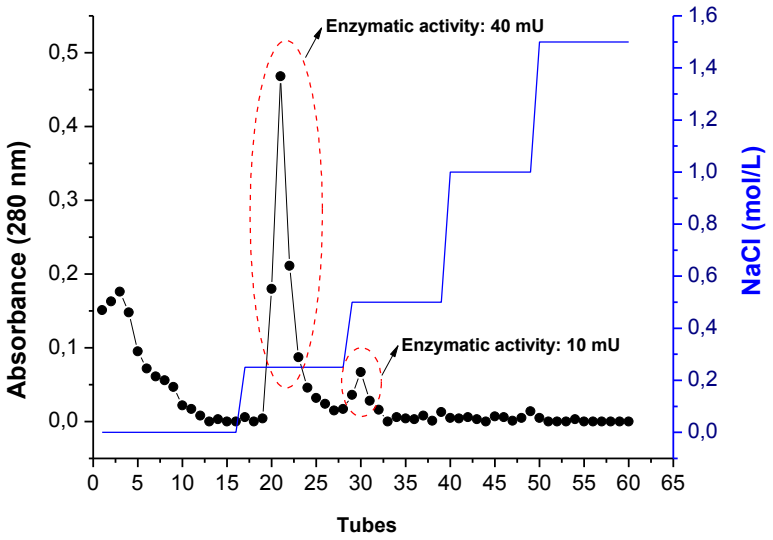
421 ion exchange chromatography was performed on DEAE-cellulose column.

422 **Table 2.** Effect of inhibitors on the activity of semi-purified acid
423 protease from *P. corruscans* stomach.

Inhibitor	Activity (%)	Inhibition (%)
Control*	100 ± 0.5[a]	0
Mercaptoethanol [4 mM]	76.8 ± 1[b]	23.2
EDTA [4 mM]	101.7 ± 0,4[a]	0
Pepstatin A [0.5 mM]	12.7 ± 1[c]	87.3

424 *Activity without presence of inhibitors. $p<0.05$

425 **Figures**



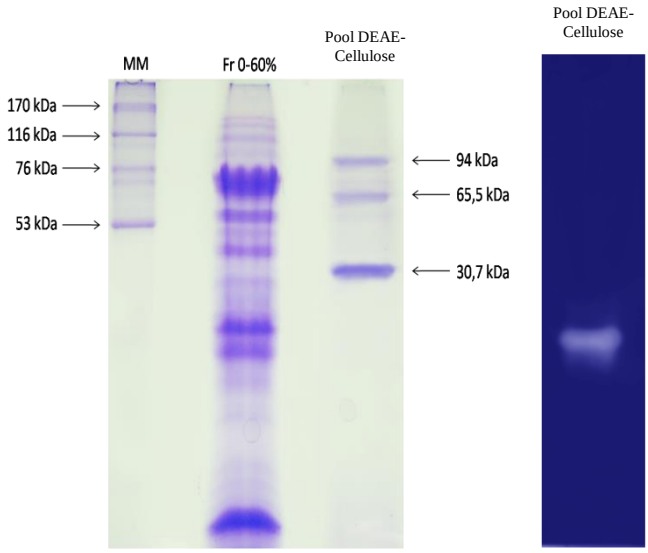
426

427 **Figure 1.** Chromatogram of purification on DEAE-Cellulose column: (●) Absorbance

428 (280 nm); (**-**) NaCl step gradient.

429

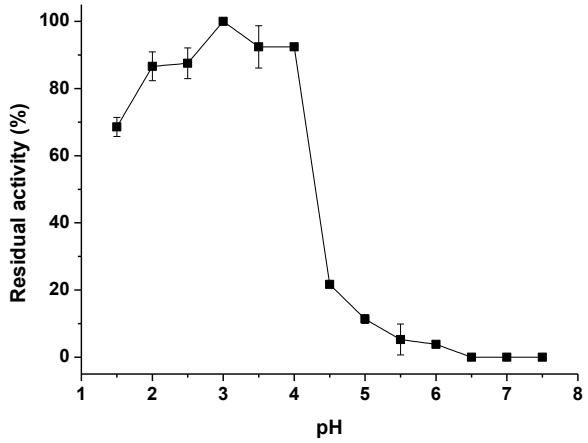
430



431

432 **Figure 2:** A) SDS-PAGE electrophoresis 12.5%. B) Native PAGE zymogram 14%

433 using 2% bovine hemoglobin as substrate.



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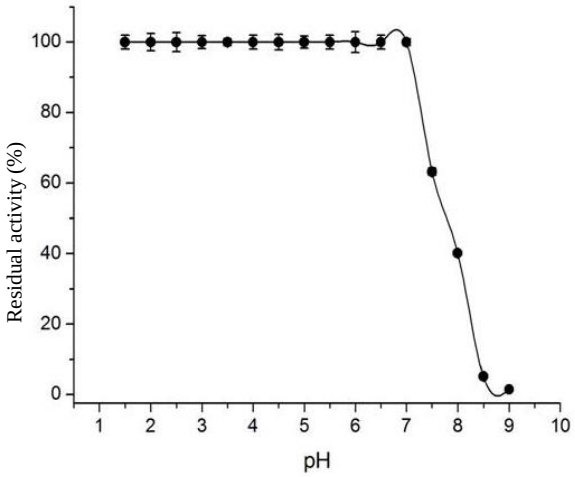
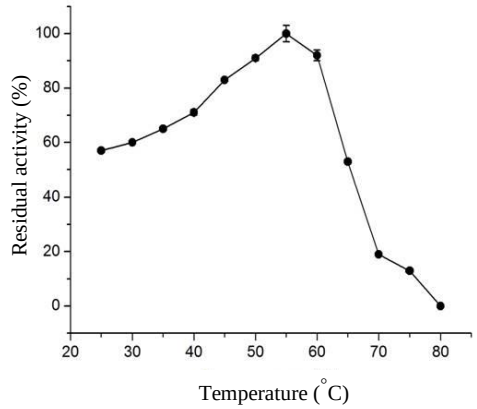


