UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

DISSERTAÇÃO DE MESTRADO

PURIFICAÇÃO E ATIVIDADE ANTIOXIDANTE DE PEPTÍDEOS OBTIDOS POR HIDRÓLISE ENZIMÁTICA DO RESÍDUO DO PROCESSAMENTO DO CAMARÃO MARINHO *Litopenaeus vannamei*

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RECIFE
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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

RECIFE
2013

Catalogação na Fonte:
Bibliotecário Bruno Márcio Gouveia, CRB-4/1788.

Firmino, Guilherme Oliveira

Purificação e atividade antioxidante de peptídeos obtidos por hidrólise enzimática do resíduo do processamento do camarão marinho *Litopenaeus vannamei* / Guilherme Oliveira Firmino. – Recife: O Autor, 2013.

69 f.: il., fig.

Orientador: Ranilson de Souza Bezerra

Coorientadora: Célia Maria Machado Barbosa de Castro

Dissertação (graduação) – Universidade Federal de Pernambuco. Centro de Ciências Biológicas. Pós-graduação em Bioquímica e Fisiologia, 2013.

Inclui bibliografia e anexos

1. **Peptídeos 2. Hidrólise 3. Camarão I. Bezerra, Ranilson de Souza II. Castro, Célia Maria Machado Barbosa de III. Título.**

572.65 CDD (22.ed.) UFPE/CCB-2013-340

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Data: 30/07/2013

Dedico este trabalho a minha mãe, **Maria Carmem de Oliveira e Silva** por todo esforço dedicado à minha formação, seja como profissional, seja como ser humano. A senhora é uma lutadora e um exemplo de vida, e sem você eu não estaria aqui.
Te amo muito.

AGRADECIMENTOS

A **Deu**s, criador, que me dá forças para seguir em frente.

À minha mãe, por toda dedicação e esforço empregados na minha formação.

Ao **CNPQ** pela concessão da bolsa

Ao Professor Dr**. Ranilson de Souza Bezerra** e à **Drª. Suzan Diniz Santos** pela dedicação a este trabalho.

À Professora **Drª. Célia Maria Machado Barbosa de Castro** por ter me iniciado e me apoiado na carreira de pesquisador.

Aos professores do Programa de Pós-graduação em Bioquímica e Fisiologia pelos conhecimentos transferidos

Aos colegas do Laboratório de Enzimologia (LABENZ), especialmente a **Janilson Félix da Silva** e **Juliett de Fátima Xavier da Silva** por toda a paciência em passar seus conhecimentos.

À Noronha Pescados por ceder o resíduo de camarão utilizado nesse estudo

À **Marina Marcuschi** pela contribuição realizada a este trabalho.

À minha irmã, **Samanta Oliveira Firmino** e minhas tias, **Maria José de Oliveira Santos** e **Maria de Fátima de Oliveira e Silva**, por todo apoio que me deram nas horas de dificuldade.

À minha namorada, **Camila Martins Gomes Morais** por toda a paciência e apoio dedicados a mim.

Aos amigos de longa data, **Denis Vitor Moreira**, **Alissom Rodrigues de Paula**, **Ícaro Nigro**, **Yuri Ayres, Malu Cavalcante** e **Renata Raele** pelo apoio e horas de divertimento.

Ao secretário da pós-graduação, **Djalma Gomes da Silva**, e aos demais funcionários do Departamento de Bioquímica, por sua competência nos serviços prestados.

“A alegria está na luta, na tentativa, no sofrimento envolvido e não na vitoria propriamente dita.”

Mahatma Ghandi

RESUMO

O cultivo de camarão representa, aproximadamente, 4,5 % da aquicultura mundial, sendo o camarão branco do pacífico *Litopenaeus vannamei* a espécie mais cultivada no planeta, entretanto 48 a 56 % do seu peso são descartados como lixo. A elaboração de hidrolisados proteicos tem sido uma forma de reaproveitamento de matéria orgânica bastante estudada nos últimos anos. Hidrolisados proteicos podem possuir em sua composição peptídeos antioxidantes, podendo ser utilizados como alimentos antioxidantes, ou na indústria, como conservante de alimentos. O objetivo desse estudo foi obter e avaliar a atividade antioxidante dos peptídeos presentes no hidrolisado proteico do camarão. O hidrolisado proteico de camarão (SPH) foi obtido por hidrolise enzimática das cabeças do camarão utilizando alcalase a 0,5% e a atividade antioxidante foi avaliada pelos ensaios do DPPH, poder redutor, ABTS+ e atividade quelante do ferro. O SPH obteve uma boa atividade antioxidante pelos ensaios do ABTS+ (52.24 % $\pm$ 1,127 a 5 mg/mL), poder redutor (0.442 $\pm$ 0.045 a 5 mg/mL) e atividade quelante do ferro (95.874 % $\pm$ 0.723 a 1mg/mL), porém apresentou uma atividade eliminadora do radical DPPH em torno de 30 % em todas as concentrações testadas. O SPH foi purificado por duas etapas cromatográficas. Primeiramente, o SPH foi submetido a uma troca iônica em uma coluna DEAE-Sepharose e a fração SPH IP2 com o maior poder redutor foi submetida a uma cromatografia por exclusão molecular, em uma coluna de Sephadex G-25 (1,5x42 cm) e três frações foram obtidas. A fração SPH EP3 obtida pela exclusão molecular apresentou maior poder redutor na concentração de 500 μg/mL (0, 437 $\pm$ 0, 017), uma alta atividade quelante do ferro (81.19 % $\pm$ 2.59 a 1mg/mL), porém esta apresentou uma baixa capacidade de eliminação do radical ABTS+ (15,31% $\pm$ 1,61 a 2mg/mL). Esses resultados sugerem que o SPH é um importante candidato a alimento com potencial antioxidante e a quelação dos íons Fe^{2+} é um dos principais mecanismos pelos quais SPH EP3 exerce sua atividade antioxidante, entretanto estudos subsequentes são necessários para melhor caracterizar esses peptídeos e os mecanismos pelos quais exercem atividade antioxidante.

Palavras-chaves: atividade antioxidante; hidrolisados proteicos; peptídeos antioxidantes; *Litopenaeus vannamei*

ABSTRACT

Shrimp production represents, near to 4.5 % of worldwide aquaculture. The whiteleg shrimp *Litopenaus vannamei* is the most cultivated species in the world, besides that 48 to 56 % of its body weight are discarded as waste. Protein hydrolysate production has been an extensively studied way of reusing of organic matter in the last years. They may have antioxidants peptides in their composition, which could be used as natural antioxidant foods or to prevent the food spoilage. The objective of this study was to obtain and evaluate the antioxidant activity of peptides within the Shrimp protein hydrolysate. The Shrimp Protein hydrolysate (SPH) was obtained by alcalase (0.5 %) enzymatic hydrolysis of Shrimp heads and the SPH antioxidant activity was evaluated by DPPH, reducing power, ABTS+ and ferrous chelant activity assays. SPH had a good antioxidant activity by ABTS+ scavenging assay (52.24 % $\pm$ 1.127 at 5 mg/mL), reducing power (0.442 $\pm$ 0.045 at 5 mg/mL) and ferrous chelant activity (95.874 % $\pm$ 0.723 at 1mg/mL), but it had a DPPH scavenging activity near of 30 % in all tested concentrations. Further, the SPH was purified by two subsequent chromatographic steps. Firstly SPH was purified by an anion exchange DEAE-Sepharose chromatography and the fraction SPH IP2, which had the best reducing power was fractionated by a size exclusion chromatography in a 1,5x42 cm Sephadex G-25 column and three fractions were obtained. The fraction SPH EP3 from size exclusion showed the highest antioxidant activity by reducing power assay (0.437$\pm$ 0.017 at 500 ug/mL). Moreover SPH EP3 showed a high Fe^{2+} chelant activity (81.19% $\pm$ 2.59 at 1mg/mL), but a weak ABTS+ scavenging activity (15.31% $\pm$ 1.61 at 2 mg/mL). These results suggest that SPH could be employed as an antioxidant food and the Fe^{2+} chelation is an important mechanism by SPH excerpts its antioxidant activity. Besides that, sequencing studies and antioxidant evaluation by other methodology are necessary in order to better characterize and understand the mechanisms by which SPH excerpts its antioxidant activity.

Key-words: antioxidant activity; antioxidant peptides; *Litopenaeus vannamei;* protein hydrolysates.

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LISTA DE ABREVIATURAS

FAO ……..Food and Agriculture Organization

pH ……Hidrogeniônic Potential

HAT …… Hidrogen Atoms Transference

ET ……Electron Transference

SPH ……Shrimp Protein Hydrolysate

BHA ……..Butylated Hydroxianisole

BHT ……Butylated Hydroxitoluene

DPPH ….....**...**1,1-diphenyl-2-pycryl-hydrazyl

ABTS .....2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

HPLC .....High Performance Liquid Chromatography

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1. INTRODUÇÃO

A indústria mundial de pescado gera uma grande quantidade de refugo ou subprodutos com alto teor nutritivo. O reconhecimento da limitação de recursos naturais e o aumento da poluição têm enfatizado a necessidade de uma melhor utilização de subprodutos da indústria pesqueira (GUERARD; SELLOS, e LE GAL, 2005). A recuperação e utilização desses materiais têm grande importância na redução de problemas ambientais e maximização dos benefícios econômicos. Além disso, os subprodutos representam importantes biorrecursos, podendo ser utilizados na produção de alimentos, produtos de cuidado à saúde e fármacos; ou ainda, como rações especiais para peixes e outros animais. (JE et al., 2009).

Segundo dados da Organização das Nações Unidas para Agricultura e Alimentação, FAO, (2012) o *L. vannamei* é a espécie de camarão mais cultivada no mundo. Entretanto, após o processamento, uma parte considerável é descartada como lixo (SOLANO et al., 2009). Este subproduto é fonte de uma grande quantidade de biomoléculas, tais como polissacarídeos, proteínas e minerais. Apresentando alguns destes compostos propriedades benéficas à saúde, tal como controle da produção de radicais livres.

