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Produção de peptídeos bioativos a partir do colágeno isolado de dourado (*Coryphaena hippurus*)

NATHALIA ALBUQUERQUE ROBERTO

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2017

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Dissertação apresentada ao programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco - UFPE, como requisito final para obtenção do título de Mestre em Ciências Biológicas.

Orientador: Prof. Dr. Ranilson de Souza Bezerra
Co-orientadora: Prof.ª Drª. Ana Lucia Figueiredo Porto

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À minha família

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos” (Isaac Newton).

RESUMO

Os resíduos de processamento de peixe são uma importante fonte de recursos subutilizados que apresentam potencial para diversas aplicações biotecnológicas. A espécie *Coryphaena hippurus*, conhecida no Brasil como dourado, representa um importante recurso pesqueiro com uma ampla distribuição global. O colágeno, é uma proteína fibrosa encontrada em todo o reino animal, que possui uma variedade de aplicações industriais, principalmente nos setores farmacêutico, de cosméticos e alimentos. Devido aos baixos riscos de transmissão de doenças, alto rendimento nos processos de extração e ausência de toxicidade, organismos aquáticos têm se destacado como uma alternativa frente ao colágeno comercial (obtido a partir de animais terrestres). Assim, o presente trabalho teve por objetivo produzir peptídeos bioativos através da hidrólise do colágeno isolado da pele de dourado (*C. hippurus)*, empregando colagenase extraída de suas vísceras intestinais. O rendimento da extração do colágeno ácido solúvel (ASC) e pepsino solúvel (PSC) foi de 43% e 3%, respectivamente. A SDS-PAGE do ASC e PSC apresentaram padrão de bandas semelhante ao encontrado na literatura para amostras de colágeno, com cadeias α 1 e α 2, bem como cadeia β. O espectro de absorção ultravioleta (UV) de ambos colágenos apresentou absorção máxima a 230 nm. A solubilidade de ASC e PSC foi maior na faixa de pH ácida, alcançando seu máximo em pH 1 e 2, respectivamente. A solubilidade do ASC foi superior em baixas concentrações de NaCl, apresentando diminuição da solubilidade em concentrações acima de 1%. Os espectros FTIR do ASC e PSC exibiram picos característicos de Amida I, II, III bem como amida A e B, semelhantes a colágenos do tipo I de outros peixes. Os resultados obtidos neste estudo indicam a possibilidade de obtenção de proteases e colágeno do tipo I a partir da pele do dourado para a produção de peptídeos bioativos.

Palavras-chave: Colágeno. *Coryphaena hippurus*. Dourado. Resíduos. Peptídeos de colágeno.

ABSTRACT

Fish processing wastes are an important source of underutilized resources that have potential for various biotech applications. The species *Coryphaena hippurus*, represents an important fishery resource with a wide global distribution. Collagen is a fibrous protein found throughout the animal kingdom, which has a variety of industrial applications, mainly in the pharmaceutical, cosmetics and food industries. Due to the low risks of disease transmission, high yields in the extraction processes and absence of toxicity, aquatic organisms have stood out as an alternative against commercial collagen (obtained from terrestrial animals). Therefore, the present work aimed to produce bioactive peptides through the hydrolysis of collagen isolated from the skin of gold (C. hippurus), using collagenase extracted from its intestinal viscera. The extraction yield of ASC and PSC was 43% and 3%, respectively. The SDS-PAGE of the ASC and PSC presented bands pattern similar to that found in the literature for samples of collagen, with α 1 and α 2 chains, as well as β chain. The ultraviolet (UV) absorption spectrum of both collagens showed maximum absorption at 230 nm. The solubility of ASC and PSC was higher in the acid pH range, reaching its maximum at pH 1 and 2, respectively. The solubility of the ASC was higher at low NaCl concentrations, with a decrease in solubility at concentrations above 1%. FTIR spectra of ASC and PSC exhibited characteristic peaks of Amide I, II, III as well as amide A and B, similar to type I collagens of other fish. The results obtained in this study indicate the possibility of obtaining proteases and type I collagen from the skin of gilt for the production of bioactive peptides.

KEYWORDS: Collagen. *Coryphaena hippurus*. Dolphinfish. Wastes. Collagen Peptides.

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LISTA DE SIGLAS E ABREVIATURAS

(t) – toneladas

µg - Micrograma

µL - Microlitro

ASC - Colágeno ácido solúvel

ATPS – aqueous two-phase system

Azocoll – Azo dye-impregnated collagen.

BSE - Encefalopatia espongiforme bovina

Da - Dalton

FAO - Organização das Nações Unidas para Alimentação e Agricultura

IUCN - União Internacional para a Conservação da Natureza e dos Recursos Naturais

kDa – Quilo Dalton

MPA - Ministério da Pesca e Aquicultura

PEG – polietilenoglicol

pH - Potencial Hidrogeniônico

PPG - polipropilenoglicol

PSC - Colágeno pepsino solúvel

SDFA – sistema de duas fases aquosas.

SDS - Sódio dodecil sulfato

SDS-PAGE - Eletroforese em gel de poliacrilamida utilizando SDS

TSE - Encefalopatia espongiforme transmissível

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

TCA - Ácido tricloroacético

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1 INTRODUÇÃO

A indústria pesqueira é uma das principais fontes de meios de subsistência para a população mundial e tem apresentado um constante crescimento, impulsionado, principalmente, pela necessidade de suprir a demanda mercado consumidor. Seguindo esta tendência, nos últimos 10 anos, o Brasil apresentou um crescimento de 16.9% na produção de pescado, e atualmente ocupa a 10ª posição do ranking mundial com um patamar de 266.042 milhões de toneladas (t) registrado, em 2012 (FAO, 2014).

O aumento da produção de pescado é concomitante a produção de resíduos, seja através da captura artesanal ou das etapas industriais de processamento, que incluem materiais como vísceras digestivas, ossos, pele, escamas, nadadeiras e cabeças, representando aproximadamente 70% do peso total do peixe (KOMKLAO, 2008). Outrora, já foram considerados de baixo valor e utilizados como alimento para animais de criação, na produção de silagem e fertilizantes ou jogados fora. Entretanto, nas últimas duas décadas, os subprodutos do pescado têm apresentado potencial para serem utilizados para diversos fins, como na produção de alimentos, biodiesel/biogás, produtos dietéticos, produtos farmacêuticos (incluindo óleos), pigmentos naturais (após extração), cosméticos, e outros processos industriais (FAO, 2016).

As vísceras digestivas dos peixes são uma excelente fonte de enzimas especializadas como pepsina, tripsina, quimotripsina e colagenases, bem como enzimas lipases, que representam uma ferramenta vantajosa nas etapas dos processos tecnológicos na indústria, seja ela alimentícia, farmacêutica e/ou biomédica, por permitirem um melhor controle dos processos de produção, por serem biodegradáveis, fáceis de serem utilizadas e eficazes em mesmo em baixas concentrações (LIMA et al., 2011; DABOOR, 2012; BOUGATEF, 2013; RASKOVIC et al., 2014; FAO, 2016). Além disso, peles, escamas e ossos de peixes são uma ótima fonte de colágeno e gelatina, cálcio e outros minerais como o fósforo que pode ser usado em alimentos, rações ou como suplementos (FAO, 2016).

Esses resíduos, geralmente, são descartados, advindo sobretudo ao alto custo para manutenção de todo processamento e, por vezes, são despejados sem o menor tratamento no meio ambiente, contribuindo para a ocorrência de danos ambientais. Nessa problemática, é de grande interesse da comunidade cientifica pesquisas que visem a recuperação e utilização desses resíduos, como fonte alternativa de ganhos, através da agregação de valor, como por exemplo, a exploração das biomoléculas presentes nestas vísceras, muitas vezes de interesse da própria indústria.

Nos últimos anos, diversas pesquisas têm focado obtenção de peptídeos bioativos a partir da hidrólise de proteínas alimentares e destacado suas potenciais ações em uma ampla gama de funções fisiológicas, como ação anti-hipertensiva, antioxidantes, agonistas de opióides, imunomoduladores, antimicrobianos, probióticos, ligação a minerais, efeitos antitrombóticos e hipocolesterolêmicos (RYAN et al., 2011). Na perspectiva de converter os resíduos da indústria pesqueira em produtos de valor agregado, pesquisadores tem destacado o potencial do colágeno obtido a partir de peles, escamas e ossos de peixes, assim como de proteases digestivas, na produção de peptídeos com atividades biológicas (antimicrobiana, antioxidante e anti-hipertensiva) (GÓMEZ-GUILLÉN et al., 2011; NGO; RYU; KIM, 2013).

Desta forma, a presente pesquisa visa a obtenção de colágeno e proteases a partir dos resíduos gerados pelo processamento do peixe *Coryphaena hippurus*, uma espécie de grande importância no setor pesqueiro, e verificar seu potencial para aplicação biotecnológica.

2 REVISÃO BIBLIOGRÁFICA

2.1 Produção de pescado

A pesca e a aquicultura são importantes fontes de alimento, nutrição, renda e meios de subsistência para centenas de milhões de pessoas em todo o mundo. Dados gerais da produção mundial de pescado mostraram que em 2014 a produção total atingiu o patamar de 167.2 t, dos quais 81.5 milhões foram provenientes da pesca extrativista (Fig. 1) (FAO, 2016).