435

436 **Figura 3.** A) Effect of pH on the activity of semi-purified enzyme from the stomach of
437 *P. corruscans*. The enzymatic activity assay was performed at different pH (1.5 to 7.5).
438 Glycine-HCl buffer pH 1.5-3.5; Citrate-phosphate buffer pH 4.0-7.5; Tris-HCl buffer
439 pH 8.0-9.0. B) Effect of pH on the stability of semi-purified enzyme from *P. corruscans*
440 stomach. The enzyme was incubated for 30 min. at various pH (1.5 to 9.0).
441 Subsequently, the enzymatic activity assay was conducted at 37°C. The activity was
442 performed using 2% bovine hemoglobin as substrate.



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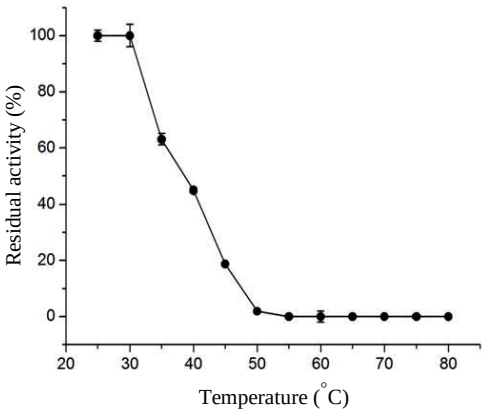

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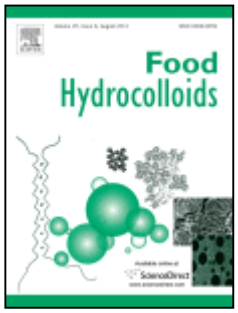
445

446 **Figura 4.** A) Effect of temperature on the activity of semi-purified acid protease from
447 *P. corruscans* stomach. Enzymatic activity assays were performed in the temperature
448 range from 25 to 75°C. B) Effect of temperature on the stability of semi-purified
449 enzyme from *P. corruscans* stomach. The enzyme was incubated for 30 min. at various
450 temperatures (25 to 75°C). After cooling enzyme was performed enzymatic activity
451 assay at 37°C. The activity was performed using 2% bovine hemoglobin as substrate.

452 .

CAPÍTULO 3

Isolation and characterization of skin collagen of spotted sorubim (*Pseudoplatystoma corruscans*)



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FOOD HYDROCOLLOIDS

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Isolation and characterization of skin collagen of spotted sorubim (*Pseudoplatystoma corruscans*)

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Abstract

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the skin of spotted sorubim (*Pseudoplatystoma corruscans*) were isolated and characterized. The total yield of collagen extraciton was 10.6% (dry weight). Both the ASC and PSC consisted of two different α chains (α 1 and α 2), their dimers (β chain) and trimmers (γ chain), and were characterized to be type I collagen. The ASC and PSC ultraviolet (UV) absorption spectrum showed a maximum absorption at 247 and 246 nm, respectively. The solubility of ASC and PSC reached maximum at pH 1 and 3, respectively. ASC was soluble in the range of NaCl concentration 0 – 2% (w/v) whereas PSC was soluble until 3% (w/v). The results obtained in this study indicate the possibility of using spotted sorubim skin as a source of biomolecules with great potential for biotechnological and industrial application.

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

1. Introduction

Collagen is the most abundant protein of animal origin and constitutes about 30% of total proteins (Muyonga et al., 2004). The structural protein of fibrous characteristic is a unique in its ability to form insoluble fibers with high resistance the traction (Gelse et al., 2003). It is main structural element of bones, cartilages, skin, tendons, ligaments, blood vessels, teeth and other present structures in the vertebrates (Kittiphattanabawon et al., 2010). At present, at least 29 variants of collagen have been identified, and each differs considerably in amino acid sequence, structure and function, more likely associated with specific genetic variants (Liu et al., 2012). All members of the collagen family are characterized by domains with repetitions of the proline-rich tripeptides, Gly-X-Y, involved in the formation of the triple helix (Muyonga et al., 2004). Among them, type I collagen is the most abundant, found in all connective tissue, such as skins and bones (Nagai et al., 2008; Duan et al., 2009).

Collagen has a wide range of applications as food, cosmetics, biomedical materials, pharmaceuticals and film industries due mainly properties as biocompatibility, biodegradability and low antigenicity (Liu et al., 2009). Commonly isolated from by-products of land-based animals, such as cows, pigs and poultry, the uses of collagen and collagen derived products of land animal origin have become of more concern due recurrent cases of zoonosis such as avian and bovine sponge encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD) and avian influenza (Binsi et al., 2009). As consequence, the search for alternative and safer sources this compound has become an attractive investment (Zhang et al., 2007; Duan et al., 2009).