O termo "radical livre" refere-se a qualquer espécie molecular com um ou mais elétrons desemparelhados, característica esta, que promove uma natureza química instável à molécula, tornando-a muito reativa. De fato, quando reagem com o DNA, RNA, proteínas e lipídeos, provocam efeitos tóxicos. Radicais livres incluem tanto as espécies reativas de oxigênio(EROs; como, superóxido (O2-), hidroxil (OH-), hidroperoxil (HOO-), peroxil (ROO-) e alcoxil (RO-), bem como as espécies reativas de nitrogênio (LEE e LEE, 2006; TUDEK et al., 2010; ZIECHA et al. 2011).

Em humanos, a oxidação de moléculas por radicais livres pode ocasionar doenças crônicas, tais como: diabetes, doenças cardiovasculares, desordens neurodegenerativas e câncer (DONG et al.,2008). Além do mais, a peroxidação lipídica é uma grande preocupação para a indústria alimentícia e ao consumidor final, pois promove alterações indesejáveis no sabor e odor do produto, além originar produtos potencialmente tóxicos (LIN e LIANG, 2002).

No sentido de prevenir a deterioração dos alimentos e proteger contra doenças, é importante inibir a oxidação dos lipídeos e a formação de radicais livres nos alimentos e no corpo (AMES, 1983). Nos últimos anos, têm se dado grande importância à procura de novos

antioxidantes naturais, para usos medicinais e alimentares, em substituição aos antioxidantes sintéticos (BOUGATEF et. al, 2010).

Recentemente, o processo de hidrólise enzimática com proteases tem recebido bastante atenção. Hidrolisados proteicos ou peptídeos podem afetar funções relacionadas à saúde do indivíduo, tais como: pressão sanguínea e função antioxidante (CLEMENTE, 2000). Além disso, estudos recentes têm demonstrado que hidrolisados proteicos de peixe, obtidos por hidrólise enzimática, podem ser utilizados como antioxidantes naturais (YOU et al., 2010; LEE, JEON E BYUN, 2011)

Embora estudos sobre os efeitos antioxidantes de peptídeos isolados de hidrolisados de carcaça das mais diversas espécies marinhas tenham sido realizados. Ainda pouco foi relatado sobre a purificação de peptídeos com atividade antioxidante de espécies da carcinicultura Além disso, a grande expressividade do *Litopenaeus vannamei* para carcinicultura mundial traz como desvantagem o aumento da produção de resíduos potencialmente danosos ao meio-ambiente. Dessa forma, este estudo justifica-se pela sua importância biotecnológica, econômica e ambiental, tendo em vista que a obtenção de peptídeos antioxidantes, além de minimizar o impacto ambiental resultante do seu processamento, resulta em um produto novo e de alto valor agregado.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. A indústria mundial de pescado e a carcinicultura

Os últimos anos trouxeram um aumento crescente na produção de recursos pesqueiros destinados ao consumo humano. Em 2010 foram produzidos, aproximadamente, 148 milhões de toneladas de pescado, representando um crescimento de 8,1% em relação ao ano de 2006 (FAO, 2012a). Apesar de a captura representar a maior parcela da produção (aproximadamente 59,7%), o principal responsável por esse aumento tem sido a aquicultura, que cresceu 26,7% no período de 2006 a 2010, ao passo que foi observado uma queda de 1,5 % na produção por captura neste mesmo período (Figura 1). Além disso, dados preliminares da Organização das Nações Unidas para a Agricultura e Alimentação (FAO) para o ano de 2011 apontam que esta relação permanecerá nos próximos anos.

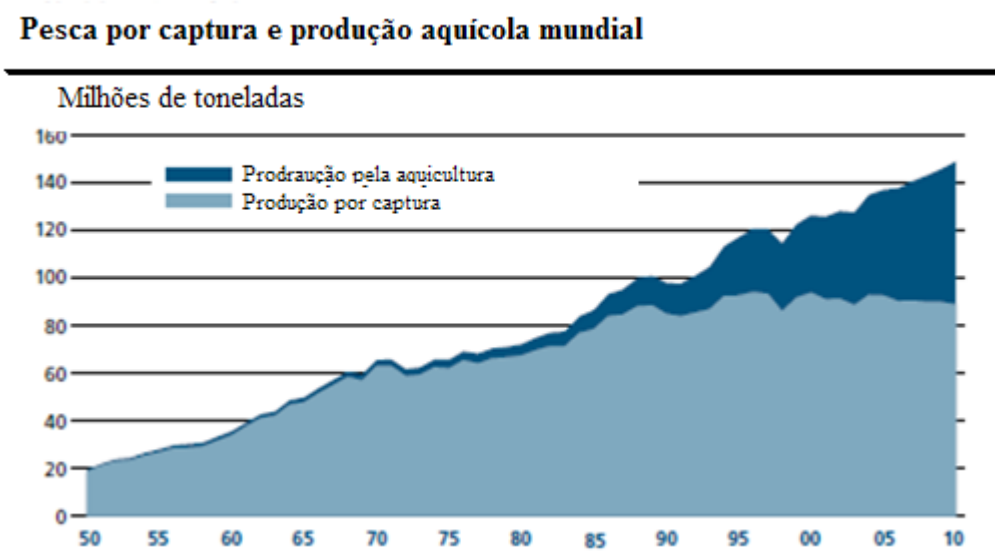

Figura 2: Pesca por captura e produção aquícola mundial (Fonte: FAO (2012a), modificado).

Segundo o último boletim da FAO, referente ao ano de 2010, a grande maioria da produção de pescado (86,4%) destinou-se ao consumo humano, sendo este comercializado na forma viva, fresca, refrigerada ou processada. Os demais 13,6%, aproximadamente 20,2

milhões de toneladas foram destinados para usos não-alimentícios. Deste grupo, 75% foram comercializados como farinha ou óleo de peixe e apenas 25% foram utilizados para outros usos, tais como matéria-prima para alimentação animal e usos farmacêuticos (FAO, 2012a).

O camarão branco do pacífico, *Litopenaeus vannamei* (Boone, 1935) (classe *Crustacea, ordem Decapoda, família Penaeidae*) (Figura 2), é uma espécie oriunda da costa sul americana do Oceano Pacífico com acentuada predominância na faixa costeira do Equador, sendo cultivado em todos os países produtores de camarão do mundo ocidental. Esta espécie apresenta em geral, uma taxa de crescimento uniforme e fácil adaptabilidade a diferentes condições físico-químicas do meio ambiente, tais como salinidade e temperatura (Dos SANTOS, 2009). Segundo dados da FAO (2012b) o *L. vannamei* é a espécie de crustácea mais cultivada no mundo, registrando uma produção de aproximadamente 2,7 milhões no ano de 2010 (Figura 3)

.

Figura 2: *Litopenaeus vannamei* Foto A.M. Arias (Fonte: http://www.ictioterm.es)

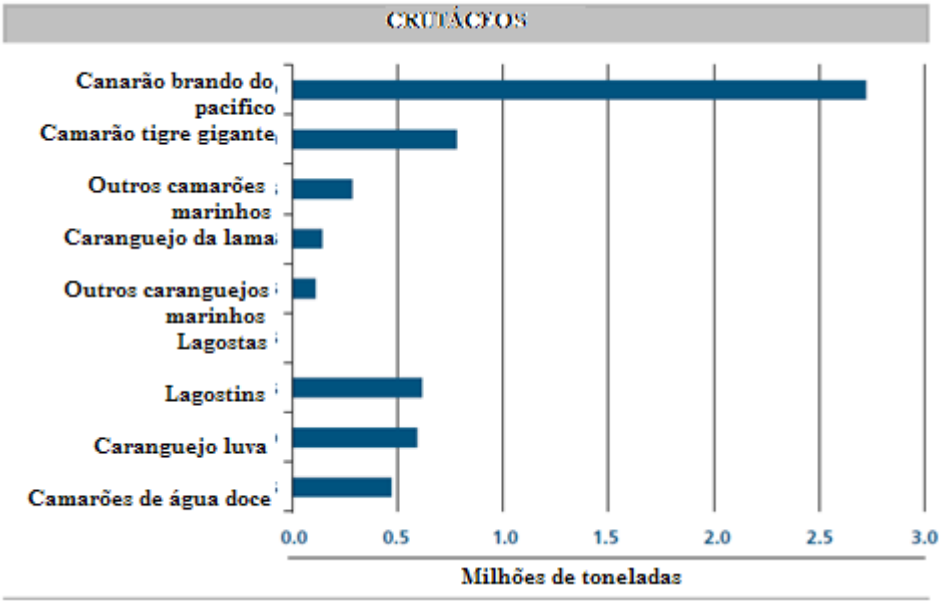

Figura 3: Produção mundial de crustáceos no ano de 2010. (Fonte: FAO (2012a), modificado)

O *L. vannamei* foi introduzido no Brasil na década de 1980 e demonstrou alta adaptabilidade às condições climáticas brasileiras, devido à sua rusticidade, rapidez no crescimento, ampla faixa de tolerância à salinidade e capacidade de aproveitar dietas com níveis proteicos variando de 20 a 40 % (COSTA, 2004). Nos dias atuais, é a espécie de camarão de produção predominante no país, que ocupa o posto de 7º produtor mundial, com sua produção concentrada na região Nordeste (MINISTÉRIO DA PESCA E AQUICULTURA, 2010)

Após o processamento do pescado, geralmente 40 % da matéria inicial é destinada ao consumo humano, o restante (60%) são considerados como resíduos sendo descartados como lixo (DEKKERS et al., 2011). Esses subprodutos contêm uma boa quantidade de material proteico que pode ser reaproveitado na forma de farinha, silagem, fertilizante e alimentação animal. Entretanto, esta atividade apresenta uma baixa rentabilidade o que tem direcionado a indústria para obtenção de produtos com maior valor agregado. (HSU, 2010; ARVANYTOIANNIS, 2008; HE, FRANCO e ZHANG, 2013). Além disso, devido a sua alta concentração de matéria orgânica, o resíduo de processamento do pescado é classificado como um dos mais dispendiosos para se eliminar (HE, FRANCO e ZHANG, 2013), sendo seu descarte uma preocupação constante da indústria. Nesse contexto, uma das principais questões a ser considerada é o melhor aproveitamento da biomassa resultante do processamento dos recursos pesqueiros (GUERARD, SELLOS e LE GAL, 2005).

