UNIVERSIDADE FEDERAL DO RIO GRANDE-FURG
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

OBTENÇÃO, AVALIAÇÃO E MICROENCAPSULAÇÃO DE HIDROLISADOS PROTÉICOS BIOATIVOS A PARTIR DE SUBPRODUTOS DA CARPA COMUM (*Cyprinus carpio*)

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Tese apresentada ao Programa de Pós-graduação em Aquicultura da Universidade Federal do Rio Grande-FURG, como requisito parcial à obtenção do título de Doutor.

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Si yo pudiera enumerar cuánto debo a mis grandes antecesores y contemporáneos, no me quedaría mucho en propiedad.

Johann W. Goethe

A mi familia, a todos y cada uno de ellos.
A Elías y Lucía.

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RESUMO GERAL

A inapropriada gestão dos subprodutos da aquicultura e a indústria de pescado pode produzir grande impacto ecológico e afetar significativamente a viabilidade econômica do setor. A hidrólise enzimática é uma técnica eficiente para agregar valor a estes subprodutos produzindo peptídeos com grande quantidade de aminoácidos essenciais e variadas atividades biológicas como antioxidante, anti-hipertensiva, e neuroprotetora entre outras. O objetivo da presente tese foi obter hidrolisados proteicos do subproduto do beneficiamento (cabeça, visceras, carcaça e pele) da carpa comum (*Cyprinus carpio*), avaliar *in vitro* e *in vivo* sua capacidade antioxidante e microencapsular os hidrolisados com atividade antioxidante. No Capítulo I foi realizado um trabalho de revisão sobre diferentes métodos para a produção de hidrolisados de proteínas assim como um exame das pesquisas atuais avaliando as propriedades bioativas dos hidrolisados de subprodutos de pescado. No Capítulo II foi avaliada *in vitro* a capacidade antioxidante dos hidrolisados protéicos. Para isto, o subproduto da carpa comum foi hidrolisado utilizando as enzimas Alcalase (A) e Protamex (P) para atingir graus de hidrólise (DH) de 10 e 15%. Foi investigada a capacidade antioxidante *in vitro* contra radicais peroxil e radicais DPPH assim como a atividade antioxidante *in vitro* em células de hipocampo HT-22. O hidrolisado A15 mostrou a maior atividade antioxidante ($p < 0.05$) contra o radical DPPH. O hidrolisado P15 apresentou a menor atividade contra os radicais peroxil ($p < 0.05$). A dosagem da concentração intracelular de espécies reativas de oxigênio (ROS) do sistema de células HT-22 revelou que P15 (1.25 mg/ml) reduziu a concentração de ROS ($p < 0.05$). No Capitulo III foi avaliada a atividade antioxidante *in vivo* dos hidrolisados de carpa. Foi realizado um ensaio de alimentação para avaliar o efeito da suplementação dietética do hidrolisado A15 sobre o estado antioxidante do zebrafish (*Danio rerio*). Os peixes-zebra foram alimentados por 44 dias com quatro dietas diferentes contendo níveis crescentes de hidrolisados (0 g Kg^{-1}; 25 g Kg^{-1}; 50 g Kg^{-1}; 100 g Kg^{-1}) e ao termino do ensaio foram coletadas as brânquias, cérebro e músculos para avaliar a capacidade antioxidante total contra radicais peroxil (ACAP) e peroxidação lipídica (TBARS). No músculo a peroxidação lipídica foi reduzida no músculo dos peixes alimentados com a dieta suplementada com 50 g Kg^{-1} de hidrolisado ($p < 0.05$). A peroxidação lipídica no cérebro foi reduzida ($p < 0.05$) em todos os grupos quando comparados com o grupo alimentado com a dieta sem hidrolisado. Finalmente,

no Capítulo IV desta tese, foi avaliado o efeito da microencapsulação dos hidrolisados por coacervação complexa com pectina e posterior secagem por atomização sobre a atividade antioxidante dos hidrolisados. Para este estudo foram avaliados hidrolisados de músculo e hidrolisados de subprodutos de carpa. Os resultados revelaram que a microencapsulação e secagem por "spray-drying" das suspensões de hidrolisados reduziu a atividade antioxidante ($p < 0.05$). Pelo contrario, a microencapsulação por coacervação complexa com pectina e posterior secagem por atomização não teve efeito significativo sobre a atividade antioxidante ($p > 0.05$). Compendiando, o presente trabalho de tese demostrou que a hidrólise enzimática dos subprodutos do beneficiamento da carpa comum é uma técnica eficaz que permite a liberação de peptídeos com atividade antioxidante e neuroprotetora com grande potencial no uso na indústria farmacêutica e de alimentos.

Palavras chave: Antioxidante, Coacervação complexa, Microencapsulação, Spray-drying, Zebrafish

ABSTRACT

The mismanagement of the fish industry and aquaculture by-products can affect the economic viability of the sector and also create serious pollution problems. Enzymatic hydrolysis is an efficient method to add value to these byproducts by releasing peptides with high quantity of essential amino acids and with several biological activities such as antioxidant, antihypertensive, and neuroprotective, among others. The objective of this thesis was to obtain protein hydrolysates from common carp (*Cyprinus carpio*) by-product (head, viscera, carcass and skin) in order to evaluate its *in vitro* and *in vivo* antioxidant capacity as well as to microencapsulate the antioxidant hydrolysates. In Chapter I was reviewed the different methods for the production of protein hydrolysates as well as the current research evaluating the bioactive properties of fish by-product hydrolysates. In Chapter II, the antioxidant capacity of protein hydrolysates was evaluated. To his purpose, common carp by-product was hydrolyzed using the enzymes Alcalase (A) and Protamex (P) to achieve degree of hydrolysis (DH) of 10 and 15%. The *in vitro* antioxidant capacity against peroxyl radicals and DPPH radicals in addition to the *in vitro* antioxidant activity in HT-22 hippocampal cells was investigated. The hydrolysate A15 showed the highest antioxidant activity ($p < 0.05$) against the DPPH radical. The hydrolysate P15 exhibited the lowest activity against peroxyl radicals ($p < 0.05$). The measurement of the intracellular concentration of reactive oxygen species (ROS) of the HT-22 cell system treated with carp by-product hydrolysates revealed that P15 (1.25 mg/ml) reduced the intracellular concentration of ROS ($p < 0.05$). In Chapter III it was evaluated the *in vivo* antioxidant activity of carp by-product hydrolysates. A feeding trial was carried out in order to evaluate the effect of dietary supplementation of the A15 hydrolysate on the antioxidant status of zebrafish (*Danio rerio*). The zebrafish were fed for 44 days with four different diets containing increasing levels of hydrolysates (0 g kg^{-1}, 25 g kg^{-1}, 50 g kg^{-1}, 100 g kg^{-1}). At the end of the feeding trial, gills, brain and muscles were collected with the purpose of assessing the antioxidant capacity against peroxyl radicals (ACAP) and lipid peroxidation (TBARS). In zebrafish muscle, the lipid peroxidation was reduced ($p < 0.05$) in fish fed with the diet supplemented with 50 g kg^{-1} of hydrolysate. Lipid peroxidation in the brain was reduced ($p < 0.05$) in all groups when compared to the group fed the diet without hydrolysate. Finally, in chapter IV, the effect of the microencapsulation of the hydrolysates by pectin

complex coacervation and subsequent spray-drying on the antioxidant activity was evaluated. In this study, hydrolysates from of carp muscle and carp by-products were assessed. The results showed that microencapsulation by spray-drying reduced the antioxidant activity of the hydrolysates ($p < 0.05$). In contrast, microencapsulation by complex coacervation with pectin and subsequent spray drying had no significant effect on antioxidant activity ($p > 0.05$). In conclusion, the present thesis demonstrated that the enzymatic hydrolysis of the common carp by-products is a competent method that allows the release of peptides with antioxidant and neuroprotective activity with great potential in the pharmaceutical and food industry.

Keywords: Antioxidant, Complex coacervation, Microencapsulation, Spray-drying, Zebrafish.

1 INTRODUÇÃO GERAL

1.1. Importância da carpa comum na aquicultura

A produção de peixes de água doce compreende atualmente 47.1 milhões de toneladas No panorama brasileiro, a produção aquícola nacional foi de 562,500 t em 2014, representando um incremento de 17.3 % em relação à produção de 2010 (FAO, 2016), podendo ser atribuído este crescimento á ampliação das politicas publicas mediante programas governamentais, o que repercutiu no desenvolvimento do setor (MPA, 2010). Particularmente, a piscicultura continental representa 84.32 % da produção nacional. A tilápia, o tambaqui e a carpa foram as espécies mais cultivadas, as quais somadas representaram 85.5 % da produção nacional (IBGE, 2015).

Entre as espécies de peixes criadas no sul do Brasil destacam-se as carpas, com ênfase na carpa comum (*Cyprinus carpio* Linnaeus, 1758). A carpa comum é um peixe de habito alimentar omnívoro, alimentando-se de invertebrados e plantas. Esta espécie pode atingir 35 kg de peso em cativeiro e mais de 60 kg na natureza (Silva et al., 2006). Entre os principais fatores que contribuem para o sucesso da criação desta espécie podem se destacar a resistência ao manejo e a doenças, facilidade de obtenção de alevinos, ótima aceitação de alimentação exógena e rápido crescimento, podendo atingir facilmente 2 kg de peso no primeiro ano de cultivo. Igualmente, tolera baixos níveis de oxigênio dissolvido e sobrevive em uma ampla faixa de temperatura da água (0 a 40 ºC) (Tölg, 1984). Adapta-se bem a sistemas de criação baseados na reciclagem de subprodutos agropecuários (Tamassia, 2004), por sua capacidade de aproveitamento de alimentos diversos. Além disso, esta espécie pode ser criada em sistemas de produção semi-intensivos, a baixo custo, com aproveitamento de resíduos agroindustriais em sua alimentação e utilizada em policultivo e cultivos consorciados com outras espécies de

carpas, como a carpa-capim (*Ctenopharyngodon idella*), a carpa-prateada (*Hypophthalmichthys molitrix*) e a carpa-cabeça-grande (*Aristichthys nobilis*) (Silva et al., 2006).

A produção global de carpa comum aumenta paralelamente ao aumento da produção mundial de aquicultura de peixes de água doce. Segundo a FAO (FAO-FIGIS), a produção de peixes de água doce foi de 31,839,573 t em 2005 e aumentou para 45,335,385 t em 2011 (um aumento de mais de 42 %). Durante esses anos, a contribuição da carpa comum para a produção global de aquicultura manteve-se em cerca de 8-9 %. Assim, estima-se que as espécies de água doce, como a carpa, o catfish (incluindo *Pangasius*) e a tilápia, serão responsáveis pela maior parte do aumento da produção aquícola e representarão cerca do 60 %da produção total da aquicultura em 2025 (FAO, 2016).

1.2. Impacto dos resíduos de pescado

A produção de pescado sustentada pela produção da aquicultura vem crescendo a uma taxa média anual de 3.2 % junto com o aumento do consumo de peixe per capita que aumentou de 9.9 kg em 1960 para 20.1kg em 2012 (FAO, 2016). Este aumento da produção e da demanda de produtos de pescado tem gerado grandes quantidades de resíduos orgânicos provenientes do descarte durante o beneficiamento ou processamento (Morales-Medina et al., 2016). Os subprodutos do processamento e beneficiamento do pescado são comumente classificados como recursos com baixo valor de mercado. Dependendo do tipo de indústria, os subprodutos e resíduos gerados a partir do processamento do pescado pode atingir valores de mais de 70 % da biomassa inicial (Halim et al., 2016). Deste modo, são geradas anualmente grandes quantidades de subprodutos de processamento, incluindo músculos, vísceras, cabeças, peles,

nadadeiras, e carcaça (Harnedy e Fitzgerald, 2012). Adicionalmente, 50 % dos resíduos acima mencionados são descartados e apenas 30 % são utilizados em atividades e produtos de baixo valor comercial, tais como alimentos para animais, agentes fertilizantes e produção de silagem. (He et al., 2013, Hsu, 2010, Je et al., 2007). A conversão destes subprodutos em ingredientes funcionais de alto valor pode proporcionar uma solução para lidar com as restrições legais, os custos elevados e os problemas ambientais associados à eliminação desses resíduos (Harnedy e Fitzgerald, 2012).

Os subprodutos de pescado são uma fonte significativa de proteína e outros componentes, como ácidos graxos poli-insaturados, fosfolipídios e vitaminas (Shirahigue et al., 2016). A maneira convencional de utilização de subprodutos do processamento de peixe é à produção de farinha de pescado, técnica mediante a qual as proteínas desta matéria prima não são recuperadas eficientemente. Portanto, em busca de alternativas viáveis para o problema de descarte da indústria pesqueira, propõe-se o desenvolvimento de processos com o intuito de recuperação ou alteração das proteínas dos resíduos do pescado visando seu uso como ingredientes que além das características nutricionais proporcionam benefícios fisiológicos e/ou reduzem o risco de doenças crônicas, também chamados ingredientes funcionais (Martins et al., 2009; Santos et al., 2009). A hidrólise proteica é uma forma eficiente de agregar valor aos subprodutos da indústria do pescado (Halim et al., 2016).

1.3. Hidrolisados proteicos de pescado

Os hidrolisados proteicos de pescado, designados pela FAO pela sigla FPH (“Fish Protein Hydrolysates”) podem ser definidos como proteínas clivadas em peptídeos de vários tamanhos ou em seus aminoácidos individuais. Assim, os FPH

submetidos a secagem podem atingir uma concentração de proteína de até 90 %, além de apresentar propriedades funcionais úteis para a indústria alimentícia (Oetterer, 2006). Os FPH podem ser obtidos industrialmente basicamente por métodos químicos (hidrólise alcalina e hidrólise ácida) ou por métodos bioquímicos (hidrolise enzimática e autólise) (Adler-Nissen, 1984).

A hidrólise química é um processo totalmente inespecífico no qual é feito uma solubilização ácida ou alcalina das proteínas, atingindo pH extremo, seguida de uma precipitação ácida ou alcalina até atingir o ponto isoelétrico da proteína. Esta hidrolise química, pode destruir os L-aminoácidos da proteína, dando lugar a D-aminoácidos e formando substâncias tóxicas como a lisino-alanina e comprometendo o valor nutricional e funcional da proteína (Lahl e Braun, 1994; Martins et al., 2009). A hidrólise química é mais comumente utilizada na prática industrial. Dessa forma, os processos biológicos que utilizam adição de enzimas são mais promissores quando se deseja produtos com alta funcionalidade e valor nutritivo (Martins et al., 2009).

A hidrólise enzimática permite melhor controle do processo e do produto resultante quando comparado com a hidrólise química e é considerado o processo mais efetivo para a produção de peptídeos bioativos (Clemente, 2000). Vermeirssen et al. (2004) definiram os peptídeos bioativos como componentes derivados de alimentos que além de seu valor nutricional exercem um efeito fisiológico no organismo. Estes peptídeos encontram-se inativos na estrutura da proteína nativa e quando liberados por proteólise (*in vivo* ou *in vitro*) podem mostrar diversas atividades biológicas tais como antioxidante, antimicrobiana, anti-hipertensiva, anti-inflamatória, neuroprotetora, antidiabética e potencial atividade anticâncer, entre outras (Ktari et al., 2012; Centenaro et al., 2014; Intarasirisawat et al., 2013; Ennaas et al., 2015; Hsu et al., 2011; Roblet et

al., 2016; Ahn et al., 2012). Desta forma, os peptídeos gerados a partir da hidrolise de subprodutos de pescado podem ajudar a promover a saúde e na prevenção de doenças crônicas (Kim e Wijesekara 2010).

Nos últimos 60 anos diversos autores têm reportado e descrito a proteólise enzimática e solubilização de proteínas provenientes de diferentes fontes (Aspmo et al 2005; Petreus et al 2011). Santos et al. (2011) utilizaram as enzimas comerciais Alcalase e Flavourzyme para a obtenção de hidrolisados de cabrinha (*Prionotus punctatus*), um pescado de baixo valor comercial, modificando as propriedades funcionais das proteínas. Em outro estudo, a pele da polaca do Alasca (*Theragra chalcogramma*), um subproduto da indústria pesqueira, foi submetido a hidrólise utilizando proteases comerciais como Neutrase, Flavourzyme, Alcalase e Protamex, além das enzimas tripsina e papaína, o qual resultou em diferentes produtos com atividade antioxidante (Jia et al., 2010). No capitulo I desta tese oferece-se uma revisão dos métodos de produção de hidrolisados a partir de subprodutos de pescado, assim como também uma revisão dos trabalhos mais recentes avaliando as atividades antioxidante, antimicrobiana, anti-hipertensiva entre outras. Adicionalmente, no Capítulo II do presente trabalho avalia-se a atividade antioxidante de hidrolisados proteicos produzidos a partir de subprodutos do beneficiamento da carpa comum utilizando diferentes enzimas comerciais.

1.3.1. Inclusão de hidrolisados proteicos em rações para aquicultura

Devido a suas propriedades funcionais e valor nutritivo, os hidrolisados proteicos de pescado tornam-se ingredientes potenciais capazes de substituir ou complementar as dietas de aquicultura à base de farinha de peixe. Adicionalmente, os hidrolisados proteicos de pescado têm sido usados em raçoes de aquicultura como

suplemento proteico, atraentes ou potenciadores de palatabilidade (Aguila et al., 2007; Hardy, 1991). Deste modo, diversos estudos têm avaliado os efeitos sobre o crescimento da inclusão de hidrolisados proteicos na dieta de diversas espécies de peixes tais como o salmão atlântico (*Salmo salar*) (Refstie et al., 2004), linguado (*Scophthalmus maximus*) (Xu et al., 2016), truta arco-íris (*Oncorhynchus mykiss*) (Aksnes et al, 2006a), bacalhao (*Gadus morhua*) (Aksnes et al, 2006b), larvas de carpa (*Cyprinus carpio*) (Carvalho et al, 1997), larvas de robalo japonês (*Lateolabrax japonicus*) (Liang et al, 2006), larvas de corvina amarela (*Larimichthys crocea*) (Cai et al., 2015), larvas de perca gigante (*Lates calcarifer*) (Srichanun et al., 2014). Estes estudos acima citados confirmam que os alimentos com suplemento de hidrolisado de proteína de peixe melhoram tanto o crescimento quanto o desenvolvimento do sistema digestivo. Contudo, a maioria dos estudos avaliando o efeito da incorporação de hidrolisados proteicos de pescado em dietas de peixes centram-se nas consequências sobre os parâmetros zootécnicos e/ou sobrevivência (Martínez-Álvarez et al., 2015). Existe uma carência de estudos *in vivo* que avaliem o efeito das propriedades bioativas dos hidrolisados proteicos sobre a saúde dos peixes. É importante ressaltar que a maioria das bioatividades benéficas descritas para os peptídeos derivados de animais foram avaliadas apenas *in vitro*. Por conseguinte, é necessário avaliar a atividade desses peptídeos em animais após a digestão para avaliar a sua eficácia, resposta a diversas concentrações e segurança antes do uso como ingrediente funcional. Visando suprir esta carência de informações, no Capítulo III do presente trabalho de tese foi realizado um estudo avaliando a inclusão de diferentes concentrações de hidrolisado de subprodutos de carpa em rações para aquicultura.

1.4. Microencapsulação

Os antioxidantes naturais são geralmente moléculas instáveis e sensíveis ao calor e à luz, o que limita a sua aplicação na indústria alimentar (Lozano-Vázquez et al., 2015; Chao et al., 2011). Além disso, alguns destes compostos apresentam outras limitações tais como sabor desagradável, reduzida biodisponibilidade e elevada suscetibilidade a condições de armazenamento e processamento, bem como resistência a diversos fluidos digestorios (Nedovic et al., 2011; Poshadri e Kuna, 2010; Wilson e Shah, 2007). A microencapsulação é uma técnica confiável que pode fornecer solução aos problemas anteriormente citados, uma vez que é capaz de melhorar o tempo de retenção do nutriente no alimento, permite liberação controlada e direcionada, preserva a estabilidade dos compostos bioativos durante o processamento e armazenamento, evita interações indesejáveis com a matriz alimentar, retarda os processos de degradação, mascara o gosto e aumenta a biodisponibilidade, enquanto mantêm a funcionalidade do componente bioativo (Aguiar et al., 2016; Agnihotri et al., 2009, Poshadri e Kuna, 2010). Outras vantagens podem incluir a facilidade de manejo, a concentração adequada e a dispersão uniforme (Lozano-Vazquez et al., 2015, Nedovic et al., 2011; Wilson e Shah, 2007).

1.4.1. Coacervação complexa

O processo de microencapsulação por coacervação complexa envolve pelo menos dois polímeros com carga diferente sob determinadas condições. Na maioria dos casos, os dois polímeros incluem uma molécula de natureza proteica e uma molécula de polissacarídeo (Jun-xia et al., 2011). Assim, os coacervados complexos são formados quando dois polieletrólitos carregados de forma oposta são misturados em solução aquosa. Este processo resulta numa separação de fase (líquido-líquido) na qual uma fase

densa rica em polímeros (coacervado) se separa da fase de solução pobre em polímeros diluído (fase aquosa) (Black et al., 2014). Neste sentido, a complexação por interações eletrostáticas pode ser influenciada por diferentes parâmetros tais como pH, força iônica, relação proteína-polissacarídeo, distribuição de cargas da proteína e hidrofobicidade relativa das moléculas (Schmitt et al., 1998).

As microcápsulas produzidas por coacervação possuem excelentes características de liberação controlada e propriedades termo-resistentes (Dong et al., 2008). Adicionalmente, as microcápsulas produzidas por coacervação podem ser desidratadas com o intuito de estender o seu tempo de estocagem e aplicação das microcápsulas em produtos sólidos.

O processo de desidratação das microcápsulas produzidas por coacervação complexa pode utilizar diferentes métodos de secagem tais como liofilização, secagem em estufa ou remoção da água por solventes. No entanto, estes métodos não permitem a obtenção de partículas individualizadas, provocando interferências com o tamanho do produto e modificando as propriedades de liberação do núcleo das microcápsulas (Thies, 1995). Consequentemente, a secagem por atomização ou *spray-drying* torna-se um método interessante para a obtenção de partículas individuais de tamanho micrométrico.

1.4.2. Spray-drying (secagem por atomização)

O *spray-drying* pode também ser usado como um método de encapsulação, quando se aprisionam materiais ativos dentro de uma matriz de proteção, que é essencialmente inerte para o material a ser encapsulado (Tewa-Tagne et al., 2007). A secagem por atomização é a mais utilizada na indústria alimentar devido à simplicidade e flexibilidade do processo (podendo trabalhar em operação continua), custo

relativamente baixo, alta estabilidade do produto seco final (devido ao baixo teor de umidade), alta redução de volume inicial e facilidade de manuseio (Ben Amara et al., 2016). Este processo envolve quatro estágios: (i) preparação da dispersão ou da emulsão; (ii) homogeneização da dispersão; (iii) atomização da emulsão, e (iv) desidratação das partículas atomizadas (Shahidi e Han, 1995). O primeiro estágio consiste na formação de uma emulsão fina e estável do material do núcleo, dentro da solução de parede. A mistura a ser atomizada é preparada por dispersão do material o núcleo que, neste caso, é normalmente de natureza hidrofóbica, numa solução do agente de revestimento com o qual é imiscível (Gharsallaoui et al., 2007).