Therefore, many scientists have been focusing their experiments to aquatic animals to produce collagen including freshwater and marine fish and mollusks (Pati et al., 2010; Jayathilakan et al., 2012; Veerurajat et al., 2014). Skin collagen from several fish species has been extracted and characterized such as silver carp (Safandowska et al., 2013), amur sturgeon (Veeruraj et al., 2014), grass carp, silver carp, bighead carp and black carp (Liu et al., 2014), black rockfish, sea bass and red sea bream (Cho et al., 2014). The number of studies with the objective of extracting collagen from sea fish has been growing continually due to advantages as great readiness; low risks of transmitting diseases; high yield in the extraction processes; toxicity absence (Senaratne et al.,

2006). Due to the large evolutionary distance between fish and humans, the risk of disease transmission by this route becomes very low (Song et al., 2006).

The observation of the fact that the increase in fish production is proportional to the residue left by this industry, and that it contribute to environmental pollution, have emphasized the need to use these products (eg as a source of biomolecules for industrial applications). Accordance to MPA (2010), Brazil produced approximately 1,2 millions tons, being among the twenty larger producing of fish of the world.

The spotted sorubim (*Pseudoplatystoma corruscans*) is a carnivorous freshwater fish distributed throughout the South American rivers (São Francisco, Amazonas and Paraná) (Crepaldi et al., 2006). This specie comprises the largest fish of the Pimelodidae family and may reach 100-120 kg. It is among the fish species that show great commercial value and potential for Brazilian aquaculture (Inoue et al., 2009). Thus the aim of this study was to isolate and extract the acid soluble (ASC) and pepsin soluble (PSC) collagen from the skin of *P. corruscans* as well as characterize and suggest its use as an alternative source of collagen to land animals.

2. Material and Methods

2.1 Collection and storage of samples

Juvenile specimens (medium length at first maturity =75 cm) of spotted sorubim (n=3) with total length of 76.3 ± 1.5 cm and weighing 3.3 ± 0.1 kg were obtained from the São Francisco River, in Pilão Arcado, Bahia, Brazil. Fish were transported to the laboratory and its skin removed. The skin was washed with cold water (5-8ºC) and cut into small pieces (0.5 cm^2). The prepared skin samples were packed in polyethylene bags and kept at -20ºC until used.

2.2 Chemicals reagents

All reagents were of analytical grade. Tipe I collagen from calf skin was purchased from Sigma chemical company (CAS Number 9007-34-5) (St Louis, MO, USA).

2.3 Pretreatment of skin

To remove non-collagenous proteins, the prepared fish skin was mixed with 0.2M NaOH at a skin/alkali solution ratio of 1:10 (w/v). The mixture was continuously stirred for 3h 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). To remove fats 10% butyl alcohol in the ratio of 1:10 (w/v) was used and the mixture was continuously stirred for 6h 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 bleached the skin and was carried out in the wash (Nagai and Suzuki, 2000).

2.4 Extraction of acid soluble collagen (ASC)

ASC was prepared by the method of Nagai and Suzuki (2008) with a slight modification. The residue was extracted with 0.5 M acetic acid at sample/acid ratio of 1:20 (w/v) for 3 days. The resulting viscous solution was centrifuged at 20,000 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 0.75 M Tris–HCl (pH 8.8). After standing overnight, the resulting precipitate was collected by centrifuging at 20,000 g for 60 min and then dissolved in 10 volumes of 0.5 M acetic acid. The solution obtained was dialyzed against 0.1M acetic acid and subsequently against distilled water. The dialysate was freeze-dried and referred to as acid soluble collagen (ASC).

2.5 Extraction of pepsin soluble collagen (PSC)

The pepsin soluble collagen (PSC) was obtained through the incubation of the insoluble material obtained in the previous steps with commercial pepsin (EC 3.4.23.1; crystallized and lyophilized, Sigma, MO) at enzyme/skin ratio of 1:20 (w/w) with constant homogenization for 24 hours at 4 °C. The pepsin-solubilized collagen (PSC) was obtained by the same method as the ASC. The yield of ASC or 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 skin used (g).

134

135 2.6 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

136

137 SDS–PAGE was performed following the method of Laemmli (1970).
138 Solubilized samples of ASC and PSC (25 μg of protein) were mixed at a ratio 1:1 (v/v)
139 with the sample buffer (containing 0.5 M Tris HCl, pH 6.8, 4% SDS, 20% glycerol and
140 10% b-ME), heated in a bath (IKA® Works Inc., China) at 85°C for 5 minutes and were
141 loaded on to polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel
142 and subjected to electrophoresis at a constant current of 15 mA/gel for 1 h and 30 min
143 using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After
144 electrophoresis, the gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15%
145 (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and
146 10% (v/v) acetic acid. Type I collagen from calf skin (Sigma-Aldrich Co., St. Louis,
147 MO) was also prepared following similar procedure and 10 μl were loaded as standard
148 collagen. High-molecular-weight protein markers (GE Healthcare UK Limited,
149 Buckinghamshire, UK) were used to estimate the molecular weight of proteins.

150

151 2.7 UV absorption spectrum

152

153 UV absorption spectra of ASC and PSC from spotted sorubim (*P. corruscans*)
154 skin was carried out according to Nalinanon et al. (2011), using a SmartSpec Plus
155 spectrophotometer Bio-Rad. The collagen samples were dissolved in 0.5 M acetic acid
156 solution with a sample/solution ratio of 1:1000 (w/v). The solutions were then placed
157 into a quartz cell with a path length of 1 mm. UV spectra were measured at wavelength
158 200 – 600 nm.