A carcinicultura representa, aproximadamente, 4,5 % da aquicultura mundial, com uma produção estimada de 3,8 milhões de toneladas em 2010, movimentando mais de U$D 16 bilhões (FAOb, 2012). Segundo Sachindra e colaboradores (2005) 48% a 56 % do peso do camarão (cefalotórax e exoesqueleto) são descartados como lixo impróprio para consumo. Todo esse subproduto gerado representa um importante problema de descarte, bem como compreende um poluente em potencial para o meio-ambiente (FERRER et al., 1996) .

Entretanto, o resíduo de camarão é uma importante fonte de biomoléculas e minerais (SACHINDRA e BASKAR, 2008), havendo um número considerável de estudos apontando os subprodutos do processamento do camarão como fontes carotenóides (SANTOS, et al., 2012), polissacarídeos, proteínas e glicosaminoglicanos (CAHÚ et al., 2012). Há ainda estudos demonstrando processos que convertem este resíduo em produtos com atividades biológicas (SACHINDRA e BASKAR, 2008; BENJAKUL et al., 2009; SANTOS et al., 2012).

Tendo em vista o variado leque de moléculas bioativas que podem ser obtidas dos subprodutos do processamento do camarão. O uso desses compostos na produção de produtos de cuidado à saúde pode representar uma solução aos problemas de descarte e à baixa rentabilidade gerada pelos métodos tradicionais de reaproveitamento. Gerando um produto novo e de alto valor agregado.

2.2. Hidrolisados proteicos

A organização mundial da saúde recomenda as proteínas do pescado como fontes de aminoácidos essenciais (USYDUS et al., 2009). Uma vez que os subprodutos do processamento do pescado são ricos nessas moléculas, umas das formas de aproveitamento desse resíduo pela indústria é na produção de hidrolisados proteicos. Compreendem-se por hidrolisados proteicos, produtos da quebra de proteínas em pequenos peptídeos por um processo de hidrólise (HE, FRANCO e ZHANG, 2013; PESUPULETI e BRAUN, 2010). Este procedimento não só mantém o teor de aminoácidos essenciais, como promove uma série de propriedades ao produto final, tornando possível sua aplicação na indústria farmacêutica e de alimentos (HE, FRANCO e ZHANG, 2013).

A hidrólise pode ser realizada por um processo químico, utilizando ácidos e bases, sob altas temperatura e pressão; ou biológico, por meio de enzimas (PESUPULETI e BRAUN,

2010). Sua ação pode modificar as propriedades do resíduo por três vias: diminuindo o peso molecular, aumentando o número de grupos ionizáveis e expondo grupos hidrofóbicos. Essas modificações são responsáveis por melhorar propriedades físico-químicas, tais como solubilidade, capacidade emulsificante e capacidade de ligação ao óleo. Exercendo efeitos diretos sobre o desempenho e o comportamento do hidrolisado em sistemas alimentares (PANYAM e KILARA, 1996; HE, FRANCO e ZHANG, 2013).

A hidrólise química apresenta como vantagem um processo de produção mais rápido, a alta recuperação de proteínas e o baixo custo. Entretanto é um processo de difícil controle de qualidade, além de gerar um produto de baixa funcionalidade, pouco palatável e com baixa qualidade nutricional (HE, FRANCO e ZHANG, 2013; CHOBERT et al., 1996). Devido à estas limitações, sua utilização está restrita, principalmente, a produtos de baixo valor agregado, como fertilizantes (KRISTINSSON e RASCO, 2000; SANMARTIN et al.,2009).

Na hidrólise biológica são usadas enzimas proteolíticas. Essas enzimas são classificadas, segundo seu mecanismo de ação em: 1) endoproteases, responsáveis por clivar ligações peptídicas no interior das moléculas, produzindo peptídeos de comprimentos relativamente grandes; e 2) exoproteases, enzimas que removem aminoácidos das regiões N- e C- terminais, hidrolisando as ligações peptídicas terminais (Clemente, 2000).

Em comparação com a hidrólise química, o uso da hidrólise enzimática é mais vantajoso por produzir hidrolisados mais homogênios, tornar o hidrolisado mais palatável, manter o valor nutritivo e reduzir o conteúdo de sal do produto final. Suas desvantagens são o alto custo e o maior tempo de processamento (KRISTINSSON e RASCO, 2000; SANMARTIN et al.,2009).

Por dispensar o custo de enzimas comerciais, a utilização de enzimas do próprio organismo (autólise) também tem sido relatado (SHAHIDI et al, 1995), principalmente na produção de molhos e silagem (KRISTINSON e RASCO, 2000), bem como ração animal (LEAL et al., 2010). Entretanto, devido ao menor tempo requerido para obter graus de hidrólise semelhantes aos da autólise e ao melhor controle das condições de hidrólise, produzindo composições e perfis peptídicos mais consistentes, o uso das enzimas comerciais tem sido, em geral, preferido, em comparação aos processos autolíticos (ANUSHA, SARAMANAYAKA e LI-CHAN, 2011).

Quanto à faixa de pH de atividade, as enzimas que trabalham em faixas próximas ao neutro, como a alcalase, neutrase e flavourzyme têm sido usadas, preferencialmente, às ácidas. Isso se deve aos efeitos indesejáveis do baixo pH sobre o hidrolisado. Dentre esses efeitos estão, a redução do teor proteico, a queda no valor nutricional, devido à destruição de

aminoácidos essenciais; e redução na funcionalidade do hidrolisado devido ao excesso de hidrólise (KRISTINSSON e RASCO, 2000).

Muitas dessas enzimas que trabalham em faixas de pH próximas ao neutro são de origem microbiana. As enzimas microbianas possuem maior estabilidade de temperatura e pH, além de serem consideradas mais eficientes na hidrólise de proteínas do resíduo de pescado (HERPANDI et.al, 2011). A alcalase é uma endopeptidase alcalina obtida do microrganismo *Bacillus licheniformis* que, por produzir um produto com gosto suave mesmo em elevados graus de hidrólise, tem sido intensivamente empregada pela indústria, sendo considerada uma das melhores enzimas para o preparo de hidrolisados proteicos (BENJAKUL e MORRISSEY, 1997; KRISTINSSON e RASCO, 2000; CENTENARO et al. 2009).

Os peptídeos bioativos obtidos pelo processo de hidrólise são fragmentos proteicos que, além de atuarem como fonte de nitrogênio e aminoácidos, possuem inúmeras funções fisiológicas em potencial, incluindo atividades antioxidante, opióide, imunomodulatória, antibacteriana, antitrombótica e anti-hipertensiva (MURRAY e FITZGERALD, 2007). Em geral, esses peptídeos possuem 2 a 20 aminoácidos de comprimento e estão presentes na sequencia da proteína de origem, sendo liberados durante a digestão gastro-intestinal e/ou durante o processamento do alimento (HARNEDY e FITZGERALD, 2012).

O tipo de peptídeo bioativo a ser gerado de uma fonte proteica em particular depende da sequência primária da proteína em questão e da especificidade da enzima a ser utilizada (HARNEDY e FITZGERALD, 2012). Além disso, a escolha cuidadosa de condições como temperatura, pH e controle de tempo de hidrólise é crucial para a obtenção de hidrolisados com as propriedades bioativas e funcionais desejáveis (SARAMANAYAKA e LI-CHAN, 2011).

Há inúmeros estudos na literatura que apontam para as diversas propriedades biológicas dos hidrolisados proteicos de organismos marinhos. Entre elas destacam-se a atividade, anti-hipertensiva (RAGHAVAN e KRISTINSON, 2009; JUNG et al., 2006), anticancer (ALEMÁN et al., 2011), antifadiga (YOU et al., 2011), bem como a atividade antioxidante (DEKKERS et al., 2011; WU, CHEN e CHIAU., 2003; DONG et al., 2008; YOU et al, 2010;). Outros estudos ainda demonstraram hidrolisados com funcionalidades antioxidantes e anti-hipertensivas mistas (KANTAPHANT, BENJAKUL e KISHIMURA, 2011; JE et al., 2009; BENJAKUL et al., 2009).

2.3. Peptídeos antioxidantes

Os radicais livres são átomos de oxigênio altamente reativos, formados durante o metabolismo aeróbio, que apresentam uma estrutura instável de elétrons desemparelhados (KLOMPONG et al., 2007). Esses radicais estão associados com a ocorrência de diversas doenças, como aterosclerose, câncer e inflamação (FRÖLICH e RIEDERER, 1995; MURAMATSU, 1995). Sob condições normais, nosso sistema de defesa pode remover esses radicais por meio de antioxidantes enzimáticos (Superoxido dismutase e glutationa peroxidase), e/ou não enzimáticos (vitaminas, co-enzimas e co-fatores) (JOHANSEN et al., 2005). Entretanto, quando radicais livres são produzidos em excesso esse sistema fica sobrecarregado, o que causa efeitos danosos para a célula, devido à oxidação de lipídeos, proteínas, DNA e enzimas (PHILANTO, 2006).