FIGURA 1 - Produção e utilização da pesca e aquicultura mundial

	2009	2010	2011	2012	2013	2014
	Milhões de toneladas					
Produção						
Captura						
Continental	10.5	11.3	11.1	11.6	11.7	11
Marinha	79.7	79.9	82.6	79.7	81.0	81
Total - Captura	90.2	89.1	93.7	91.3	92.7	93
Aquicultura						
Continental	34.3	36.9	38.6	42.0	44.8	47
Marinha	21.4	22.1	23.2	24.4	25.5	26
Total - Aquicultura	55.7	59.0	61.8	66.5	70.3	73
TOTAL	**145.9**	**148.1**	**155.5**	**157.8**	**162.9**	**167**
Utilização						
Consumo humano	128.3	128.1	130.8	136.9	141.5	146
Outros usos	22.0	20.0	24.7	20.9	21.4	20
População Mundial	6.8	6.9	7.0	7.1	7.2	7
Abastecimento per capta	18.1	18.5	18.6	19.3	19.7	20

Fonte: FAO, 2016

Seguindo a tendência do mercado mundial, o Brasil também tem demostrado avanços na indústria pesqueira. Nos últimos 10 anos, o país apresentou um crescimento de 16,9% na produção de pescado (BRASIL, 2013), Dentro os tipos de pesca exercidas no país, a pesca extrativista é principal fonte de recursos pesqueiros, principalmente a do tipo marinha que em 2011 foi responsável por cerca de 38,7% da produção total de pescado, seguida pela aquicultura continental (38,0%), pesca extrativa continental (17,4%) e aquicultura marinha (6%). Nesse mesmo ano, a produção nacional total foi de 1.431.974,4 t, com um incremento de aproximadamente 13,2% em relação à 2010 (BRASIL, 2013). A região Nordeste brasileira é umas das principais produtoras de pescado do país, seguida pelas regiões Sul e Norte. Em 2011 a região foi responsável por 31,7% da produção nacional, porcentagem que equivale a cerca de 454 mil t, já as regiões Sul, Norte, sudeste e Centro-Oeste registraram 336.451,5 t (23,5%), 326.128,3 t (22,8%), 226.233,2 t (15,8%) e 88.944,5 t (6,2%), respectivamente. Esse panorama

de produção entre as regiões brasileiras tem sido constante nos últimos anos (Fig. 2) (BRASIL, 2013).

FIGURA 2 - Produção de pescado nas Regiões brasileiras no período de 2007 a 2010

Regiões	2007					2008				
	Total	Pesca extrativa		Aquicultura		Total	Pesca extrativa		Aquicultura	
		marinha	continental	marinha	continental		marinha	continental	marinha	continental
Brasil	1.071.393,50	539.966,50	243.210,00	78.405,00	209.812,00	1.156.364,00	529.714,20	261.282,80	83.358,60	282.008,50
Norte	238.340,50	72.036,50	139.966,00	200,00	26.138,00	270.458,90	89.065,60	151.216,10	265,20	29.912,00
Nordeste	331.538,50	155.625,50	68.497,00	63.500,50	43.915,50	374.815,40	182.444,50	68.084,50	67.740,40	56.546,00
Sudeste	195.919,00	137.666,00	22.201,00	838,00	35.214,00	173.457,90	99.248,70	24.222,20	800,80	49.186,20
Sul	255.080,50	174.638,50	2.092,00	13.866,50	64.483,50	273.849,20	158.955,50	4.138,10	14.552,20	96.203,50
Centro-Oeste	50.515,00	-	10.454,00	-	40.061,00	63.782,60	-	13.621,90	-	50.160,80
Regiões	2009					2010				
	Total	Pesca extrativa		Aquicultura		Total	Pesca extrativa		Aquicultura	
		marinha	continental	marinha	continental		marinha	continental	marinha	continental
Brasil	1.240.813,50	585.671,50	239.492,60	78.296,30	337.353,00	1.264.764,90	536.454,90	248.911,40	85.058,60	394.340,00
Norte	263.814,40	97.095,00	130.691,00	246,10	35.782,30	274.015,60	93.450,20	138.726,40	257,90	41.581,10
Nordeste	411.463,10	210.965,90	69.994,80	62.859,10	67.643,30	410.532,10	195.842,20	68.783,50	67.327,90	78.578,50
Sudeste	177.248,70	96.364,20	21.265,30	780,10	58.839,00	185.635,90	90.588,70	23.276,50	855,50	70.915,20
Sul	316.257,10	181.246,40	5.516,20	14.411,00	115.083,50	311.700,00	156.573,80	5.083,70	16.617,40	133.425,10
Centro-Oeste	72.030,20	-	12.025,30	-	60.004,90	82.881,40	-	13.041,30	-	69.840,10

Fonte: Banco do Nordeste - BNB/ETENE/CIES (2012)

Os peixes são um dos produtos alimentares mais comercializados em todo o mundo, por isso o principal destino desta produção é suprir a demanda do mercado, cujo o consumo per capta tem sido estimado em cerca de 20 Kg (FAO, 2016a). Segundo Larsen e Roney (2013), no ano de 2012, com uma produção mundial de 66 milhões de t, a aquicultura superou a produção de carne bovina cujo a produção foi de em 63 milhões t. Os autores justificam tal feito pela baixa demanda de insumos necessários para manter a produção e também, em virtude das considerações ambientais e sobre a saúde que têm levado as pessoas a reduzirem o consumo de carne bovina, pois esta tem sido associada ao um maior risco de desenvolvimento de doenças cardíacas e digestivas, e adotarem o consumo de peixes como alternativa mais saudável. O pescado se destaca do ponto de vista nutricional, quando comparado a outros alimentos de origem animal, sobretudo por conter grandes quantidades de vitaminas A e D, minerais cálcio, fósforo, ferro, elevada proporção de ácido graxos poliinsaturados de cadeia longas e cerca de 50-60% das proteínas e aminoácidos essenciais que um adulto necessita diariamente para o seu bem-estar e saúde (SARTORI e AMANCIO, 2012).

2.2 Recuperação de resíduos do processamento do pescado

De acordo com a Organização das Nações Unidas para Alimentação e Agricultura (FAO) (2014), cerca de 20-25% da captura mundial de pescado não é empregada para o consumo direto na alimentação humana, seguindo para elaboração de rações, óleos ou é desperdiçada como resíduos que representam cerca de 70% do peso total dos peixes e incluem principalmente materiais que não são adequados para a elaboração de produtos de valor agregado destinados à alimentação humana, tais vísceras digestivas, ossos, pele, escamas, nadadeiras e cabeças. Estes resíduos geralmente são descartados ou utilizados na produção de farinhas, óleos, silagens e compostagens de peixes, destinados à alimentação animal, na produção fertilizantes e produtos químicos (ARRUDA, 2004; KOMKLAO, 2008).

A indústria do pescado representa um vasto potencial econômico, pois seus descartes podem ser facilmente transformados em produtos com aproveitamento mercadológico. Todavia, apesar da variedade de vias de destino disponíveis para recuperação dos resíduos, devido ao elevado custo necessário para realização da mesma, essa atividade não se mostra economicamente atrativa para indústria e por isso esses resíduos são descartados de forma irregular, acarretando sérios problemas ambientais (ARRUDA, 2004).

Afim de contribuir para resolução dessa problemática, atualmente, a comunidade cientifica tem se voltado às pesquisas que visam recuperação e utilização desses resíduos, como fonte alternativa de ganhos, através da agregação de valor, como por exemplo, a exploração das biomoléculas presentes nesses materiais (BEZERRA et al., 2005; DABOOR et al., 2012; BOUGATEF, 2013; MEDEIROS et al., 2015; ARAÚJO et al., 2016; FRANÇA et al., 2016; SANTOS et al., 2016;).

2.3 Produção do *Coryphaena hippurus*

A espécie *C. hippurus* representa um importante recurso pesqueiro mundial, cujo a taxa anual de captura tem se apresentado em constante crescimento (Fig. 3), atingindo um patamar de 115.658 t (FAO, 2016b). No Brasil, os últimos registros contabilizados nos anos de 2009, 2010 e 2011, foram de 8.588,0 7.999,3 e 4.379,2 t, respectivamente. A região Nordeste, destaca-se nesse cenário com as produções de Alagoas, Pernambuco e Rio Grande do Norte, constituindo-se um importante recurso para a pesca artesanal, devido a qualidade e o valor comercial de sua carne (NÓGREGA et al., 2009). Comercialmente, apresenta-se inteiro, eviscerado, sem cabeça e eviscerado, e filés com ou sem pele. Após o processamento, a pele e as vísceras de *C. hippurus* podem representar até 16% do seu peso seco (SANTIVAÑEZ, 2016).