159

160 2.8 Solubility

161

162 The solubility of ASC and PSC was determined by the method of Montero et al.
163 (1991) with a slight modification. Collagens were dissolved in 0.5 M acetic acid to
164 obtain a final concentration of 3 mg/ml and the mixture was stirred at 4°C for 24h.
165 Thereafter, the mixture was centrifuged at 20,000 g for 60 min at 4°C. The supernatant
166 was used for solubility study.

167

168 2.8.1 Effect of pH on solubility
169
170 Sample solution (0.8 ml) was transferred to a 1.5 ml centrifuge tube and the pH
171 was adjusted with either 6 M NaOH or 6 M HCl to obtain the final pH ranging from 1 to
172 12. The volume of solution was made up to 1 ml by deionized water previously adjusted
173 to the same pH as the sample solution. The solution was centrifuged at 20,000 g for 60
174 min at 4°C. For all the samples, protein content in the supernatant was measured. Then
175 the relative solubility was calculated in the comparison with that of be obtained at the
176 pH giving the highest solubility.
177
178 2.8.2 Effect of NaCl on solubility
179
180 Collagen solution (0.5 ml) was mixed with 0.5 ml of NaCl in 0.5 M acetic acid
181 at various concentrations to give the final concentrations of 0%, 1%, 2%, 3%, 4%, 5%
182 and 6% (w/v). The mixture was stirred continuously at 4°C for 60 min, followed by
183 centrifuging at 20,000 g for 60 min at 4°C. Protein content in the supernatant was
184 measured and the relative solubility was calculated as previously described.
185
186 2.9. Statistical analyses
187
188 All experiments were performed in triplicate and expressed in means ± standard
189 deviation and a probability value of < 0.05 was considered significant. The mean values
190 were evaluated by analysis of variance (ANOVA) followed by Tukey test. The analyzes
191 were performed using the statistical program MicroCal® Origin® Version 8.0
192 (MicroCal, Northampton, MA, USA).
193
194 **3. Results and Discussion**
195
196 3.1 Yield of ASC and PSC from the skin of spotted sorubim
197
198 ASC e PSC were isolated from the skin of spotted sorubim with yields of 3.1%
199 and 7.5% (dry weight), respectively (Table 1). The skin was not completely solubilized
200 by 0.5 M acetic acid, as shown by the low yield of ASC. Pepsin-solubilized collagen
201 was readily solubilized from the residue from the acetic acid extraction. This result was

in agreement with Jongjareonrak et al. (2005a) who reported the incomplete solubilisation of bigeye snapper skin in 0.5 M acetic acid. The fact that the PSC showed a greater extraction yield, when compared to ASC, can be due to possibility of the collagen molecules in the *P. corruscans* skin are linked through crossed covalent bonds that they happen through the condensation of groups aldehydes in the telopeptides areas and intermolecular of the collagen fact that causes a decrease in solubility of the protein in acetic acid (Zhang et al., 2007). When submitting the residue of the acid acetic extraction to the pepsin action, the material obtained is a result of cleavage of the telopeptide regions and crossed covalent bonds (Foegeding et al., 1996).

The total yield of collagen from the skin of spotted sorubim (10.6%) was higher than that from brown stripe red snapper skin (9%) and lower than that from the skins of japanese sea-bass (51.4%), chub mackerel (49.8%), and bullhead shark (50.1%) (Nagai et al., 2000). The pepsin was able to cleave specifically at the telopeptide region of collagen from the skin of *P. corruscans*. Thus pepsin could be used as an aid for increasing the extraction yield of collagen from the skin of spotted sorubim.

Others works reported in the literature showed the following yield for the extraction of collagen from skin (wet weight basis): *Lutjanus lutjanus* (10.9%) (Kittiphattanabawon et al., 2005), *Aluterus monoceros* (7.6%) (Ahmad et al., 2010), *Priacanthus tayenus* and *Priancanthus macracanthus* (7.7% and 7.1%) (Benjakul et al., 2010), *Doryteuthis singhalensis* (81.4%) (Veeruraj et al., 2014). The yields of ASC and PSC from bigeye snapper skin were 6.4% and 1.1%, respectively (Jongjareonrak et al., 2005a) and 9.3% and 8.8%, respectively, for ASC and PSC from brownbanded bamboo shark (*Chiloscyllium punctatum*) (Kittiphattanabawon et al., 2010). Whereas for brownstripe red snapper skin, the yields of ASC and PSC were 9% and 4.7% respectively (Jongjareonrak, et. al., 2005b). The yield of ASC (dry weight) from the skin of ornate threadfin bream (*Nemipterus hexodon*) was 24.9% (Nalinanon et al., 2011). ASC and PSC from skin of *Diodon holocanthus* (dry weight) showed yields of 4% and 19.5% (Huang et al., 2011) and 80% and 7.1% from skin of *Evenchelys macrura* (Veeruraj et al., 2013), respectively.