Os radicais livres produzidos não provocam apenas prejuízos ao organismo, mas também a indústria alimentícia, por meio da peroxidação lipídica, a qual promove alterações indesejáveis no sabor e odor do produto, além originar produtos potencialmente tóxicos (LIN e LIANG, 2002). Antioxidantes são úteis em retardar a peroxidação lipídica e seus produtos secundários. Com isso, mantêm o sabor, a textura e, em alguns casos, a cor do produto durante seu período de estocagem. (ANUSHA, SARAMANAYAKA e LI-CHAN, 2011). Além disso, antioxidantes reduzem a oxidação das proteínas, bem como, sua interação com grupos carbonil, responsáveis por alterações na funcionalidade das mesmas (ELIAS, KELLERBY E DECKER, 2008).

Antioxidantes sintéticos, tais como BHT, BHA e TBHT têm sido usados pela indústria para prolongar o tempo de prateleira dos alimentos. Entretanto, tem sido observado que esses compostos produzem efeitos danosos à saúde, com isso, muitos estudos estão sendo realizados no sentido de se obter novos antioxidantes naturais (HALLIWELL e WHITEMAN, 2004).Nos últimos anos, foram purificados peptídeos antioxidantes de hidrolisados de subprodutos de diversas espécies marinhas, tais como *Hypoptychus dybowskii* (LEE, JEON e BYUN, 2011), *Otolithes ruber* (NAZEER, KUMAR e GANESH, 2012), *Sardinella aurita* (BOUGATEFF et al, 2010), *Gadus macrocephalus* (NGO et al., 2011), *Oreochromis niloticus* (NGO et al, 2010 e), *Chlorella ellipsoidea* (KO, KIM e JEON, 2012). Esses peptídeos podem exercer sua atividade eliminando radicais livres, quelando metais de transição ou inibindo a peroxidação lipídica (ERDMANN, CHEUNG e SCHRÖDER, 2008; SARMADI e ISMAIL, 2010). Apesar de sua relação estrutura-atividade antioxidante ainda não estar estabelecida,

estudos sugerem que o tipo, a posição e o grau de hidrofobicidade dos aminoácidos presentes parecem exercer um papel essencial sobre a atividade antioxidante (HARNEDY e FITZGERALD, 2012).

A atividade antioxidante não pode ser mensurada baseada em apenas um modelo e, na prática, mais de um teste é utilizado na avaliação da atividade antioxidante de interesse (ALAN, BRISTI e RAFIQUZZMAN, 2012). Os ensaios para a avaliação *in vitro* da atividade antioxidante são divididos em dois grupos de reações: reações de transferência de átomos de hidrogênio (HAT); e reações de transferência de elétrons (ET) (HUANG, OU e PRIOR, 2005).

Os ensaios de HAT quantificam a habilidade de um antioxidante de doar átomos de hidrogênio, por meio de uma reação de competição entre o composto antioxidante e um substrato (HUANG, OU e PRIOR, 2005). Os ensaios de ET mensuram a capacidade redutora do composto, relacionando-a a mudanças na coloração da reação (HUANG, OU e PRIOR, 2005). Nessa categoria se enquadram os ensaios de DPPH e de poder redutor total.

Além disso, como dito anteriormente, os peptídeos antioxidantes também podem atuar como quelantes de metais de transição. A redução da oxidação resultante da quelação de metais de transição pode ser avaliada por uma reação espectrofotométrica na qual a interação do antioxidante com o íon Fe^{2+} presente no meio é determinada pela formação de um complexo colorido com a ferrozina (NAJAFIAN e BABJI, 2012).

Na análise da atividade antioxidante *in vivo*, a amostra é testada em animais de laboratório, como camundongos e ratos, utilizando um regime dosagem definido para o respectivo teste a ser empregado. Após esse período, os animais são normalmente sacrificados e as amostras de sangue e tecidos utilizadas para os ensaios. Nesta categoria estão incluídas a avaliação das enzimas envolvidas na defesa contra o estresse oxidativo, tais como catalase e glutationa-s-transferase, bem como a análise de substâncias derivadas do ácido tiobarbitúrico (ALAN, BRISTI e RAFIQUZZMAN, 2012).

A maioria dos peptídeos antioxidantes possui pesos moleculares variando de 500 a 1800 Da, além de, frequentemente, apresentarem resíduos hidrofóbicos, como valina e leucina, no seu N-terminal, e resíduos de prolina, histidina, tirosina, triptófano, metionina e cisteína na sua sequencia (SARAMANAYAKA e LI-CHAN, 2011). Resíduos hidrofóbicos, como valina ou leucina facilitam a interação de peptídeos na interface óleo-água, facilitando a eliminação de radicais livres na fase lipídica (RANATHUNGA et al., 2006)

Uma vez que a oxidação de lipídeos promove o desenvolvimento de um sabor indesejável e produtos potencialmente tóxicos, a atividade antioxidante dos hidrolisados

proteicos pode ser aplicada a produtos alimentícios para estender sua vida de prateleira (HE, FRANCO e ZHANG, 2013). Em um estudo realizado por Dekkers e colaboradores (2011) foi possível aumentar o tempo de prateleira do músculo vermelho do Dourado (*Coryphaena hippurus*) após sua imersão em hidrolisado proteico de tilápia.

Segundo SARMADI e ISMAIL (2010), por estarem inativos na sequência nativa da proteína, os peptídeos contidos nos hidrolisados proteicos podem ser utilizados como produtos farmacêuticos. HOU e colaboradores (2009), em estudo realizado em camudongos ICR, machos, observaram efeito protetor de peptídeos contidos na gelatina da pele do *Gadus macrocephalus* contra o foto envelhecimento induzido pela radiação. NAZEER, KUMAR e GANESH (2012) observaram que o peptídeo isolado do hidrolisado proteico de *Otolithes ruber* reduziu o estresse oxidativo em ratos e o dano, *in vitro*, ao DNA.

2.4. Purificação de peptídeos

De maneira a obter um produto mais puro e com maior atividade, algumas estratégias de separação e purificação podem ser empregadas. Membranas seletivas para baixos pesos moleculares são úteis em concentrar peptídeos antioxidantes dos componentes de maior massa molecular, como cadeias polipeptídicas não-digeridas e enzimas (SARAMANAYACA e LI-CHAN, 2011). Entretanto, para purificar peptídeos com atividades específicas, esses processos devem ser acoplados às técnicas cromatográficas (POULIOT, GAUTHIER e GROLEAU, 2006). A cromatografia por exclusão molecular é utilizada para obter frações peptídicas mais puras e com maior atividade. Esse nível de pureza é necessário ao desenvolvimento de produtos farmacêuticos (HE, FRANCO e ZHANG, 2013).

Atualmente, o método de separação de peptídeos mais utilizado é a cromatografia líquida de alta eficiência (HPLC), permitindo rápida separação e detecção de suas características hidrofílicas e hidrofóbicas (SHAHIDI e ZHONG, 2008). Neste tipo de cromatografia são utilizadas bombas de alta pressão que aceleram o movimento das moléculas pela coluna, aumentando significativamente a resolução (LEHNINGER, 2011). Além disso, a cromatografia líquida associada à espectrometria de massas (LC-MS/MS) é útil na identificação das sequências peptídicas.

Há um grande número de trabalhos utilizando a ultra-filtração, bem como diversos processos cromatográficos na purificação de peptídeos de diversas espécies marinhas (JE,

PAEK e KIM, 2005; KUMAR, NAZEER e JAIGANESH, 2011; REN et al., 2008)). Entretanto, devido ao alto custo de produção, tanto o fracionamento por membrana, quanto o processo de purificação ainda são difíceis de aplicar em escala industrial (HE, FRANCO e ZHANG, 2013).

3. OBJETIVOS

3.1. Geral

Obter, purificar e caracterizar peptídeos antioxidantes do hidrolisado proteico do subproduto do processamento do camarão branco do pacífico (*Litopenaeus vannamei*) por técnicas cromatográficas.

3.2. Específicos

- Obter peptídeos por hidrólise proteolítica do resíduo do *Litopenaeus vannamei;*
- Avaliar a atividade antioxidante do hidrolisado proteico de camarão;
- Separar o hidrolisado proteico por cromatografia por traça iônica em uma coluna de DEAE–Sepharose;
- Avaliar a atividade antioxidante das frações obtidas pela troca iônica pelo ensaio do poder redutor;
- Separar a fração com maior atividade obtida na troca Iônica numa coluna de exclusão molecular Sephadex G-25;
- Avaliar a atividade antioxidante das frações obtidas pela exclusão molecular pelo ensaio do poder redutor;
- Caracterizar a atividade antioxidante da fração com melhor poder redutor obtida pela cromatografia por exclusão molecular pelos ensaios do ABTS+ e atividade quelante do ferro.

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5. ARTIGO

Purification and antioxidant activity of peptides produced by enzimatic hydrolysis of pacific white shrimp Litopenaeus *vannamei* by-products.

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ABSTRACT

Shrimp production represents 4.5% of worldwide aquaculture, but 48 to 56% of its weight is discarded as waste. Protein hydrolisates production has been extensively studied way of reusing of organic matter. The objective of this work was to purify and evaluate antioxidant activity of the shrimp protein hydrolysate of *Litopenaeus vannamei* waste.Heads were hydrolysed by alcalase and a Shrimp protein hydrolysate (SPH) was obtained. The SPH showed a good antioxidant activity by ABTS+ assay (52.24±1.127%) reducing power (0.442±0.045) and ferrous chelant activity (95.874±0.723) at 5 mg/mL. SPH was fractionated by anion exchange DEAE-Sepharose and a size exclusion G-25 chromatography and the fractions were evaluated by reducing power assay. SPH EP3 from G-25 chromatography showed the highest reducing power (0.437±0.017 at 500 μg/mL). SPH EP3 was also evaluated by its ABTS+ and Ferrous chelant activity. SPH EP3 showed a weak ABTS+ scavenging activity (15.309±1.608% at 2 mg/mL), but a powerful Fe^{2+} chelating activity (81.19±2.59 at 1mg/ml), suggesting that chelation of metals plays a pivotal role in its antioxidant activity. However other experiments are necessary to better clarify the SPH EP3 antioxidant nature.