No entanto, devido à exposição a altas temperaturas, a secagem por atomização pode alterar irreversivelmente a propriedades das proteínas. O aumento de temperatura acima de 60 °C pode acarretar alterações estruturais irreversíveis da proteína, resultando em estruturas menos ordenadas e uma maior exposição dos grupos hidrofóbicos e sulfidrila em comparação com a estrutura inicial (Bernard et al., 2008).

No Capitulo IV do presente trabalho de tese será comparada a eficiência de retenção de atividade antioxidante dos hidrolisados proteicos de carpa comum após microencapsulação e secagem pelos métodos anteriormente citados.

2. OBJETIVO

2.1 Objetivo geral

Produzir, avaliar e microencapsular hidrolisados proteicos com atividade antioxidante, obtidos a partir do músculo e dos subprodutos do beneficiamento da carpa comum (*Cyprinus carpio*) produzida em cativeiro.

2.2 Objetivos específicos

- Submeter o músculo e os subprodutos do beneficiamento da carpa comum a hidrólise enzimática utilizando enzimas comerciais e estimar a eficiência da hidrólise usando como referencia o grau de hidrólise atingido em cada caso.
- Avaliar a atividade antioxidante *in vitro* dos hidrolisados proteicos do subproduto do beneficiamento da carpa obtidos com diferentes enzimas e diferentes graus de hidrólise.
- Estimar o efeito sobre a viabilidade celular e produção de espécies reativas de oxigênio (ROS) *in vitro* dos hidrolisados proteicos de subproduto de carpa em células de hipocampo de rato (HT-22).
- Estudar a influencia da inclusão de níveis crescentes de hidrolisado proteicos de subproduto de carpa sobre o metabolismo antioxidante do cérebro, músculo e brânquias de peixe-zebra (*Danio rerio*).
- Microencapsular os hidrolisados proteicos de carpa mediante coacervação complexa e secagem por atomização, e estudar a morfologia das microcápsulas por microscopia eletrônica de varredura.
- Estudar o efeito dos diferentes métodos de microencapsulação sobre a atividade antioxidante dos hidrolisados.

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CAPÍTULO I

PEPTIDES FROM FISH BY-PRODUCT PROTEIN HYDROLYSATES AND ITS FUNCTIONAL PROPERTIES: AN OVERVIEW

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Peptides from fish by-product protein hydrolysates and its functional properties: An overview

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Resumo

O manejo inadequado dos resíduos do processamento do pescado ou subprodutos é um dos maiores problemas que a indústria do pescado tem que encarar atualmente. A má administração desta matéria prima pode causar perdas econômicas e problemas de poluição ambiental. Uma maneira eficiente de agregar valor à proteína de resíduos de pescado é a hidrólise proteica. Os hidrolisados proteicos podem melhorar as propriedades funcionais do produto e permitem a liberação de peptídeos de diferentes tamanhos com diversas bioatividades tais como antioxidante, antimicrobiana, anti-hipertensiva ou anti-hiperglicémica entre outras. Este trabalho faz uma revisão dos diferentes métodos de produção de hidrolisados proteicos assim como também uma revisão das pesquisas atuais sobre as propriedades bioactivas de diversos hidrolisados proteicos de subprodutos de pescado; almejando um duplo objetivo: adicionar valor a esses subprodutos e minimizar o negativo impacto no meio ambiente decorrente do seu manejo ineficiente.

Palavras chave: Anti-hipertensivo, Antimicrobiano, Antioxidante, Peptídeos bioativos, Hidrólise enzimática.

Abstract

The inadequate management of fish processing waste or by-products is one of the major problems that fish industry has to face nowadays. The mismanagement of this raw material leads to economic loss and environmental problems. The demand for the use of these by-products has led to the development of several processes in order to recovery biomolecules from fish by-products. An efficient way to add value to fish waste protein is protein hydrolysis. Protein hydrolysates improve the functional properties and allow the release of peptides of different sizes with several bioactivities such as antioxidant, antimicrobial, antihypertensive or antihyperglycaemic among others. This paper reviews different methods for the production of protein hydrolysates as well as current research about several fish by-products protein hydrolysates bioactive properties, aiming the dual objective: adding value to these underutilized by-products and minimizing their negative impact on the environment.

Keywords: Antihypertensive, Antimicrobial, Antioxidant, Bioactive peptides, Enzimatic hydrolysis.

1. Introduction

Many countries worldwide have the fish industry as a pillar of their economy with an annual production of approximately140 million tons, of which about 80% is destined for human consumption (Benhabiles et al. 2012). It has been estimated that 1 billon people depend directly or indirectly on the trade and fish production (Oosterveer 2008). Nevertheless, fish trade presents underutilized fish by-products which include head, skin, trimmings, fins, frames, viscera and roe that account for more than 60% of total biomass. Fish processing by-products are fish material left over from the primary processing of fish manufacturing process (He et al. 2013). In most cases, these fish by-products are discarded without intention of recovery (Halim et al. 2016). Moreover, discards of fish by-products have a great ecological impact and also considerably affect the economic viability of the fishing and aquaculture sector. The European Commission is carrying out modifications in the common fishery policy in order to the complete elimination of discards. Technical solutions are required to use fish by-products as raw materials for the production of added-value compounds (Morales-Medina et al. 2016).

Fish processing by-products are a great source of high quality compounds that may be used for human consumption. These by-products can be a great source of value added products such as proteins, amino acids, collagen, gelatin, oils, and enzymes (Ghaly et al. 2013). Moreover, up to 10-20% (w/w) of total fish protein can be found in fish by-products. In addition, the crude protein content of fish by-products varies from 8-35% (Sila and Bougatef 2016). The essential amino acids and bioactive peptides found in fish proteins have great potential for their use in the production of drugs and functional foods (Sila and Bougatef 2016). In order to recovery protein and peptides from fish by-products several methods such as acid or alkaline hydrolysis, autolysis and

enzymatic hydrolysis have been developed. Non-hydrolysed fish proteins do not possess these properties due to the poor accessibility to the functional peptide sequence (Ghaly et al. 2013; Kim and Wijesekara 2010).

The first reported acid hydrolysis was reported by Braconnot in 1820 (Hill 1965). Acid hydrolysis process is carried out using high values of temperature and pressure. It is based on the prolonged boiling of the protein with strong acid solutions. This method completely destroys tryptophan and parts of serine and threonine (Pasupuleti and Braun 2010). However, protein hydrolysates produced by acid hydrolysis process are commonly used as flavour enhancers and in the manufacture of animal feeds (Bucci and Unlu 2000; Nagodawithana et al. 2010). During alkaline hydrolysis, proteins are solubilized by the addition of basic agents and elevating the temperature. Although through alkaline hydrolysis tryptophan remains intact, threonine and serine are damaged or destroyed (Pasupuleti and Braun 2010). Furthermore, proteins can be hydrolysed into peptides by endogenous enzymes of the fish muscle and viscera; this process is called autolysis (Halim et al. 2016).

Enzymatic hydrolysis is a process carried out under moderate conditions of pH and temperature. Furthermore, it is a specific process with an easy control of the degree of hydrolysis as well as allows retaining the nutritional value of the source protein. In this process, several proteolytic enzymes are commonly used to hydrolyse the proteins and converting them into high added-value products with functional, biological, and nutritional properties (Kristinsson and Rasco 2000; Shahidi et al. 1995). Protein hydrolysates are proteins broken into peptides that contain between 2-20 amino acids. In this process the source not only maintains a high content of essential amino acids but also generate other activities with potential use as food additives (García-Moreno et al.

2014; Pasupuleti and Braun 2010) such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, antimicrobial, among others (Kim and Wijesekara 2010). Several proteolytic enzymes are commonly used to hydrolyse proteins by-products which include Alcalase, papain, pepsin, trypsin, a-chymotrypsin, pancreatin, Flavourzyme, Pronase, Neutrase, Protamex, bromelain, cryotin F, protease N, protease A, Orientase, thermolysin, and Validase (Hsu 2010; Je et al. 2007; Ngo et al. 2010; Raghavan and Kristinsson 2008). In order to obtain bioactive peptides with functional properties, it is essential to control the hydrolysis time as well as to establish ideal values of pH and temperature to optimize enzyme activity.

The molecular weights and sizes of the peptides have a significant impact on their bioactive properties. Therefore, the purification and characterization of the peptides resulting from protein hydrolysis is a common practice nowadays in order to study the properties of the hydrolyzed product (Halim et al. 2016). Due to the molecular complexity of peptides, it is difficult to use similar methods to those applied in the purification of other organic compounds such as crystallization. The high performance liquid chromatography (HPLC) is widely used for the separation, purification and identification of bioactive peptides. Moreover, reversed phase chromatography allows rapid separation and detection of peptide fractions while normal phase chromatography is used for separation of hydrophilic peptides. Furthermore, the ion exchange chromatography can separate peptides based on their charge; while the gel filtration chromatography (in aqueous systems) and gel permeation chromatography (in non-aqueous systems) allows a separation based on the molecular weight. However, industrial production of purified peptides is hampered by low production yields. On the other hand, the industry has in consideration the purification of peptides for having

lower production costs when compared with chemical synthesis of peptides (Agyei and Danquah 2011).

Recently, several authors have purified fish by-product hydrolysates and have reported sequences of peptides with different bioactivities such as antioxidant (Ahn et al. 2014; Cai et al. 2015; Chi et al. 2015a, b), antihypertensive activity (Intarasirisawat et al. 2013), antibacterial activity (Ennaas et al. 2015), cholecystokinin release activity (Cudennec et al. 2008) and antiproliferative (Picot et al. 2006). Therefore, fish processing by-products can be used as a source for producing nutraceuticals and food additives for functional foods for human consumption. The present paper provides an overview about specific characteristics, production and purification as well as current and future trends of fish and shellfish by-product protein hydrolysates and bioactive peptides. Recent research of bioactive functionalities will be also briefly discussed.

2. Fish protein hydrolysates production methods

Today, the production of protein hydrolysates is massive worldwide. The protein hydrolysis involves the chemical or enzymatic breakdown of protein molecules into a mixture of peptides with different molecular weight and amino acids composition and sequence, various sizes and amino acids. The most widely used methods for the production of protein hydrolysates in industrial practices are chemical and biological methods. However, some authors have also produced protein hydrolysates via thermal hydrolysis by retorting the raw material in an autoclave at 121 °C (Wang et al. 2013) or by bacterial fermentation (Jemil et al. 2014). Figure 1 summarizes hydrolysis production methods.

2.1. Chemical hydrolysis

Chemical methods involve acid and alkaline hydrolysis. Since these are methods relatively inexpensive and easy to operate, they have been the preferred practices to produce protein hydrolysates at industrial scale. However, chemical hydrolysis is difficult to control due to its harsh reaction and unspecific peptide bonds cleaving, giving a heterogeneous yield of peptides and reduces the nutritional quality of products (Celus et al. 2007).

2.1.1. Acid hydrolysis

The most frequent acids used to carry out an acid hydrolysis are hydrochloric and sulfuric acid. In this process, the proteins are completely hydrolysed at high temperature (for example, 18 h, 118 °C, 6 N hydrochloric acid) and often high pressure, the hydrolysate is then neutralized to pH 6.0 - 7.0 and concentrated (Kristinson and Rasco 2000; Pasupuleti and Braun 2010). During this process, the proteins are completely hydrolysed leading the yield of individual amino acids. Moreover, during acid hydrolysis process all peptide bonds of the protein are non-specifically attacked, resulting in the destruction of some individual amino acids such as tryptophan, methionine, cystine and cysteine. Also, asparagine and glutamine are converted into glutamic acid and aspartic acid respectively. This destruction of amino acids results in a final product with poor nutritional value. However, they are widely used as a flavour enhancer in pet food and as fertilizer (Kristinson and Rasco 2000; Nagodawithana et al. 2010).

2.1.2. Alkaline hydrolysis

Alkaline hydrolysis is a simple process in which the protein is solubilized by heating followed by the addition of calcium, sodium or potassium hydroxide as alkaline

agents and maintaining the temperature at a range between 25-55 °C (Pasupuleti and Braun 2010). During alkaline hydrolysis of proteins, some amino acids like serine and threonine are damaged during alkaline hydrolysis, however, tryptophan remains intact. Also, this process causes racemization of amino acids, by modifying its structure and converting L-amino acid in its isomeric forms D-amino acids (Wisuthiphaet and Kongruang 2015). Disulphide bonds are also fragmented with loss of cysteine, serine, and threonine and formation of lysinoalanine, ornithinoalanine, and lanthionine (Kristinson and Rasco 2000). Some of these elimination and addition reactions could lead to the formation of toxic substances such as lysinoalanine that are highly undesirable in foods (Lahl and Braun 1994).

2.2. Biochemical hydrolysis

2.2.1. Autolysis

Autolysis of animal tissues is the process responsible of modifications of the quality attributes of flesh foods during post-mortem stages (Visessanguan et al. 2001). This process involves the action of endogenous proteolytic enzymes (endo- and exo-proteases) on the animal proteins. In this process, there are no enzyme costs involved. The end product of autolytic hydrolysis is generally a fairly viscous liquid rich in free amino acids and small peptides (Kristinsson and Rasco 2000). Autolytic degradation of proteins in fish and meat has been widely studied due to its applicability in the production of added value products such as protein hydrolysates and silages (Ledward and Lawrie 1984; Rao et al. 1996). Moreover, endogenous protease activity is attributed predominantly to digestive enzymes of the viscera. In this way, the by-product of Pacific whiting surimi was hydrolysed with endogenous enzymes, resulting in fish sauce fish with several biochemical properties (Tungkawachara et al. 2003), as well as

other sources such as capelin (Shahidi et al. 1995).The seasonal variations affect the presence or concentration of some endogenous enzymes. This fact makes autolysis a very difficult controllable process and affects directly the molecular profile of the end product (Kristinson and Rasco 2000). The main limitation of the production of bioactive peptides or added value products by autolytic hydrolysis is the reduced functionality and the difficulty to obtaining a homogenous hydrolysate.

2.2.2. Enzymatic hydrolysis

The hydrolysis of proteins by exogenous enzymes or enzymatic hydrolysis allows a better control of the hydrolysis process and the resulting product. Therefore, enzymatic hydrolysis is considered as the most effective way to obtain protein hydrolysates with bioactive properties (Clemente 2000; Shahidi et al. 1995). Any hydrolysis process involves at least five variables: (i) protein substrate concentration , (ii) enzyme-substrate ratio (E/S), (iii) pH, (iv) temperature, and (v) degree of hydrolysis (DH) (Adler-Nissen 1984). Generally, there is an optimum combination of both pH and temperature, where the enzyme shows the highest activity. Physicochemical conditions of the hydrolysis reaction such as temperature, pH and enzyme/substrate ratio must be adjusted to optimize the activity of the proteolytic enzyme (Kim and Wijesekara 2010; Santos et al. 2011). At the beginning of hydrolysis process the mixture must be heated to about 85-95 °C for 5-20 min in order to terminate the endogenous enzyme activity respectively. Some Industrial food-grade proteinases derived from microorganisms have been used to produce bioactive peptides by enzymatic hydrolysis such as Alcalase®, Flavourzyme®, and Protamex®, as well as enzymes from plants such as papain or bromelain and animal sources such as pepsin and trypsin (Samaranayaka and Li-Chan 2011).

Protease specificity affects the size, the amount, the composition of free amino acid and peptides and their amino acid sequence which influences the bioactivity of the obtained hydrolysate (Sarmadi and Ismail 2010). Proteases are classified by their principal functional group in their active site (serine, thiol, carboxyl and metallo) and their hydrolysing mechanism (endo or exon-proteases). Endo-proteinases are more widely used in food protein hydrolysis than exo-proteases. Moreover, endo-proteinases produce relatively large peptides while exopeptidases hydrolyse the terminal peptide bonds removing amino acids from either the N terminus (aminopeptidases) or the C terminus, (carboxypeptidases) (Kristinsson and Rasco 2000). The degree of hydrolysis (DH) is a fundamental parameter for the characterization and the production and of protein hydrolysates. It is defined as the percentage of broken peptide bonds in relation to the original protein. The degree of hydrolysis achieved in the hydrolysis is determined by the conditions used in the process such as substrate concentration, enzyme / substrate ratio, incubation time as well as the physicochemical conditions such as pH and temperature. Moreover, another factor that will determine the degree of hydrolysis is the nature of the enzyme, characterized by its specific activity and type of activity. Thus, the nature of the enzyme used will not only influence the degree of hydrolysis but also the type of peptides produced (Wang et al. 2013). The higher the DH, more number of peptides would be produced in the solution that will result in an increase of protein solubility and the possibility to recover the protein to be used as a food additive (Sheriff et al. 2014).

When the desired DH is attained, it is necessary to terminate the enzymatic reaction by heating the slurry to 85-95 °C for 5-20 min or by acidifying the hydrolysate mixture to an extreme acidic pH value to inactivate the enzyme activity, this step is

often used for the preparation of antimicrobial peptides. In industry, the production process of protein hydrolysates can be coupled to membrane technology, reducing the cost associated with enzyme inactivation at the end of the hydrolysis process (Guerard 2007). Subsequently, it is necessary to separate the different fractions (sludge of solids and non-soluble proteins at the bottom, aqueous layer at the middle and lipid phase at the top) by centrifugation. The oil phase over the aqueous phase is removed and the soluble fraction collected (Kristinsson and Rasco 2000). Commonly, after this step the hydrolysates are freeze dried and stored until further analysis or application.

3. Bioactive properties of fish by-products protein hydrolysates

Bioactive or biologically active peptides have been defined as "food derived components (naturally occurring or enzymatically generated) that, in addition to their nutritional value exert a physiological effect in the body" (Vermeirssen et al. 2004). Moreover, protein hydrolysates and peptides from waste processing fish by-products can promote human health, and may help in the prevention of chronic diseases (Kim and Wijesekara 2010). In order to promote the biological activity of peptides, which are inactive in the structure of the native protein, they must be liberated by proteolysis (digestion *in vivo*) or hydrolysis (*in vitro* by enzymes). Thus, the resulting peptides may possess biological activities such as antioxidant, antimicrobial, antihypertensive, anti-inflammatory and antidiabetic potential activity against cancer, among others. In this section we will provide a review of studies hydrolysed fish products have bioactive properties

3.1. Antioxidant activity

The paradox of the oxygen is the fact that oxygen is an essential for energy production in most of living organisms but at the same time, reactive oxygen species (ROS) are continuously generated in cellular metabolism. High ROS concentrations can be extremely deleterious to cell constituents (Amado et al. 2009). Oxidative damage is caused by the depletion of antioxidants in the body due to the formation of ROS by physiological processes or by exogenous molecules (Shackelford et al. 2000; Valavanidis et al. 2006). Organisms protect themselves from such harmful effects with a complex antioxidant defense system that include a number of enzymatic and non-enzymatic defenses (Monserrat et al. 2008). In this regard, the oxidative stress starts when the formation of ROS exceeds the antioxidant defenses capacity (Amado et al. 2009; Jones 2006). In fact, the oxidative stress is related to a number of deleterious processes, such as protein damage, lipid peroxidation, enzyme inactivation and DNA breakage. These processes favour the occurrence of various diseases or pathologies such as the formation of tumours or cancer, heart disease, rheumatoid arthritis and aging (Sohal 2002; Klaunig and Kamendulis 2004).

Lipid oxidation mediated by free radicals, oxidative stress and antioxidants have been widely discussed in many research areas (Sila and Bougatef 2016). Although synthetic antioxidants as butylated hydroxy-anisole (BHA) and butylated hydroxytoluene (BHT) show stronger antioxidant activities than natural antioxidant, as α-tocopherol, ascorbic acid, are used in foods to prevent deterioration. However, the use of these synthetic compounds has begun to be restricted due to their potential health hazards and toxicity (Centenaro et al. 2014; Sabeena Farvin et al. 2014). Protein hydrolysates and peptides from fish by-products have shown antioxidant activities and

they can be considered as potential substitutes of synthetic antioxidants to reduce oxidative processes as well as ingredients for producing functional foods (Chi et al. 2015a; Frankel and Meyer 2000; Wiriyaphan et al. 2012)

Some authors reported that the hydrophobic amino acids as alanine, phenylalanine, isoleucine, leucine, valine and glycine and proline, methionine, tyrosine, histidine, lysine and cysteine may improve the efficiency of antioxidant peptides. These amino acids can act as proton donors or electron and / or as lipid radicals scavengers (Je et al. 2007; Samaranayaka and Li-Chan 2011; Sarmadi and Ismail 2010). In the same way, it has been demonstrated that acidic amino acids such as glutamic acid and aspartic acid as well as basic amino acids such as arginine, lysine and histidine present antioxidant capacity as chelator of metal ions due to carboxyl and amino groups in the side chains (Sarmadi and Ismail 2010; Udenigwe and Aluko 2012). Also, amino acids with aromatic residues can act as proton donors to radicals with electron deficiency (Sarmadi and Ismail 2010)

Several recent works have proved the antioxidant activity of fish by-product protein hydrolysates (Table 1). Lassoued et al. (2015) hydrolysed thornback ray (*Raja clavata*) skin gelatin with four different proteases to obtain peptides with antioxidant activity (proteolytic proteases from *Bacillus subtilis* A26, *Raja clavata* crude alkaline protease extract, Alcalase and Neutrase). Results showed that the highest antioxidant activity was obtained with protein hydrolysates generated by *Bacillus* A26 proteases. Further, Lassoued et al. (2015) purified the hydrolysates to identify the sequence of peptides. The pentapeptide Ala-Val-Gly-Ala-Thr purified from *Raja clavata* skin gelatin showed the highest antioxidant activity using the DPPH (2,2-Diphenyl-1-Picrylhydrazyl) radical-scavenging assay. The octapeptide Phe-Leu-Asn-Glu-Phe-Leu-

His-Val isolated from salmon by-product protein hydrolysate exhibited DPPH and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging activity and strong ferric reducing activity (Ahn et al. 2014). In a recent study Chi et al. (2015b) hydrolysed skin from Bluefin leatherjacket (*Navodon septentrionalis*) processing by-product with several proteolytic enzymes. The Bluefin leatherjacket skin hydrolysate produced with the enzyme Alcalase showed the highest antioxidant activity against $DPPH^{\cdot}$, $HO^{\cdot}$, and $O2^{\cdot -}$ radicals. In the same study, the antioxidant peptides Gly-Ser-Gly-Gly-Leu, Gly-Pro-Gly-Gly-Phe-Ile, and Phe-Ile-Gly-Pro were purified. The authors assumed that the isolated peptides could exert the antioxidant activities due to the hydrophobic nature and/or the aromatic residues of some amino acids contained in the peptidic chains. Recently, peptides from *Raja porosa* cartilage have shown excellent antioxidant properties. In this study Pan et al. (2016) isolated three bioactive hexapeptides (Phe-Ile-Met-Gly-Pro-Tyr, Gly-Pro-Ala-Gly-Asp-Tyr and Ile-Val-Ala-Gly-Pro-Gln) of skate cartilage that demonstrated good scavenging activities against $DPPH^{\bullet}$, $HO^{\bullet}$, $O_2^{\bullet -}$ and $ABTS^{\bullet +}$ due to their small molecular structure and the presence of hydrophobic amino acid residues within the peptide sequences..