These variations in yields are related both to different biological conditions, which each species is subject, as well as the conditions and methods of extraction (Regenstein et al., 2007; McCormick, 2009). Also worth pointing out that structural differences in collagen is directly related to the performance of the extraction, because if the molecules in telopeptide region are highly cross-linked the collagen solubility in

236 acid tends to decrease (Foegeding et al., 1996). The difference in efficacy of pepsin in
237 extracting collagen might be governed by fish species, collagen composition and
238 configuration or amount of pepsin used.
239
240 3.2. SDS–PAGE patterns of collagen
241
242 The acid and pepsin-solubilized collagens from skin of *P. corruscans* were
243 examined by SDS–PAGE, using a 7.5% gel (Figure 1). The subunit composition of
244 collagens exhibited a similar distribution of the bands observed in type I commercial
245 collagen obtained from bovine skin, used as standard, and in most collagen obtained
246 from skin and fish scales related in the literature (Singh et al., 2011; Li et al., 2013;
247 Veeruraj, et al., 2013). All collagens comprised at least two different α chains (α1 and
248 α2) and their dimers (β chain) and trimmers (γ chain), and the intensity of α1 chain was
249 about double than that of α2 chain. According to Singh et al. (2011), one of the
250 characteristics observed in the electrophoretic profile of type I collagen is a 2:1 ratio in
251 the intensity of bands α1 and α2, respectively.
252 The estimated molecular weight of α chain, using globular protein standards,
253 was approximately 120 kDa, which might be overestimated because of the difference
254 between globular protein and collagenous protein with high content of relatively small
255 amino acid residues (Gly and Ala) (Muyonga et al., 2004). The β and γ subunits have
256 molecular mass above 200 kDa, as also observed according to Foegeding et al. (1996).
257 These results suggested that the collagens were type I collagen mainly as many other
258 fish species. Similar electrophoretic patterns of type I collagen from the skin of
259 brownstripe red snapper were reported by Jongjareonrak et al. (2005b). Type I collagen
260 also was found in the skins of Nile perch (Muyonga et al., 2004), bigeye snapper
261 (Kittiphattanabawon et al., 2005), brownbanded bamboo shark (Kittiphattanabawon et
262 al., 2010), unicorn leatherjacket (Ahmad et al., 2010), striped catfish (Singh et al.,
263 2011), balloon fish (Huang et al., 2011), ornate threadfin bream (Nalinanon et al., 2011)
264 and marine eel-fish (Veeruraj et al., 2013).
265 Moreover, it can observe the differences in size of the bands corresponding to
266 the β chain between ASC and PSC. The intensity of γ chain was greater in ASC.
267 According to Sato et al. (2000) the increase on intensity and size of these chains reveal
268 the presence de large amounts of crossed bond protein. This may explain the better
269 extraction yield using pepsin, because the hydrolysis of the telopeptide and crosslinks

regions present in the β chain increases the acid solubility of collagen. The result suggested that the intra- and inter-molecular cross-links of collagens were richer in ASC than in PSC. Similar electrophoretic protein patterns were found in ASC and PSC from the skin of large fin long barbel catfish (*Mystus macropterus*) (Zhang et al., 2009).

3.3 UV-vis spectra

ASC and PSC isolated from *P. corruscans* skin showed a maximum absorption at 247 and 246 nm, respectively. It can be observed that both ASC as PSC exhibited a higher absorption rate of ultraviolet rays in the range 230-247 nm. The absorption spectrum UV of collagen was obtained by the skin of *P. corruscans* at the wavelengths 200 - 600 nm. Most proteins have a maximum ultraviolet absorption at 280 nm. 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 and Liu, 2006). Most work on processes of extraction and characterization of collagen reports a small amount of these amino acids in this protein (Liu and Liu, 2006; Yan et al., 2008; Huang et al., 2011; Singh et al., 2011). Others works exhibited a higher absorption rate of ultraviolet rays at 230 nm for collagen from skin of ornate threadfin bream (*Nemipterus hexodon*) (Nalinanon et al., 2011), frog skin collagen, 236 nm (Li et al., 2004); *Ictalurus punctaus*, 236 nm (Liu et al., 2007), *Theragra chalcogramma*, 220 nm (Yan et al., 2008). ASC isolated from *Oreochromis niloticus* skin showed a maximum absorption at 220 nm (Zeng et al., 2009). Both the ASC and PSC from the balloon fish skin (*Diodon holocanthus*) have maximum absorptions near 210 and 240 nm (Huang et al., 2011). ASC and PSC isolated from skin of marine eel-fish (*Evenchelys macrura*) showed maximum absorption at 225 and 228 nm (Veeruraj et al., 2013) whereas the both extracted collagens from outer skin of squid (*Doryteuthis singhalensis*) showed as a maximum absorption at 230 and 222 nm (Veeruraj et al., 2014). Furthermore, according to Liu and Liu (2006) due to the characteristics of collagen, can be expressed 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 collagen solubility.

304

305 3.4 Effect of pH on collagen solubility

306

307 The effect of pH on the solubility of ASC and PSC from *P. corruscans* skin was
308 shown in Fig. 2. It was observed that both samples showed higher solubility in acidic
309 pH range. The solubility of ASC and PSC reached maximum at pH 1 and 3,
310 respectively. In general, both collagens are solubilized in the acidic pH range (1-4)
311 (Jongjareonrak et al., 2005b). Marked decreases in solubility were observed in the
312 neutral and alkaline pH ranges. Futhermore, a minimum solubility was observed at pH
313 9.0 to ASC and pH 10.0 to PSC.

314 Profiles similar to collagens extracted from byproducts of other fish processing
315 have been found in the literature (Nalinanon et al., 2007; Li et al., 2013). Acid-soluble
316 collagens extracted from the skins of several underutilized fishes showed the highest
317 solubility at pH 2 (*Siganus fuscescens*), (*Myliobatis tobijei*), (*Dasyatis akajei*),
318 (*Dasyatis laevigata*) (Bae et al., 2008). PSC from the skin of unicorn leatherjacket
319 (*Aluterus monocerous*) was solubilized in the pH range of 1-6, with the highest
320 solubility at pH 2 (Ahmad et al., 2010). ASC and PSC extracted from the balloon fish
321 skin were solubilized to the highest extent in acidic pH range between 1 and 5. (Huang
322 et al., 2011). Maximum stability of ASC and PSC was found to be at pH 4 and 3,
323 respectively for collagen from the *Evenchelys macrura* (Veeruraj et al., 2013).