FIGURA 3 - Produção global do *Coryphaena hippurus* de 1950 a 2014

Fonte: FAO (2016b)

2.4 Classificação taxonômica do Dourado

Reino: Animalia

Filo: Chordata

Classe: Actinopterygii

Ordem: Perciformes

Família: Coryphaenidae

Gênero: Coryphaena

Espécie: *C. hippurus*

2.5 Biologia do *Coryphaena hippurus*

A família Coryphaenidae é constituída por duas espécies, o dourado (*Coryphaena hippurus*) e a palometa (*Coryphaena equiselis*). Esses peixes de hábitos epipelágicos, caracterizam-se por apresentar corpo alongado, comprimido lateralmente e elevado na altura da região posterior da cabeça, estreitando-se gradativamente em direção à cauda. Além disso, os representantes possuem dimorfismo sexual acentuado; no qual os machos adultos apresentam a cabeça tipicamente alta com curvatura superior abrupta e as fêmeas possuem um perfil superior da cabeça relativamente curvo e mais suave (ROCHA; COSTA, 1999). O dourado *C.*

hippurus (Fig. 4) se diferencia de *C. equiselis* principalmente pelo tamanho, que comumente é entre 80 cm e 1,2 m, e o peso que varia entre 12 e 25 kg, podendo chegar até 2 metros de comprimento e a 35 kg. Além disso o dourado apresenta as nadadeiras peitorais relativamente longas e maiores que metade do comprimento da cabeça; corpo mais baixo e alongado; coloração azul-esverdeado metálico com tonalidades amarelo-ouro na cabeça, ventre e nas nadadeiras anal e caudal; e também pequenas pintas negras circulares distribuídas pelo corpo (COLLETTE, 1984; ROCHA; COSTA, 1999).

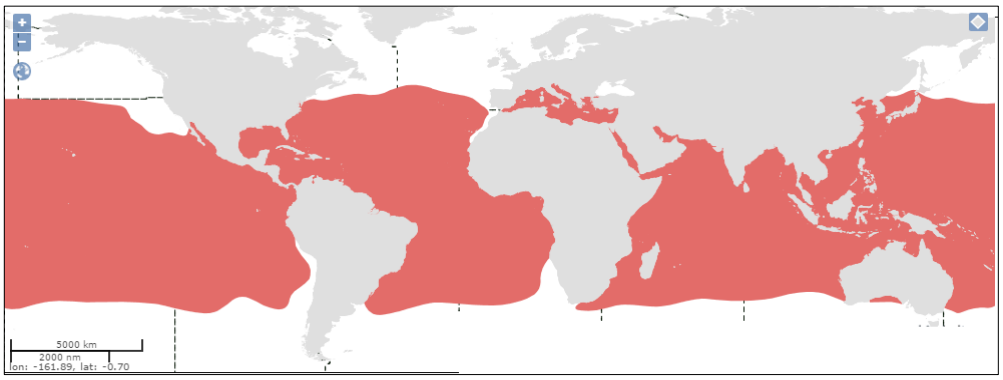
FIGURA 4 - *Coryphaena hippurus Linnaeus*, 1758

Fonte: Do autor, 2017

O dourado alimenta-se de quase todas as formas de peixes e zooplâncton, mas também crustáceos e lulas. A maturidade sexual é alcançada em 4-5 meses. Os ovos pelágicos e larvas; desova ocorre em mar aberto, geralmente durante o verão (FAO, 2016b).

O *C. hippurus* é um peixe ósseo marinho, epipelágico com ampla distribuição ao redor do mundo, principalmente em a águas tropicais e subtropicais (Fig. 4 com temperaturas entre 21º a 30ºC, do Atlântico Ocidental (Golfo do México e Caribe), Atlântico Oriental, e também há registros no Mar Mediterrâneo, Oceano Indico e Pacífico Ocidental. (NÓBREGA et al., 2009; FARRELL et al., 2014). Segundo os critérios da IUCN, o estado de conservação (COLLETE, 2011).

FIGURA 5 - Mapa de distribuição mundial de *Coryphaena hippurus*



Fonte: FAO (2016b).

2.6 Colágeno

Os colágenos compõem a superfamília de proteínas fibrosas estruturais e insolúveis presentes em todos os organismos multicelulares (LEE et al., 2001; DUARTE, 2012). O colágeno era considerado apenas elemento com funções estruturais nos tecidos conjuntivo e intersticial, onde contribuíam para a estabilidade e integridade estrutural dos tecidos e órgãos. Todavia, atualmente sabe-se que os colágenos apresentam uma grande variedade de funções, que são determinadas pela a presença de domínios adicionais não helicoidais em sua estrutura (GELSE; PÖSCHL; AIGNER, 2003; SILVA E PENNA, 2012). Até o presente momento, um total de 29 tipos de colágenos foram geneticamente codificados dos tecidos de vertebrados e nomeadas por algarismos romanos de I a XXIX (CHUNG; UITTO, 2010; RICARD-BLUM, 2011; DUARTE, 2012). A descoberta da expressão e função dos diferentes tipos de colágeno contribuiu compreensão do papel dessas moléculas no organismo e também como os defeitos em sua síntese podem resultar em doenças como: condrodisplasias, osteogênese imperfeita, síndrome de Alport, síndrome de Danlos-Ehler ou epidermólise bolhosa (GELSE; PÖSCHL; AIGNER, 2003; RICARD-BLUM, 2011).

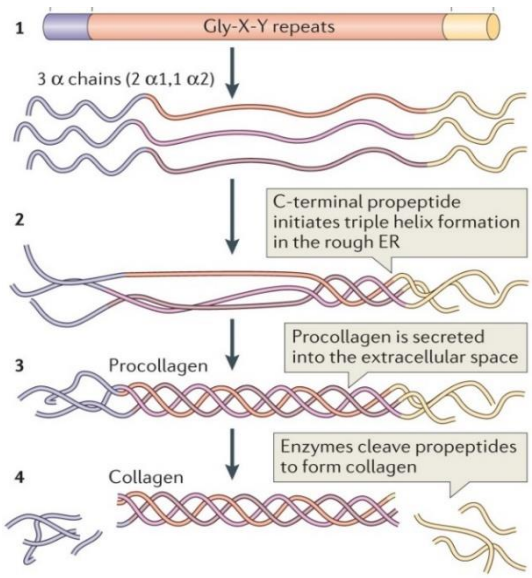
No seu aspecto estrutural, os colágenos possuem três cadeias polipeptídicas helicoidais, chamadas de cadeia α, superenoveladas, que podem ser idênticas (homotrímeros) ou formadas por duas ou mais cadeias diferentes (heterotrímeros). A formação do procolágeno se dá pelo enovelamento das cadeias α individuais numa conformação de tripla hélice baseado na característica da sequência primária, que consiste numa sequência tripeptídica repetitiva de glicina-X-Y, onde Y geralmente é um prolina ou 4-hidroxiprolina e X pode ser qualquer um dos 20 aminoácidos-padrão (MAYNE; BURGESON, 1987; DUARTE, 2012). O procolágeno

é secretado pelos fibroblastos no espaço extracelular e convertido em colágeno pela remoção dos propeptídeos contendo nitrogênio (N-) e carbono (C-) terminais (Fig. 6) pela metaloproteínases, levando a formação do tropocolágeno, a unidade básica do colágeno (SILVA E PENNA, 2012; MOUW; OU; WEAVER, 2014).

O colágeno tipo I é o principal colágeno presente nos ossos, tendões, pele, ligamentos, córnea e muitos tecidos conjuntivos intersticiais, com exceção de muito poucos tecidos, como cartilagem hialina, cérebro e corpo vítreo. Sua tripla hélice é normalmente formada como um heterotrímero por duas cadeias α1 idênticas e uma cadeia α2. Suas fibras helicoidais triplas (in vivo), são principalmente incorporadas a compostos que contêm colágeno tipo III (na pele e fibras reticulares) ou colágeno tipo V (em osso, tendão, córnea). O colágeno tipo I proporciona rigidez à tração na maioria dos órgãos e notadamente nos tendões e fáscia, e define propriedades biomecânicas consideráveis relativas ao suporte de carga, resistência à tração e rigidez torcional em particular após a calcificação dos ossos (GELSE; PÖSCHL; AIGNER, 2003).

De uma forma geral, os colágenos são compostos interessantes para diversos setores

FIGURA 6 - Estrutura do colágeno.



Fonte: MOUW; OU; WEAVER, 2014

industriais principalmente por serem, biodegradáveis, fracamente antigénicos e com biocompatibilidade superior em comparação com outros polímeros naturais, tais como albumina e gelatina (GELSE; PÖSCHL; AIGNER, 2003). Na indústria biomédica, tem sido frequentemente utilizado para reparação cirúrgica, curativo para tratamento de queimaduras e úlceras, na administração e liberação controlada de fármacos, na formulação de comprimidos para liberação de proteínas, e também na engenharia de tecidos (LEE; SINGLA; LEE, 2001).

O consumo do colágeno hidrolisado pode proporcionar benefícios como melhoria da firmeza da pele; proteção de danos nas articulações; melhoria no tratamento da osteoporose; prevenção do envelhecimento; proteção contra úlcera gástrica além de prevenção e o tratamento das disfunções gastrointestinais. Assim, por suas propriedades emulsificantes, agentes espumantes, estabilizantes coloidais, formadores de películas biodegradáveis, o colágeno tem atraído o interesse da indústria de alimentos para sua aplicação como ingrediente funcional em alimentos (SILVA E PENNA, 2012; FAN et al., 2016).