3.2. Antimicrobial activity

Currently, the research focusing on the characterization and isolation of antimicrobial peptides from fish processing by-products is lower when compared to research on antioxidant peptides (Di Bernardini et al. 2011). Antimicrobial peptides are chains of amino acids with a molecular weight below 10 kDa that usually contain less than 50 amino acids of which nearly a half are hydrophobic (Najafian and Babji 2012). Moreover, through modification of the net charge or the hydrophobicity ratio, the antibacterial activity of cationic peptides can be modulated (Sila et al. 2014). The

interaction of these peptides with the bacterial membrane could form pores or block the membrane ion gradients leading to the destruction of the cell constituents. Moreover, several peptides could also generate bacterial depletion without membrane lysis probably by modifying the cellular metabolism (Wald et al. 2016). However, the mechanism through the peptides exert antibacterial activity is not yet completely understood.

Almost all antimicrobial peptides from fish possess antibacterial activities against numerous Gram-negative and Gram-positive strains. These antimicrobial peptides are potential candidates for new antibiotic development in the pharmaceutical field as well as antimicrobial agents for the food industry. In this manner, these antimicrobial peptides may be used as antibacterial, antiviral, antifungal, immunomodulatory, and antitumor agents (Kim and Wijesekaraa 2010; Rajanbabu and Chen 2011).

Table 2 shows recently published works that reported fish by-product protein hydrolysates with antimicrobial activity. Ennaas et al. (2015) hydrolysed mackerel by-products using Protamex, Neutrase, papain, and Flavourzyme as proteolytic enzymes. These hydrolysates showed antibacterial activity against Gram-positive (*L. innocua*) and Gram-negative (*E. coli*) strains. The highest antibacterial activity of these hydrolysates was showed when they were fractionated with acetone, which suggests the hydrophobic nature of these bioactive peptides. In the other hand, trout pepsin was used to produce antibacterial trout by-products protein hydrolysates against food contaminants and fish farming pathogens (Wald et al. 2016). Hydrolysates with hydrolysis degree of 30% showed the highest activity against *Flavobacterium psychrophilum* and *Salmoninarum renibacterium*. Moreover, the amino acids lysine,

leucine, alanine, arginine, glycine, aspartic acid and glutamic acid were the most abundant in this hydrolysate. Furthermore, head, frames and viscera from tilapia were submitted to enzymatic hydrolysis using Protamex enzyme by Robert et al. (2015). These hydrolysates possessed well-balanced amino acid profile and showed antimicrobial activity against *Edwardsiella tarda* and *Bacillus megaterium*.

3.3. Antihypertensive activity

As reported by World Health Organization (WHO 2010) about 30% of the deaths in the world are owed to cardiovascular diseases and it is estimated that by 2020 stroke and heart disease will be the major cause of death worldwide. The renin-angiotensin system is associated with the regulation of blood pressure (Skeggs et al. 1956). Angiotensin-I converting enzyme (ACE, EC 3.4.15.1) can increase blood pressure by catalysing the conversion of the decapeptide angiotensin-I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to the vasoconstrictor octapeptide angiotensin-II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Moreover, the ACE action contributes to increase blood pressure by the degradation of the nonapeptide vasodilator bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (Bougatef et al. 2008; Ghassem et al. 2011). Therefore, inhibition of ACE has become the main target in the treatment of hypertension (Himaya et al. 2012). Although several synthetic ACE inhibitors such as enalapril, alacepril or lisinopril have an effective result against hypertension, it is reported that they have side effects including inflammatory response, dry cough, taste disturbance, skin eruptions or angioneurotic oedema (Intarasirisawat et al. 2013). Hence, food derived ACE inhibitory peptides are being considered as an alternative.

The relationship between structure and activity of food-derived ACE inhibitory peptides has not been fully established. However, ACE inhibitory peptides generally

contain zinc-binding ligands, a hydrogen-bond donor and carboxyl terminal group (Andrews et al. 1985). Also, ACE activity could be inactivated by the presence of hydrophobic amino acids at the C-terminal tail by the alteration of the catalytic site of ACE (Kang et al. 2003). Peptides containing branched-chain aliphatic amino acids at N-terminal end are also suggested to have strong activity as ACE inhibitors (Wijesekara and Kim 2010).

Recently, several studies have highlighted ACE inhibitor activity of fish by-products protein hydrolysates. Intarasirisawat et al. (2013) hydrolysed skipjack (*Katsuwonus pelamis*) roe with Alcalase to 5% DH. They purified the hydrolysate by ultrafiltration, cation exchange column chromatography, and reverse phase high performance liquid chromatography (RP-HPLC). The hexapeptide Met-Leu-Val-Phe-Ala-Val showed the higher ACE inhibitory activity. Although the ACE inhibitory capacity of this hexapeptide was weaker than the commercial synthetic ACE inhibitors, the authors assumed that it could be potentially used as a functional food ingredient against hypertension symptom. In other study, ACE inhibitory capacity of protein hydrolysates from salmon pectoral fin was evaluated (Ahn et al. 2012b). Salmon by-product proteins were hydrolysed using Alcalase, Flavourzyme, Neutrase, pepsin, Protamex and trypsin. The hydrolysates produced with the enzyme Alcalase showed the highest ACE inhibitory activity. The authors identified three peptides (Val-Trp-Asp-Pro-Pro-Lys-Phe-Asp, Phe-Glu-Asp-Tyr-Val-Pro-Leu-Ser-Cys-Phe and Phe-Asn-Val-Pro-Leu-Tyr-Glu). They supposed that the presence of Phe, Leu, and Tyr residues at the C-terminal play an important role in their ACE inhibition activity. Collagen extracted skin is a normally discarded by-product in the Atlantic salmon industry. Gu et al. (2011) hydrolysed Atlantic salmon skin protein using Alcalase and Papain in a two-step

hydrolysis process. Two dipeptides, Ala-Pro and Val-Arg, were isolated from Atlantic salmon skin protein hydrolysates. These peptides were found to be the major contributors to the ACE inhibitory capacity peptides in the protein hydrolysate. Gu et al. (2011) suggested that salmon skin collagen peptides might be useful as functional foods and antihypertensive agents. Several other recent studies that have produced fish by-product protein hydrolysates with ACE inhibitory activity are listed in Table 3.

3.4. Other bioactivities

Some researchers have demonstrated that fish by-product protein hydrolysates possess other bioactivities with promissory applications in the pharmaceutical field or as food additives in functional foods. At this point, although fish by-product protein hydrolysates have been reported to show antitumor or antiproliferative activities, the research in this field remains limited compared to vegetative peptides (Suarez-Jimenez et al. 2012). Hsu et al. (2011) produced and identified two antiproliferative peptides active against human breast cancer cell line MCF-7 from tuna dark muscle by using papain and Protease XXIII. The isolated amino acid sequences for both peptides were Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr and Glu-Gly-Gly-Val-Tyr-Met-Val-Thr, respectively. The authors concluded that tuna dark muscle by-product would be a good source to produce antiproliferative peptides. Moreover, proteins from tuna cooking juice, a by-product produced during the processing of canned tuna hydrolysates by using protease XXIII, have shown antiproliferative activity (Hung et al. 2014). Tuna cooking juice hydrolysates showed antiproliferative activities up to 25% against MCF-7 cells without affecting normal breast epithelial cells. Two peptides were identified as Lys-Pro-Glu-Gly-Met-Asp-Pro-Pro-Leu-Ser-Glu-Pro-Glu-Asp-Arg-Arg-Asp-Gly-Ala-Ala-Gly-Pro-Lys and Lys-Leu-Pro-Pro-Leu-Leu-Leu-Ala-Lys-Leu-Leu-Met-Ser-Gly-

Lys-Leu-Leu-Ala-Glu-Pro-Cys-Thr-Gly-Arg. Although the anti-proliferation activity demonstrates a correlation with antioxidant activities, it seems that there is no correlation between the peptides molecular weight and their antiproliferative activity and more works should be carried out to clarify this concern (Hsu et al. 2011; Hung et al. 2014; Lee et al. 2003).

Fish by-products protein hydrolysates have also demonstrated potential activities against several diseases or health disorders that concern world population nowadays. Indeed, salmon frame and tilapia skin gelatin protein hydrolysates have shown potential applications as antihyperglycaemic agent as potent as other antidiabetic drugs (Roblet et al. 2016; Wang et al. 2015). In their work, Wang et al. (2015) stated that the gelatin hydrolysates of warm-water fish skin had more potential for the production of antidiabetic drugs as precursors of DPP-IV inhibitors than those of cold-water fish. Li-Chan et al. (2012) identified two tetrapeptides (Gly-Pro-Ala-Glu, Gly-Pro-Gly-Ala) obtained from Atlantic salmon skin gelatin hydrolysed with Flavourzyme with high DPP-IV inhibitory activity and could be used for the treatment or prevention of type 2 diabetes.

In an inflammatory process, activated macrophages of the immune system secrete nitric oxide in the inflammation sites to repair tissue and to remove the cause of the inflammation (Ahn et al. 2012a; Guastadisegni et al. 2002). However, various inflammatory diseases are related to the overproduction of nitric oxide. In this way, salmon by-products were hydrolysed by Ahn et al. (2012a), and the obtained hydrolysates showed an anti-inflammatory activity by inhibiting nitric oxide production and proinflammatory cytokines.

Collagen and gelatin are excellent functional ingredients for the cosmetics industry for the manufacture of anti-aging and anti-wrinkle products due to their excellent moisturizing property. Traditionally, these compounds were obtained from terrestrial animals but owing to certain animal diseases and ethnic and religious barriers, collagen and gelatin from fish are becoming more important as a preference of the cosmetic industry for the preparation of functional cosmeceuticals (Kim 2014).

Table 4 shows some recent works of fish by-products protein hydrolysates presenting antiproliferative, antidiabetic, antiinflamatory or immunomodulatory activity

4. Conclusion

In this work, we briefly reviewed some important aspects about the production of fish by-products protein hydrolysates, as well as some recent works testing their bioactive properties. Fish waste is presented as an important source of proteins, peptides and amino acids with high potential to develop novel nutraceuticals that may replace or minimize the potential deleterious effects of synthetic drugs. Additionally, the use of this technology would serve the dual purpose of developing a high added value product from a cheap and abundant raw material and minimizing the polluting potential of fish waste. The knowledge about the recovery of fish by-products and their potential bioactivities has hugely increased during the past decades. Nevertheless, more studies are needed to lead a better understanding of the mechanism through which fish by-product protein hydrolysates exert their biological activities. Moreover, further *in vivo* studies must be carried out to answer the questions of their absorption in the gastrointestinal track and bioavailability, in order to develop food additives like nutraceuticals for human functional foods and natural drugs against diverse diseases.

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Table 1 - Antioxidant activity of fish by-product protein hydrolysates (works published since 2012)

Fish	Source	Enzyme	Purified Sequences	Activity	Reference
Thornback ray (*Raja clavata*)	Skin gelatin	*Bacillus subtilis* A26, *R. clavata* crude alkaline protease extract, Alcalase 2,4L ®, Neutrase 0,5L®	-	DPPH radical-scavenging, reducing power, preventing β-carotene bleaching, total antioxidant capacity, Inhibition of supercoiled plasmid DNA scission induced by hydroxyl radicals	Lassoued et al. (2015)
Salmon (Scientific name not specified)	Pectoral Fin	Alcalase, Flavourzyme, Neutrase, pepsin, Protamex, trypsin	Phe-Leu-Asn-Glu-Phe-Leu-His-Val (1018,48 Da)	DPPH and ABTS radical-scavenging activity, Ferric reducing power, protection of plasmid pBR322 DNA against hydroxyl radical-induced damage, Protection effect on hydrogen peroxide-induced hepatic damage in Chang liver cells	Ahn et al. (2014)
Bluefin leatherjacket (*Navodon septentrionalis*)	Skin	Trypsin, Flavourzyme, Neutrase, papain, Alcalase, pepsin,	Gly-Ser-Gly-Gly-Leu (389,41 Da), Gly-Pro-Gly-Gly-Phe-Ile (546,63 Da), Phe-Ile-Gly-Pro (432,52 Da)	DPPH, superoxide and hydroxyl radical-scavenging activity, lipid peroxidation inhibition	Chi et al. (2015a)
Bluefin leatherjacket (*Navodon septentrionalis*)	Heads	Papain	Trp-Glu-Gly-Pro-Lys (615,69 Da), Gly-Pro-Pro (269,33 Da), Gly-Val-Pro-Leu-Thr (485,59 Da)	DPPH, hydroxyl, superoxide and ABTS radical-scavenging activity, lipid peroxidation inhibition	Chi et al. (2015b)

Fish	Source	Enzyme	Purified Sequences	Activity	Reference
Skate (*Raja porosa*)	Cartilage	Trypsin and Alcalase	Phe-Ile-Met-Gly-Pro-Tyr (726,90 Da), Gly-Pro-Ala-Gly-Asp-Tyr (578,58 Da), Ile-Val-Ala-Gly-Pro-Gln (583,69 Da)	DPPH, hydroxyl, superoxide and ABTS radical-scavenging activity, lipid peroxidation inhibition	Pan et al. (2016)
Giant catfish (*Pangasianodon gigas*)	Skin gelatin	Visceral alkaline-proteases from Giant catfish, commercial trypsin, Izyme AL®	-	ABTS radical-scavenging, Ferric reducing antioxidant power (FRAP) and metal (ferrous) chelating ability	Ketnawa et al. (2016)
Blue shark (*Prionace glauca*)	Skin gelatin	Protamex	Fraction with highest antioxidant capacity: Glu-Gly-Arg, Gly-Pro-Arg, Gly-Tyr, Gly-Phe and four amino acids of Arg, Leu,Tyr and Phe.	DPPH and Hydroxyl radical-scavenging activity	Weng et al. (2014)
Salmon (Scientific name not specified)	Pectoral Fin	Alcalase, Flavourzyme, Neutrase, Protamex, pepsin, trypsin	Fraction with mollecular weight between 1000-2000 Da	DPPH and hydrogen peroxyde scavenging activity, inhibition of intracellular reactive oxygen species generation and lipid peroxidation, enhanced the level of glutathione in Chang liver cells	Ahn et al. (2012)
Smooth hound (*Mustellus mustellus*)	Viscera	*M. mustellus* crude alkaline protease extract, Neutrase®, Esperase®, Purafect®, and combinations between endogenous and commercial proteases	-	DPPH scavenging activity, FRAP, iron chelating activity, β-carotene bleaching prevention, lipid peroxidation inhibition, DNA breakage protection	Abdelhedi et al. (2016)

Fish	Source	Enzyme	Purified Sequences	Activity	Reference
Amur sturgeon (*Acipenser schrenckii*)	Skin gelatin	Alcalase, Flavourzyme	-	Lipid peroxidation inhibition, protein oxidation prevention, loss in sulfhydryl content prevention	Nikoo et al. (2015)
Tuna (Scientific name not specified)	Dark muscle	Alcalase	-	DPPH, superoxide and hydroxyl radical-scavenging activity, reducing power, ferrous ion chelating activity, Inhibition of linoleic acid autoxidation	Saidi et al. (2014)
Grass carp (*Ctenopharyngodon idella*)	Skin	Alcalase	Pro-Tyr-Ser-Phe-Lys (640,74 Da), Gly-Phe-Gly-Pro-Glu-Leu (618,89 Da), Val-Gly-Gly-Arg-Pro (484,56 Da)	DPPH, hydroxyl, and ABTS radical-scavenging activity, lipid peroxidation inhibition	Cai et al. (2015)
Indian mackerel (*Rastrelliger kanagurta*)	Backbones	Pepsin, papain	-	DPPH and Hydroxyl radical-scavenging activity, reducing power, lipid peroxidation inhibition	Sheriff et al. (2014)
Asian seabass (*Lates calcarifer*)	Skin	Protease from hepatopancreas of Pacific white shrimp, Alcalase	-	DPPH and ABTS radical-scavenging activity, Ferric reducing antioxidant power, metal (ferrous) chelating activity, inhibition of lipid peroxidation	Senphan and Benjakul (2014)
Threadfin bream (*Nemipterus spp.*)	Surimi by-products	Protease from *Virgibacillus sp.* SK33, Alcalase, pepsin, tripsin	-	ABTS radical-scavenging, Ferric reducing antioxidant power, β-carotene bleaching prevention, Cytoprotective activity	Wiriyaphan et al. (2012)

Fish	Source	Enzyme	Purified Sequences	Activity	Reference
Unicorn leatherjacket (*Aluterus monoceros*)	Skin gelatin	Partially purified glycyl endopeptidase (Autolysis)	-	Protection effect against H2O2-induced DNA damage, on induction of antioxidant enzyme activities,	Karnjanapratum et al. (2016)
Unicorn leatherjacket (*Aluterus monoceros*)	Skin gelatin	protease from *Bacillus amyloliquefaciens* H11, Alcalase	-	ABTS radical-scavenging activity, Ferric-reducing antioxidant power, metal (ferrous) chelating activity, inhibition of lipid peroxidation	Sai-Ut et al. (2014)
Cod (*Gadus morhua*)	Backbones	Protease P "Amano" 6	-	Oxygen radical absorbance capacity, metal chelating ability, intracellular antioxidant activity in HepG2 cell	Halldorsdottir et al. (2014)
Tilapia (*Oreochromis niloticus*)	Frame	Flavourzyme 1000 L	-	DPPH radical-scavenging activity, Metal chelating activity, inhibition of lipid peroxidation	Chuesiang and Sanguandeekul (2015)
Smooth hound (*Mustelus mustelus*)	Heads and viscera	*M. mustellus* gastric protease extract, *M. mustellus* intestine protease extract, porcine pancreatine	-	DPPH radical-scavenging activity, Ferric reducing power, β-carotene bleaching prevention	Sayari et al. (2015)
Whitemouth croaker (*Micropogonias furnieri*)	Carcasses	Flavourzyme 1000 L	-	Lipid peroxidation inhibition	Zavareze et al. (2014)
Asian seabass (*Lates calcarifer*)	Skin gelatin	Alcalase	-	Protection against H2O2-induced DNA damage	Sae-leaw et al. (2016)

Fish	Source	Enzyme	Purified Sequences	Activity	Reference
Tilapia (*Oreochromis niloticus*)	Skin gelatin	Bromelain, papain, trypsin,Flavourzyme, Alcalase, Neutrase	-	ABTS radical-scavenging activity, Ferric-reducing antioxidant power, metal (ferrous) chelating activity, inhibition of lipid peroxidation	Choonpicharn et al. (2014)
Skipjack (*Katsuwana pelamis*)	Roe	Alcalase	Asp-Trp-Met-Lys-Gly-Gln, Met-Leu-Val-Phe-Ala-Val (678 Da), Met-Cys-Tyr-Pro-Ala-Ser-Thr, Phe-Val-Ser-Ala-Cys-Ser-Val-Ala-Gly (839 Da), Leu-Ala-Asp-Gly-Val-Ala-Ala-Pro-Ala, Tyr-Val-N-Asp-Ala-Ala-Thr-Leu-Leu-Pro-Arg, Asp-Leu-Asp-Leu-R-Lys-Asp-Leu-Tyr	ABTS, hydrogen peroxide and singlet oxygen scavenging-activity, metal chelating activity	Intarasirisawat et al. (2013)
Skate (*Raja porosa*)	Skin gelatin	Alcalase, flavourzyme, Neutrase, Protamex	Met-Val-Gly-Ser-Ala-Pro-Gly-Val-Leu (829 Da), Leu-Gly-Pro-Leu-Gly-His-Gln (720 Da)	Intracellular radical-scavenging effects, enhanced expression of antioxidative enzymes (superoxide dismutase and glutathione)	Ngo et al. (2014)

Fish	Source	Enzyme	Purified Sequences	Activity	Reference
Pacific cod (*Gadus macrocephalus*)	Skin gelatin		Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro (1301 Da)	Hydroxyl radical-scavenging activity, protective effects on cell membrane lipid peroxidation, protective effects on cell membrane protein oxidation, protective effects on cellular DNA oxidation, intracellular radical-scavenging activity, enhanced expression of antioxidative enzymes (superoxide dismutase, glutathione and catalase)	Himaya et al. (2012)
Horse mackerel (*Magalaspis cordyla*)	Skin	Pepsin + trypsin + α-chymotrypsin	Asn-His-Arg-Tyr-Asp-Arg (856 Da)	DPPH and hydroxyl radical-scavenging activity, ferric-reducing antioxidant power, metal (ferrous) chelating activity, inhibition of lipid peroxidation	Sampath Kumar et al. (2012)
Croaker (*Otolithes ruber*)	Skin	Pepsin + trypsin + α-chymotrypsin	Gly-Asn-Arg-Gly-Phe-Ala-Cys-Arg-His-Ala (1101.5 Da)	DPPH and hydroxyl radical-scavenging activity, ferric-reducing antioxidant power, metal (ferrous) chelating activity, inhibition of lipid peroxidation	Sampath Kumar et al. (2012)

Table 2 - Antimicrobial activity of fish by-product protein hydrolysates (works published since 2012).

Fish	Source	Enzyme	Purified Sequences	Microbial strains	Reference
Atlantic mackerel (*Scomber scombrus*)	Viscera, digestive gland, stomach gonads, heart, intestines, liver and spleen	Protamex	Ser-Ile-Phe-Ile-Gln-Arg-Phe-Thr-Thr (1094,7 Da), Arg-Lys-Ser-Gly-Asp-Pro-L-Gly-Arg (1042,5 Da) , Ala-Lys-Pro-Gly-Asp-Gly-Ala-Gly-Ser-Gly-Pro-Arg (1111,6 Da), Gly-Leu-Pro-Gly-Pro-Leu-Gly-Pro-Ala-Gly-Pro-Lys (1102,5 Da)	Gram-positive (*Listeria innocua*), Gram-negative (*Escherichia coli*)	Ennaas et al. (2015)
Rainbow trout (*Oncorhynchus mykiss*)	Viscera	Trout pepsin, porc pepsin	-	Gram-positive (*R. salmoninarum, W. minor, W. paramesentoides, Micrococcus luteus, B. cereus, Ent. faecalis*), Gram-negative (*A. media, A. salmonicida, F. araucananum, F. psychrophilum, C. freundii, E. Coli, Pro. Mirabilis, P. flureszens*)	Wald et al. (2016)
Tilapia (*Oreochromis niloticus*)	Heads, frames and viscera	Protamex	-	Gram-positive (*Bacillus megaterium*), Gram-negative (*Yersinia ruckeri, Edwardsiella tarda*)	Robert et al. (2015)
Anchovy (*Engraulis japonicus*)	Cooking wastewater	Protamex	Gly-Leu-Ser-Arg-Leu-Phe-Thr-Ala-Leu-Lys (1104,66 Da)	Gram-positive (*Staphylococcus aureus, Bacillus subtilis, S. pneumoniae*), Gram-negative (*E. coli, S. dysenteriae, P. aeruginosa, Salmonella typhimurium*)	Tang et al. (2015)

Fish	Source	Enzyme	Purified Sequences	Microbial strains	Reference
Smooth hound (*Mustellus mustellus*)	Viscera	*M. mustellus* crude alkaline protease extract, Neutrase®, Esperase®, Purafect®, and combinations between endogenous and commercial proteases	-	Gram-positive (*Staphylococcus aureus , Micrococcus luteus, Bacillus cereus*), Gram-negative (*Escherichia coli, Klebsiella pneumoniae, Salmonella enterica, Salmonella typhi, Enterobacter sp.*)	Abdelhedi et al. (2016)

Table 3 - Antihypertensive activity of fish by-product protein hydrolysates (works published since 2012).