324 However the solubility profile of PSC was slightly greater than ASC at neutral
325 and alkaline pH. It is know that when the pH of the collagen solution reaches a value
326 equal or close to the isoelectric point (pI), there is a decrease in solubility caused by a
327 reduction in the amount of charges molecular (Vojdani, 1996). Foegeding et al. (1996)
328 report that the pI of type I collagen ranging between pH 6.0 and pH 9.0. At this point,
329 the total net charges of protein molecules are zero and hydrophobic-hydrophobic
330 interaction increases, thereby leading to the precipitation and aggregation of protein
331 (Singh et al., 2011). In contrast, when the pH is lower or higher than pI, the net charge
332 of protein molecules are greater and the solubility is increased by the repulsion forces
333 between chains. Therefore, this result is in accordance with the collagen solubility from
334 fish skin reported by literature.

335

336 3.5 Effect of NaCl concentration on collagen solubility

337

The effect of NaCl on the solubility of ASC and PSC extracted from skin of spotted sorubim is shown in Fig. 3. For ASC diluted in 0.5 M acetic acid, the protein starts to precipitate at a concentration of 1% (w/v) and has a large percentage precipitate when the NaCl concentration reaches 3% (w/v). The PSC remained more than 80% solubility under large part 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.

The solubility of collagens from the skin of brownstripe red snapper (*Lutjanus vitta*) and bigeye snapper (*Priacanthus tayenus*) in acetic acid solution decreased with increasing NaCl concentration (Jongjareonrak et al., 2005a; Kittiphattanabawon et al., 2005). Similar result was reported by Zeng et al. (2009) in which a solubility of ASC decreased sharply when NaCl content reached 2% and remained at constant low level at 4% NaCl or above. PSC from the skin of unicorn leatherjacket (*Aluterus monocerous*) had drastic decrease in solubility at NaCl concentration higher than 2% (Ahmad et al., 2010). Considerable decrease also was noticeable when the concentration was increased to more than 4% for acid and pepsin-solubilized collagens from the skin of balloon fish (*Diodon holocanthus*) (Huang et al., 2011). Expressive decrease in ASC and PSC solubility was observed with 3% NaCl or above for collagen extracted from the skin of *Evenchelys macrura* (Veeruraj et al., 2013).

The decrease in solubility of collagens could be described as being due to a "salting out" effect, which occurred at relatively high NaCl concentrations (Jongjareonrak et al., 2005a). 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 et al., 2008). Similar behaviours were found for ASC and PSC extracted from spotted sorubim skin.

However, PSC showed a greater solubility than ASC at NaCl concentrations used. Trend curves following a pattern where the ASC is more susceptible variations in the concentration of NaCl of what the PSC are commonly reported in the literature, brownstriped snapper fish (Jongjareonrak et al., 2005b); striped catfish (Singh et al., 2011); fish balloon (Huang et al., 2011); mackerel (Li et al., 2013). A greater solubility of PSC could be due to the partial hydrolysis of high MW cross-linked molecules by pepsin (Singh et al., 2011). In addition, the differences in amino acid compositions and structure between ASC and PSC might result in such differences.

4. Conclusion

Both ASC and PSC were successfully isolated from the skin of *Pseudoplatystoma corruscans*. Pepsin could be used as an aid for increasing the extraction yield of collagen. Based on SDS–PAGE patterns and absorption spectrum UV of collagen it may be suggested that collagenous material extracted in this work in both cases is type I, most commercially important. It was observed that both ASC and PSC showed higher solubility in acidic range of pH. However the solubility profile of PSC was slightly greater than ASC at neutral and alkaline pH. The solubility decreased in the presence of NaCl at concentrations above 3% although PSC showed a greater solubility than ASC at NaCl concentrations used. The use of pepsin showed useful due to the yield achieved and also to degree of preservation of structures, proving it caused little changes in collagen. Therefore, the skin of this piscivorous catfish could be an alternative source of collagen for further applications.

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577

Figure captions

Figure 1: SDS-PAGE patterns of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the *Pseudoplatystoma corruscans* skin. M, high-molecular weight markers; I, type I collagen from calf skin.

Figure 2: Relative solubility (%) of ASC and PSC from *Pseudoplatystoma corruscans* skin in 0.5 M acetic acid at different pHs.

Figure 3: Relative solubility (%) of ASC and PSC from *Pseudoplatystoma corruscans* skin in 0.5 M acetic acid with different NaCl concentrations.