O colágeno pode ser utilizado em sua estrutura bruta, na forma de fibras ou pó, e também pode ser submetido à reação de hidrólise, que leva à produção da gelatina e do colágeno hidrolisado. A preparação industrial de gelatina envolve a hidrólise parcial da estrutura nativa de colágeno para se obter a forma solúvel em água. Através de uma degradação enzimática adicional da gelatina resulta no que chamamos de "colágeno hidrolisado", que contém peptídeos com massa molecular que variam de 3 a 6 kDa. Recentemente, pesquisas tem mostrado que alguns peptídeos de colágeno possuem atividade anti-hipertensiva, inibindo o efeito da enzima de conversão da angiotensina I, promovendo a inibição de danos cardiovasculares às células endoteliais através da sua atividade inibitória da ACE e regulação do óxido nítrico e da molécula de adesão intercelular, além de uma potente atividade antioxidante em diferentes sistemas oxidativos (ZHANG et al., 2010; ZAGUE et al., 2011; HUANG et al., 2017).

Peles e tendões de espécies de vertebrados como porco, bezerro e boi, tem sido as principais fontes de colágeno utilizadas para as aplicações industriais. Entretanto, devidos aos riscos de envolvidos na transmissão de doenças como: encefalopatia espongiforme bovina (EEB), encefalopatia espongiforme transmissível (EET) e febre aftosa (FMD); e a existência de barreiras religiosas envolvidas no consumo de produtos de origem bovina ou suína, conduziram a busca por novas fontes de colágeno com o intuito de suprir a decorrente valorização dos subprodutos industriais do colágeno (VEERURAJ; ARUMUGAM; BALASUBRAMANIAN, 2013). Como consequência, as fontes alternativas de colágeno, especialmente as provenientes de organismos aquáticos, tais como água doce e peixes marinhos e moluscos têm recebido atenção crescente na última década (LIU et al., 2012; VEERURAJ et al., 2015).

As peles, ossos, nadadeiras e escamas de peixes, representam uma rica fonte de colágeno e são resíduos gerados em grandes quantidades após o processamento de peixe, podendo servir como fontes do colágeno. Embora as físico-químicas do colágeno das espécies de peixes sejam diferentes dos mamíferos, não há associação destas proteínas a doenças, por isso o emprego

desses resíduos para extração de colágeno além de representar um destino sustentável, também é uma oportunidade de incrementar o processo produtivo do pescado, agregando valor ao produto final (PATI; DHARA; ADHIKARI, 2010; GÓMEZ-GUILLÉN et al., 2011; OLIVEIRA, 2015).

2.7 Colagenases

As colágenas são enzimas altamente específicas para a degradação do colágeno (nativo ou desnaturado) tornando-o um alvo fácil para outras enzimas proteolíticas (YANSHUN et al., 2016) e são classificadas em dois grandes grupos: as metalocolagenases e as serinocolagenases (OLIVEIRA, 2015).

As metalocolagenases são enzimas zinco-dependentes, que desempenham um papel importante, clivando principalmente colágeno dos tipos I, II, III, VII e X, entre os resíduos de Gly-IIe ou Gly-Leu das cadeias da tripla hélice. Essas enzimas desempenham papéis essenciais no controle de uma ampla gama de processos biológicos e patológicos associados à degradação da matriz extracelular de células animais, remodelação de tecidos, tais como embriogênese, morfogênese e cicatrização de feridas (DABOOR et al., 2010; DUARTE; CORREIA; ESTEVES, 2014; ALIPOUR et al., 2016).

As serinocolagenases apresentam um resíduo de serina nos seus sítios catalíticos e massas moleculares que variam na gama de 24.000-36.000 kDa. Capazes de clivar tripla hélice de colágeno, essas enzimas normalmente estão associadas a órgão digestivo, e são muitas vezes envolvidos com a produção de hormônios, degradação da proteína, coagulação do sangue e fibrinólise (DABOOR et al., 2010; OLIVEIRA, 2015).

Quanto à aplicabilidade, essas proteases podem ser utilizadas em diversos processos industriais, tais como amaciamento dos alimentos, formulação de fármacos, medicamentos e também no desenvolvimento de cosméticos (RASKOVIC et al., 2014; SUPHATHARAPRATEEP et al., 2011). As colagenases têm sido utilizadas principalmente no tratamento de cicatrizes hipertróficas e queimaduras como componente de pomadas cicatrizantes, e também no desenvolvimento de agentes para o tratamento da osteoporose, ulceração gástrica, hipertensão, isolamento e cultivo de células (LIMA, 2013). No setor alimentício apresentam potencial de atuarem como amaciadoras de carne, como foi evidenciado por Foegeding e Larick (1986) e por Desmond e colaboradores (2001). (KANTH et al., 2008; RASKOVIC et al., 2014).

A colagenase empregada na indústria é proveniente de diversas fontes, principalmente fúngica e bacteriana, como as fornecidas por *Candida albicans* (LIMA et al., 2009), *Bacillus*

cereus (LIU et al., 2010), *Bacillus licheniformis* (BAEHAKI et al., 2012) e *Penicillium aurantiogriseum* (LIMA, 2014). Fontes vegetais também são descritas na literatura (KIM et al., 2007; RASKOVIC et al., 2014).

Os resíduos do processamento do pescado, principalmente as vísceras digestivas, são ricas fontes de proteases, inclusive das com propriedades colagenolíticas. Diversos estudos realizados com espécies de importância econômica como: *Novoden modestrus* (KIM et al., 2002); *Scomber japonicus* (PARK et al., 2002); *Pagrus major* (WU et al., 2010), *Sardinella aurita* (HAYET et al., 2011), têm destacado as propriedades e o potencial dessas enzimas para aplicação biotecnológica.

2.8 Extração e purificação de proteases com propriedades colagenolíticas

As colagenases podem ser obtidas através de métodos de extração enzimática tradicionais, que incluem extração de tampão Tris-HCl (bicarbonato de sódio, entre outros), precipitação com sulfato de amónio, ultrafiltração. Ademais, o extrato pode ser purificado métodos cromatográficos como: filtração em gel, troca iónica, interação hidrofóbica ou afinidade (DABOOR et al., 2010; LIMA et al., 2013). As purificações por métodos cromatográficos tendem a ser demoradas, difíceis e exigem alguns reagentes caros, o que contribui para elevar o custo do processamento (LIMA et al., 2013). Um método alternativo e coerente para purificação de proteases obtidas a partir dos resíduos do pescado, é a extração líquido-líquido (ELE) a partir do sistema de duas fases aquosas (SDFA).

O sistema de duas fases aquosas é um processo de bioseparação que permite um elevado grau de purificação e recuperação de materiais biológicos, além de uma série de vantagens em relação aos métodos convencionais para o isolamento e purificação de proteínas: (a) o equilíbrio de partição é atingido muito rapidamente, (b) o alto teor de água das duas fases (70-80% v/v), que significa biocompatibilidade, baixa tensão interfacial e minimização da degradação de biomoléculas, (c) a facilidade de extração em grande escala; (d) a utilização de material de baixo custo, (e) a possibilidade da reciclagem dos polímeros; entre outras (DUARTE, 2012; LIMA, 2013; OLIVEIRA, 2015). A purificação da molécula-alvo, se dá por sua partição seletiva em umas das duas fases liquidas, que podem ser formadas por polímeros, polieletrólitos, ou ainda, polímeros em combinação com solutos de baixa massa molar. Os sistemas mais usados geralmente são constituídos por polietilenoglicol (PEG) /dextrana, polipropilenoglicol (PPG) /dextrana; PEG/fosfato de potássio; PEG/sulfato de magnésio e PEG/citrato de sódio (PESSOA JÚNIOR; KILIKIAN, 2005). Rosso et al. (2012) e Lima et al.

(2013) mostraram que o uso de SDFA formado por PEG e fosfato, apresentou eficiência do na extração de colagenase de microrganismos.

3 OBJETIVOS

3.1 Geral

Produzir peptídeos bioativos através da hidrólise do colágeno isolado da pele de dourado *Coryphaena hippurus*, empregando colagenase extraída de suas vísceras intestinais.

3.2 Específicos

- Extrair colágeno ácido solúvel (ASC) e pepsino solúvel (PSC) a partir da pele de do dourado *Coryphaena hippurus*
- Determinar o rendimento da extração do colágeno extraído da pele de *C. hippurus*;
- Identificar o tipo de colágeno isolado, caracterizando-o por seu perfil bioquímico, através de SDS-PAGE;
- Verificar a melhor solubilidade relativa do colágeno obtido, frente a variações de pH e concentrações de NaCl;
- Caracterizar fisicamente o colágeno isolado, através de espectroscopia na região do infravermelho.
- Extrair a colagenase a partir de vísceras intestinais de *C. hippurus*;
- Pré purificar uma protease colagenolítica a partir de vísceras intestinais de *C. hippurus* por meio precipitação por sulfato de amônia e do Sistema de Duas Fases Aquosas (SDFA);
- Investigar a capacidade da enzima obtida na hidrólise do colágeno extraído a partir da pele C. *hippurus.*

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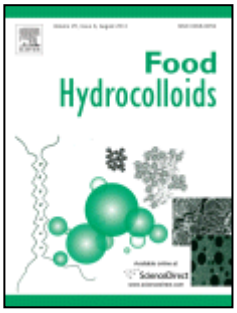

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Production of bioactive peptides from collagen isolated from common dolphinfish (*Coryphaena hippurus*)

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Abstract

The aim of this study was to isolate and extract the acid soluble (ASC) and pepsin soluble (PSC) collagen from the skin of dolphinfish *Coryphaena hippurus* as well as characterize and conduct a biotechnological application in the production of collagen peptides. The yield of ASC and PSC were 43% and 3% (dry weight), respectively. Both the ASC and PSC consisted of two different α chains (α 1 and α 2), their dimers (β chain) and were characterized to be type I collagen. The ASC and PSC ultraviolet (UV) absorption spectrum showed a maximum absorption at 230 nm, respectively. The solubility of ASC and PSC reached maximum at pH 1 and 2, respectively. The solubility of the ASC was higher at low NaCl concentrations, with a decrease in solubility at concentrations above 1%. FTIR spectra of ASC and PSC exhibited the characteristic peaks of Amide I, II, III as well as amide A and B similar to others type I collagen of fishes. The results obtained in this study indicate the possibility of using dolphinfish skin as a source type I collagen and biomolecules with great potential for biotechnological and industrial application.