Fish	Source	Enzyme	Purified Sequences	Activity	Reference
Skipjack (*Katsuwana pelamis*)	Roe	Alcalase	Asp-Trp-Met-Lys-Gly-Gln, Met-Leu-Val-Phe-Ala-Val (678 Da), Met-Cys-Tyr-Pro-Ala-Ser-Thr, Phe-Val-Ser-Ala-Cys-Ser-Val-Ala-Gly (839 Da), Leu-Ala-Asp-Gly-Val-Ala-Ala-Pro-Ala, Tyr-Val-N-Asp-Ala-Ala-Thr-Leu-Leu-Pro-Arg, Asp-Leu-Asp-Leu-R-Lys-Asp-Leu-Tyr	ACE inhibitory	Intarasirisawat et al. (2013)
Salmon (Scientific name not specified)	Pectoral fin	Alcalase, Flavourzyme, Neutrase, pepsin, Protamex, trypsin	Val-Trp-Asp-Pro-Pro-Lys-Phe-Asp, Phe-Glu-Asp-Tyr-Val-Pro-Leu-Ser-Cys-Phe, Phe-Asn-Val-Pro-Leu-Tyr-Glu	ACE inhibitory	Ahn et al. (2012b)
Skate (*Okamejei kenojei*)	Skin gelatin	Alcalase, flavourzyme, Neutrase, Protamex	Met-Val-Gly-Ser-Ala-Pro-Gly-Val-Leu (829 Da), Leu-Gly-Pro-Leu-Gly-His-Gln (720 Da)	ACE inhibitory	Ngo et al. (2014)
Pacific cod (*Gadus macrocephalus*)	Skin gelatin	Pepsin + trypsin + α-chymotrypsin	Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro (1301 Da)	ACE inhibitory	Himaya et al. (2012)

Fish	Source	Enzyme	Purified Sequences	Activity	Reference
Smooth hound (*Mustelus mustelus*)	Heads and viscera	*M. mustellus* gastric protease extract, *M. mustellus* intestine protease extract, porcine pancreatine	-	ACE inhibitory	Sayari et al. (2015)
Tilapia (*Oreochromis niloticus*)	Skin gelatin	Bromelain, papain, trypsin,Flavourzyme, Alcalase, Neutrase	-	ACE inhibitory	Choonpicharn et al. (2014)
Tilapia (*Oreochromis niloticus*)	Frame	Flavourzyme 1000 L	-	ACE inhibitory	Chuesiang and Sanguandeekul (2015)

Table 4 –Fish by-product protein hydrolysates with antiproliferative, antidiabetic, antiinflamatory or immunomodulatory activity

Fish	Source	Enzyme	Purificated Sequences	Bioactivity	Reference
Tuna (*Thunnus tonggol*)	Dark muscle	Papaine, Protease XXIII	Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr (1206 Da), Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr (1124 Da)	Antiproliferative	Hsu et al. (2011)
Tuna (*Thunnus tonggol*)	Cooking juice	Protease XXIII	Lys-Pro-Glu-Gly-Met-Asp-Pro-Pro-Leu-Ser-Glu-Pro-Glu-Asp-Arg-Arg-Asp-Gly-Ala-Ala-Gly-Pro-Lys (2449,29 Da), Lys-Leu-Pro-Pro-Leu-Leu-Leu-Ala-Lys-Leu-Leu-Met-Ser-Gly-Lys-Leu-Leu-Ala-Glu-Pro-Cys-Thr-Gly-Arg (2562,41 Da)	Antiproliferative	Hung et al. (2014)
Atlantic salmon (*Salmo salar*)	Frames	Pepsin + trypsin + chymotrypsin	-	Antidiabetic	Roblet et al. (2016)
Tilapia (*Oreochromis niloticus*)	Skin	Flavourzyme 1000 L	Ile-Pro-Gly-Asp-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro (919,53 Da), Leu-Pro-Gly-Glu-Arg-Gly-Arg-Pro-Gly-Ala-Pro-Gly-Pro (1026,58 Da), Gly-Pro-Lys-Gly-Asp-Arg-Gly-Leu-Pro-Gly-Pro-Pro-Gly-Arg-Asp-Gly-Met (1358,76 Da)	Antidiabetic	Wang et al. (2015)
Atlantic salmon (*Salmo salar*)	Skin gelatin	Alcalase, bromelain, Flavourzyme	Gly-Pro-Ala-Glu (372,4 Da), Gly-Pro-Gly-Ala (300,4 Da)	Antidiabetic	Li-Chan et al. (2012)

Fish	Source	Enzyme	Purificated Sequences	Bioactivity	Reference
Salmon (Scientific name not specified)	Pectoral fin	Alcalase, Flavourzyme, Neutrase, Protamex, pepsin, trypsin	Fraction with mollecular weight between 1000-2000 Da	Antiinflamatory	Ahn et al. (2012a)
Unicorn leatherjacket (*Aluterus monoceros*)	Skin gelatin	Partially purified glycyl endopeptidase (Autolysis)	-	Inmunomodulatory and antiproliferative	Karnjanapratum et al. (2016)
Asian seabass (*Lates calcarifer*)	Skin gelatin	Alcalase	-	Inmunomodulatory and antiproliferative	Sae-leaw et al. (2016)

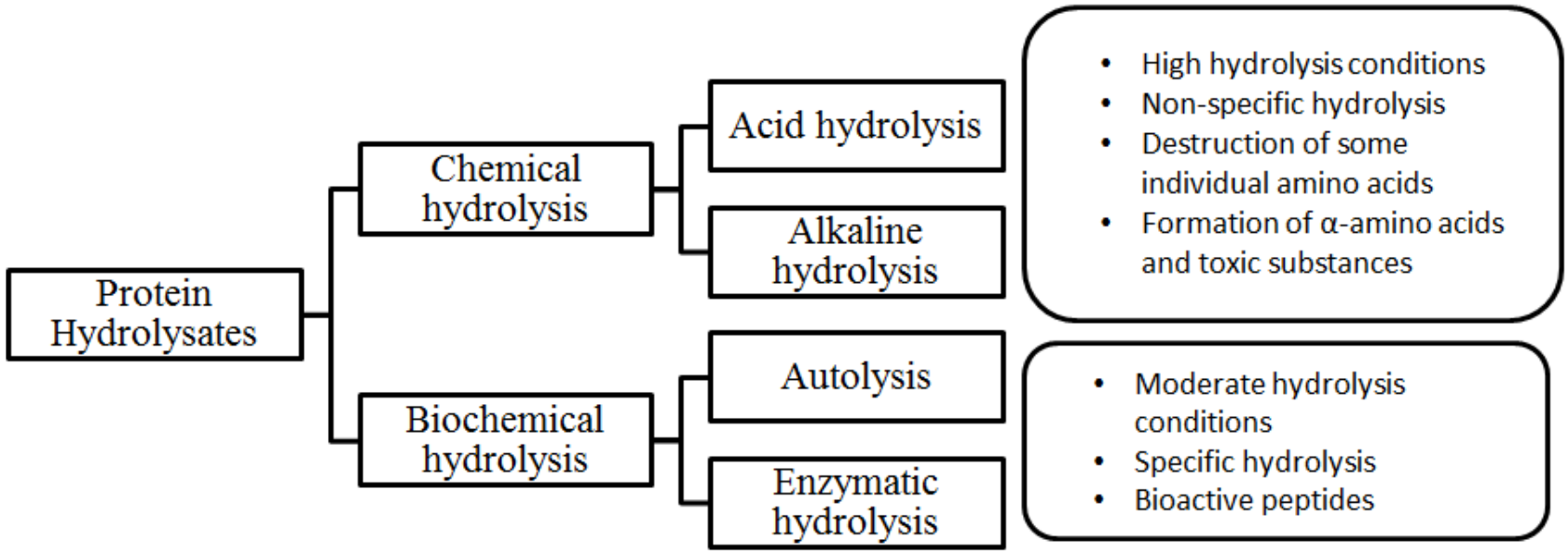

Figure 1. Hydrolysis production methods. (Zamora-Sillero et al., 2017)

CAPÍTULO II

EVALUATION OF THE ANTIOXIDANT ACTIVITY *IN VITRO* AND IN HIPPOCAMPAL HT-22 CELLS SYSTEM OF PROTEIN HYDROLYSATES OF COMMON CARP (*Cyprinus carpio*) BY-PRODUCT

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Evaluation of the antioxidant activity *in vitro* and in hippocampal HT-22 cells system of protein hydrolysates of common carp (*Cyprinus carpio*) by-product

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Resumo

Os subprodutos do processamento do pescado podem representar mais do 50% do material de partida. Se o manejo deste material não é o adequado, essas grandes quantidades de pescado podem causar problemas de poluição e gerar custos associados a sua eliminação. A hidrólise enzimática é uma das técnicas que está sendo desenvolvida atualmente com o intuito de recuperar e adicionar valor a essas biomoléculas. Há um interesse crescente nos antioxidantes naturais devido a que eles devem ser mais seguros para os consumidores do que antioxidantes sintéticos. Neste estudo, subproduto da carpa comum foi hidrolisado usando as enzimas Alcalase (A) e Protamex (P) até alcançar graus de hidrólise (DH) de 10 e 15%. Foi investigada a capacidade antioxidante contra radicais peroxil, radical DPPH (1,1-diphenyl-2-picrylhydrazyl) e a concentração de espécies reativas de oxigénio (ROS) intracelular após exposição aos hidrolisados proteicos de carpa comum. Os resultados mostraram que o hidrolisado A15 mostrou a maior atividade antioxidante ($p<0.05$) contra radicais DPPH. A15 mostrou a maior atividade antioxidante *in vitro* contra radicais peroxil, enquanto que P15 mostrou a menor atividade contra radicais peroxil ($p<0.05$). Os hidrolisados que mostraram maior e menor atividade antioxidante *in vitro* (A15 e P15 respectivamente) foram selecionados para a determinação da atividade antioxidante em cultura celular de células Ht-22. A dosagem mostrou que P15 na concentração de 1.25 mg/ml conseguiu reduzir significativamente ($p<0.05$) a concentração de ROS intracelular. Estes resultados demostraram que hidrolisados proteicos de subprodutos de carpa comum são fonte de peptídeos antioxidantes com potencial para as indústrias de alimentos e farmacêuticas para produzir novos nutracêuticos ou alimentos funcionais.

Palavras chave: Alcalase, DPPH, Grupos sulfidrila, Protamex, Radicais peroxil.

Abstract

Fish processing by-products may become more than 50% of the starting material. If mismanaged, these large quantities of discarded fish can create serious pollution problems and can also generate cost associated to disposal. Enzymatic hydrolysis is one of the techniques that is currently being developed nowadays in order to recuperate and add value to these biomolecules. There is an increasing interest in natural antioxidants as they should be safer for consumers than synthetic antioxidants. In this study, common carp by-product was hydrolysed using the enzymes Alcalase (A) and Protamex (P) to reach degrees of hydrolysis (DH) of 10 and 15%. Antioxidant capacity against peroxyl radicals, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and measurement of intracellular reactive oxygen species concentration after exposure to common carp protein hydrolysates were investigated. The results revealed that the hydrolysate A15 exhibited significant ($p<0.05$) higher antioxidant activity against DPPH radical. A15 shown the highest *in vitro* antioxidant competence against peroxyl radicals, while P15 showed the lowest activity against peroxil radicals ($p<0.05$). Hydrolysates having the highest and the lowest *in vitro* antioxidant activity (A15 and P15 respectively) were selected for the determination of antioxidant activity in HT-22 cells system. Measurement of intracellular ROS concentration revealed that P15 at the concetration of 1.25 mg/ml significantly ($p<0.05$) reduced the intracellular ROS concentration. These results showed that common carp by-product protein hydrolysates are a source of antioxidant peptides with high potential for food and pharmaceutical industries to develop new nutraceuticals and functional foods.

Keywords: Alcalase, DPPH, Peroxyl radicals, Protamex, Thiols.

1. Introduction

Fish industry is one of the most important resources in many countries worldwide. It has been estimated that 1 billon people depend directly or indirectly on the trade and fish production (Oosterveer, 2008; Centenaro et al., 2014). Besides, fish processing industry produces more than 60% of by-products as waste, which includes head, skin, trimmings, fins, frames, viscera and roes (Dekkers et al., 2011). These large quantities of fish processing by-product from fisheries could create serious pollution and disposal problems in both developed and developing countries (Chalamaiah et al., 2012).

Fish processing by-products are fish material left over from the primary processing of fish manufacturing process (He et al., 2013). The percentage of by-products generated in this process is about 50 % of the weight of the starting material and generates expenses associated with the disposal. Due to its high content of organic material, these by-products are classified as waste which is more costly to dispose (Peter and Clive, 2006). Taking in account the limited biological resources and increasing environmental pollution, exist a great need to find a solution for better management and use of by-products generated (Guérard, 2007; Neves et al., 2015).

Fish processing by-products are normally marketed as low-value products such as fishmeal, animal feed and fertilizer (Hsu, 2010). In this way, several techniques have been developed to recuperate essential nutrients and bioactive compounds to add value to these by-products, solving in this way the problems associated with disposal and pollution (Santos et al., 2011). Enzymatic hydrolysis is one of the techniques that is currently being developed nowadays in order to recuperate these biomolecules (Zavareze et al., 2014). In this process, several proteolytic enzymes are commonly used

to hydrolyze fish proteins to produce fish protein hydrolysates (FPH) and transforming them into high added-value products with functional, biological and nutritional properties (Kristinsson and Rasco, 2000; Halim et al., 2016). FPH are defined as proteins that are broken into peptides that contain between 2-20 amino acids. Due to the protein size reduction and their good functional properties, protein hydrolysates are used as readily available source of proteins for human and animal nutrition (Harnedy and Fitzgerald, 2012). The enzymatic hydrolysis produces peptides not only with a high content of essential amino acids but with biological activities with potential use for food or pharmaceutical fields (Garcia-Moreno et al., 2014) such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, antimicrobial, among others (Ktari et al., 2012; Centenaro et al., 2014; Intarasirisawat et al., 2013; Ennaas et al., 2015; Hsu et al., 2011; Roblet et al., 2016; Ahn et al., 2012). Numerous proteolytic enzymes are frequently used to hydrolyze the proteins from fish byproducts for the production of FPH. Some Industrial food-grade proteinases derived from microorganisms have been used to produce bioactive peptides by enzymatic hydrolysis such as Alcalase, Flavourzyme, and Protamex, as well as enzymes from plant (e.g. papain) and animal sources (e.g., pepsin and trypsin) (Samaranayaka and Li-Chan, 2011).

The production of reactive oxygen species (ROS) is inherent to aerobic life and possesses a dualistic role in the sense that ROS can induce deleterious effects on several macromolecules but they also participate in cell signal processes (Bhattacharyya et al., 2014; Monserrat et al., 2008;). Oxidative stress is involved in the occurrence of several diseases such as hypertension, cancer, diabetes, Alzheimer's and ageing (Garcia-Moreno et al., 2014, Hajieva and Behl, 2006). Some synthetic antioxidants, such as butylated

hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate, are usually used as food additives. However, these antioxidants possess potential risks to human health, and their use as food additives is restricted in some countries (Barlow and Schlatter, 2010; Kaur and Kapoor, 2001). Hence, there is a growing interest in identifying antioxidants substances from natural sources. Furthermore, in the last decade, several authors have reported a strong antioxidant activity for fish protein hydrolysates obtained from different species such as Black scabbardfish (*Aphanopus carbo*) (Batista et al., 2010), sardinelle (*Sardinella aurita*) (Bougatef et al., 2010), spotless smoothhound (*Mustelus griseus*) (Wang et al., 2014), cod (*Gadus morhua*) (Sabeena Farvin et al., 2014), amur sturgeon (*Acipenser schrenckii*) (Nikoo et al. 2015), silver carp (*Hypophthalmichthys molitrix*) (Malaypally et al. 2015) or cobia (*Rachycentron canadum*) (Fonseca et al., 2016). These studies demonstrated that protein hydrolysates have effective antioxidant activities and could be further been investigated for potential use as food additives or nutraceutics. Another important aspect to consider is the choice of the enzyme since it has a great impact on the type of peptides released by hydrolysis of fish protein (Laroque et al., 2008) and thus, peptides with different antioxidant properties can be obtained.

Another bioactive property of fish protein hydrolysates that is gaining more attention is their neuroprotective ability. Due to its high energy demand, high celular, lipid and protein content as well as low levels of endogenous scavengers, the brain is and organ extremely susceptible to oxidative stress damage (Gao et al., 2012). In this way, oxidative stress plays a role in the development of neurodegenerative diseases (Chen et al., 2012; Reed, 2011). The neuroprotective activity of fish protein

hydrolysates has been examined previously (Cai et al., 2015). However, the antioxidant effect of FPH on mouse hippocampal cells HT-22 has not previously been reported.

Aquaculture has been the engine driving growth in total fish production as global capture production has leveled off. Production of freshwater fishes has always been dominated by carps (71.9%, 24.2 million tonnes, in 2010) (FAO, 2016). Contrasting the seafood processing sector, freshwater industries are generally disorganized, particularly in developing countries. Therefore, in these countries there are abundant problems associated to fish processing by-product management or disposal (Bhaskar et al., 2008). Common carp (*Cyprinus carpio* Linnaeus, 1758) is one of the most important species of freshwater fish, currently cultured in many countries and frequently consumed. This species is native to Eastern Europe and Central Asia. Moreover, common carps have been introduced to more than 120 countries (Winker et al., 2010). Also, *C. carpio* is reared in polyculture with grass carp (*Ctenopharyngodon idella* Valenciennes, 1844), silver carp (*Hypophthalmichthys molitrix* Valenciennes, 1844) and bighead carp (*Aristichthys nobilis* Richardson, 1854) in low cost semi-intensive systems (Ritter et al., 2013).

The objective of the present work was to evaluate the antioxidant activity of protein hydrolysates prepared from common carp by-products using different proteolytic enzymes and degree of hydrolysis and to evaluate the antioxidant activity in *in vitro* and in cell assays with the generated peptides from this source.

2. Material and methods

2.1 Raw material

Common carp (*C. carpio*) were supplied by Piscicultura Andreghetto (Ajuricaba, Rio Grande do Sul State, Brazil). The fish were transported on ice within 24 h to the Laboratory of Food Technology of the Federal University of Rio Grande – FURG (Rio Grande do Sul, Brazil) where they were beheaded, eviscerated and filleted. Subsequently, the by-product consisting in head, viscera, bones and skin were blended and packaged in sealed plastic bags and stored at -20°C till further use.

2.2 Proximate analysis

The moisture, protein, lipid and ash contents of common carp by-product were determined according to the AOAC methods (AOAC, 2005). Briefly, moisture content was obtained by keeping the samples at 105°C for 5 h. Ash content was determined after sample incineration at 600°C for 5 h. Lipid content was determined using ether extraction with a Soxhlet extractor. Crude protein content was determined using the Kjeldahl method (N x 6.25).

2.3 Hydrolysis procedure

Raw material was thawed overnight at 4°C. The raw material was homogenized with two volumes of distilled water (w/v) in a jacketed bioreactor (MA502, Marconi, SP, Brazil) at 270 rpm following by heating the slurry at 85°C in order to inactivate the endogenous enzymes. Subsequently, the slurry was hydrolyzed using Alcalase 2.4 L® (Novozymes, Bagsvaerd, Denmark) or Protamex® (Sigma Aldrich, MO, USA). The hydrolysis were conducted at pH 8 and 50°C, while enzyme-substrate ratio was set at

2% (w/w) for both enzymes to determine the maximum degree of hydrolysis for each enzyme. Afterward, new hydrolysis processes were conducted to reach degrees of hydrolysis of 10 and 15% for both enzymes to produce the samples used in the subsequent analysis (Alcalase 10% = A10, Alcalase 15% = A15, Protamex 10% = P10, Protamex 15% = P15). The DH defined as the percent ratio of the number of peptide bonds broken (h) to the total of peptide bonds per unit of weight (h_{tot}). h_{tot} was assumed to be 8.6 meq/g for fish protein. DH was calculated from the amount of NaOH added to keep the pH constant during the hydrolysis (Adler-Nissen, 1984) as given in the equation 1:

$$\text{DH}\ (\%) = h/h_{tot} = [(B \times N_b) / MP] \times (1 / \alpha) \times (1/h_{tot}) \times 100 \qquad [1]$$

where B is the volume (mL) of NaOH consumed, N_b is the normality of NaOH, MP is the mass of protein and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as presented in the equation 2:

$$\alpha = (10^{pH-pK}) / (1+ 10^{pH-pK}) \qquad [2]$$

where the pH is the values at which the hydrolysis process was conducted and the pK value according Beychok and Steinhart (1964 apud Kristinsson and Rasco, 2000), it can be estimated by equation 3:

$$pK = 7.8 + [(298 - T) / (298 \times T)] \times 2400 \qquad [3]$$

where T is the temperature expressed in kelvin T at which the hydrolysis was performed.

After incubation the samples were submitted in a water bath at 90°C for 15 min to inactivate the enzyme. Then, samples were centrifuged at 16,300 x g for 15 min,

supernatants were frozen (Indrel, SP, Brazil) at -84°C and subsequently freeze dried (L108, Liotop, SP, Brazil) and stored at -20°C until analysis were performed.

2.4 In vitro determination of antioxidant activity

2.4.1 1,1-Diphenyl-2-picrylhydrazyl (DPPH˙) radical scavenging activity

The free radical method using 1,1-diphenyl-2-picryl-hydrazyl (DPPH˙) is a well-established assay for the *in vitro* determination of antioxidant activity in protein hydrolysates due to its reliability and reproducibility (Laroque et al., 2008).

In order to determine the DPPH˙ radical scavenging activity of the hydrolysates the method reported by Nicklisch and Waite (2014) and adapted for our laboratory was used. Briefly, the freeze dried hydrolysates were dissolved in a 0.1M citrate phosphate buffer (pH 7.0) supplemented with 0.3% (v/v) Triton X-100 to reach a concentration of 4 mg protein/ml. Afterward, an aliquot (190 µL) of each sample was mixed with 10 µL of a daily-prepared solution of DPPH˙ at 2 mM in methanol. The reduction of DPPH˙ radical was measured at 490 nm in a spectrophotometer with a microplate reader (ELx808, Biotek Instruments Inc., Winooski, Vermont). A control was run in the same way by using citrate phosphate buffer (pH 7.0) supplemented with 0.3% (v/v) Triton X-100 without sample, milli-Q water instead of sample, and sample blank was also made for each sample by adding methanol instead of DPPH˙ solution.

Then, DPPH˙ radical scavenging activity was calculated following equation 4:

$$\text{DPPH}^{\cdot}\ \text{inhibition}\ (\%) = ((A_{Control} - A_{Sample}) / A_{Control}) \times 100 \qquad [4]$$

Where $A_{control}$ is the absorbance of control and A_{sample} is the difference between the absorbance of the sample and the absorbance of the sample with methanol instead of $DPPH^{\cdot}$ solution.