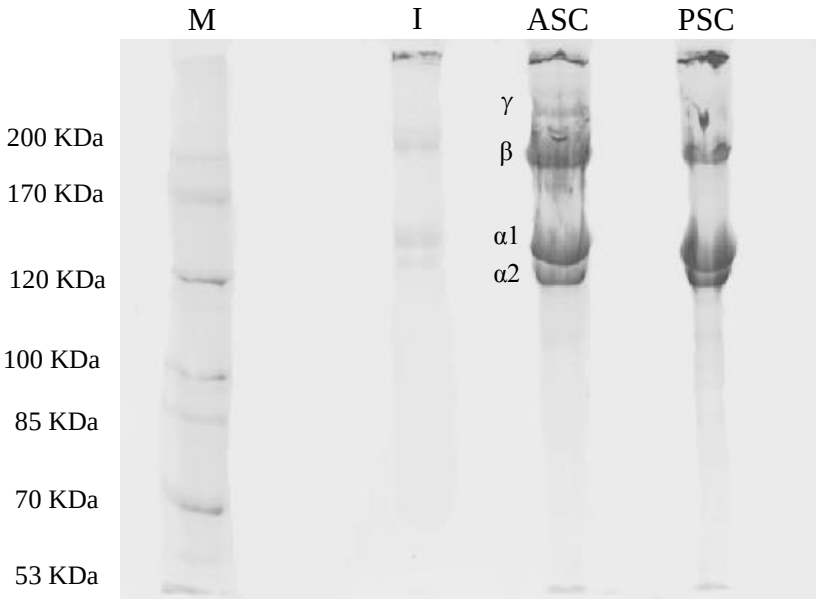
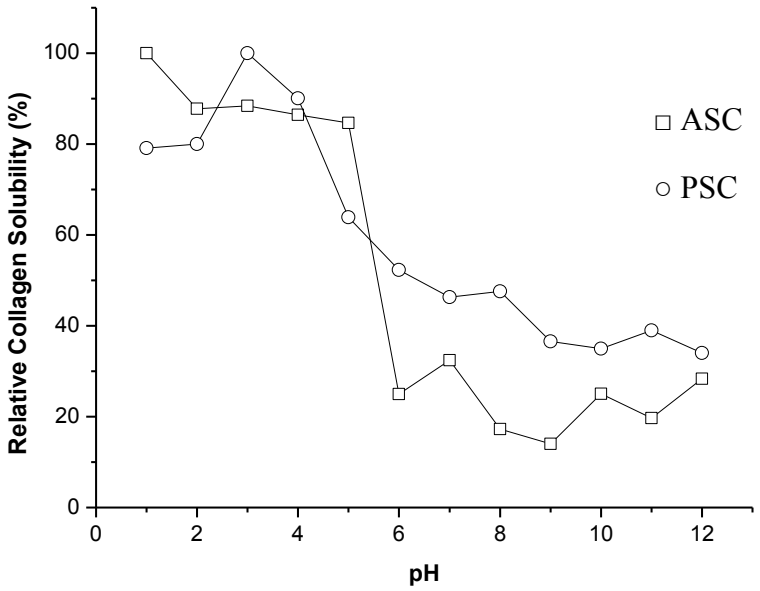


Figure 1



599

600 **Figure 2**

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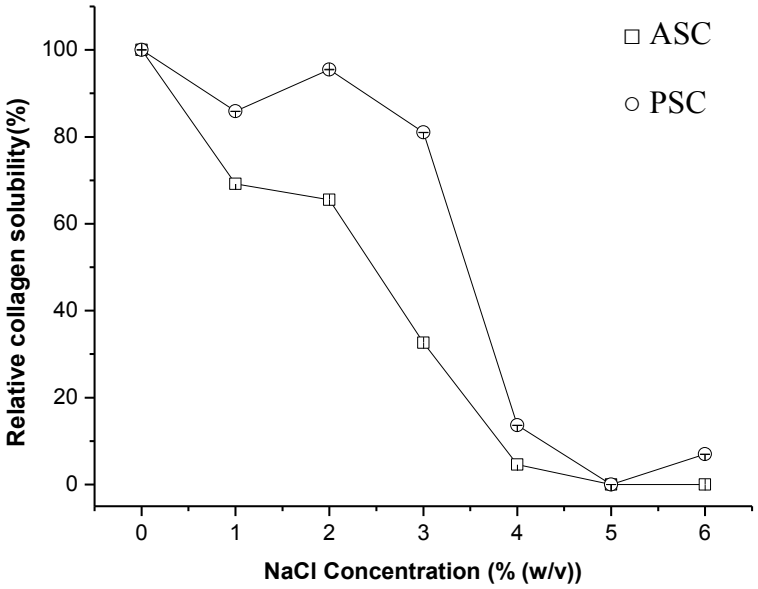

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604 **Figure 3**

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606 **Table 1.** The extraction yields of acid and pepsin soluble collagens from fish skin.
607

Species	Extract yield (%)		References
	ASC	PSC	
	(dry weight)		
Pseudoplatystoma corruscans	3.1	7.5	present study
Diodon holocanthus	4.0	19.5	Huang et al., 2011
Nemipterus hexodon	24.9	-	Nalinanon et al., 2011
Evenchelys macrura	80.0	7.1	Veeruraj et al., 2013
Amur sturgeon	37.4	52.8	Wang et. al., 2014
	(wet weight)		
Lutjanus lutjanus	6.4	1.1	Jongjareonrak et al., 2005a
Lutjanus vitta	9.0	4.7	Jongjareonrak, et. al., 2005b
Chiloscyllium punctatum	9.4	8.8	Kittiphattanabawon et al., 2010
Pangasianodon hypophthalmus	5.1	7.7	Singh et al., 2011

CONSIDERAÇÕES FINAIS

A presente tese reportou a purificação parcial e caracterização de uma protease ácida (pepsina símile) do estômago do pintado (*P. corruscans*), bem como o aproveitamento de resíduos do peixe (viscera e pele) para aplicação na extração de colágeno. O processo de purificação parcial, juntamente com as etapas de caracterização enzimática evidenciaram características interessantes da protease ácida estudada como acentuada atividade enzimática, estabilidade em pH neutro e elevada temperatura ótima, reforçando o grande potencial de utilização das vísceras do pintado como fonte de obtenção de proteases ácidas para aplicação industrial.