Keywords: by-products. wastes. biomaterial. collagen fish.

1. Introduction

Collagen is the most abundant protein representing nearly 30% of total proteins in the animal body. It is the major component of extracellular matrix and is vital for mechanical protection of tissues, organs, and physiological regulation of cellular environment (Pati, Adhikari, & Dhara, 2010). Because of its excellent physical properties and the health benefits, collagen has been widely and applied in many industries, including supplements, food and pharmaceutical industries all over the world (Bhagwat & Dandge, 2016; Li et al., 2013; Wu et al., 2010; Zhang, Duan, Tian, & Konno, 2009). Among various existing types, type I collagen has been extensively used and are mainly isolated from the skins of land-based animals, such as cow and pig (Pati et al., 2010). However, due to outbreak of Bovine Spongiform Encephalopathy (BSE), Transmissible Spongiform Encephalopathy (TSE), Foot and Mouth Disease (FMD) in pigs, cattle, use of collagen and collagen derived products from these sources have been limited (Li et al., 2013). Therefore, the develop of alternative and safer collagen sources become necessary and attractive.

Marine captured fisheries contribute about 49 percent of total world fish production and recently, there has been much interest in investigating ways to make more efficient use of underutilized resources and waste generated in fisheries processing (FAO, 2016). More than 30% of fish processing wastes consist of skin, scale and bone, which are very rich in collagen and have received increasing attention as collagen sources (Pati et al., 2010). In the last few years, attempts have been made to isolate and characterize collagens from skin of several fish species (Li et al., 2013; Moreno et al., 2016; Pal et al., 2016; Sionkowska, Kozłowska, Skorupska, & Michalska, 2015; Tan et al., 2014; Tang et al., 2015; Zeng et al., 2012). Collagen obtained from fish waste has also been focused as a source of biologically active peptides with promising health benefits for nutritional or pharmaceutical applications such as antihypertensive activity, antioxidant activity, ability to promote the proliferation of human keratinocytes (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011; Huang, Wu, Yang, Li, & Kuo, 2015).

Dolphinfish (*Coryphaena hippurus*) is a mid-trophic level pelagic fish of significant importance to artisanal, recreational, and commercial fisheries throughout the world's tropical and subtropical oceans are consumed in high quantities and are commercially relevant resources for national and international markets, records for dolphinfish suggest a yearly catch of around 115,650 tons (FAO, 2016; Merten, Appeldoorn, & Hammond, 2016; Nelson et al., 2016).

The aim of this study was to isolate and extract the acid soluble (ASC) and pepsin soluble (PSC) collagen from the skin of *C. hippurus* as well as characterize and conduct a biotechnological application in the production of collagen peptides.

2. Material and Methods

2.1. Collection and storage of samples

The Intestinal waste and skin of dolphinfish (*C. hippurus*) were obtained from the fishermen's colony of Janga, located in Paulista, Pernambuco, Brazil. The materials were stored in ice and transported to the Laboratório de Enzimologia (Federal University of Pernambuco (UFPE), Recife/PE, Brazil) where they were washed with cold distilled water and stored at -27°C for further processing.

2.2. Pretreatment of skin

To remove non-collagenous proteins, the prepared fish skin was mixed with 0.2 M NaOH at a skin/alkali solution ratio of 1:10 (w/v). The mixture was continuously stirred for 6 h at 4 ºC. 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 4 ºC and the skin was washed as previously described. Then was added 3% hydrogen peroxide at a ratio of 1:10 (w/v) for whitening the skin and was carried out in the wash (Singh, Benjakul, Maqsood, & Kishimura, 2011).

2.3. Extraction of acid soluble collagen (ASC)

ASC was prepared by the method of Nagai and Suzuki (2000). The residue was extracted with 0.5 M acetic acid at sample/acid ratio of 1:2 0 (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 for precipitation of the collagen by the addition of 0,9 M carbonate-bicarbonate buffer (pH 10) with 0,9 M NaCl, to titrate the acetic acid with collagen until the pH has reached the value 8.88. After standing overnight, the resulting precipitate was collected by centrifuging at 20,000 g for 30 min. 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).

2.4. 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 porcine pepsin 1% (w/w) (EC 3.4.23.1, Sigma-Aldric, MO) in a solution containing in 0.5M acetic acid with constant homogenization for 24h 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).

2.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

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

2.6. UV absorption spectrum

UV absorption spectra of ASC and PSC from *C. hippurus* skin was carried out according to Nalinanon et al. (2011), using a Thermo Scientific Multiskan™ GO Microplate Spectrophotometer. The collagen sample (1mg/mL) was dissolved in 0.5M acetic acid solution. UV spectra were measured at wavelength 200 – 600 nm.

2.7. FTIR analysis

Both ASC and PSC were subjected to attenuated total reflectance-Fourier transform infrared spectroscopy (FTIR) according to the method of Singh et al. (2011). FTIR spectrometer (Cary 630 FTIR Spectrometer, AgilentTechnologies, United States). For spectra analysis, the collagen samples were placed onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 400–4000 cm^{-1} with automatic signal gain were collected in 8 scans at a resolution of 16 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ºC.

2.8. Effect of pH on solubility

The collagen samples (3mg/mL) were dissolved in distilled water with pH adjusted from 1.0 to 12.0 with HCl and NaOH 6M and shake at 4°C for 24h. The solution was centrifuged at 20,000 g for 30 min at 4 °C (Singh et al., 2011). For all the samples, protein content in the supernatant was measured (Smith et al., 1985) and the relative solubility was calculated in comparison with that obtained at the pH rendering highest solubility.

2.9. Effect of NaCl on solubility

Collagen solution (0.5 ml) was mixed with 0.5 ml of NaCl in 0.5 M acetic acid at various concentrations to give the final concentrations of 0 %, 1 %, 2 %, 3 %, 4 %, 5 % and 6 % (w/v). The mixture was stirred continuously at 4 °C for 60 min, followed by centrifuging at 20,000 g for 60 min at 4 °C. Protein content in the supernatant was measured and the relative solubility was calculated as previously described (Singh et al., 2011).

2.10.Collagenolytic protease extraction steps and protein determination

The processing of intestinal waste was realized in accordance with the methodology described Teruel & Simpson (1995). The ratio of viscera to extraction buffer (0.05 M Tris-HCl pH 7.5, containing 5 mM $CaC1_2$) was 1:3 (w/v). The extraction method followed systematic processes (Steps I, II, III and IV) for later analysis by which fractions were obtained. In step I, the material was homogenized and centrifuged. The resulting waste was again homogenized (Step II) and subsequently centrifuged through new maceration and homogenization (Step III). Then, the material was centrifuged and filtered in sterile1 syringe 0.22 µm and the resulting material was defined as step IV. In each maceration and homogenization step, all the viscera collected were homogenized separately for 5 minutes at an adjusted speed of 10,000 – 12,000

rpm (4°C) (homogenizer IKA RW 20D S32, China). The homogenate was then centrifuged (Sorvall Superspeed Centrifuge RC-6, North Carolina, USA) at 12,000 x *g* for 30 min at 4°C.

The best fraction of the supernatant was precipitated by ammonium sulfate (60-90%) and passed through a recovered process in aqueous two-phase system (ATPS), using pH 8.0, 17.5% (w/w) PEG 8000 and 5.0% (w/w) sodium sulfate. The protein concentration was determined according to Smith (1985).

2.11. Hydrolysis of collagen by collagenolytic protease

The digestion measure of *C. hippurus* native collagen was according to the method of Moore & Stein (1954) and Park, Lee, Byun, Kim & Kim (2002). The test was performed using protease with collagenolytic properties obtained from intestinal viscera of *C. hippurus.* The analysis was performed with crude extract, pre-purified extract by ammonium sulfate precipitation (60-90%) and PEG- collagenolytic protease recovered by the process in aqueous two-phase system (ATPS).

A reaction mixture contained 25 mg of skin collagen, 5 mL of 50 mM Tris-HCl (pH 7.5) that contained 5 mM $CaCl_2$ and 0.5 mL of each extract, was incubated at 37 °C for 18,24, 48, 66 hours. The reaction was stopped by adding 0.2 mL of 50% trichloroacetic acid. After 10 min (at room temperature), the solution was centrifuged at 1800 xg for 20 min. The supernatant (0.2 mL) was mixed with 1.0 mL of a ninhydrin solution, incubated at 100 °C for 20 min and then cooled to room temperature. Subsequently, the mixture was diluted with 5 ml of 50% 1-propanol for an absorption measurement at 570 nm. A buffer (50 mM Tris-HCl, pH 7.5) that contained 5 mM $CaCl_2$ was used instead of an enzyme solution as reference. The concentration of hydrolyzed amino acids was determined by a standard curve based on a solution of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme that is required for the hydrolysis of 1mmole of substrate per hour.