2.4.2 Antioxidant capacity against peroxyl radicals (ACAP)

This is a simple rapid and reliable method reported by Amado et al. (2009) based on the detection of ROS by fluorometry (ex/em: 485/520nm) using 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) as substrate. Peroxyl radicals are generated by thermal decomposition at 35 °C of 2,2′-azobis (2 methylpropionamidine) dihydrochloride (ABAP) in different samples of hydrolysates dissolved in citrate phosphate buffer 0.1M (pH 7) to get a final protein concentration of 4 mg/mL. Samples antioxidant capacity against peroxyradicals is monitored by the fluorescence signal emitted by the reaction between ROS and deacetylated H_2DCF using a fluorescence microplate reader (Victor 2, Perkin Elmer). The thermal decomposition of ABAP and ROS formation was monitored for 90 min, with readings every 5 min.

Total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second order polynomial function. The results were expressed as area difference of FU×min in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). Antioxidant capacity was considered as the relative difference between ROS area with and without ABAP (higher area difference meaning low antioxidant capacity). Relative area was calculated as follows in equation 5:

$$(\text{ROS area}_{\text{ABAP}} - \text{ROS area}_{\text{background}}) / \text{ROS area}_{\text{background}} \quad [5]$$

In this method, the lower the relative area, the higher the antioxidant capacity and viceversa.

2.4.3 Determination of thiols and disulfide bond linkages

For the determination of thiols and disulfides we used a protocol based on the methods reported by White et al. (2003) and Chen et al. (2008) with modifications proposed by Enamorado et al. (2015). Briefly, the hydrolysates dissolved (4 mg protein/mL) in citrate-phosphate buffer (pH 7) was split in two aliquots of 30 µL. One of these aliquots was reduced with 10.4 µL of a solution of $NaBH_4$ (reducing agent) in sodium phosphate (0.15 M, pH 7.5) while the other was completed with the same volume of sodium phosphate. Subsequently, 4.5 µL of ethanol absolute was added to all samples. All samples were incubated 30 min at 50 °C. Afterward, 20 µL de each aliquot and 180 µL of a solution of 10 mM 2,3-naphthalenedicarboxaldehyde (NDA, Sigma Aldrich, MO, USA) was placed in a white 96 wells microplate for fluorimeter. The mixtures were incubated for 30 min at room temperature. Concurrently, a calibration curve with solutions of reduced glutathione (GSH) was prepared ranging from 0.015 to 40 nM. The intensity of fluorescence was measured using a fluorimeter at 480 ex.-525 em. The difference in peptides sulfydhryl groups (P–SH) concentration between the aliquot that received the reductive agent and the other that did not was considered the concentration of peptides disulfide bonds (P–S–S).

2.5 Assays with cell line HT-22

2.5.1 Cell culture

HT-22 cells, mouse hippocampal cell line (kindly provided by Salk Institute for Biological Studies, La Jolla, CA), were grown on bottles for cell culture in Dulbecco's

modified Eagle's medium (DMEM) containing high glucose (Gibco, USA) supplemented with sodium bicarbonate (1.5 g/L) (Vetec), 10% fetal bovine serum (FBS) (Invitrogen, Brazil) and 1% of antibiotics and antimicotic (penicilin [100 U/mL], streptomycin [100U/mL] and amphotericin [0.25 μg/mL]) (Gibco, USA). Cells were cultured at 37 °C in 5% CO_2. Growth media was changed every two days. The cells used in assays were previously transferred to 24-well or 96-well plates for cell culture, followed by a 24 h period grip. For the cell culture assays, the hydrolysates A15 and P15 were selected. The treatments were carried out in sextuplicate for cell viability assay and in quadruplicate for intracellular ROS determination. Experiments were performed at 70–80% confluence.

2.5.2 Cell viability assay

Cell viability was measured with the MTT method (3(-4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolium-Sigma-Aldrich, USA). HT-22 cells ($5x10^4$ cells/mL) were seeded into 96-well culture plates and incubated for 24h with the different traitments (A15 and P15) at different concentrations (0.03 mg/mL, 0.06 mg/mL, 1.25 mg/mL). After add 20 μL of MTT (5mg.mL-1) and three hours of incubation at 37 °C, the resultant formazan salt crystals were solubilized in 200 μL of DMSO (dimethyl sulfoxide). The amount of purple formazan was assessed by measuring the absorbance at 540 nm on a multiwall plate reader (ELX 800 Universal Microplate Reader, Bio-TEK).

2.5.3 Measurement of intracellular ROS concentration

For measurement of intracellular ROS levels, HT-22 cells ($5x10^4$cells/mL) were seeded onto 24-well culture plates. After 24 h incubation with the treatments. Cells were incubated for 30 min at 37 °C with the fluorogenic compound H_2DCF-DA (Sigma,

Germany) (40μM). Cellular fluorescence was quantified during 90 min at 37 °C using a fluorometer (Victor 2, Perkin Elmer) equipped with a microplate reader, at 472 ex.-528 em. The ROS area was standardized by the number of viable cells and ROS levels were expressed in terms of fluorescence area.

2.6 Statistics

All data were expressed as the mean ± standard error (SE). The differences between the treatments were evaluated using a two-factor analysis of variance (ANOVA), where the factors were enzyme (Alcalase or Protamex) and degree of hydrolysis (10% or 15%). For the determination of antioxidant activity in HT-22 cells system the differences were evaluated using one way ANOVA. Post-hoc tests were employed using Newman-Keuls method. In all cases the significance level adopted was 5% (α=0.05). The ANOVA assumptions (normality by Shapiro-Wilks and variance homogeneity by Levene) were previously met (Zar, 1999).

3. Result and discussion

3.1 Proximate composition

Proximate composition of common carp was determined following the methods of AOAC (2005). Common carp by-products (in 100 g raw material) had a moisture content of 69.04 ± 1.07 g, protein content of 15.19 ± 1.27 g, lipid content of 13.73 ± 1.79 g and ash content of 4.23 ± 0.41 g. The protein content found in common carp by-product is higher than the reported by Šližytė et al. (2014) for herring (*Clupea harengus*) by-product. Bhaskar et al. (2008) hydrolyzed visceral waste of catla (*Catla catla*), and reported a fat content of 12.46 ± 1.45 (g 100^{-1}) which is in concordance with

the results obtained in this study. The relative high ash content may result from the presence of high proportion of bones in the raw material.

3.2 Hydrolysis of common carp by-products

The maximum degree of hydrolysis (DH) for each enzyme were determined (Fig. 1). The DH is an indicator for cleavage of peptide bonds and is commonly used as an important parameter that characterizes a protein hydrolysate (Wang et al. 2013). The higher the DH, more number of peptides would be produced in the solution that will result in an increase of protein solubility and the possibility to recover the protein to be used as food grade additive (Sheriff et al. 2014). *C. carpio* by-products treated with the enzyme Alcalase showed higher degree of hydrolysis (23.86 %) when compared with the by-product treated with Protamex (15.13 %). For both enzymes the hydrolysis was characterized by a high rate of hydrolysis for the first 100 min. After this time the rate of hydrolysis was decreased until a steady-state phase. This characteristic pattern of hydrolysis was reported by different investigators earlier (Amado et al., 2013; Kong et al., 2007).The DH reached in this work was similar that the reported by Guérard et al. (2001) for the hydrolysis of yellowfin tuna (*Thunnus albacares*) wastes using Alcalase but lower than the reported by Bhaskar et al. (2008). These authors found a DH close to 50% hydrolyzing visceral waste protein from indian carp (*Catla clatla*) with an enzyme to substrate level of 1.5% (v/w), pH 8.5, temperature of 50 °C and a hydrolysis time of 135 min using Alcalase. Alcalase 2.4 L is a serine endo-protease from *Bacillus licheniformis* while Protamex is a complex endo-protease from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*. It could be possibly be inferred that Alcalase and Protamex have different proteolytic affinity for substrate protein. The degradation of short-chain protein tends to release more free amino acids or small peptides than the

cleavage of long-chain protein and leads to larger DH due to the enhanced base consumption (Wang et al., 2013).

3.3 In vitro antioxidant activity of common carp hydrolysates

3.3.1 (DPPH˙) radical scavenging activity

A popular strategy to determine the *in vitro* antioxidant activity of a given compound is to directly measure the ability to scavenge specific free radicals. DPPH˙ radical-scavenging ability of common carp by-product hydrolysate was evaluated. DPPH˙ is a stable free radical which accepts an electron or hydrogen radical to become a stable molecule. Hence, this method is often used as a substrate to evaluate the scavenging potency of protein hydrolysates due its reliability and reproducibility (Laroque et al., 2008; Sabeena Farvin et al., 2013). Fig. 2 shows the DPPH radical-scavenging activity of common carp by-product hydrolysates (4 mg of protein/mL) produced with Alcalase 2.4 L and Protamex with DH 10 and 15 %. The results indicated that A15 exhibited the highest DPPH˙ scavenging activity ($p<0.05$) showing 71.64 % of scavenging activity while there were not significant differences ($p>0.05$) between the DPPH˙ radical scavenging activity of A10 (51.13 ± 2.43 %), P10 (51.68 ± 3.33 %) and P15 (45.50 ± 1.65 %.

Some studies reveal that significant differences are found in the DPPH˙ scavenging activity of the protein hydrolysates when employing different enzymatic treatments (García-Moreno et al., 2014; Fonseca et al., 2016). The results of this study reveal that common carp by-product hydrolysate may contain peptides that act as electron donors and may possibly react with free radicals to transform them to more stable molecules.

3.3.2 Antioxidant capacity against peroxyl radicals (ACAP)

Recently, the measurement of antioxidant responses has been conducted in terms of the overall capacity to scavenge different forms of ROS (Amado et al., 2009). Peroxyl radicals are physiological highly reactive radicals that are involved in lipid peroxidation and tissue injury in biological systems (Ambigaipalan et al., 2015). In this method, H_2DCF-DA must be deaceylated through esterases activity that are present in biological samples. It can be hypothesized that esterases are present in common carp by-products protein hydrolysates since H_2DCF were formed and oxidized by ROS, giving a fluorescence signal.

To the best of our knowledge, there are no previous works using this method to evaluate the peroxyl radical scavenging capacity of protein hydrolysates. However, several authors have reported the competence of fish protein hydrolysates against oxygen radicals (Girgih et al., 2015; Nasri et al., 2013; Halldorsdottir et al., 2014). Those radicals have the ability to abstract hydrogen from polyunsaturated fatty acid, thereby inducing lipid oxidation (Yarnpakdee et al., 2014). Lipid peroxidation is an oxidative chain reaction in which one lipid molecule after another becomes oxidised to the maximum possible extent so as to form lipid peroxides (Alashi et al. 2014). The role of antioxidants during lipid peroxidation is to reduce the peroxyl radical to the hydro-peroxide before it can propagate the radical chain (Alashi et al. 2014). Lipid peroxidation proceeds via a free radical-mediated process in biological systems and thereby resulting in an oxidative alteration of polyunsaturated fatty acids in the cell membranes. In particular, lipid peroxidation in foods led to the development of undesirable off-flavors, potentially toxic substances and deterioration (Park et al., 2001).

Fig. 3 shows the values (in relative area) of the competence of common carp by-product protein hydrolysates to reduce peroxil radicals. Remembering that high relative area means low antioxidant competence and viceversa, it can be seen in Fig. 3 that P15 showed significantly ($p<0.05$) lower antioxidant capacity when compared with A10, A15 and P10. These results indicate the capacity of the common carp protein hydrolysates to scavenge peroxyl radicals.

3.3.3 Determination of thiols and disulfide bond linkages

The imbalance between the oxidation and reduction reactions due to an increase in ROS or a decrease in detoxification capacity contributes to the alteration of the thiol/ disulfide redox status (Moriarty-Craige and Jones, 2004).

The amino acid cysteine has a thiol group that can act as an electron donor against peroxyl radicals through interconversions between the sulfhydryl and disulfide groups (Atmaca, 2004). The sulfhydryl group is present in the amino acid cysteine and it may act as radical scavenger as an electron donor (Flora, 2009). Moreover, cysteine is a limiting amino acid in the synthesis of reduced glutathione (GSH). This tripeptide possesses its active site in the sulfhydril group and acts in the cells as one the main antioxidant defense and also participates in the detoxification of chemical agents and the elimination of lipoperoxidation products, among others (Birben et al., 2012). In this way, the decrease in sulfhydryl groups results from the formation of disulfide bonds through oxidation of these sulfhydryl groups or through disulfide interchanges (Leelapongwattana et al., 2005).

The results of the determination of thiols and disulfide bond linkages are shown in Fig. 4. P10 exibited the highest concentration of P-SH ($p<0.05$). However, common carp by-products hydrolyzed using Protamex exhibited higher percentage of P-S-S

($p<0.05$). This results could indicate a prooxidant condition during the hydrolysis process with the enzyme Protamex. On the other hand, A10 and A15 evidenced peptides with higher sulfhydryl groups retained. These results suggest that the higher antioxidant activity presented by A15 in the DPPH radical scavenging and ACAP assays could be related to its higher concentration of sulfhydryl bonds.

3.4 Cell culture

In this study, the hydrolysates A15 (due to its high DPPH radical scavenging activity, high antioxidant competence against peroxil radicals and higher concentration of sulfhydryl groups retained after hydrolysis process) and P15 used for comparison purposes, as it was the sample that presented the lowest antioxidant competence against peroxil radicals, were selected for the cell culture assays.

3.4.1 Effect of protein hydrolysates on HT-22 cells viability

Oxidative stress has been implicated in the progression of many neurodegenerative diseases including Alzheimer's disease (Chen et al. 2012). Following this concept, HT22 is an immortalized mouse hippocampal neuronal cell line that can be employed in the study of neuroprotection by antioxidant molecules. The hippocampus is a vertebrate brain structure, which is responsible for consolidating short-term memories into long-term memories. Therefore, the hippocampal cell line HT-22 is considered an extremely useful model for the study of diseases related to the death of neuronal cells by oxidative damage (El-Orabi et al., 2011; Ramos et al., 2016).

Results of cell viability are shown in Figure 5. Carp by-product protein hydrolysates A15 and P15 presented no cytotoxicity. Moreover, it was found that A15 and P15, even at the lowest concentration (0.03 mg/mL) greatly promoted the growth of HT-22 cells and/or mitochondria dehydrogenases activity. The MTT assay measures the

cell viability by the formation of formazan cristals (product that results from cellular mitochondrial dehydrogenases activity). In this way, increasing cell viability were reported in HepG2 cells treated with oat protein derived peptides (Du et al., 2016) as well as in in hamster ovary cells treated with yeast, soy, and broadbean hydrolysates (Lee et al., 2009). Therefore, the common carp by-product protein hydrolysates could be used for further analysing the intracellular ROS concentration.

3.4.2 Measurement of intracellular ROS concentration

Reactive oxygen species are highly reactive molecules generated continuously during aerobic metabolism. In excessive amounts, ROS can lead to lipids, protein and DNA oxidation, protein cross-linking and cell death. Thus, cell culture models provide a valuable tool for understanding the mechanisms that lead to cell death and the mechanisms that contribute to the generation of different diseases (Uy et al., 2011).

Results of ROS intracellular concentration are represented in Figure 5b. HT-22 cell exposure to the hydrolysates A15 and P15 for 24 h increased ROS concentration at the lowest concentration ($p<0.05$). It is known, that a pro-oxidant condition generates an antioxidant response (Rahal et al., 2014) and it is possible that the lower concentration of hydrolysates needed more time to generate an antioxidant response. However, at higher concentrations of hydrolysates, the intracellular concentration of ROS in HT-22 cells returned to baseline levels and in the case of P15 there was a significant ($p<0.05$) decrease in intracellular ROS production at the highest hydrolysate concentration. This decrease in intracellular ROS could be due to the amino acid constituents and the sequence of the peptides of the hydrolysate. Some authors reported that the presence of hydrophobic amino acids and histidine, proline, methionine, tyrosine, lysine, phenylalanine, and cysteine has been shown to enhance the potency of antioxidant

peptides through proton-donation ability, electron-donation ability, and/or direct lipid radical scavengers (Je et al., 2007; Samaranayaka and Li-Chan, 2011; Sarmadi and Ismail, 2010). Moreover, it has been demonstrated that acidic amino acids such as glutamic acid and aspartic acid exhibit strong antioxidant ability due to carboxyl and amino groups in the side chains as chelator of metal ions (Sarmadi and Ismail, 2010; Udenigwe and Aluko, 2012). Several amino acids, such as Trp, Tyr, His, Leu, and Cys, have been reported to exhibit radical-scavenging activities (Chen et al., 1996; Park et al., 2001). In addition, the carboxylic and amino groups in branches of acidic (Asp and Glu) and basic (Arg, Lys, and His) amino acids have been reported to enhance metal chelation through their charged properties (Liu et al., 2010).

However, an antioxidant regulation in biological systems is not limited to the elimination of ROS by radical scavenging or reducing compounds. but also includes the upregulation of antioxidant enzymes, modulation of redox cell signaling and gene expression, among others mechanisms (Jones, 2006). After the determination of the antioxidant activity with an *in vitro* chemical method it is very important to proceed with the cellular assays in order to address aspects such as the bioavailability of the antioxidant potential, absorption, partition in the membranes, which are crucial factors for determining the antioxidant activity *in vivo* (López-Alarcón and Denicola, 2013).

This study demonstrrated that *in vitro* assays showed different results than the assays with HT-22 cell line, where P15 showed the highest ($p<0.05$) antioxidant activity which possibly is associated with differences in the permeability and uptake of the different types of peptides.

4 Conclusion

This study has shown that protein hydrolysates of common carp by-products possess *in vitro* antioxidant properties as evidence their strong DPPH radical scavenging and also by their antioxidant capacity against peroxyl radicals. Although both enzymes have undergone good extent of protein degradation by Alcalase and Protamex mediated hydrolysis, the enzyme Alcalase showed higher substrate affinity leading to higher DH. Moreover, the hydrolysate produced with Alcalase with 15% DH presented higher *in vitro* antioxidant capacity. On the other hand, the hydrolysate produced with Protamex with 15% DH showed the lowest ROS production in HT-22 cells after 24h exposure. Further purification and characterization of these hydrolysates could elucidate the discrepance between *in vitro* and cell culture methods for the determination of the antioxidant activity.

Finally, this work demonstrated that it is feasible to produce hydrolysates that demonstrate antioxidant activity from common carp by-products. These hydrolysates are molecules with less potential risk than synthetic antioxidants and could be suitable for the use in the food industry in order to prevent oxidation of lipids as well as they present potential for the production of functional or nutraceutical foods. Animal models and human studies are more appropriate but more costly and time-consuming, making cell culture assays very attractive as an intermediate test method. However, for a better understanding of the processes and their application in products, further *in vivo* studies in animal models would be necessary to evaluate the possible health benefits of hydrolysates as well as their effect on the sensory properties in food products.

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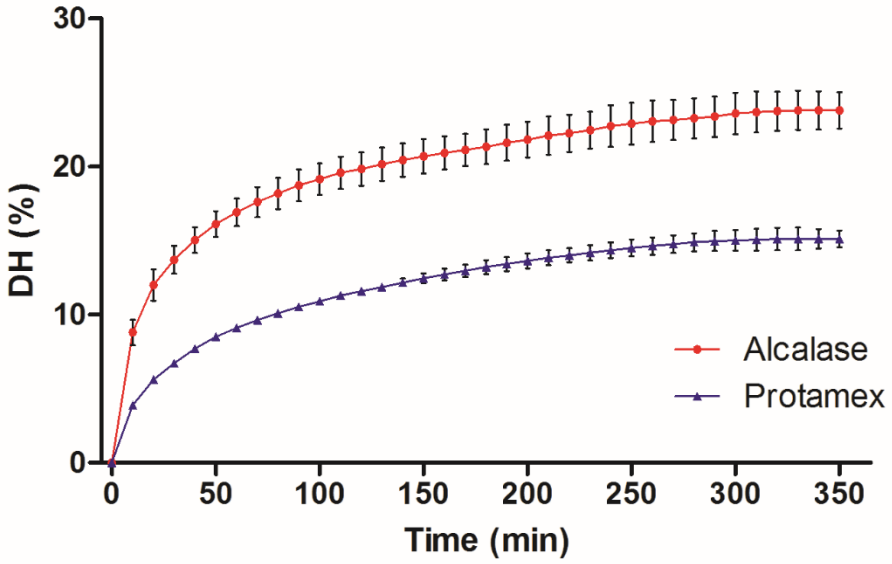

Fig. 1 Degree of hydrolysis (DH) curve from common carp by-products treated with Alcalase and Protamex (pH 8.0; 50 °C). Values presented are the mean value ± standard desviation of triplicate hydrolysis procedures.

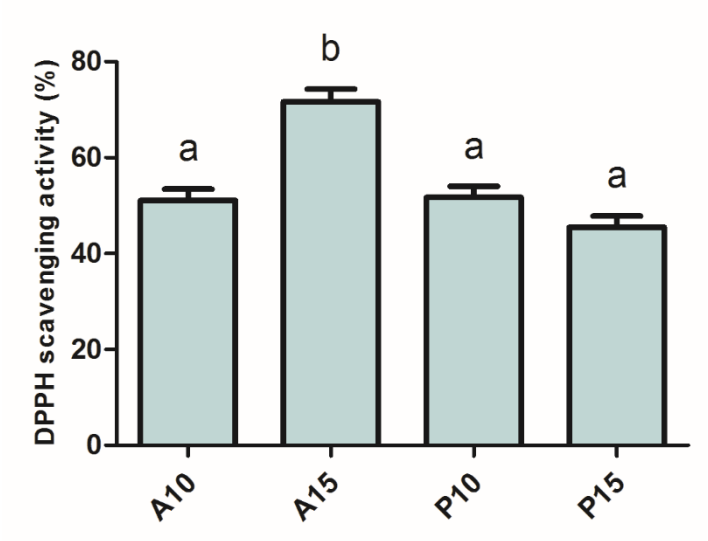

Fig. 2. DPPH radical scavenging activity of protein hydrolysates (4 mg protein/mL) obtained from *C. carpio* by-product using the enzymes Alcalase (A) or Protamex (P) to degree of hydrolysis of 10 and 15%. Data are expressed as mean±1 SE (n=4). Identical letters indicate absence of statistical differences ($p>0.05$).

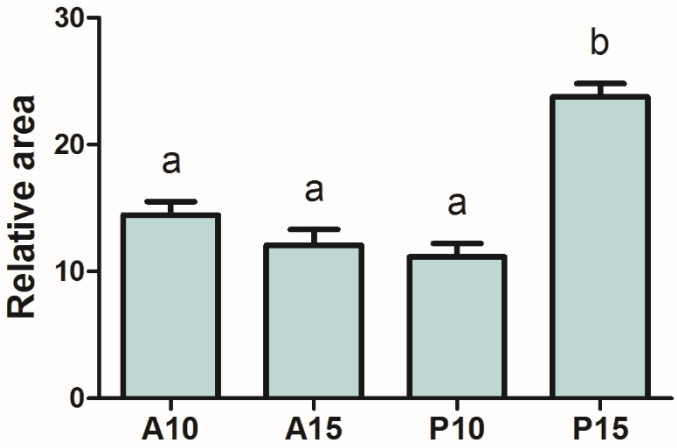

Fig 3. Total antioxidant capacity against peroxyl radicals (expressed as relative area) of protein hydrolysates (4 mg protein / mL) obtained from *C. carpio* by-product using the enzymes Alcalase (A) or Protamex (P) to degree of hydrolysis of 10 and 15%. Data are expressed as mean±1 SE (n=4). Identical letters indicate absence of statistical differences ($p>0.05$).