Em relação à aplicação biotecnológica dos resíduos do peixe, foi possível realizar a extração de colágeno proveniente da pele, bem como proceder com a utilização de protease ácida para aumentar o rendimento da extração. Colágeno ácido solúvel e pepsino solúvel tipo I foram isolados e caracterizados com rendimentos de 3,1% e 7,5% respectivamente. O biomaterial obtido apresentou propriedades físico-químicas similares a colágenos extraídos de pele de outros peixes citados na literatura.

Através das análises de caracterização dos colágenos obtidos (ASC e PSC) também se verificou a importância da utilização da pepsina no processo de obtenção de colágeno, pois além do aumento no rendimento, a estrutura da proteína manteve-se preservada, com pequenas mudanças. Assim, é possível afirmar que proteases ácidas provenientes do pintado possuem um grande potencial para aplicação em processos industriais, como o de aditivo no processo de extração de colágeno.

ANEXOS

ANEXO 1:

Guide for authors

***LWT*- Food Science and Technology**

INTRODUCTION

LWT - Food Science and Technology is an official journal of the Swiss Society of Food Science and Technology (SGLWT/SOSSTA) and the International Union of Food Science and Technology (IUFoST). LWT - Food Science and Technology is an international journal that publishes innovative papers in the fields of food chemistry, biochemistry, microbiology, technology and nutrition. The work described should be innovative either in the approach or in the methods used. The significance of the results either for the science community or for the food industry must also be specified. Contributions that do not fulfil these requirements will not be considered for review and publication. Submission of a paper will be held to imply that it presents original research, that it has not been published previously, and that it is not under consideration for publication elsewhere.

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b. Title (120 characters or less)

c. Short abstract (≤ 300 words).

d. Identify the address and contact information for the contact author. The contact information should include author name, postal address, telephone number, fax number, and email.

e. Anticipated time needed to complete the proposed work once the initial abstract has been approved

Manuscript Preparation

a. All lines and pages must be continuously numbered.

b. All text should be double-spaced.

c. Total manuscript length ≤ 3,000 words (text portion).

d. Total number of Tables ≤ 5.

e. Total number of figures ≤ 5.

f. Maximum number of references (including those cited in tables and figures) not to exceed 50.

g. In the reference list identify five (5) key references (indicated by an * in front of the reference in the reference section). In two to three sentences explain why this reference is a key reference.

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Results should be clear and concise.

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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.

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Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

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ANEXO 3:

Normas da revista: FOOD HYDROCOLLOIDS

AUTHOR INFORMATION PACK

TABLE OF CONTENTS.

DESCRIPTION.

Food Hydrocolloids only publishes original and novel research that is of high scientific quality. Research areas include basic and applied aspects of the characteristics, properties, functionality and use of **macromolecules** in **food systems. Hydrocolloids** in this context include **polysaccharides**, modified polysaccharides and **proteins** acting alone, or in mixture with other food components, as thickening agents, gelling agents, film formers or surface-active agents. Included within the scope of the journal are studies of real and model **food colloids** - dispersions, emulsions and foams – and the associated **physicochemical stability** phenomena - creaming, sedimentation, flocculation and coalescence.

In particular, *Food Hydrocolloids* covers: the full scope of **hydrocolloid behaviour**, including isolation procedures, chemical and physicochemical characterization, through to end use and analysis in finished food products; structural characterization of established food hydrocolloids and new ones ultimately seeking food approval; **gelling mechanisms**, syneresis and polymer synergism in the gelation process; rheological investigations where these can be correlated with hydrocolloids functionality, colloid stability or **organoleptic** properties; theoretical, computational or simulation approaches to the study of **colloidal stability**, provided that they have a clear relationship to food systems; surface properties of absorbed films, and their relationship to foaming and emulsifying behaviour; phase behaviour of low-molecular-weight surfactants or soluble polymers, and their relationship to food colloid stability; droplet and bubble growth, bubble nucleation, thin-film drainage and rupture processes; fat and water crystallization and the influence of hydrocolloids on these phenomena, with respect to stability and texture; direct applications of hydrocolloids in finished food products in all branches of the food industry, including their interactions with other food components;and toxicological, physiological and metabolic studies of hydrocolloids.

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AUDIENCE.

Food scientists and technologists, R&D managers, concerned with the application of science in the use, development and manufacture of food hydrocolloids.

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In particular, *Food Hydrocolloids* covers: the full scope of hydrocolloid behaviour, including isolation procedures, chemical and physicochemical characterization, through to end use and analysis in finished food products; structural characterization of established food hydrocolloids and new ones ultimately seeking food approval; gelling mechanisms, syneresis and polymer synergism in the gelation process; rheological investigations where these can be correlated with hydrocolloids functionality, colloid stability or organoleptic properties; theoretical, computational or simulation approaches to the study of colloidal stability, provided that they have a clear relationship to food systems; surface properties of absorbed films, and their relationship to foaming and emulsifying behaviour; phase behaviour of low-molecular-weight surfactants or soluble polymers, and their relationship to food colloid stability; droplet and bubble growth, bubble nucleation, thin-film drainage and rupture processes; fat and water crystallization and the influence of hydrocolloids on these phenomena, with respect to stability and texture; direct applications of hydrocolloids in finished food products in all branches of the food industry, including their interactions with other food components;and toxicological, physiological and metabolic studies of hydrocolloids.

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