3. Results and Discussion

3.1. Yield of ASC and PSC from the skin of *C. hippurus*

ASC and PSC were isolated from the skin of spotted dolphinfish with yields of 43% and 3% (based on the dry weight), table 1 shows different yields in different species of fish. These variations in yields relate both to different biological conditions to which each species is

conditioned, as well as to the species used and the extraction methods. Some authors report a decrease in solubility of collagen in acid media due to the high number of cross-links by covalent bonds through the condensation of aldehyde groups at the telopeptide region, being necessary the use of pepsin to extraction of residue collagen from the acetic acid extraction, and they achieved significant yields (Wang et al. 2008; Zeng et al. 2009; Zeng et al., 2012; Li et al. 2013). However, for skin of dolphinfish, the acid extraction was sufficient to obtain almost all collagen, that also may indicate a low level of these cross-links.

3.2. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

The electrophoretic patterns revealed that both the ASC and PSC were consisting of α1 and α2-chains at a ratio of approximately 2:1 (Fig. 1) and high-molecular-weight components including β chain and similar to that observed in typical composition of type I collagen derived from bovine skin, used as standard, and collagen derived from fish skin and scale described currently by (Mori et al., 2013; Pati, Adhikari, & Dhara, 2010; Wu, Wang, Lin, Chen, & Wu, 2014).

3.3. UV absorption spectrum

According with Kumar et al. (2012) a basic and simple way to characterize the collagen is to wave scan the sample from 200 to 400 nm, due the triplehelical collagen has maximum peak at 230 nm and a negative peak near 204 nm. The maximum absorption wavelength of protein for ASC and PSC samples was 230nm (Fig.2). Generally, this value is close to 280 nm, due to the presence of aromatic side chains of tyrosine and phenylalanine in the collagen. Tyrosine and phenylalanine amino acids absorb light in the region of 240-300 nm (maximum absorption for tyrosine is 275 nm and for phenylalanine is 258 nm), so this low level of absorbance may indicate low levels of these amino acids in *C. hippurus* collagen.

Moreover, the maximum absorbance at 220-240 nm may be associated with the groups C=O, -COOH, $CONH_2$ in polypeptides chains of collagen (Kozlowska, Sionkowska, Skopinska-Wisniewska, & Piechowicz, 2015; Nalinanon et al., 2011; Pal, Nidheesh, & Suresh, 2015; Veeruraj, Arumugam, & Balasubramanian, 2013).

3.4. FTIR analysis

FTIR spectra of ASC and PSC exhibited the characteristic peaks of Amide I, II, III as well as amide A and B (Fig. 3). The absorption characteristics of Amide A, commonly associated with N-H stretching vibration, occurs in the wavenumber range 3400–3440 cm^{-1}. The amide B pick representing the asymmetrical stretching of CH_2 the absorption peaks of ASC and PSC were found nearly at 3300 cm^{-1}. When the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies as observed by other authors (Liu et al., 2012; Singh et al., 2011; Wang et al., 2014). The amide I band which usually in the range from 1600 cm^{-1} to 1700 cm^{-1} is mainly associated with stretching vibrations of the carbonyl groups (C=O bond) present along the polypeptide backbone which is a sensitive marker of the peptide secondary structure. Normal absorption range of the amide II bands position is 1550-1600 cm^{-1}, as observed in both types of collagen, cause by NH in-plane bend and the CN stretching vibration (Jeong, Venkatesan, & Kim, 2013; Wu, Wang, Lin, Chen, & Wu, 2014). Amide III bands were found at wavenumber of next to 1200, this peak is complex with intermolecular interactions in collagen, consisting of components from C–N stretching and N–H in plane bending from amide linkages, as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Matmaroh, Benjakul, Prodpran, Encarnacion, & Kishimura, 2011).

3.5. Effect of pH and NaCl concentration on collagen solubility

The effect of pH on the solubility of ASC and PSC from dolphinfish (*C. hippurus*) skin was shown in Fig. 4. It was observed that both samples showed higher solubility in acidic pH range. The solubility of ASC and PSC reached maximum at pH 1 and 2, respectively. Generally, these types of collagen were exhibited higher solubilization in acidic pH from 1 to 4 and the lesser in pH 7 (Veeruraj et al., 2013). When the pH of the collagen solution is equal to or close to the isoelectric point (pI), there is a decrease in solubility caused by a reduction in the amount of molecular charges. The pI of type I collagen ranging between pH 6.0 and pH 9.0. At this point, the total net charges of protein molecules are zero and hydrophobic - hydrophobic interaction increases, thereby leading to the precipitation and aggregation of protein (Singh et al., 2011; Li et al., 2013). In contrast, when the pH is lower or higher than pI, the net charge of protein molecules is greater and the solubility is increased by the repulsion forces between chains. Therefore, this result is in accordance with the collagen solubility from fish skin

reported by literature (Zeng et al., 2012; Li et al., 2013; Veeruraj et al., 2013; Wu et al., 2014; Thuy et al., 2014).

A drastic decrease in solubility of the PSC was observed with 1% and 2% NaCl, followed by a slight increase in the following concentrations. Considering that, a solubility of ASC was reduced by 3% with a slight increase of 4% and a minimum solubility was observed in NaCl 6% (Figure 5). A decrease in collagen solubility could have been described by a salt release phenomenon that occurred at a low NaCl concentration. An increase in ionic strength causes a reduction in the solubility of the protein by a hydrophobic-hydrophobic interaction reinforced between as protein chains, and competing for salt water, leading to protein precipitation (Li et al., 2013; Wu, Wang, Lin, Chen, & Wu, 2014). This solubility behavior of collagens with changes in pH and NaCl concentrations may play a crucial role in their extraction and the choice or your application (Montero et al., 1991).

3.6. Assay for substrate specificity: pre-purified enzyme

Collagenolytic protease isolated by ATPS was able to cleave native collagen only after 18 hours of incubation (Fig. 6). For samples of crude extract and pecipitated by ammonium sulphate, no activity was detected in the collagen cleavage before 24 h. Collagen hydrolysis was observed mainly by the activity of the pecipitated sample (Fig. 7). Enzymatic specificity test using collagenolytic protease have been described by Teruel & Simpson (1995) for winter flounder (*P. americanus*), Park et al. (2002) for mackerel (*S. japonicus*) and Herreiro-Hernandez, Duflos, Malle & Bouquelet (2003) for iced cod (*G. morhua*).

Conclusion

Based on SDS-PAGE and the different techniques of physicochemical characterization, it can be concluded that the collagen extracted from the skin of the gold is type I collagen. The high yield from the acid extraction can represent a saving in the process, dispensing the use of pepsin to extract the remaining collagen, making it an interesting and economic alternative source. The hydrolysis experiment needs to be improved, however the intestinal visceras of *C. hippurus* also present as a source of proteases with collagenolytic activities, capable of hydrolyzing the native molecule of collagen producing peptides. Therefore, the results support the proposal of the use of processing residues of *C. hippurus* as an alternative source of collagen as well as collagenolytic proteases.

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Figure captions

Figure 1: SDS-PAGE patterns of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the *Coryphaena hippurus* skin. M: high-molecular weight markers; 1: type I collagen from achilles tendon bovine 2: ASC of *C. hippurus* skin; 3: PSC of *C. hippurus* skin.

Figure 2: UV-Vis spectra of collagen isolated from the skin of *C. hippurus* in 0.5 M acetic acid (1:1)

Figure 3: (a) Infrared spectrometry of pepsin soluble collagen (ASC) from the skin of *Coryphaena hippurus*. (b) Infrared spectrometry of pepsin soluble collagen (PSC) from the skin of *C. hippurus*.

Figure 4: Relative solubility (%) of ASC and PSC from *Coryphaena hippurus* skin in 0.5 M acetic acid at different pHs.

Figure 5: Relative solubility (%) of ASC and PSC from *Coryphaena hippurus* skin in 0.5 M acetic acid with different NaCl concentrations.

Figure 6: PEG-collagenolytic protease on extracted from digestive viscera of *Coryphaena hippurus* against collagen type I, various incubation times.

Figure 7: Crude extract and extrat pecipitated by ammonium sulphate, extracted from digestive viscera of *Coryphaena hippurus* against collagen type I , various incubation times.