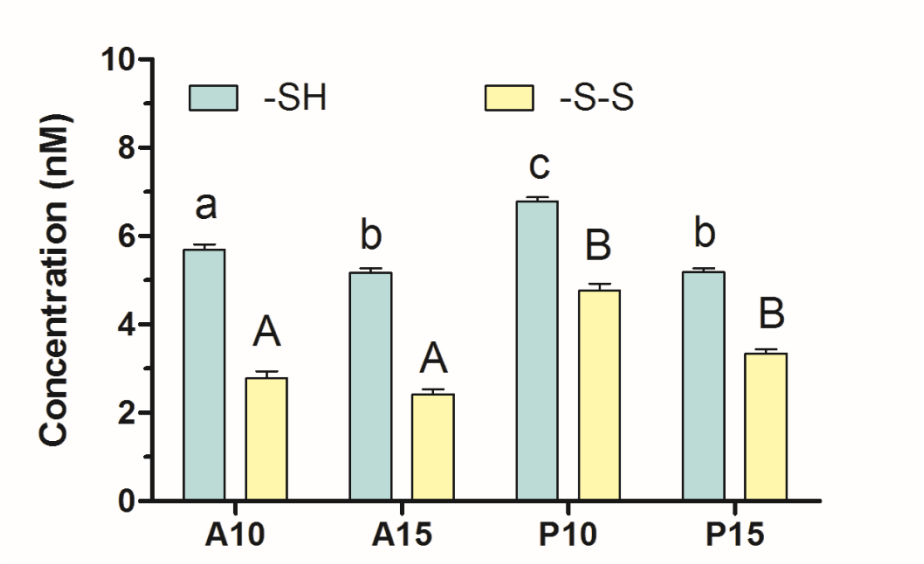

Fig. 4. Total concentration of sulfhydryl (P-SH) groups and percentage of disulfide bonds (P-S-S) present in protein hydrolysates (4 mg protein / mL) obtained from C. carpio by-product using the enzymes Alcalase (A) or Protamex (P) to degree of hydrolysis of 10 and 15%. Data are expressed as mean±1 SE (n=4). Upper cases are referred to total disulfide bonds. Lower cases are referred to percentage of sulfhydryl groups Identical letters indicate absence of statistical differences ($p>0.05$)

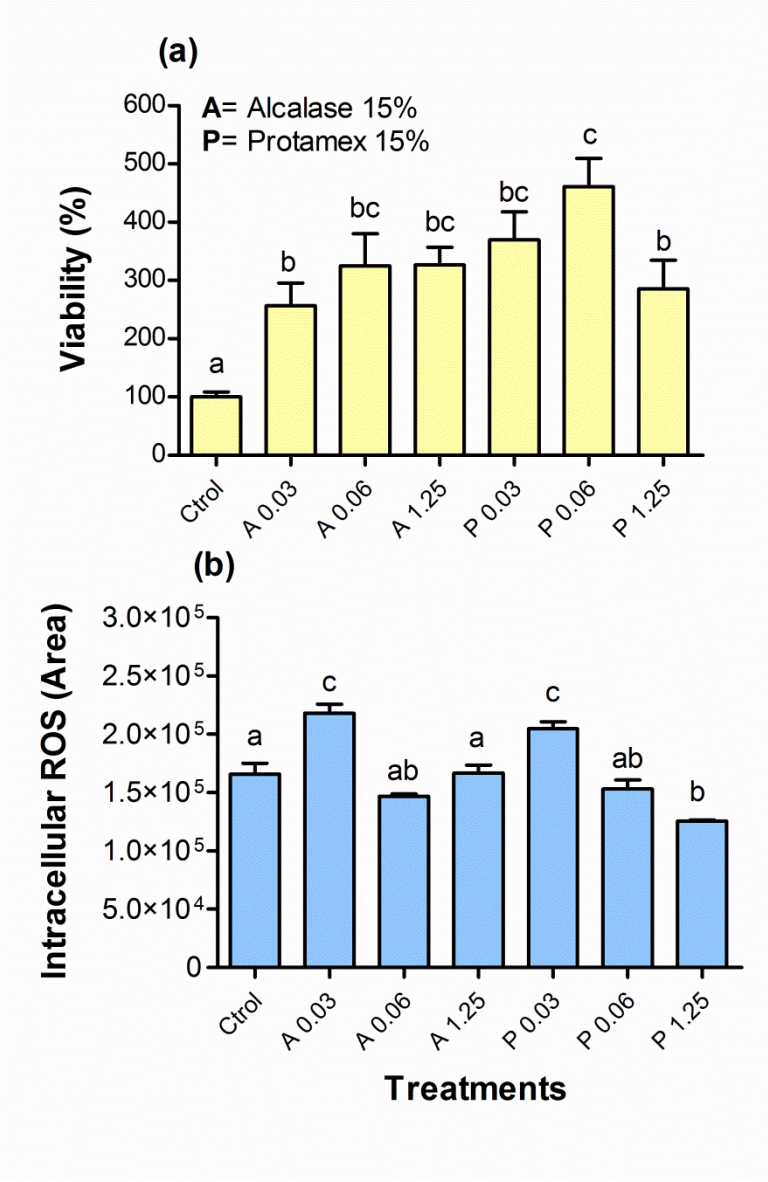

Fig. 5. (a) Cell viability (%) measured by the 3(-4,5-dimethylthiazol-2-yl)-2,5-difenyltetrazolium (MTT) and **(b)** intracellular reactive oxygen species (ROS) concentration in HT-22 cells after exposures of 24 h. Data are expressed as mean ± standard error. Indenticalletters indicate absence of significant difference ($p > 0.05$). Ctrol = control group; A = Alcalase 15% DH; P= Protamex 15% DH. Numbers 0.03, 0.06 and 1.25 (mg/ml) are the hydrolysate concentrations used in the assays.

CAPÍTULO III

EFFECT OF DIETARY COMMON CARP BY-PRODUCT PROTEIN HYDROLYSATES ON ANTIOXIDANT STATUS IN DIFFERENT ORGANS OF ZEBRAFISH (*Danio rerio*)

Artigo formatado para submissão segundo as normas da revista *Aquaculture Nutrition*

Obs: Foi adicionado resumo em portugues

Effect of dietary common carp by-product protein hydrolysates on antioxidant status in different organs of zebrafish (*Dario rerio*)

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Resumo

Foi realizado um ensaio de alimentação para avaliar o efeito da suplementação dietética de hidrolisados proteicos de subprodutos da carpa comum (Cyprinus carpio) sobre o estado antioxidante do zebrafish (Danio rerio). O subproduto da carpa comum foi hidrolisado utilizando Alcalase até um grau de hidrólise de 15%. Os zebrafish foram alimentados durante 44 dias com quatro dietas com níveis crescentes de hidrolisados de subprodutos de carpa (CBH0: 0g Kg^{-1}; CBH25: 25g Kg^{-1}; CBH50: 50g Kg^{-1}; CBH100: 100g Kg^{-1}). Ao término do ensaio de alimentação foram coletadas as brânquias, cérebro e músculos dos peixes-zebra para avaliar a capacidade antioxidante total contra radicais peroxil (ACAP) e peroxidação lipídica (TBARS). Embora no músculo a atividade antioxidante total não mostrasse diferenças ($p > 0.05$), a peroxidação lipídica foi reduzida no músculo dos peixes alimentados com a dieta CBH0. A peroxidação lipídica do cérebro foi reduzida ($p <0,05$) em todos os grupos quando comparada com a dieta controle CBH0. A redução na peroxidação lipídica no músculo do peixe-zebra é um resultado promissor com aplicações potenciais na formulação de rações para aquicultura que ajudem a melhorar o tempo de validade dos files de pescado. Além disso, a diminuição da peroxidação lipídica cerebral destaca o potencial dos hidrolisados proteicos de subprodutos de pescado como nutracêuticos e/ou como ingredientes para alimentos funcionais para consumo humano que podem ajudar na prevenção de doenças neurodegenerativas.

Palavras chave: Atividade antioxidante contra radicais peroxil (ACAP), Brânquias, Cérebro, Músculo, TBARS.

Abstract

A feeding trial was conducted to evaluate the effect of dietary protein hydrolysates from common carp (*Cyprinus carpio*) by-products on the antioxidant status of zebrafish (*Danio rerio*). Common carp by-product was hydrolyzed using Alcalase to degree of hydrolysis of 15%. The zebrafish were fed for 44 days with four different diets with increasing levels of carp by-product hydrolysates (CBH0: 0g Kg^{-1}; CBH25: 25g Kg^{-1}; CBH50: 50g Kg^{-1}; CBH100: 100g Kg^{-1}). The gills, muscle and brain was dissected at the end of the feeding trial in order to evaluate the total antioxidant capacity against peroxyl radicals (ACAP) and lipid peroxidation (TBARS). Although total antioxidant capacity did not show differences in muscle ($p > 0.05$), lipid peroxidation was reduced in the muscle of fish fed CBH50 diet. Brain lipid peroxidation was reduced ($p < 0.05$) in all groups when compared with the control diet CBH0. The reduction in muscle lipid peroxidation in zebrafish is a promissory result with potential applications in the formulation of aquaculture diets that could enhance the quality and shelf of fish fillets. The decrease in brain lipid peroxidation highlights the potential of fish protein hydrolysates as nutraceutics for functional foods that may help the prevention for neurodegenerative diseases.

Keywords: Antioxidant capacity against peroxyl radicals (ACAP), Brain, Gills, Muscle, TBARS.

1. Introduction

Fish industry, including aquaculture, is one of the most important economic and alimentary resources and in many countries around the world with approximately 140 million tons of fish production, in which 110 million tons are for human consumption (Benhabiles et al. 2012). The sector of fisheries and aquaculture generates large amounts of food grade products, also called by-products, after primary production of food products such as steaks, snacks, etc. The common carp (*Cyprinus carpio*) is a freshwater species whose production in ponds for human consumption began in China 3,000 years ago, and nowadays is widely produced in more than 100 countries around the world, due to its rapid growth rate and easy cultivation (Guler et al. 2008). Moreover, is estimated that freshwater species, such as carp, catfish and tilapia, will account for most of the increase in aquaculture production and represent about 60 % of total aquaculture production in 2025 (FAO 2016).

Currently, in the industrial processes most of the products are disposed as waste or used in low value food applications (Aspevik et al. 2016). Moreover, these underutilized fish by-products which include head, skin, trimmings, fins, frames, viscera and roe account for more than 60% of total biomass (He et al. 2013). Furthermore, discards of fish by-products have a great ecological impact and also significantly affect the economic viability of the fishing and aquaculture sector. In this regard, the European Commission is carrying out modifications in the common fishery policy in order to the complete elimination of discards. Technical solutions are required to use fish by-products as raw materials for the production of added-value compounds (Morales-Medina et al. 2016).

Fish processing by-products can be a great source of added value products such as proteins, amino acids, collagen, gelatin, oils, and enzymes (Ghaly et al. 2013). The crude protein content of fish by-products varies from 8-35% which is up to 10-20% (w/w) of total fish protein (Sila & Bougatef 2016). In order to recovery protein fish by-products and the release of bioactive peptides that benefits human health, several methods such as acid or alkaline hydrolysis, autolysis and enzymatic hydrolysis have been developed (Kim & Wijesekara 2010; Ghaly et al. 2013). However, enzymatic hydrolysis is the preferred technique to produce bioactive protein hydrolysates since is a fast and controllable method in which several proteolytic enzymes are used to cleave the protein of the source, releasing amino acids and peptides with functional, nutritional, and biological properties such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, antimicrobial, among others (Hsu et al. 2011; Ahn et al. 2012 , Intarasirisawat et al. 2013; Centenaro et al. 2014; Ennaas et al. 2015; Abdelhedi et al. 2016; Roblet et al., 2016).

A great variety of physiological disorders and diseases are correlated to the disequilibrium between the accumulation of reactive oxygen species (ROS) and the capacity of the antioxidant defenses (Roberts & Sindhu 2009). In this regard, the oxidative stress starts when the formation of ROS exceeds the antioxidant defenses capacity (Amado et al. 2009). In fact, oxidative stress is related to a number of deleterious processes such as protein damage, lipid peroxidation, enzyme inactivation and DNA breakage, which favor the manifestation of various diseases (Sohal 2002; Klaunig & Kamendulis 2004). The utilization in processed food of synthetic antioxidants as butylated hydroxy-anisole (BHA) and butylated hydroxytoluene (BHT) has begun to be limited due to their potential health hazards and toxicity (Centenaro et

al. 2014; Sabeena-Farvin et al. 2014). Protein hydrolysates and peptides from fish by-products have shown antioxidant activities and they can be considered as potential substitutes of synthetic antioxidants and as food supplements to reduce oxidative processes as well as ingredients for producing foods that have a potentially positive effect on health beyond basic nutrition, also known as functional foods (Frankel & Meyer 2000; Wiriyaphan et al. 2012; Chi et al. 2015a). In this regard, nutraceutics from fish by-product are gaining more and more attention nowadays. However, only a limited number of studies have been conducted to date for assessing the biological antioxidative potential of fish protein hydrolysates using cell cultures, animal models or human clinical trials. Nevertheless, results from these kind of studies provide great promise that these peptidic antioxidants could have an impact on reducing oxidative stress as well as the risk of various degenerative diseases such as cancer, cardiovascular disease, inflammatory diseases, etc. associated with oxidative stress (Samaranayaka & Li-Chan, 2011).

Moreover, due their high nutritional value and bioactive properties, fish protein hydrolysates have been used to complement or partially replace the fishmeal in aquaculture diets. In this way, during the last decade several works have been conducted to investigate the effect of the addition of fish protein hydrolysates in the diets of larvae and juveniels of different fish species such as yellow croaker (*Larimichthys crocea*) (Cai et al. 2015), European sea bass (*Dicentrarchus labrax*) (Kotzamanis et al. 2007) and Asian seabass (*Lates calcarifer*) larvaes (Srichanun et al. 2014) as well as red sea bream (*Pagrus major*) (Khosravi et al. 2015) and turbot (*Scophthalmus maximus*) juveniles (Zheng et al., 2013; Xu et al. 2016) among others.

The characteristics of zebrafish (*Danio rerio*) make it an attractive model organism for *in vivo* research studies. One of the great advantages of this biological model is that various organ systems from zebrafish are remarkably similar to those of humans as well as it has a very similar genetic structure to humans (Dooley & Zon 2000; Howe et al. 2013). Moreover, the zebrafish's blood-brain, the major obstacle for drug delivery to the brain as well as to protect the brain from toxic substances, is similar to that of higher vertebrates (Jeong et al. 2008). Also, zebrafish is inexpensive to maintain when compared to other vertebrate model organisms (i.e. mice), produce a large progeny, and they have a fast development.

The objective of this study was to evaluate the effect of the addition of different dietary levels of common carp by-product protein hydrolysate (CBH) on the antioxidant and energetic metabolism and lipid metabolism of zebra fish, with the dual aim of testing the applicability of common carp by-product protein hydrolysate in aquaculture diets as well as their potential application as nutraceutics or ingredients for the formulation of functional food for human consumption.

2. Material and methods

2.1 Protein hydrolysates preparation

Common carp (*Cyprinus carpio*) by-products consisting in head, viscera, bones and skin were obtained from the fish producer Piscicultura Andreghetto (Ajuricaba, RS, Brazil). After arrival to the laboratory, the by-product were blended and packaged in sealed plastic bags and stored at -20 °C till further use. Before the hydrolysis process, raw material (by-product) was thawed overnight at 4 °C. The raw material was homogenized with two volumes of distilled water (w/v) in a jacketed bioreactor (MA502, Marconi, SP, Brazil) at 270 rpm following by heating the slurry at 85 °C for

15 minutes in order to inactivate the endogenous enzymes. Subsequently, the slurry was hydrolyzed using Alcalase 2.4 L® (kindly donated from Novozymes, Bagsvaerd, Denmark). The hydrolysis was conducted at pH 8.0 and 50 °C, while enzyme-substrate ratio was set at 2 % (w/w) to reach degree of hydrolysis (DH) of 15 % to produce the sample used in this work. The DH was calculated from the amount of NaOH added to keep the pH constant during the hydrolysis (Adler-Nissen 1984).

After incubation the samples were submitted in a water bath at 90 °C for 15 min to inactivate the enzyme. Then, samples were centrifuged (Hanil 22K, Gwangju, South Korea) at 16,300 x g for 15 min at 4 °C, and the supernatants were frozen (Indrel, SP, Brazil) at -84 °C and subsequently freeze dried (L108, Liotop, SP, Brazil) and stored at -20 °C.

Total protein content of common carp by-product hydrolysate was assayed according the Hagen et al. (1989). The total amino acid composition of common carp protein hydrolysate (except tryptophan) was measured by method described by White et al. (1986). Tryptophan concentration was measured according Lucas & Sotelo (1980).

2.2 Experimental diets

The formulation and proximate composition of the experimental diets for the zebra fish are shown in Table 1.

Four isolipidic (11% lipids) and isoproteic (50% crude protein) diets were formulated to have increasing levels of common carp by-product protein hydrolysates (CBH0: 0 g kg^{-1}; CBH25: 25 g kg^{-1}; CBH50: 50 g kg^{-1}; CBH100: 100 g kg^{-1}). Diets were prepared by initially mixing and homogenizing the dry ingredients and subsequently adding the oils. The mixture was moisturized and the homogenate forced

through a 2 mm meat grinder and subsequently it was oven-dried at 50 °C for 24 h. The dry pellet was manually crushed to 1.0 mm and stored at -20 °C until used.

Diet dry matter (DM) was obtained by keeping the samples at 105 °C for 5 h. Ash content was determined after sample incineration at 600 °C for 5 h. Lipid content was determined using ether extraction with a Soxhlet extractor. Crude protein content was determined using the Kjeldahl method (N x 6.25). All analyses followed standard procedures from the Association of Official Analytical Chemists (AOAC 2005).

2.3 Fish, rearing conditions and feeding trial

This work agrees with ethic norms of animal experimentation and was previously evaluated and approved by the Ethical Committee of Federal University of Rio Grande - FURG (CEUA, protocol number P036/2015).

The zebrafish used in the feeding trial were purchased from Red Fish (Porto Alegre, RS, Brazil). Prior to the feeding trial, the fish were acclimated to laboratory conditions and to control (CBH0) diet for 2 weeks. Afterward, a total of 240 adult zebrafish (initial body weight 0.51 ± 0.13 g) were randomly distributed into 12 tanks containing 10 L of water at a stock density of 20 fish per tank (1 g L^{-1}). The experimental tanks were arranged in an indoor recirculating aquaculture system. Experimental tanks were connected to a filter tank filled with zeolite, bio ceramic, an UV lamp (75 W) and a heater thermostate. The water exchange rate was 3 L min^{-1} $tank^{-1}$. Each diet was randomly assigned to triplicate tanks. The fish were fed twice daily. A prior feed intake test was carried out. Since the feed intake was not affected by the diets as well as did not show statistical differences between the treatments, the quantity of food offered was adjusted to the amount consumed by the group with lower consumption (1.25 g d^{-1}). The accumulations of feces at the tank bottoms were siphoned

twice daily. The photoperiod was adjusted to 14:10 h, light/dark regime. During the rearing period, water temperature was kept constant at 28 ± 0.4 °C. Water parameters pH (7.25 ± 0.13) and dissolved oxigen (> 6 mg L^{-1}) were measured daily in all tanks. Chemical parameters were measured once per week. The amounts of nitrogenous compounds (0.09 ± 0.18 mg ammonium L^{-1}, 0.12 ± 0.32 mg nitrite L^{-1} and 10.65 ± 2.43 mg nitrate L^{-1}) were maintained within the requirements for zebrafish reported by Westerfield (1995).

2.4 Sample collection

At the end of the feeding trial, all fish were fasted for 24h before harvest. Subsequently, all zebrafish were anaesthetized with tricaine methanesulfonate (50 mg/L) (Sigma-Aldrich, MO, USA) and weighed. Afterwards, the fish were euthanized by cervical dislocation, and the brain, gills and muscle from all specimens were dissected and weighed. Pools of tissue samples (brain, gills and muscle) of five zebrafish were homogenized (1:5 w/v) in Tris–HCl (100 mM, pH 7.75) buffer plus EDTA (2 mM) and Mg^{2+} (5 mM) (da Rocha et al. 2009). The homogenate pools were centrifuged at 10,000 g for 20 min at 4 °C and the supernatant was collected and stored at -80 °C for further analysis. Previously, total protein content of all pools was determined using a commercial kit based on the Biuret method (Doles®), using a microplate reader (Biotek ELX 800®) at a wavelength of 550 nm.

2.5 Antioxidant analysis

2.5.1 Determination of lipid peroxidation (TBARS)

The degree of lipid peroxidation was determined by quantification of the thiobarbituric acid-reactive species (TBARS) according to Oakes & Van der Kraak (2003). The method is based in the derivatization of malondialdehyde (MDA) (a by-

product from lipid peroxidation) with thiobarbituric acid, followed by determination of the fluorescence. The homogenized extract (40 μL, 80 μL and 200 μL for brain, gills and muscle respectively) was added to a reaction mixture containing 150 μL of 20 % (mass concentration) acetic acid, 150 μL of thiobarbituric acid (0.8 %), 50 μL of Milli Q water and 20 μL of sodium dodecyl sulfate (SDS, 8.1 %). The samples were heated to 95 °C for 30 min, and after cooling for 10 min, 100 μL of Milli Q water and 500 μL of n-butanol were added. After centrifugation (3,000 × g for 10 min at 15 °C), the organic phase (150 μL) was placed in a microplate reader and the fluorescence was registered after excitation at 520 nm and emission at 580 nm. The concentration of TBARS (nmol mg^{-1} of wet tissue) was determined using tetramethoxypropane (TMP, Acros Organics) as a standard.

2.5.2 Antioxidant capacity against peroxyl radical (ACAP)

This is a simple rapid and reliable method reported by Amado et al. (2009) based on the detection of ROS by fluorometry (ex/em: 485/520nm) using 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) as substrate. Peroxyl radicals were generated by thermal decomposition at 35 °C of 2,2′-azobis (2-methylpropionamidine) dihydrochloride (ABAP) in 50 μL of homogenized extract of gills or muscle. Samples antioxidant capacity against peroxyl radicals is monitored by the fluorescence emission caused by the reaction between ROS and deacetylated H_2DCF using a fluorescence microplate reader (Victor 2, Perkin Elmer). The thermal decomposition of ABAP and ROS formation was monitored for 30 min, with readings every 5 min.

Total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second order polynomial function. The results were expressed as area difference of FU×min in the

same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). Antioxidant capacity was considered as the relative difference between ROS area with and without ABAP (higher area difference meaning low antioxidant capacity). Relative area was calculated as follows:

$$(ROS\ area_{ABAP} - ROS\ area_{background}) / ROS\ area_{background}$$

2.6 Statistical analysis

All data were expressed as the mean ± standard error (SE). The differences between the treatments were evaluated using a one-way analysis of variance (ANOVA), with the treatments (CBH0, CBH25, CBH50 and CBH100) applied as factor. Post-hoc tests were employed using Newman-Keuls method. In all cases the significance level adopted was 5% (α=0.05). The ANOVA assumptions (normality by Shapiro-Wilks and variance homogeneity by Levene) were previously evaluated and tested with the same significance level (5%). Mathematical transformations were applied if at least one of the assumptions were not verified. A minimum of three biological replicates were performed for all analyses presented in this study.