Key words: Antioxindant *in vitro* assays, Antioxidant peptides, *Litopenaeus vannamei*, Protein hydrolysates.

1. Introduction:

In recent years an increase in the production of fishery resources for human consumption has been observed and aquaculture is the mainly responsible for this growth (FAO 2012a). Shrimp production represents, approximately 4.5 % of worldwide aquaculture and in 2010 a production of 3.8 millions of tones was observed (FAO 2012b). The White Pacific shrimp (*Litopenaeus vannamei*) is the most cultivated specie in the world and according to the last FAO bulletin, in 2010 nearly of 2.7 millions of tones were produced around the world (FAO, 2012b), however about 48 to 56 % of its production (cephalothoraxes and exoskeleton) are discarded as non edible waste (Sachindra et al., 2005). Due to its high concentration of organic matter the waste generated by fishery processing is taken as one o mostly onerous to eliminate and the better utilization of the fishery processing generated biomass is an important matter to be considered (Guerard, Sellos & le gal, 2005; He, Franco & Zhang, 2013)

Shrimp by-products are important sources o biomolecules and minerals (Sachindra & Baskar, 2008) and its importance as sources of carothenoisds (Santos et al., 2012), polysaccharide, proteins and glycosaminoglicans (Cahú et al., 2012) was observed. Furthermore, other studies have demonstrated biological processes which convert shrimp by-products in a final different product with biological activities (Sachindra & Baskar, 2008; Santos et al., 2012; Faithong et al., 2010; Binsan et al., 2008; Benjakul et al., 2009). And the reuse of shrimp residue may be a profitable alternative for dealing with disposal problems, yielding high added value products with biomedical applications.

Protein hydrolysates are breakdown products of protein digestion by a hydrolysis process (He, Franco & Zhang, 2013; Pesupuleti & Braun, 2010). This process may result in the production of bioactive peptides, peptides with 2-20 amino acids of length, which are

present in the native sequence of the protein and liberated after gastro-intestinal digestion or during the food processing (Harnedy & Fitzgerald, 2012). Furthermore, in addition to acting as a source of protein and amino acids, these bioactive peptides have potential physiologic functions, including opioid, immunomodulatory, antibacterial, antithrombotic, anti-hypertensive and antioxidant (Murray & Fitzgerald, 2007).

Free radicals are highly reactive oxygen atoms formed during aerobic metabolism, which have an unstable structure of unpaired electrons (Klompong et al., 2007). When free radicals are produced in excess it causes deleterious effects to the cells, due to the oxidation of lipids, proteins, DNA and enzymes (Philanto, 2006). Therefore, free radicals are associated to many chronic diseases, such as atherosclerosis, cancer and inflammation (Frölich e Riederer, 1995; Muramatsu, 1995). Moreover, free radicals entail losses to industry by lipid peroxidation process, which promotes undesirable changes in odor and flavor of food and generates toxic products (Lin & Liang, 2002). In order to avoid these undesirable changes and increase shelf life of food, synthetic antioxidants, such as butylated hydroxitoluene (BHT) and butylated hydroxianisole (BHA) have been applied. However studies have pointed to the harmful effects of these compounds to health and many studies have been conducted to obtain antioxidants from natural sources (Halliwell e Whiteman, 2004).

A growing number of studies have been demonstrated the purification of antioxidant peptides from many marine sources (Lee,Jeon e Byun, 2011; Nazeer, Kumar e Ganesh, 2012; Ko, Kim e Jeon, 2012; Ngo et al, 2010; Ngo et al., 2011; Bougatef et al, 2010). These natural antioxidants can perform their activities by scavenging free radicals, chelating transition metals or inhibiting the lipid peroxitation (Erdmann, Cheung e Schröder, 2008; Sarmadi & Ismail, 2010).

There are several reports about the use of shrimp by-product to obtain new products with antioxidant activities. Sachindra and Bhaskar (2008) and Faithong et al. (2010) obtained

protein hydrolysates with antioxidant activities using fermentation hydrolysis of shrimp by-products. Benjakul et al. (2009) employed flavourzyme hydrolysis to improve antioxidant and other biological activities from Mugoong, an extract paste from the Cephalotorax of white shrimp. Besides that, limited information is available about the purification of antioxidant peptides from shrimp sources. So, the aim of this study was to purify and characterize antioxidant peptides from a protein hydrolysate produced by an enzymatic hydrolysis of pacific white shrimp, *Litopenaeus vannamei*, by-products.

2. Materials and methods:

2.1. Materials:

Litopenaeus vannamei cephalotorax were provided by Noronha Pescados, Recife, PE, Brazil and stored at -20º C until use. Alcalase was purchases from Novo Nordisk (Bagsvaerd, Denmark). 1,1-diphenyl-2-pycryl-hydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Potassium persulfate and 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate (ferrozine) were purchased from Sigma Chemical Co (St. Louis, MO). All other reagents used in this study were of analytical grade.

2.2. Preparation of shrimp protein hydrolysates (SPH):

Briefly, shrimp heads were mixed with distilled water with 1:1 (w/v) ratio and boiled at 100ºC for 10 minutes in order to inactivate the endogenous enzymes. Then the mixture was cooled in room temperature and the Alcalase in a final concentration of 0.5 % (v/v) was added. The hydrolysis was performed for three hours at 40ºC. After that, the mixture was

boiled for 10 minutes to stop the reaction. The SPH was centrifuged for 10000 x *g* for 10 minutes and the supernatant were collected and stored at -20ºC for posterior antioxidant activities. All hydrolysis and antioxidant activities were performed at least in triplicate.

2.4. Antioxidant activities:

2.4.1. DPPH scavenging activity:

DPPH scavenging activity was performed as Je et al. (2009). Briefly, 100 µL of hydrolysate samples at 0.25; 0.5; 1.0; 2.0 and 5.0 mg/mL were mixed with 100 µL of 150 µM of DPPH in ethanol 99,5 % solution. This mixture was kept in the dark at room temperature for 30 minutes and the absorbances were measured at 517 nm using a Bio-rad xMark™ spectrophotometer. The radical scavenging activity (RSA) was measured using the following equation: DPPH inhibition = $[1-(A_{sample} - A_{blank})/A_{control}]*100$. Where the blank was the sample mixed with ethanol and the control was the DPPH solution without sample.

2.4.2. ABTS scavenging activity:

The ABTS+ radical scavenging was performed as Wang and Xiong (2005). A solution of ABTS+ was prepared at the final concentration of 7 mM of ABTS and 2.45 mM of potassium persulfate. This mixture was incubated for 12 to 16 hours at room temperature before the assay and thrn was diluted in 0.2 M of PBS (pH 7.4) until the desired absorbance of 0.70 $\pm$ 0.02 at 734 nm. After that, 20 µL of the samples were added to 1980 µL of ABTS+ diluted solution. The mixture was homogenized for 30 seconds, left at dark for 6 minutes. After that the absorbancies were measured at 743 nm in a Bio-rad SmartSpec™ 3000

spectrophotometer and the ABTS+ scavenging activity was measured according to the following equation: ABTS inhibition (%) = $[1-(A_{sample}/A_{control})]*100$.

2.4.3. Reducing power assay

Reducing power assay was made as described by Oyaizu (1986) with modifications. Two hundred microliters of the samples were mixed to 200 µL of 0,2M fosfate buffer (pH 6.6) and 200 µL of potassium ferricyanide. This mixture was incubated at 50ºC by 20 minutes and the reaction was stopped adding 200 µL of a 10% TCA solution. Further, 125 µL of these samples were mixed with 125 µL of distilled water in a microplate and 20 µL of $FeCl_3$ was added to each well. The reducing power was observed by an increase in absorbance at 700 nm.

2.4.4. Ferrous chelant activity:

The Fe^{2+} ion chelant activity was determined by Benjakul et al. (2005) with modifications. Samples (200 µL) were mixed to 800 µL of distilled water. Then 100 µL of 2 mmol/L $FeCl_2$ solution and 200 µL of 5 mmol/L ferrozine solution were added. This mixture was kept at room temperature for 20 minutes and the absorbance was read at 562 nm. Distilled water was used as control. The hydrolysed chelant activity (HCA) was expressed by the following equation: HCA (%) = $[1-(A_{sample}/A_{control})]*100$.

2.5. Purification SPH antioxidant peptides

2.5.1. Anion exchange chromatography

In order to separate the fraction with highest antioxidant activity, 50 mg of lyophilized SPH were loaded onto a DEAE-Sepharose anion exchange column (8mL) equilibrated with distilled water in a linear gradient of NaCl (0 – 1.5 M) at a 1mL/min flow. Each fraction was collected as 2 mL and the peaks were monitored at 280 nm. Each peak was lyophilized and the antioxidant activities were measured by reducing power at 1mg/mL.

2.5.2. Size exclusion chromatography

The fraction number 2 (SPH IP2 - 8 mg) with highest antioxidant activity obtained by DEAE-Sepharose column was further loaded onto a Sephadex G25 molecular exclusion column (1.5 x 42 cm) equilibrated with distilled water. Each fraction was collected as 2 mL at 0.4 mL/min rate flow and the peaks monitored at 280 nm. The peaks were lyophilized and concentrated at 500 µg/mL and evaluated by reducing power assay. The most active fraction from reducing power assay was also evaluated by ABTS+ scavenging assay and ferrous chelant activity assay.

2.6. Statistical analysis

SPH production and antioxidant assays were made at least in triplicate and antioxidant assays from SPH and its purified fraction were expressed by means $\pm$ SD. Significant. Statistical comparisons of the means values were evaluated by analysis of variance

(ANOVA), using the GraphPad Prism (version 5; GraphPad Software Inc., San Diego, U.S.A.) and a *P* value of <0.05 was considered as the level of statistical significance.