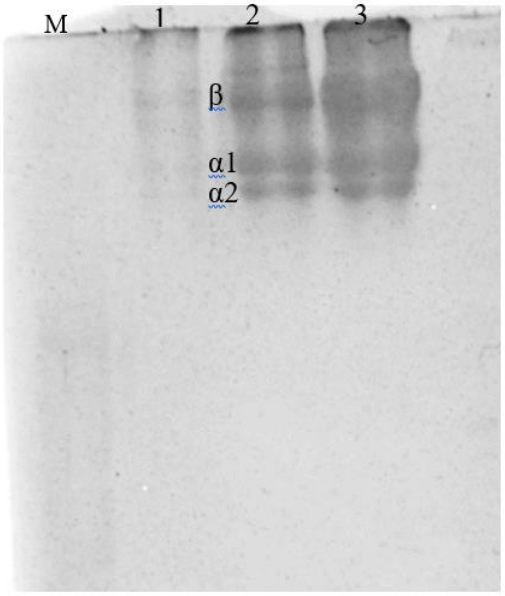

Figure 1

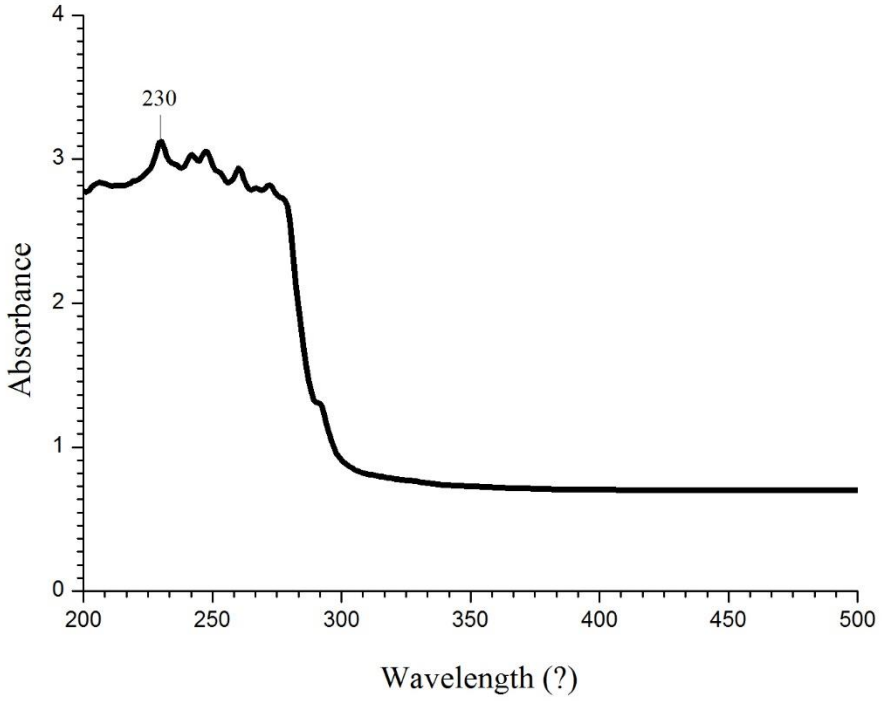

Figure 2

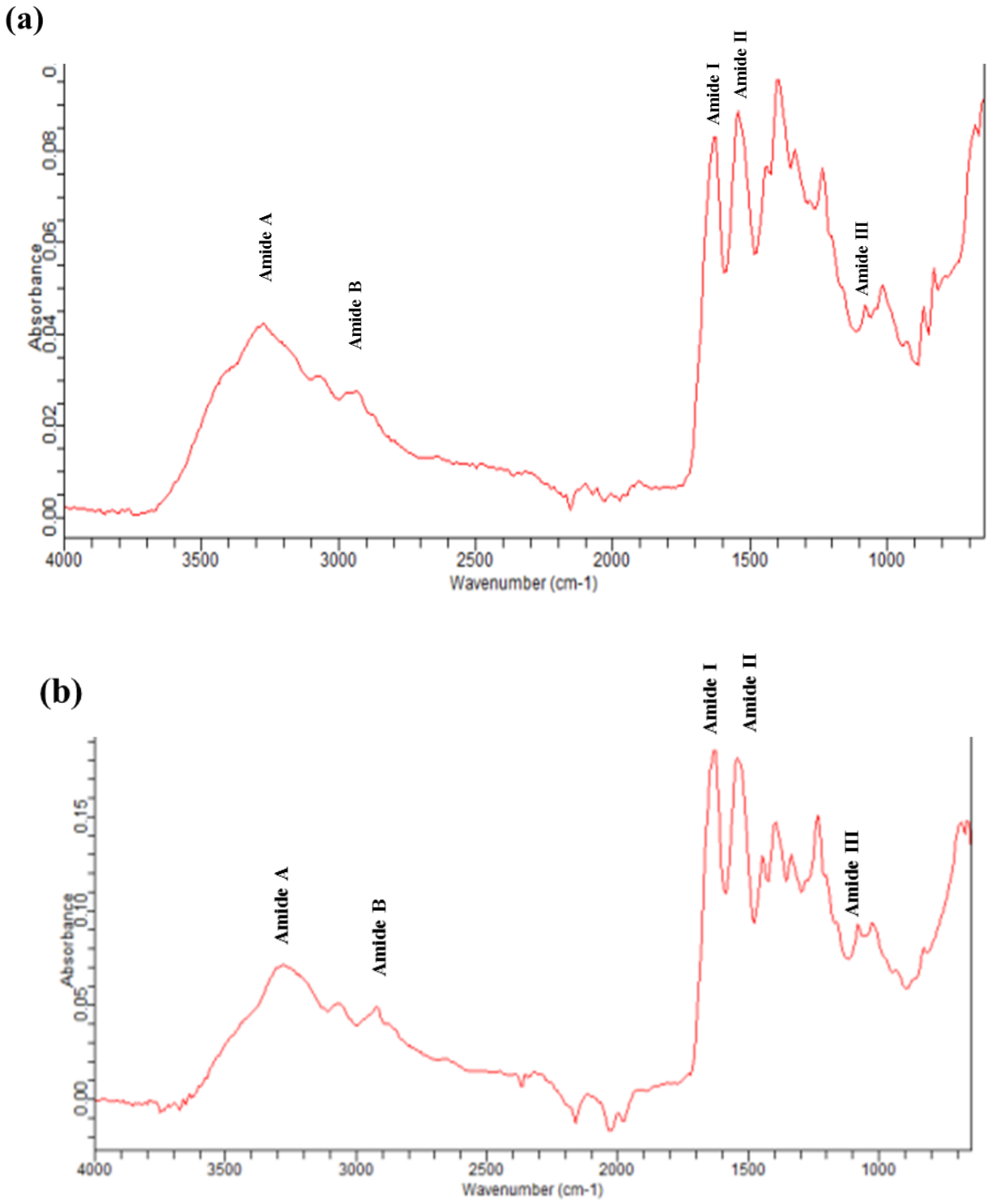
(a)
Amide A
Amide B
Amide I
Amide II
Amide III
Absorbance
0.00
0.02
0.04
0.06
0.08
0
4000
3500
3000
2500
2000
1500
1000
Wavenumber (cm-1)
(b)
Amide A
Amide B
Amide I
Amide II
Amide III
Absorbance
0.00
0.05
0.10
0.15
4000
3500
3000
2500
2000
1500
1000
Wavenumber (cm-1)

Figure 3

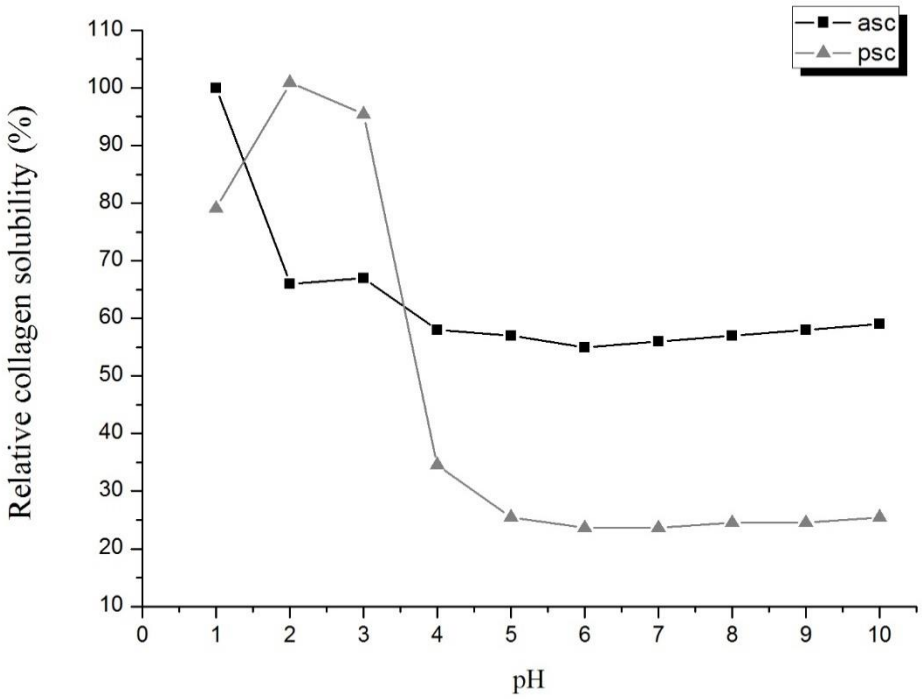
asc
psc
Relative collagen solubility (%)
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
pH