3 Result and discussion

Most of the previous works focusing on the nutritive value of fish protein hydrolysates have been mainly carried out on larvae of different fish species (Masuda et al. 2013; Ovissipour et al. 2014; Cai et al., 2015). Thus, there is a lack of studies based on the application of fish protein hydrolysates in diets for juvenile and growing-out stages (Xu et al. 2016). However, as far as we know, there are no previous studies evaluating the antioxidant activity of protein hydrolysates in the fish diet.

The amino acid constituents and the sequence of the peptides are very important factors that determine their antioxidant activity (Je et al. 2007). Besides from the

properties of the substrate hydrolyzed, the choice of the enzymatic treatment and the processing conditions as temperature, pH and enzyme/substrate ratio, the extent of the hydrolysis reaction has an important significance on the release of antioxidant peptides (Laroque et al. 2008; García-Moreno et al. 2014). Hydrophobic amino acids as well as His, Met, Tyr, Lys and Cys possess the ability to act as electron or proton donors as well as the ability to scavenge lipid radicals, thereby enhancing the antioxidant capacity of the peptides (Je et al. 2007; Sarmadi & Ismail 2010; Samaranayaka & Li-Chan 2011). The amino acid content of the common carp by-product protein hydrolysate is shown in Table 2. In this study, the presence of high contents of hydrophobic amino acids (35.8 %) in the hydrolysate offers structural properties that may enhance interactions with lipids radicals. Moreover, it has been demonstrated that acidic amino acids such as glutamic acid and aspartic acid exhibit strong antioxidant ability due to carboxyl and amino groups in the side chains as chelator of metal ions (Sarmadi & Ismail 2010; Udenigwe & Aluko 2011). In addition, the carboxylic and amino groups in branches of acidic (Asp and Glu) and basic (Arg, Lys, and His) amino acids are able to increase metal chelation through their charged properties (Liu et al. 2010). As can be seen in Table 2, the hydrolysate from common carp by-products showed high amounts of essential amino acids such (up to 42.22 %). Thus, common carp hydrolysates represent an excellent nutritional source.

Some studies have shown that partial substitution at low levels of fishmeal by protein hydrolysates may promote growth in Atlantic salmon (Espe et al.1999; Refstie et al. 2004; Hevrøy et al. 2005). However, by exceeding a certain level of substitution, growth and feed efficiency can be compromised (Espe et al. 1999; Hevrøy et al. 2005). In addition, it has also been shown that soy protein hydrolysates may also help to

replace fish meal without adverse effects on starry flounder growth and even a substitution of 85 % did not affect growth compared to a whole fish meal-based diet (Song et al. 2014). In this work, all experimental diets were well accepted by the fish and no mortality was observed in any of the groups during the experimental period. Moreover, at the end of the 44 days feeding trial, growth performance of zebrafish were no significantly influenced by dietary treatments ($p>0.05$).

As a dynamic respiratory and osmorregulatory organ, fish gills are expected to possess a high capacity to produce ROS (Sinha et al. 2014). Total antioxidant capacity against peroxil radicals and lipid peroxidation in zebrafish fed with increasing levels of common carp by-product protein hydrolysates are shown in Fig. 1a and Fig. 1b. An increase (lower relative area) in the total antioxidant capacity was observed in the gills of all groups fed with dietary protein hydrolysates of common carp by-products ($p<0.05$). However, it was observed an increase in gill lipids peroxidation in fish fed with CBH50 and CBH100 ($p<0.05$). In this way, common carp by-product protein hydrolysates increased total antioxidant capacity ACAP in gills but this increase did not prevent the lipid peroxidation that, directly or indirectly, seems to be triggered by protein hydrolysates.

As can be seen in Fig. 2a and Fig. 2b, the reduction in the lipid peroxidation of the muscle of zebrafish fed with the CBH50 diet was not related to an increase in total antioxidant capacity. It could be hypothesized that this decrease in muscle lipid peroxidation could be due to changes in the saturation of fatty acids profile. In this regard, dietary proteins have a broad spectrum of biological functions that affect the metabolism of protein, glucose and lipid protein, as well as immunological and hormonal functions (Chou et al. 2012). In particular, dietary proteins play major a role

in mechanisms affecting fatty acid composition (Leveille et al. 1963; Bjørndal et al. 2013). Moreover, Vik et al. (2015) reported a decrease in liver n-3 PUFA and n-6 PUFAs in mice fed with a diet containing peptides obtained by hydrolyzing salmon by-products. Unsaturated fatty acids are the main molecules affected by oxidation reactions. Particularly, PUFAs in membrane phospholipids are the main targets in cellular redox reactions, causing irreversible cellular damage (Sahidi & Zhong 2010). The degree of unsaturation and the composition of their fatty acids are the major factors that influence the oxidative susceptibility of lipids (deMan 1999). Moreover, fish lipids are among the most oxidizable oils, mainly due to their high PUFA content (Sahidi & Zhong 2010). Furthermore, oxidation of lipids is one of the main causes of deterioration of fish muscle quality. Oxidation of lipids also causes rancidity, off-odors and off flavors which decrease the quality of the product and shorten the shelf life of the fillet during storage time (Secci & Parisi 2016). Fillet shelf-life may vary in virtue of the pro- and antioxidant levels of the fillet, which can be influenced through the diet. At present seems reasonable to suggest that fish fed with a diet containing common carp hydrolysates should increase the fillet storage time and shelf life. Nevertheless, further studies should be conducted to verify the effect of dietary protein hydrolysates in the degree of saturation of lipids in zebrafish muscle.

The results of brain lipid peroxidation of zebrafish fed increasing levels of CBH are presented in Fig. 3. Unfortunately, there was not enough tissue to measure the total antioxidant activity against peroxil radicals in brain of fish fed with experimental diets. In this regard, it was prioritized the measurement of lipid peroxidation in brain to evaluate the effective consequence of the inclusion of protein hydrolysates. Brain lipid peroxidation was lower in all groups fed with dietary protein hydrolysates ($p < 0.05$).

The central nervous system is relatively poor in antioxidant enzymes and is susceptible to lipid peroxidation due to its high oxygen consumption and high concentration of polyunsaturated fatty acids (PUFA) (Grotto et al. 2009). Moreover, it is demonstrated that lipid peroxidation causes deleterious effects in brain cell membranes (Stefanello et al. 2005; Adegunlola et al. 2012). Furthermore, brain cell membranes are rich in PUFA which may be oxidized by the association of free radicals released and lipid peroxidation; destroying the spatial arrangement of membranes and impairing the normal functioning of indispensable membrane enzymes in nerve cells as Na^+/K^+ ATPase (Kurella et al. 1999; Assis et al. 2007). Following this concept, lipid peroxidation could lead to several neurodegenerative diseases. Bourdel-Marchasson et al. (2001) found plasma malondialdehyde (MDA) levels higher in Alzheimer's disease patients than in healthy controls. Our previous studies demonstrated that protein hydrolysates produced from common carp by-products were able to reduce ROS formation in mouse hippocampal HT-22 cells (unpublished results). The *in vivo* approach of this work effectively showed that antioxidant dietary protein common carp by-product hydrolysates have the ability to cross the blood-brain barrier and to reduce the brain lipid peroxidation in zebrafish.

4 Conclusion

The diets containing increasing levels of CBH (2.5%, 5% and 10%) in substitution of fish meal did not compromise the growth. Moreover, this study showed that the diets supplied with CBH improved significantly the total antioxidant capacity ACAP in gills of zebrafish. Besides, the results demonstrated that the hydrolysis of common carp by-product had a positive effect in muscle and brain by lowering lipid peroxidation. The fact of having reduced the lipid peroxidation in zebrafish muscle is a

promissory result with potential applications in the formulation of aquaculture diets with natural antioxidant ingredients that could enhance the shelf life of the final products or increase storage time of fish fillets. In this regard, further research is needed in order to a better understanding of these effects.

Furthermore, since zebrafish is a model organism to study oxidative mechanisms and search for effective therapies to reduce detrimental effects of oxidative damage, we have to highlight the results of the decrease in brain lipid peroxidation as a precedent for future studies evaluating common carp protein hydrolysates as a nutraceutics or an ingredient for functional foods for human consumption that may help the prevention for neurodegenerative diseases as Parkinson or Alzheimer.

Finally, incorporating up to 5% of common carp by-product protein hydrolysates in zebrafish diets did not hamper the growth and decreased lipid peroxidation in brain and muscle. The present study mainly provides evidence of potential of utilization of fish by-products protein hydrolysates as functional ingredients, aiming the dual purpose of adding value to a low value by-product and reducing the environmental pollution related to a mismanagement of this raw material.

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Table 1. Ingredients and proximate composition of experimental diets for *Danio rerio*

Ingredients (g Kg^{-1})	**CBH0**	**CBH25**	**CBH50**	**CBH100**
Casein [a]	500	490	480	460
Fish meal [b]	150	125	100	50
Carp hydrolysate	0	25	50	100
Soybean oil	50	50	50	50
Rapeseed oil	50	50	50	50
Corn starch	200	200	200	200
Cellulose [c]	40	50	60	80
Min. and Vit. premix [d]	10	10	10	10
Proximate Composition				
Dry matter (DM)	93.16	93.75	93.96	93.46
Crude protein (%DM)	49.24	49.77	49.51	49.88
Crude lipid (%DM)	11.64	11.91	11.12	11.35
NFE (%DM) [e]	32.85	32.24	33.96	35.34
Ash (%DM)	6.27	6.08	5.41	3.43
Metabolizable Energy (MJ g^{-1}) [f]	18.09	18.17	18.12	18.50

a Synth (São Paulo, SP, Brazil).

b Fish meal analysed values (as % of dry matter): crude protein, 53.86; lipid, 6.25; ash, 35.28. Torquato Pontes (RS, Brazil).

c Rhoster (São Paulo, SP, Brazil).

d Premix M. Cassab, SP, Brazil (Vitamin A (500,000 UI kg^{-1}), Vit. D3 (250,000 UI kg^{-1}), Vit. E (5,000 mg kg^{-1}), Vit. K3 (500 mg kg^{-1}), Vit. B1 (1,000 mg kg^{-1}), Vit. B2 (1,000 mg kg^{-1}), Vit. B6 (1,000 mg kg^{-1}), Vit. B12 (2,000 mcg kg^{-1}), Niacin (2,500 mg kg^{-1}), Calcium pantothenate (4,000 mg kg^{-1}), Folic acid (500 mg kg^{-1}), Biotin (10 mg kg^{-1}), Vit. C (10,000 mg kg^{-1}), Choline (100,000 mg kg^{-1}), Inositol (1,000 mg kg^{-1}). Trace elements: Selenium (30 mg kg^{-1}), Iron (5,000 mg kg^{-1}), Copper (1,000 mg kg^{-1}), Manganese (5,000 mg kg^{-1}), Zinc (9,000 mg kg^{-1}), Cobalt (50 mg kg^{-1}), Iodine (200 mg kg^{-1})).

e Nitrogen free extract (including fibre): calculated by difference (100 - crude protein - crude lipid - ash).

f Calculated from the physiological standard values, where 1 kg of carbohydrate (N-free extract), protein and lipid yields 16.7, 16.7, and 37.6 MJ, respectively (Garling and Wilson, 1976)

Table 2. Total amino acid composition of common carp by-product hydrolysate (CPH) produced with Alcalase (degree of hydrolysis 15%) (unit: g/100 g protein)

Amino acids	CPH
Aspartic acid	10.04
Glutamic acid	14.23
Serine	4.54
Glycine	10.90
Histidine	2.11
Taurine	0.78
Arginine	6.84
Threonine	4.07
Alanine	7.12
Proline	6.71
Tyrosine	2.64
Valine	4.48
Methionine	2.56
Cysteine	0.88
Isoleucine	3.76
Leucine	6.80
Phenylalanine	3.49
Lysine	7.39
Tryptophan	0.58

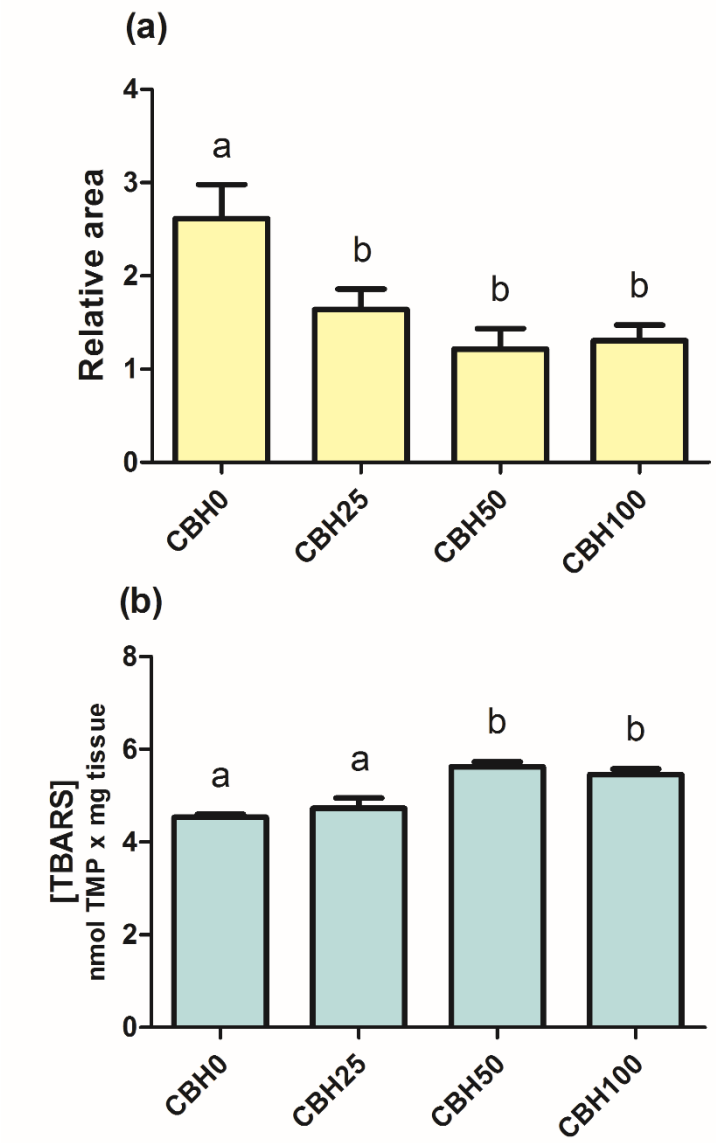

Fig. 1. a) Gill antioxidant activity against peroxyl radicals and **b)** Gill lipid peroxidation (TBARS) in of zebrafish (*Danio rerio*) fed diets with increasing common carp by-products protein hydrolysates (CBH0: 0g Kg^{-1}; CBH25: 25g Kg^{-1}; CBH50: 50g Kg^{-1}; CBH100: 100g Kg^{-1}). Data are expressed as mean±1 SE. Identical letters indicate absence of statistical differences ($p>0.05$).

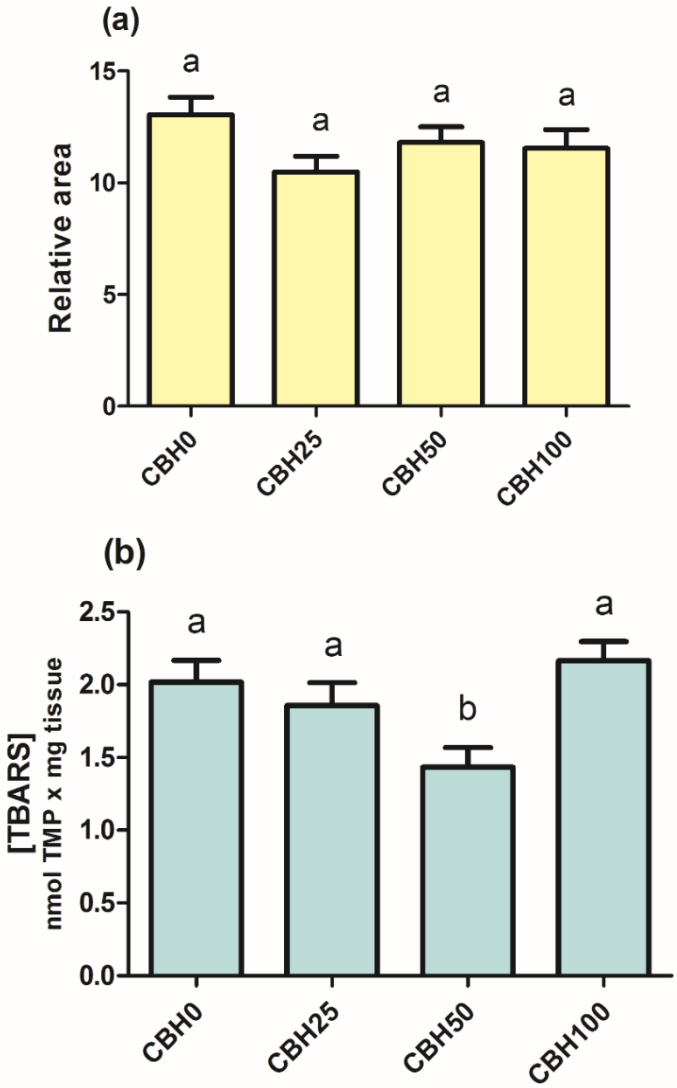

Fig 2. a) Muscle antioxidant activity against peroxyl radicals and **b)** Muscle lipid peroxidation (TBARS) of zebrafish (*Danio rerio*) fed diets with increasing common carp by-products protein hydrolysates (CBH0: 0g Kg^{-1}; CBH25: 25g Kg^{-1}; CBH50: 50g Kg^{-1}; CBH100: 100g Kg^{-1}). Data are expressed as mean±1 SE. Identical letters indicate absence of statistical differences ($p>0.05$).

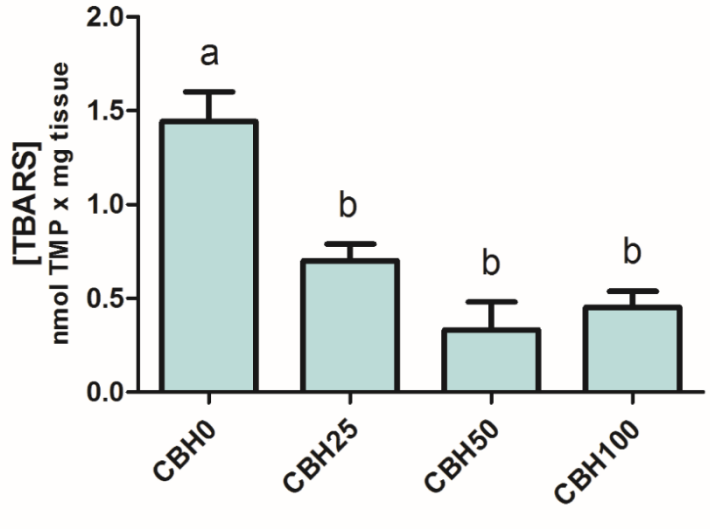

Fig. 3. Brain Lipid peroxidation (TBARS) in zebrafish (*Danio rerio*) fed diets with increasing common carp by-products protein hydrolysates (CBH0: 0g Kg^{-1}; CBH25: 25g Kg^{-1}; CBH50: 50g Kg^{-1}; CBH100: 100g Kg^{-1}). Data are expressed as mean±1 SE. Identical letters indicate absence of statistical differences ($p>0.05$).

CAPÍTULO IV

USING CITRUS PECTIN FOR THE PRESERVATION OF THE ANTIOXIDANT ACTIVITY OF COMMON CARP PROTEIN HYDROLYSATES DURING SPRAY-DRYING ENCAPSULATION

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Using citrus pectin for the preservation of the antioxidant activity of common carp protein hydrolysates during spray-drying encapsulation

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Resumo

O objetivo deste estudo foi avaliar o efeito da complexação com pectina de baixo grau de metoxilação (LM) e posterior microencapsulação por secagem por atomização na atividade antioxidante dos hidrolisados proteicos de carpa comum (*Cyprinus carpio*). Os hidrolisados proteicos mostraram máxima atividade antioxidante em condições acidas (pH 2.4) e foi avaliada a coacervação entre hidrolisados proteicos (1 g/L) e pectina LM (0-7 g/L) a esse pH. Quando a relação pectina LM/hidrolisados aumentou foi observado um aumento de turbidez em todas as amostras. As medidas de tamanho de partícula indicaram que os complexos formados com hidrolisados de subproduto de carpa tendiam a formar agregados maiores (de 261.23 ± 3.71 μm a 374.96 ± 11.29 μm) quando comparados com os complexos formados com hidrolisados de musculo (de 165.83 ± 2.59 μm a 236.18 ± 5.71 μm). Os complexos com maior e menor atividade antioxidante e as suas respetivas suspensões de hidrolisados sem pectina foram selecionadas para secagem por atomização e foi avaliada a posterior atividade antioxidante. Os resultados revelaram que a secagem por atomização não teve efeito significativo sobre a atividade antioxidante ($p > 0.05$) dos hidrolisados quando estes foram complexados com pectina LM. Pode ser concluído que a complexação de hidrolisados de carpa com pectina LM e posterior microencapsulação por secagem por atomização pode ser uma maneira eficiente de proteger a sua bioatividade.

Palavras chave: Coacervação complexa, DPPH, Hidrolisados proteicos de pescado, Microencapsulação, Subproduto.

Abstract

The aim of this study was to evaluate the effect of the complexation with low methoxyl (LM) pectin and subsequent microencapsulation by spray-drying, on the antioxidant activity of common carp (*Cyprinus carpio*) protein hydrolysates. Protein hydrolysates showed maximum antioxidant activity at acidic condition (pH 2.4) and the coacervation between protein hydrolysates (1 g/L) and LM pectin (0-7 g/L) at this pH was investigated. An increase in the ratio LM pectin/hydrolysates resulted in increased turbidity in all samples. Particle size measurements indicated that the complexes formed with carp by-product hydrolysates tended to form larger aggregates (ranging from 261.23 ± 3.71 μm to 374.96 ± 11.29 μm) when compared with complexes formed with carp muscle hydrolysates (ranging from 165.83 ± 2.59 μm to 236.18 ± 5.71 μm). Complexes having the highest and the lowest antioxidant capacity and their respective hydrolysates solutions without pectin were chosen for the spray-drying microencapsulation process and the antioxidant activity was measured. The results revealed that spray-drying did not have a significant effect ($p > 0.05$) on the protein hydrolysates antioxidant activity when they were complexed with pectin. It can be concluded that the complexation of carp protein hydrolysates with LM pectin and subsequent microencapsulation by spray-drying could be an efficient way to protect their antioxidant activity.

Keywords: Complex coacervation, By-product, DPPH, Fish protein hydrolysates, Microencapsulation.