3. Results and Discussion

3.1. SPH Production and antioxidant activity

In this work the SPH was produced by Alcalase hydrolysis. Alcalase is a microbian endopeptidase derived from *Bacillus licheniformis* which, due to produce hydrolysates with mild taste even in high degrees of hydrolysis, has been extensively used by industry and is considered one of the best enzymes to produce protein hydrolysates (Benjakul and Morrissey, 1997; Kristinsson & Rasco, 2000; Centenaro et al. 2009). Furthermore, Alcalase works at a pH range next to the neutral, avoiding the low pH undesirable effects, such as destruction of essential amino acids and loss of functionality due to excess of hydrolysis (Kristinsson & Rasco, 2000).

The antioxidant activity should not be concluded based in only one methodology and several antioxidant *in vitro* assays are carried out for evaluating antioxidant activity (Alan, Bristi & Rafiquzzman, 2012). In this work the antioxidant activity of SPH was evaluated by DPPH scavenging assay, reducing power assay, ferrous chelant activity and ABTS+ scavenging assay (Figure 1).

The DPPH is a stable free radical, which gives rise to a deep violet color due to a delocalization in a spare electron in the molecule. When this solution is mixed with a substrate that can donate an electron or a hydrogen atom, it gives rise to the reduced form of DPPH and, consequently, loss of violet color, leading to the formation of a yellow pale compound (Alan, Bristi & Rafiquzzman, 2012). In this work, five different crescent

concentrations were evaluated in order to obtain a dose-response curve, besides that, statistical significance was only observed between 0.25 and 1 mg/mL; and 0.25 and 2.0 mg/mL. All other concentrations had no significant difference on DPPH scavenging assay (Figure 1A). This result does not corroborate with the evidence of growing of DPPH scavenging activity with the increase of concentration (Je et al., 2009; Ngo et al., 2010; Ko et al., 2012) of the antioxidant compound..

The SPH free radical scavenging activity was also tested against the radical cation ABTS+. This kind of free radical scavenging assay can be applied to both lipophilic and hydrophilic compounds, and it has been widely used as antioxidant activity assay in several natural compounds (Miliauskasa, Venskutonisa, & van Beek, 2004). Different to the observed from DPPH scavenging assay, the SPH scavenging activity against ABTS+ free radical increased with increasing concentration (Figure 1B) and a ABTS+ scavenging activity of 52 $\pm$ 1.127% at 5 mg/mL was observed. Demonstrating that the SPH was effective in scavenge this free radical in a dose-dependent manner.

In order to clarify the results about the free radical scavenging activity, the SPH reducing power was evaluated. Reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron or a hydrogen atom (Yildirim et al., 2000). In this method the antioxidant compounds causes the reduction of the Fe3+/ferricyanide complex to the ferrous form, which is measured at 700 nm and increases in absorbance of the reaction mixture indicate the reducing power of the samples (Jayaprakash et al., 2001). The Fig 1 C demonstrates the increasing in SPH electron donating ability among the different concentrations. Thus can suggest that Shrimp protein hydrolysate has antioxidant activity and aspects of the samples may have interfered in the DPPH scavenging assay, which confirm the need to evaluate *in vitro* antioxidant activity by two or more assays. Besides that DPPH still is one of the most widely used test as the initial evaluation of antioxidant activity and several

studies has used this assay associated to reducing power and other assays in the avaluation of antioxidant activity (Li et al., 2008; Je et al., 2009; Galla et al., 2012).

The iron Fe^{2+} Chelant activity of SPH was also evaluated and the decrease of the red color ferrozine-Fe^{2+} complex reflected the efficacy of the samples to compete against the ferrozine for the Fe^{2+} ion (Figure 1D). The chelation of metal ions can reduce the amount Fe^{2+} or Cu^{2+} available to take part in the Fenton reaction and decrease the formation of the hydroxyl radical from the superoxide anion (Oboh, Raddatz & Henle, 2008). Zhu, Zhou and Qian (2006) obtained a hydrolysate with a high ferrous ion chelating activity of 89 % at 1 mg/mL using alcalase hydrolysis of wheat germ. In this study it was observed that SPH chelated near to 96% of Fe^{2+} in the system at 1 mg/mL. This result demonstrates that SPH has a remarkable Fe^{2+} chelating activity and it can contribute to prevent the formation of hydroxyl radical and, ultimately prevent lipid and DNA oxidation.

Several reports in literature have associated the improvement of antioxidant activity with the alcalase hydrolysis of different protein sources. Li et al., (2008) obtained a protein hydrolysate with antioxidant and radical scavenging activities from chickpea protein hydrolyzed by alcalase. Galla et al., (2012) obtained protein hydrolysates with antioxidant and good functional properties from alcalase-hydolysis of *Channa striatus* and *Labeo rohita* roe protein. Xie et al., (2013) observed that hydrolysis by alcalase enhanced the antioxidant activity and bioavailability of casein hydrolysates compared with gastrointestinal-hydrolysis. These authors also observed a cytoprotective effect of casein hydrolysates against H_2O_2 injury in a HepG2 cell culture. The antioxidant and radical scavenging activities of shrimp protein hydrolysate obtained by alcalase hydrolysis corroborate with these results and suggest that SPH is a good candidate for a functional ingredient in commercial products.

3.2. Purification of Shrimp protein hydrolysate:

In order to obtain a purer product, the shrimp protein hydrolysate was subjected to two purification processes. Firstly, 50 mg of SPH were dissolved in distillated water and loaded in an anion exchange DEAE-Sepharose column. As shown in Figure 2, SPH was fractionated into 3 sub-fractions, one unbounded and two bounded fractions (SPH IP1, SPH IP2 and SPH IP3, respectively). All the fractions was pooled, lyophilized and measured for antioxidant activity by reducing power assay. The fraction SPH IP2 showed the highest reducing power (0.330 ± 0.033 at 1 mg/mL) and was further purified by size exclusion chromatography in a Sephadex G-25 column. Figure 3 shows the elution profile of SPH IP2 fraction separated by molecular size exclusion chromatography. Three sub fractions were obtained (SPH EP1, SPH EP2 and SPH EP3, respectively). All these fractions were pooled, lyophilized and tested for reducing power assay at 500µg/mL and SPH EP3 fraction showed the highest reducing power activity($0,437 \pm 0,017$). This result is in agreement with the finding that peptides with small molecular weights have better antioxidant activity (Cheung et al., 2012; Lee, Jeon & Byun, 2011; Ko, Kim & Jeon, 2012; Zhong et al., 2011; Li et al., 2008 and Wang et al., 2012). Furthermore, the increase in reducing power of SPH EP3and the decrease in concentration compared to SPH IP2 reflects the efficiency of chromatographic processes for the purification of peptides antioxidants.

3.3. Antioxidant activities of SPH EP3:

Since SPH EP3 fraction had the best antioxidant activity, it was tested for ferrous chelant activity and its scavenging activity against ABTS+, in order to elucidate the different mechanism by which SPH EP3 exercises its antioxidant activity (Figure 4). After the

purification process SPH EP3 retained its iron chelant ability, showing a Fe^{2+} chelant activity higher than 81 % and IC_{50} near of 0,6 mg/mL. However, despite the high Fe^{2+} chelant activity, SPH EP3 showed a weak free radical scavenging at the concentrations tested by ABTS+ scavenging assay (15.31±1.61 %). It suggests that the chelation of transition metals is a pivotal way to SPH EP3 exerts its antioxidant activity. Previously reports have associated amino acid residues of Ser and His to iron chelant activity in fish proteins (Wu et al., 2012; Guo et al., 2013). Lee and Song (2009) associated the chelant activity of the nonapeptide Asp–Leu–Gly–Glu–Gln–Tyr–Phe-Lys–Gly from porcine serum albumin to the presence of Asp, Glu and Lys within its sequence. So, based on these evidences we can suggest that Shrimp protein hydrolysate of *Litopenaeus vannamei* and its purified fraction SPH EP3 can be rich in one or more of these amino acids residues.Besides that, many reports have revealed the correlation between antioxidant activities and the reducing power of bioactive compounds (Bougatef et al., 2010) and the antioxidant and radical scavenging activity of SPH EP3 should be evaluated by other assays.

4. Conclusions:

This study demonstrates that pacific white shrimp by-products hydrolysates by alcalase hydrolysis can be employed as antioxidant peptides source. Furthermore, it was purified an antioxidant peptide fraction with a high reducing power and Fe^{2+} chelant activity, but a weak free radical scavenging activity. Furthermore, it is necessary to evaluate the sequence of peptides within SPH EP3 and another antioxidant studies *in vitro* and *in vivo* should be conducted in order to better characterize SPH EP3 antioxidant activity.

Acknowledgments:

This research was supported by CAPES and CNPq. And we would like to thanks to Noronha Pescados for providing the Shrimps by-products.