Figure 4

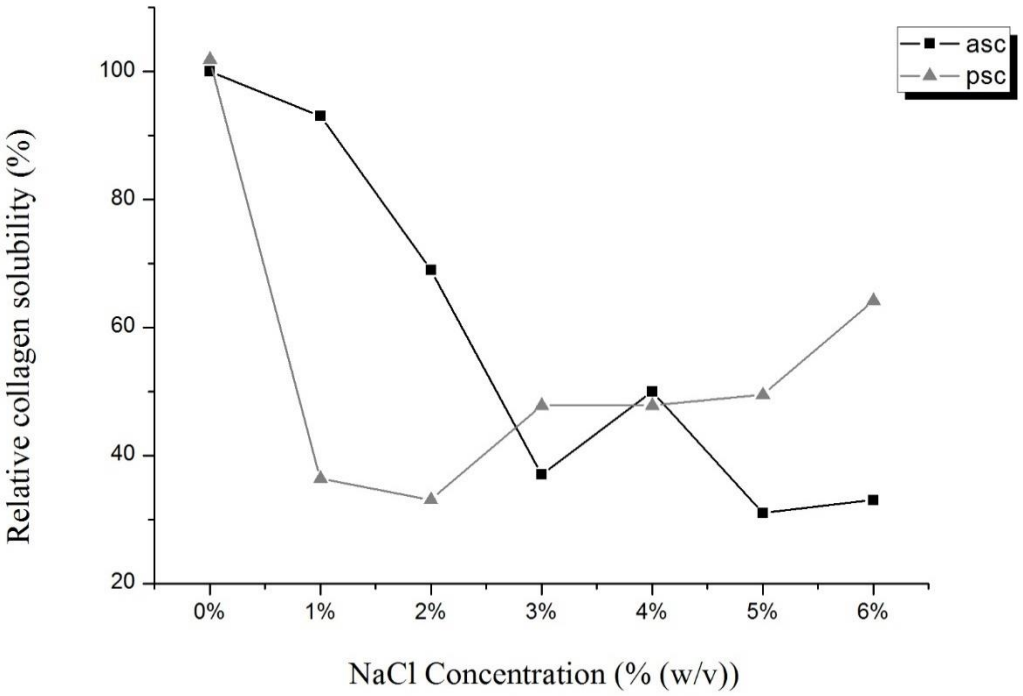
asc
psc
Relative collagen solubility (%)
100
80
60
40
20
0%
1%
2%
3%
4%
5%
6%
NaCl Concentration (% (w/v))

Figure 5

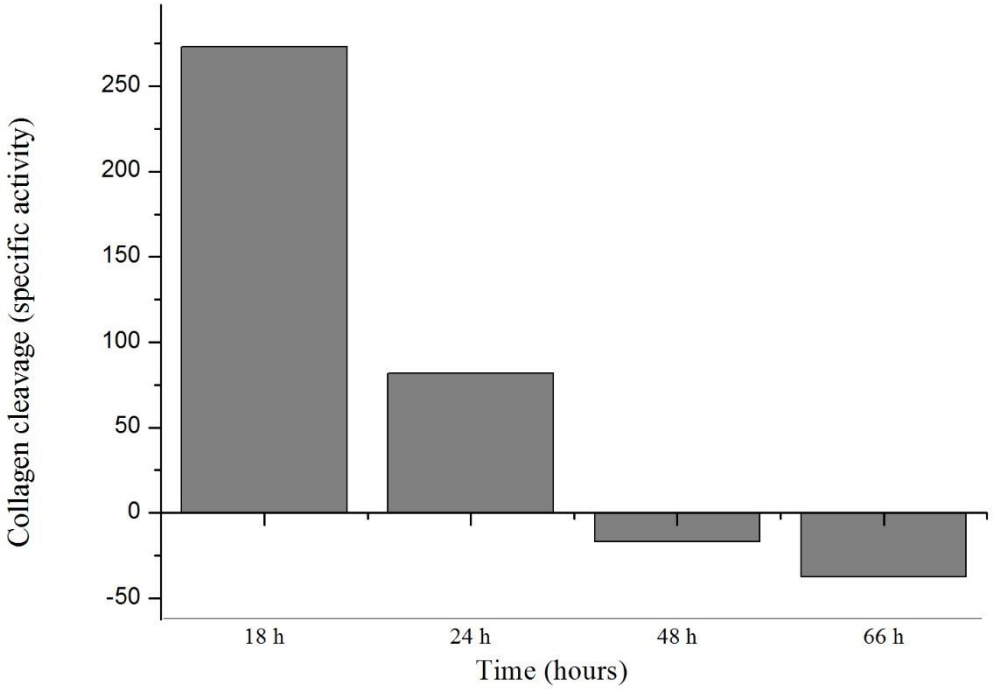
Collagen cleavage (specific activity)
250
200
150
100
50
0
-50
18 h
24 h
48 h
66 h
Time (hours)

Figure 6

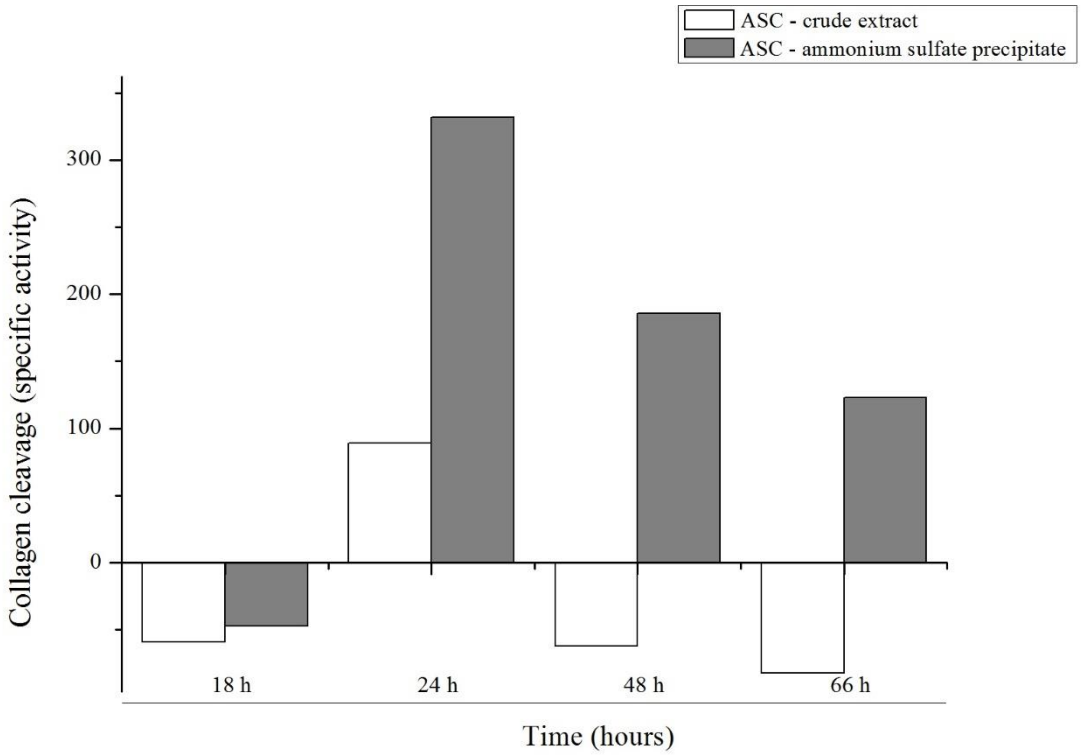
ASC - crude extract
ASC - ammonium sulfate precipitate
Collagen cleavage (specific activity)
300
200
100
0
18 h
24 h
48 h
66 h
Time (hours)

Figure 7

Table 1 – The extraction yields of acid and pepsin soluble collagen from fish skin.

Species	Extract yield (%)		
	ASC	PSC	
Coryphaena hippurus	43	3.0	Present study
Sebastes mentella	47.5	-	Wang et al., 2008
Oreochromis niloticus	39.4	-	Zeng et al., 2009
Diodon holocanthus	4.0	19.5	Huang et al., 2011
Nemipterus hexodon	24.9	-	Nalinanon et al., 2011
Hypophthalmichthys nobilis	-	60.3	Liu et al., 2012
Rachycentron canadum	35.5	12.3	Zeng et al., 2012
Scomberomorous niphonius	58.6	14.4	Li et al., 2013
Amur sturgeon	37.4	52.8	Wang et. al., 2014

.

CONSIDERAÇÕES FINAIS

O colágeno da pele de *Coryphaena hippurus* foi extraído com sucesso, obtendo-se 43% de rendimento de colágeno ácido solúvel (ASC) e 3% de colágeno pepsino solúvel. Ambos colágenos extraídos consistiam em duas cadeias α diferentes (α 1 e α 2) e seus dímeros (cadeia β). No ensaio ultravioleta (UV) do espectro de absorção, o colágeno mostrou uma máxima absorção em 230 nm. Ambos colágenos apresentaram uma maior solubilidade na faixa ácida de pH e diminuição da sua solubilidade na presença de concentrações de NaCl superiores a 1%. O padrão de picos apresentado pela análise FTIR e os demais procedimentos de caracterização da molécula indicam que o colágeno extraído é do tipo I.

Os resultados obtidos neste estudo se assemelham aos relatados na literatura para outras espécies de peixes, indicando a possibilidade de utilização da pele do Dourado como uma fonte potencial de colágeno para aplicações industriais e biotecnológicas. O alto rendimento obtido pela extração ácida pode prescindir o uso da pepsina, possibilitando uma economia nas etapas extração da molécula. Além disso o colágeno extraído também pôde ser utilizado como parâmetro para testes de especificidade e hidrólise por proteases com propriedades colagenolíticas, obtidas a partir das vísceras intestinais da mesma espécie de peixe e através de precipitação por sulfato de amônia e SDFA. Os resultados para estes testes indicaram um potencial para a produção de peptídeos bioativos de colágeno.

ANEXOS – Normas do periódico "Food Hydrocolloids"

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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-molecularweight 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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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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GUIDE FOR AUTHORS.**INTRODUCTION**

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.

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