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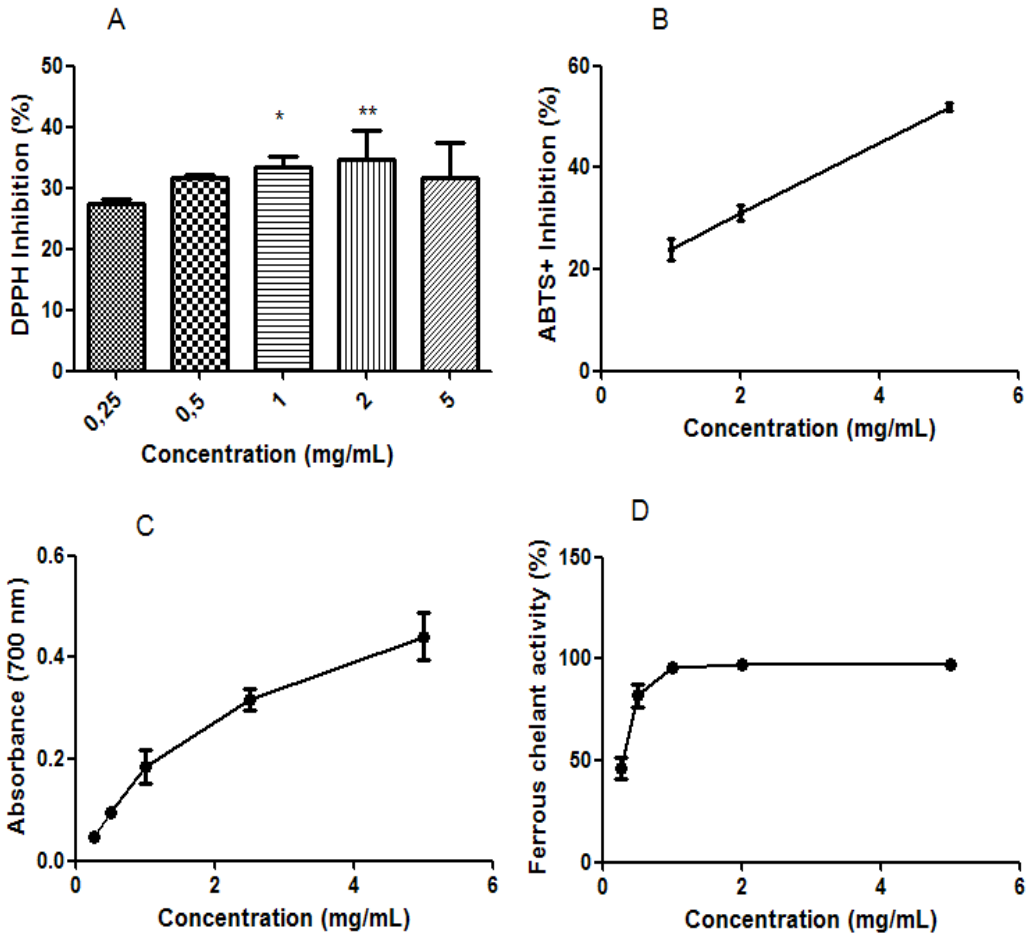

Figure 1: Antioxidant activities of SPH at different concentrations. A) DPPH scavenging assay; B) ABTS+ scavenging assay; C) Reducing power; D) Ferrous chelant activity.The assays were made at least in triplicate and the results are expressed as means ± SD. The significance among the different concentration was analysed by ANOVA test and P value of < 0.05 was considered significant.

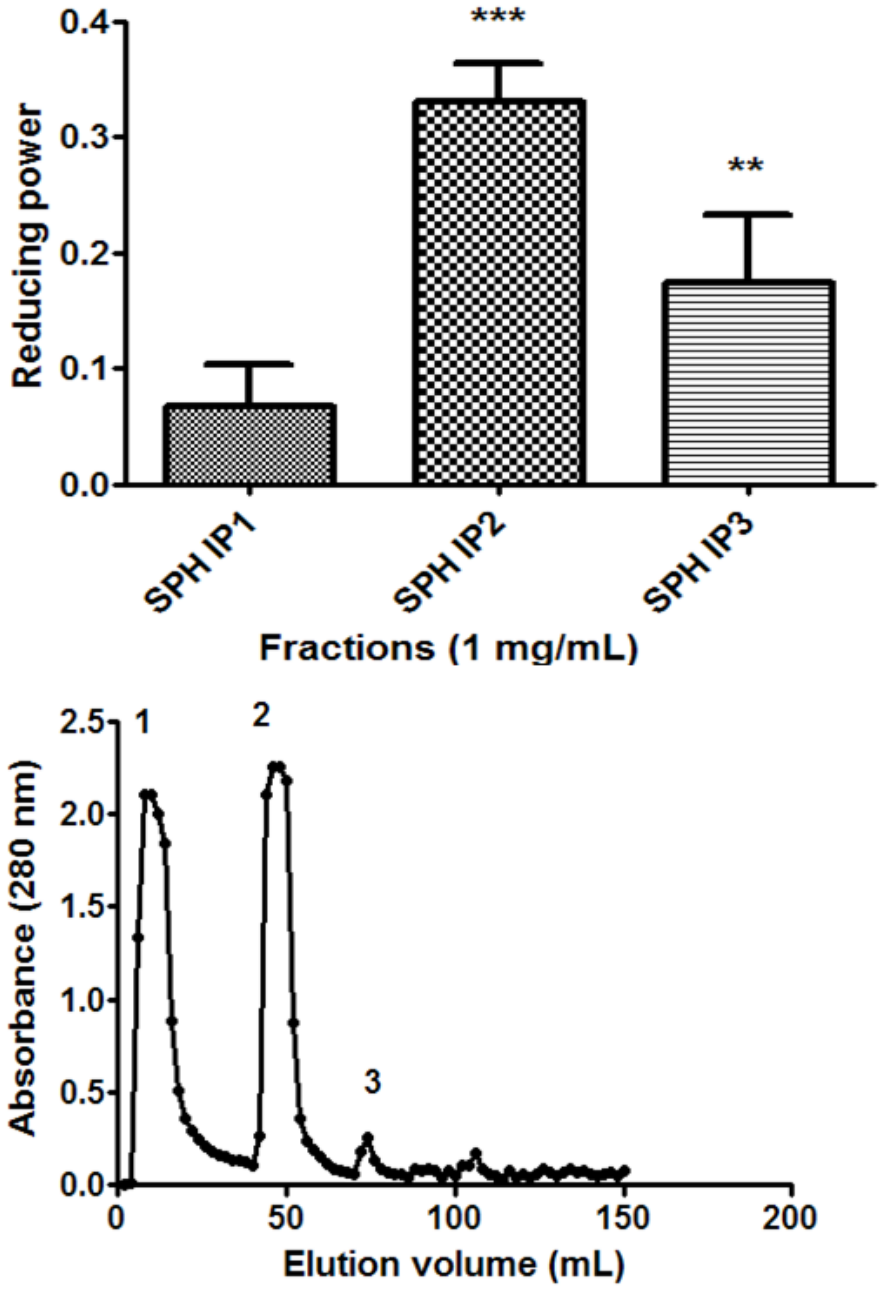

Figure 2: Elution profile of SPH separeted by DEAE-Sepharose anion exchange chromatografy and reducing power of the different fractions at 1 mg/mL (upper panel).

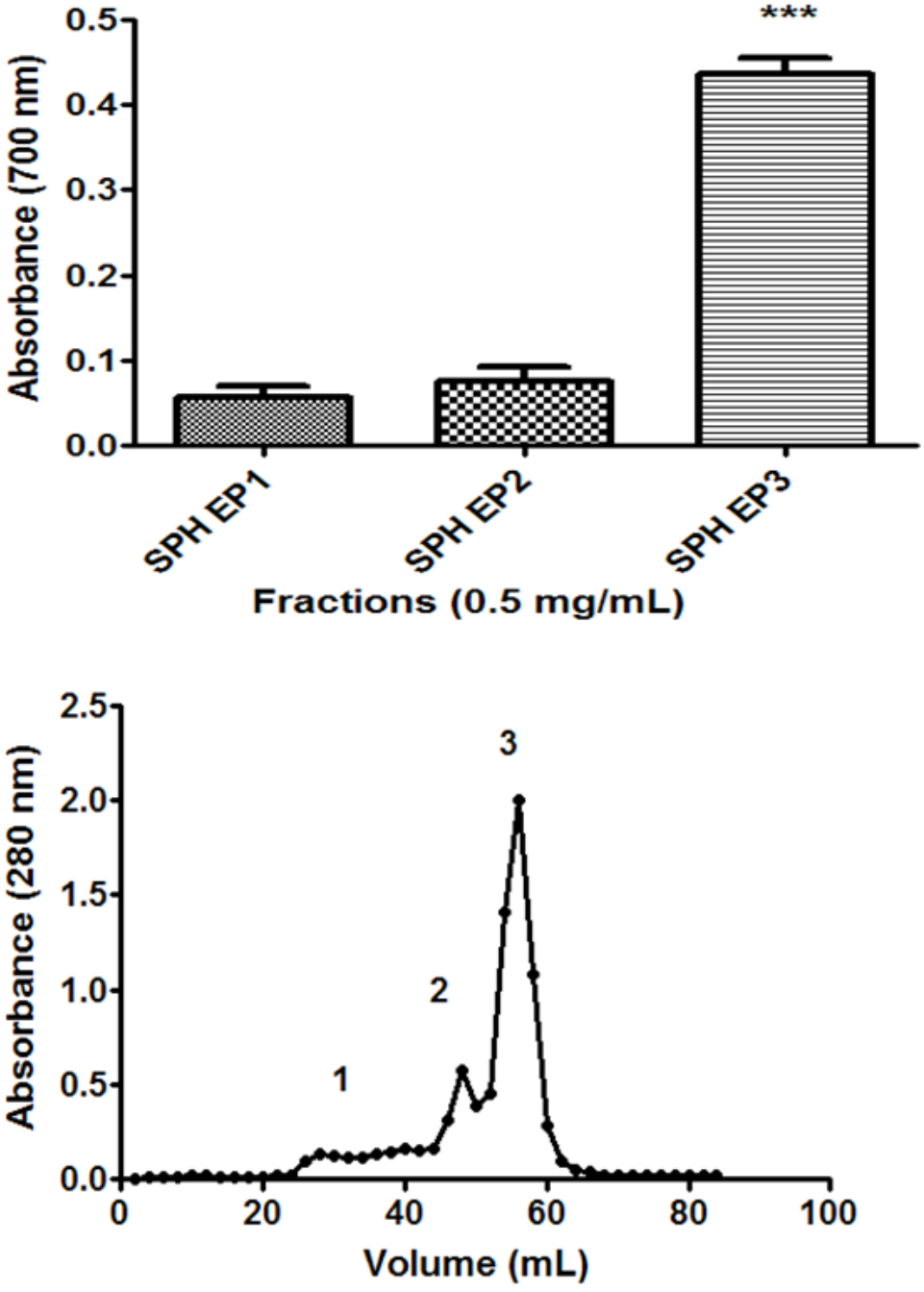

Figure 3: Elution profile of SPH IP2 separated by Sephadex G-25 size exclusion chromatography and reducing power of the different fractions at 500 μg/mL(upper panel).

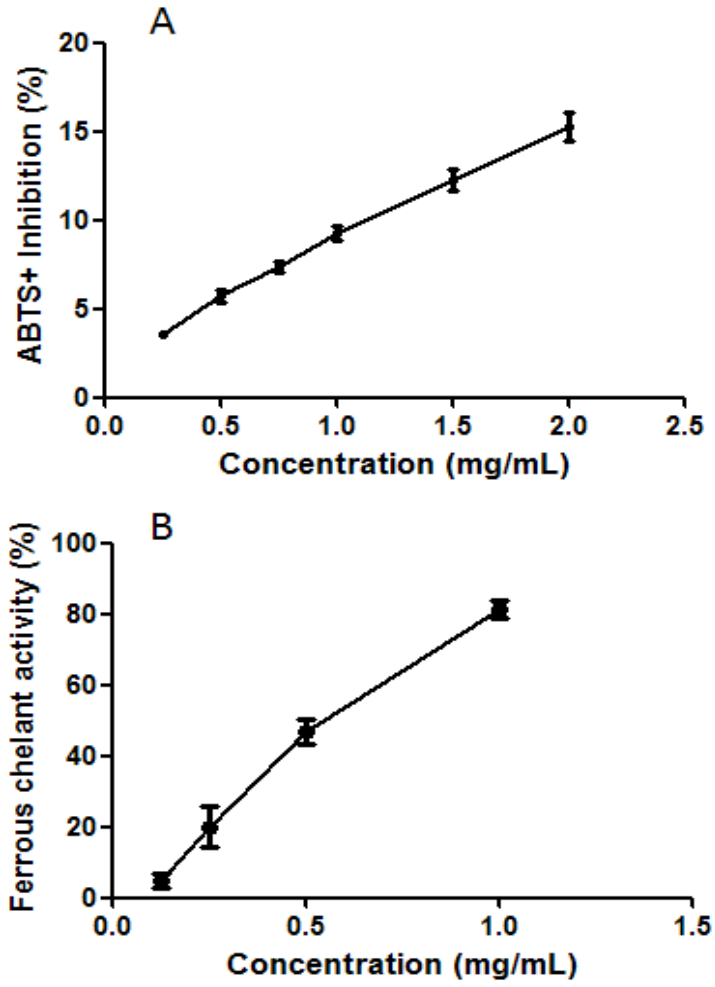

Figure 4: Antioxidant activities of SPH EP3 at different concentrations. A) ABTS+ scavenging assay; B) Ferrous chelant activity. All assays were made at least in triplicate and the results are expressed as means ± SD. The

6. CONCLUSÕES

- Os hidrolisados proteicos obtidos pela hidrólise enzimática dos sub-produtos do processamento do camarão branco do pacífico *Litopenaeus vannamei* podem ser empregados como fonte de peptídeos com atividade antioxidante;

- O processo de purificação foi eficaz na obtenção de uma fração peptídica com maior poder redutor;

- A fração SPH EP3 possui uma notável capacidade de quelação do íon Fe^{2+}, sugerindo que este seja um dos principais mecanismos envolvidos na atividade antioxidante do SPH;

- Estudos de sequenciamento e a avaliação da atividade antioxidante do SPH se fazem necessários para uma melhor caracterização das frações peptídicas presentes no hidrolisado.

7. ANEXO

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8. TRABALHOS PUBLICADOS PARALELAMENTE:

8.1. Artigo:

Shrimp Waste Extract and Astaxanthin: Rat Alveolar Macrophage, Oxidative Stress and Inflammation

Suzan D. Santos, Thiago B. Cahú, Guilherme O. Firmino, Célia C.M.M.B. de Castro, Luiz B. Carvalho Jr., Ranilson S. Bezerra, and José L. Lima Filho

Abstract: Astaxanthin is a carotenoid known to have anticoxidant and antiinflammatory properties. This study examined if shrimp astaxanthin modulates the production of superoxide (O^{-}_{2}), nitric oxide (NO), and tumor necrosis factor-α (TNF-α) in rat alveolar macrophages. The oxidative effect was induced by phorbol myristate acetate and lipopolysaccharide. The treatment was compared with superoxide dismutase, butylated hydroxytoluene, commercial astaxanthin, N-nitric-L-arginine methyl ester and L- canavanine, all administered as a 43.5-μg/mL dose in the presence of 1% EtOH/0.5% DMSO. All treatments maintained cell viability, as observed in the MTT assay, and shrimp extract increased the viable alveolar macrophages to 168%. Shrimp extract and commercial astaxanthin showed a suppressive effect on the generation of both free radicals O^{-}_{2} and NO, while purified shrimp astaxanthin was specific to NO. TNF-α secretion was correlated with NO production. However, in this correlation, the shrimp extract completely inhibited TNF-α. In the light of these findings, the anticoxidant action demonstrated in this study suggests that the shrimp extract could be considered as a promising source of bioactive substances with anticoxidant and anti-inflammatory activity.

Keywords: alveolar macrophages, anticoxidant, astaxanthin, shrimp waste, TNF-α

Practical Application: The hydrolysis process of shrimp waste generates bioactive products that add economic value to shrimp processing, mainly because they may have applications in nutraceutical and animal feed industry.

Introduction

Recently, in Brazil there has been a rapid growth of commercial shrimp farming based mainly on the species *Litopenaeus vannamei*, representing an important commodity whose production reached 65000 t in 2009 (Rocha 2010). However, it generates high levels of discarded solid waste (from 50% to 70% of total wet weight) in the form of head and abdominal exoskeleton (Binsan and others 2008). The recovery of biomolecules from this waste can be an alternative to add economic value to shrimp processing (Handayani and others 2008). Production of carotenoids has become the object of industrial interest, with global market figures of US $766 million in 2007, of which astaxanthin represented 28% (BCC Research 2008).

The antioxidant activity of carotenoids arises primarily as a consequence of the ability of the conjugated double-bonded structure to delocalize unpaired electrons (Valko and others 2006). Astaxanthin is a reddish-colored C-40 compound, with powerful broad-ranging antioxidant effect that occurs naturally in a wide variety of living organisms, such as microalgae, fungi, complex plants, and crustaceans (Hussein and others 2006; Campoio and others 2011). Furthermore, a neuroprotective (Lu and others 2010), antitumor (Yasui and others 2011) carotenoid effect and its benefic role on the inflammation induced by lipopolysaccharide (LPS) in murine macrophages (Ohgami and others 2003) have been shown.

Alveolar macrophage (AM) generates reactive oxygen species (ROS) such as superoxide anion (Tiwari and Kakkar 2009). It is the first line of defense against inhaled particles. The imbalance between the exposure to oxidants and the endogenous antioxidants is found to play an important role in various pathological conditions including asthma, acute respiratory distress syndrome, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, cardiovascular dysfunction, neurodegenerative diseases, immunosuppression, cancer, premature ageing, and so on (Tiwari and Kakkar 2009).

Nitric oxide (NO) is also produced by activated macrophages and has been shown to be involved in the pathogenesis of acute and chronic inflammatory diseases (Moilanen and others 1997). During the inflammatory process, NO production increases to levels that make the compound cytotoxic to the cells by reacting with superoxide anions to produce peroxynitrite ($ONOO^{-}$; Sautebin 2000). This NO production by macrophages depends upon induced NO synthase (iNOS), which can be activated by various agents, including LPS, interferon-gamma, tumor necrosis factor (TNF-α), and so on (Palmer and others 1993). Therefore, large amounts of NO production induced by bacterial LPS or cytokines play an important role in endotoxemia and inflammatory conditions (Bellot and others 1996).

MS 20111429 Submitted 11/27/2011, Accepted 4/11/2012. Authors Santos, Cahú, Firmino, Castro, Carvalho, and Filho are with Laboratório de Imunopatologia Keizo Asami—LIKA, Univ. Federal de Pernambuco—UFPE. Author Bezerra is with Laboratório de Enzimologia—LABENZ, Dept. de Bioquímica/UFPE. Authors Santos, Cahú, and Firmino are also with Laboratório de Enzimologia—LABENZ, Dept. de Bioquímica/UFPE. Direct inquiries to author Filho (E-mail: jourlaiz60@gmail.com).

doi: 10.1111/j.1750-3841.2012.02762.x

8.2. Patente:

RELATÓRIO DESCRITIVO DA PATENTE DE INVENÇÃO

PROCESSO PARA PRODUÇÃO POR RADIAÇÃO DE UM PRODUTO PROTEICO E LIPÍDICO A PARTIR DO HIDROLISADO PROTEICO DE PEIXE

CAMPO DA INVENÇÃO

A presente invenção diz respeito a um processo para a produção de hidrolisados proteicos de peixes e dos seus óleos de forma estéril. O produto é obtido a partir de resíduos da indústria pesqueira e pode ser utilizado pela as indústrias de cosmética, alimentícia e nutricêutica. A presente invenção refere-se, mais particularmente, a aplicação de um processo de separação e conservação com a aplicação de radiação para aumentar o tempo de prateleira de hidrolisados proteicos e óleos de peixes de forma estéril com aplicações biotecnológicas.

DESCRIÇÃO DO ESTADO DA TÉCNICA

O filé é o item de maior valor econômico, o seu rendimento varia de acordo com o domínio tecnológico das empresas processadoras de pescado e pode chegar entre 30 e 40% do peso do animal dependendo da espécie, a outra parcela é considerado como resíduo sendo composto por restos de carne, cabeça, pele, ossos, escamas e vísceras. Logo, os resíduos da indústria de processamento de pescado, por exemplo, da tilápia, representam de 60 a 70% da matéria-prima. Atualmente, estes resíduos gerados são

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