1. Introduction

The formation of complexes coacervates by the electrostatic interactions of oppositely charged molecules is a fundamental phenomenon existing in numerous biological processes. Since biopolymer complexes combine the functional properties of each component, the resulting complexes functional properties are generally improved (Schmitt et al., 1998). Complex coacervates of proteins and polysaccharides are often used simultaneously in the food industry in order to enhance structure and stability of processed foods. The interactions between proteins and negatively charged biopolymers favour the formation of complexes which possess advantageous functional properties such as micro- and nano-encapsulation processes, multi-layers structures designing, formation of new food gels and stabilization of food emulsions as well as the recovery of proteins from industrial by-products (Turgeon et al., 2007; Lutz et al., 2009). Several authors demonstrated that mainly electrostatic interactions are responsible for the complex coacervation of oppositely charged biomolecules (Weinbreck et al., 2004; Aryee & Nickerson, 2012; Bayarri et al., 2014; Ben Amara et al, 2016). Nevertheless, these electrostatic interactions may be influenced by different factors such as pH, ionic strength, concentration, protein to polysaccharide ratio, charge distribution on the protein and relative hydrophobicity of molecules (Schmitt et al., 1998).

Pectin is a an anionic polysaccharide found in primary cell walls of terrestrial plants and the major citrus processing by-products with useful properties in many applications (Bierhalz et al., 2012). The chemical structure of this polysaccharide is a linear polymer of D-galacturonic acid units with their methyl esters connected through α-(1,4)-glycosidic bonds (Sriamornsak & Kennedy, 2008). Several recent works have reported complex coacervation of pectin and proteins such as lysozyme-pectin (Ben

Amara et al., 2016), β-lactoglobulin-pectin (Wang et al., 2007), bovine serum albumin-pectin (Ru et al., 2012), among others.

Fish processing by-products are fish material discarded from the primary processing of fish manufacturing process. The percentage of by-products generated in this process is about 50 % of the weight of the starting material (He et al., 2013). These by-products are an important source of high quality compounds that may be used for human consumption as well as they can be a great source of added value products such as proteins, amino acids, collagen, gelatine, oil and enzymes (Ghaly et al., 2013). In addition to their functional, technological, and nutritional properties, some fish proteins may exhibit antioxidant activity, which is associated with bioactive peptides present in certain protein sequences. In this way, enzymatic hydrolysis is one of the techniques that is currently being developed nowadays in order to release bioactive peptides (Zavareze et al., 2014). In this process, several proteolytic enzymes are commonly used to hydrolyse fish proteins to produce fish protein hydrolysates (FPH) and transforming them into high added-value products with functional and bioactive properties such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, antimicrobial, among others (Santos et al., 2011; Harnedy & FitzGerald, 2012 ; Centenaro et al., 2014). Particularly, protein hydrolysates and peptides from fish by-products have shown antioxidant activities, and can be used as alternatives to synthetic antioxidants to reduce the potential risks of synthetic antioxidants in the body and foods (Chi et al., 2015). Moreover, the addition of bioactive peptides from protein hydrolysates in a food matrix would give to the final product the characteristics of functional food (Harnedy & FitzGerald, 2012).

Microencapsulation is often used for the isolation of active substances in order to obtain products with spherical shape and micrometric size, in which the active material is shielded by a membrane that serves as protection from the surrounding environment (Nesterenko et al., 2013). Moreover, one of the benefits of encapsulation is the ability to control the release of incorporated ingredients and the delivery to a certain target at an appropriate time. The controlled release of ingredients can improve the efficiency of food additives, expanding the application range and ensuring optimal dosage (Zavareze et al., 2014). Moreover, the coating also protects the core material from moisture, light, oxygen, other food ingredients and additional external agents (Gharsallaoui et al., 2007). Encapsulation has been traditionally used in the food industry for delivery of bioactive compounds as polyphenols, carotenoids, antioxidants and fatty acids that are sensitive to environmental factors (Longaray-García et al., 2013; Fathi et al., 2014). However, encapsulation is not yet applied to the commercial production of protein hydrolysates and bioactive peptides. The protection of bioactive peptides from physiological alterations (such as the action of digestive enzymes) is essential for their promissory application in animal and human models. Therefore, encapsulation has become an important and relevant technology for the improvement of bioactive peptides to promote human health (Mohan et al., 2015).

Polysaccharides such as maltodextrin, gum arabic, chitosan and cyclodextrin are generally used for the encapsulation of proteins as inert carriers due to their abundance, low market price and stability during production and processing of encapsulated products. In this concern, spray-drying is the most common and cheapest technique to micro-encapsulate food additives due its low production costs and the availability of equipment and materials when compared to other production methods (Gharsallaoui et

al., 2007). On the other hand, exposure to high temperature, distribution of air-water interfaces, shearing, and dehydration can lead to irreversible structural changes of the protein and possibly alter peptide structure.

In this study, common carp (*Cyprinus carpio*) by-product and muscle (used for comparison purposes) were hydrolysed using the commercial proteases Alcalase® or Protamex® to degree of hydrolysis (DH) of 10% and 15%. Common carp is one of the most important species of freshwater fish, currently cultured in many countries and frequently consumed (Nihn et al., 2011). Unlike in the seafood industry, the freshwater fish processing industry presents numerous problems associated to by-product discarding (Bhaskar et al., 2008). The aim of this work was to evaluate the effect on the antioxidant activity of the complex coacervation of common carp hydrolysates using low methoxyl (LM) pectin and the microencapsulation by spray-drying.

2. Materials and methods

2.1. Materials

Common carp (*C. carpio*) was obtained from the fish producer Piscicultura Andreghetto (Ajuricaba, Rio Grande do Sul State, Brazil). After arrival to the laboratory, the fish were beheaded, eviscerated and filleted. Subsequently, and independently, the muscle and the by-product consisting in head, viscera, bones and skin were blended and packaged in sealed plastic bags and stored at -20 °C until further use. Alcalase 2.4 L® was kindly donated by Novozymes (Bagsvaerd, Denmark). Protamex® was purchased from Sigma Aldrich (MO, USA) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) from Alfa Aesar-Thermo Fisher Scientific (Lancashire, United Kingdom). Analytical grade absolute ethanol was purchased from Chimie-plus Laboratoires (Saint Paul de Varax, France). Low methoxyl amidated pectin

(Unipectine™ OF 305 C SB, degree of esterification from 22% to 28% and degree of acetylation from 20% to 23%) was purchased from Cargill (Baupte, France). Analytical grade imidazole ($C_3H_4N_2$), acetic acid, sodium hydroxide (NaOH), and hydrochloric acid (HCl) were purchased from Sigma-Aldrich Chimie (St Quentin Fallavier, France). Maltodextrin DE 19 was obtained from Roquette-Frères SA, (Lestrem, France). Deionized water was used for the preparation of all solutions.

2.2. Preparation of protein hydrolysates

Raw material (muscle or by-product) was thawed overnight at 4°C. The raw material was homogenized with two volumes of distilled water (w/v) in a jacketed bioreactor (MA502, Marconi, SP, Brazil) at 270 rpm following by heating the slurry at 85 °C for 15 minutes in order to inactivate the endogenous enzymes. Subsequently, the slurry was hydrolyzed using Alcalase 2.4 L® or Protamex®. The hydrolysis was conducted at pH 8 and 50 °C, while enzyme-substrate ratio was set at 2% (w/w) for both enzymes and raw materials to determine the maximum degree of hydrolysis for each enzyme. Afterward, new hydrolysis processes were conducted to reach degrees of hydrolysis of 10 and 15% for both enzymes to produce the samples used in this work (Muscle Alcalase 10% = MA10, Muscle Alcalase 15% = MA15, Muscle Protamex 10% = MP10, Muscle Protamex 15% = MP15, By-product Alcalase 10% = BA10, By-product Alcalase 15% = BA15, By-product Protamex 10% = BP10, By-product Protamex 15% = BP15). The DH defined as the percent ratio of the number of peptide bonds broken (h) to the total of peptide bonds per unit of weight (h_{tot}). h_{tot} was assumed to be 8.6 meq/g for fish protein. DH was calculated from the amount of NaOH added to keep the pH constant during the hydrolysis (Adler-Nissen, 1984) as given in the equation 1:

$$DH\ (\%) = h/h_{tot} = [(B \times N_b) / MP] \times (1 / \alpha) \times (1/h_{tot}) \times 100 \qquad [1]$$

where B is the volume (mL) of NaOH consumed, N_b is the normality of NaOH, MP is the mass of protein and α is the average degree of dissociation of the α-NH2 groups released during hydrolysis expressed as presented in the equation 2:

$$\alpha = (10^{pH-pK}) / (1 + 10^{pH-pK}) \qquad [2]$$

where the pH is the values at which the hydrolysis process was conducted and the pK value calculated according Beychok and Steinhart (1964) (apud Kristinsson & Rasco, 2000), as showed in equation 3:

$$pK = 7.8 + [(298 - T) / (298 \times T)] \times 2400 \qquad [3]$$

where T is the temperature expressed in kelvin at which the hydrolysis was performed.

After incubation, the samples were heated in a water bath at 90 °C for 15 min to inactivate the enzyme. Then, samples were centrifuged (Hanil 22K, Gwangju, South Korea) at 16.300 x g for 15 min at 4 °C, supernatants were frozen (Indrel, SP, Brazil) at -84 °C and subsequently freeze dried (L108, Liotop, SP, Brazil) and stored at -20 °C until analysis were performed.

2.3. Antioxidant properties measurement

The free radical method using $DPPH^{\cdot}$ is a well-established assay for the *in vitro* determination of antioxidant activity in protein hydrolysates due to its reliability and reproducibility. $DPPH^{\cdot}$ scavenging activity was measured on protein hydrolysates (5 g/L) dissolved in imidazole-acetate buffer (5 mM, pH 2.4-8), complex coacervates of hydrolysates (1 g protein hydrolysates/L) / LM pectin (0-7 g/L) and MA15 and BP15 microcapsules (1 g protein hydrolysates/L).

In order to determine the DPPH· radical scavenging activity the method reported by Bersuder et al. (1998) was used. Briefly, an aliquot (500 μL) of each sample was mixed with 375 μL of 99% ethanol and 125 μL of a daily-prepared solution of DPPH· (0.02% in 99% ethanol solution). The mixtures were shaken and then incubated for 60 min in dark at room temperature. The reduction of DPPH· radical was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm in a spectrophotometer (Jenway 7305, Villepinte, France). DPPH radical-scavenging activity was calculated as follows in equation 4:

$$\text{DPPH radical-scavenging activity (\%)} = (A_{blank} - A_{sample}) / A_{blank} \times 100 \qquad [4]$$

where A_{blank} is the absorbance of the reaction containing all reagents (except that distilled water was used instead of the sample), and A_{sample} is the absorbance in the presence of sample. The experiment was carried out in triplicate and the results were mean values.

2.4. Preparation of LM pectin/protein hydrolysates complexes

Biopolymer mixtures containing common carp protein hydrolysates (1 g/L) and LM pectin (0-7 g/L) were prepared by mixing different ratios of the stock solutions with imidazole-acetate buffer at pH 2.4. The resulting suspensions were mixed for 1 min using a vortex mixer and their pH was adjusted again.

2.5. Zeta potential measurement

The zeta potential (ζ-potential) of common carp protein hydrolysates and common carp protein hydrolysates/LM pectin complexes was determined using a Zetasizer NanoZS90 (Malvern Instruments, Malvern, UK). The samples were diluted

(0.5% (w/w)) with imidazole-acetate buffer adjusted to the suitable pH value. The mean ζ-potential (ZP) values (±SD (standard deviation)) were obtained from the instrument.

2.6. Turbidity measurement

Turbidity measurements were performed with a spectrophotometer (Jenway 7305, Villepinte, France). The absorbance was measured at 600 nm at room temperature (25 °C) against pure protein hydrolysate solutions (without LM pectin) in imidazole-acetate buffer (5 mM; pH 2.4).

2.7. Particle size measurement

Particle size distributions of protein hydrolysates/LM pectin complexes formed at various LM pectin concentrations were measured by a laser diffraction instrument (Mastersizer 3000, Malvern Instruments, Malvern, UK). The complexes were stirred continuously throughout the measurement to ensure the samples were homogeneous. The volume particle diameter (D_{43}) was calculated from the three injections of three separate samples with three readings per sample.

2.8. Spray-drying of protein hydrolysates/LM pectin complexes

The suspensions of LM pectin/protein hydrolysates complexes (LM pectin/hydrolysates ratio of 5) prepared in imidazole-acetate buffer (5 mM; pH 2.4) containing 20 wt.% maltodextrin DE 19 as well as solutions of the same hydrolysates without pectin were dried in a laboratory scale spray-drier equipped with a 0.5 mm nozzle atomizer (Mini spray-dryer B-290, BUCHI, Switzerland). Suspensions were pumped to the spray-drier at a feed rate of 0.5 L/h at room temperature and dried at an inlet temperature of 180 °C and an outlet temperature of 90 °C. The dried powders were collected and stored in airtight containers at 4 °C. Dry powders were then weighted and mixed with imidazole/acetate buffer (5 mM, pH 2.4) to obtain reconstituted suspensions

of complexes and hydrolysates with the same dry matter as before drying (the residual moisture was taken into account). After 1 h of rotation at approximately 200 rpm, samples were analysed.

2.9. Scanning Electron microscopy (SEM)

Spray-dried microcapsules were attached to a sample stub with double-sided sticky tape and examined using scanning electron microscopy (Hitachi electron microscopy, TM 3030, Japan) at 2000 or 4000 × magnification and an accelerating voltage of 15 kV.

2.10. Statistical analysis

All experiments were performed using at least three freshly prepared samples. The results presented are the averages and standard deviations that were calculated from these replicate measurements. The differences in the antioxidant activity between the samples before and after the drying process were evaluated using a one-way analysis of variance (ANOVA), Post-hoc tests were employed using Newman-Keuls method. The significance level adopted was 5% ($\alpha = 0.05$). The ANOVA assumptions (normality by Shapiro-Wilks and variance homogeneity by Levene) were previously evaluated, and tested with the same significance level (5%).

3. Results and discussion

3.1. Characterization of protein hydrolysates

In order to find the optimum conditions and to select the most suitable material for the formation of complexes, the antioxidant activity and zeta potential of the hydrolysates were characterized as a function of pH. The determination of the ability to scavenge specific free radicals is a common *in vitro* strategy to determine the antioxidant activity of a given compound. $DPPH^{\cdot}$ is a stable free radical often used as a

substrate to evaluate the scavenging potency of protein hydrolysates that accepts an electron or hydrogen radical to become a stable molecule (Sabeena Farvin et al., 2013). Fig. 1 shows the DPPH radical-scavenging activity of common carp muscle or by-product hydrolysates (5 g/L) produced with Alcalase 2.4 L® and Protamex® with a DH of 10 and 15 %. MA10 showed the highest radical-scavenging activity (93.09 ± 0.4 %) at pH 2.4. All samples showed the minimum activity at pH 8.0 (ranging from 40.4 to 54.72 %). As the pH was increased from 2.4 to 8.0, the radical scavenging activity of all samples was decreased. The mechanism through which the protein hydrolysates exert their antioxidant activity is not fully understood. However, it has been reported that hydrophobic amino acids may enhance the potency of antioxidant peptides through proton-donation ability, electron-donation ability, and/or direct lipid radical scavengers. In addition, carboxyl and amino groups in the side chains of some aminoacids (i.e. glutamic acid and aspartic acid) could act as chelators of metal ions that could catalyse the generation of hydroxyl radicals (Quian et al., 2008; Sarmadi & Ismail, 2010).

Zeta potential as a function of pH was determined to characterize the influence of pH on the electrostatic charge of protein hydrolysates and LM pectin (Fig. 2). At pH 8.0 all protein hydrolysates exhibit negative zeta potential. However, as the pH decreases from 8.0 to 2.4 the zeta potential of protein hydrolysates increases. Protein hydrolysates from muscle and by-product of common carp presented isoelectric points (where ζ-potential was zero) ranging from 2.58 to 3.69. At pH 2.4, all protein hydrolysates presented positive zeta potential. LM pectin was negatively charged from 2.4 to 8.0 due to the presence of ionized carboxylic acid groups along its backbone. Nevertheless, the magnitude of the charge of LM pectin increased significantly above

pH 5.0. According to Ben Amara et al. (2016) this increase can be caused by an intensive deprotonation of the carboxylic groups of the pectin backbone.

At pH 2.4 (condition used in this work to produce the complex coacervates) the protein hydrolysates exhibit maximum DPPH radical scavenging activity. Therefore, at this pH, it would be expected that the difference of charges between the positive protein hydrolysates and negative LM pectin permit the formation of electrostatic complexes.

3.2. Properties of pectin/protein hydrolysates complexes

Protein to polysaccharide ratio is one of the main factors that affect the formation of complex coacervates due to the balance of charges between protein and polysaccharides (Eghbal et al., 2016). In this work, the formation of LM pectin/hydrolysates complexes at pH 2.4 was studied. Increasing LM pectin concentrations (0–7 g/L) were added to common carp protein hydrolysates at 1 g/L concentration. As shown in Fig. 3, an increase in the ratio LM pectin/hydrolysates resulted in increased turbidity in all samples. This increase in turbidity can be caused by the formation of an increasing number of complexes and/or the formation of large complexes. Moreover, LM pectin/hydrolysates ratio from 0 to 7 caused the increase of the turbidity values in all samples except the samples MP15 and BP10 that experienced a decrease in turbidity at a ratio pectin/hydrolysate of 6. Thus, it can be inferred that all negative charges of LM pectin interacted electrostatically with positively charged hydrolysates resulting in the formation of a maximum amount of complex from which there was a gradual dissociation of complexes since negatively charged pectin molecules inhibited aggregation of complexes due to electrostatic repulsion by opposite charges.

The turbidity measurements were supplemented with information about particle size (Fig. 4) and zeta potential (Fig. 5) of the LM pectin/protein hydrolysates complexes. Fig. 4 shows the particle mean diameter (D_{43}) of complex coacervates formed with mixtures of negatively charged LM pectin (0–7 g/L) in the presence of a fixed positively charged protein hydrolysates concentration (1 g/L) at pH 2.4. Particle size measurements indicated that the complexes formed with carp by-product hydrolysates tended to form larger aggregates (ranging from 261.23 ± 3.71 μm to 374.96 ± 11.29 μm) when compared with complexes formed with carp muscle hydrolysates (ranging from 165.83 ± 2.59 μm to 236.18 ± 5.71 μm) probably due to a high aggregation of individual complexes which result in a distribution of larger particles. Both type of complexes (those formed with by-product hydrolysates as those formed with muscle hydrolysates) showed relatively constant particle size at LM pectin/hydrolysate ratio from 1 to 7, indicating that they did not experienced extensive changes in their self-association with different concentrations of pectin. According to the results obtained in this study, LM pectin concentration does not affect the formation of complexes of LM pectin/protein hydrolysates. Moreover, variations in the concentration of LM pectin had probably an effect only on the number of complexes, without apparent changes in the size of the particles.

To monitor how the net charge of formed complexes changes upon titration with pectin, the zeta-potential of the complexes was measured as a function of LM pectin concentration at pH 2.4 (Fig. 5). The zeta potential of the complexes changed from positive values (when only the protein hydrolysates were in solution) to negative values with increasing concentrations of pectin, presumably due to the adsorption of the amine groups of cationic proteins and/or peptides by the anionic carboxyl groups of the

polysaccharide caused by electrostatic attractions. All complexes with exception of BA15 and BP10 reached a plateau at LM pectin/protein hydrolysates ratio of 1 with intermediate zeta potential between the polysaccharide and individual protein at pH 2.4, confirming that around this concentration the protein molecules were saturated with LM pectin chains (Harnsilawat et al., 2006). Complex coacervates formed with protein hydrolysates BP10 and BA15 suffered a decrease close to the zeta-potential value of LM pectin alone. It can be thought that all positive sites of these samples were saturated with negative sites of LM pectin at the LM pectin/protein hydrolysates ratio of 5 above which the excess amount of LM pectin in the medium resulted in decreasing of zeta potential. Therefore, it can be deduced from Figs. 3-5 that the increasing of the turbidity along with reduction of the zeta potential after the addition of LM pectin to protein hydrolysates solutions at pH 2.4 caused effectively the binding of the two molecules mainly by electrostatic interactions.

The effect of LM pectin complexation on the antioxidant activity of the protein hydrolysates is shown in Fig. 6, which gives the DPPH radical scavenging activity as a function of the LM pectin concentration (0–7 g/L) at a fixed protein hydrolysates concentration (1 g/L). According to this figure, the DPPH radical scavenging activity of LM pectin/protein hydrolysates complexes was lower than the protein hydrolysates alones (Fig. 1) due to the lowest concentration of hydrolysates used in this assay (1 g/L). However, in both assays evaluating the antioxidant activity (hydrolysates and LM pectin/hydrolysates complexes), the samples MA15 and MA10 showed the highest antioxidant activity while MP15 and BP15 exhibited the weakest activity among all samples. Moreover, increasing levels of pectin (0-7 g/L) did not affect the antioxidant activity of protein hydrolysates, which remained relatively constant at LM

pectin/protein hydrolysates ratio from 0 to 7. It can be inferred that increasing levels of LM pectin did not affect the antioxidant activity of the protein hydrolysates. This is in line with the preliminary test carried out in our laboratory to confirm the lack of antioxidant activity against $DPPH^{\cdot}$ of LM pectin alone which showed to have a negligible activity against this radical (data not shown).

Therefore, in this study, the complexes formed with the samples MA15 (due to its high antioxidant capacity and high turbidity values) and BP15 used for comparison purposes, as the sample that presented the lowest antioxidant capacity, were selected for the spray-drying microencapsulation.

3.3. Spray-dried microcapsules containing antioxidant protein hydrolysates

Bioactive peptides may need to be isolated with matrices that are more complex since unlike other bioactive food components (i.e. polyphenols, vitamins, etc.) bioactive peptides are composed of highly heterogeneous chemical species. Encapsulation of bioactive peptides can be performed primarily to mask the bitter taste resulting from hydrophobic amino acid residues generated by protein hydrolysis as well as to reduce hygroscopicity to ensure their structural stability to maintain their bioactive properties (Mohan et al., 2015). Polysaccharides derived from plant, animal or microbial sources, such as gum arabic, chitosan, cyclodextrin and maltodextrin have been used for the encapsulation of proteins and peptides because the large molecular structure of the polysaccharides provides stability of encapsulated products during the production processing and storage (Gharsallaoui et al., 2007; Mohan et al., 2015).

Table 1 shows the effect of the spray-drying process on the antioxidant activity of complex coacervates formed with the protein hydrolysates MA15 and BP15, as measured by the capacity to scavenge the DPPH free radical before and after spray-

drying. The antioxidant activity against DPPH radical after spray-drying for powders that were spray-dried were 50.53 ± 2.67% and 12.31 ± 1.43% for the complexes formed with pectin and MA15 and BP15 respectively. The results were not significantly different ($p > 0.05$) when compared with the DPPH radical scavenging capacity of the samples before spray-drying. On the other hand, the antioxidant activity of spray-dried solutions of non-complexed hydrolysates MA15 and BP15, was significantly lower (11.43 ± 4.31 % and 2.25 ± 0.7 respectively). Therefore, it can be concluded that spray-dried complex coacervates of common carp protein hydrolysates under these conditions did not affect the antioxidant capacity significantly maybe due to the low contact time with the hot air of the material in the chamber. This contact time (related to the aspiration rate) is therefore a critical parameter in the spray-drying process when the objective is to minimize the damage to the antioxidant compounds (Medina-Torres et al., 2016).

Fig. 7 presents scanning electron microscopic photographs of dry microcapsules obtained by spray-drying of protein hydrolysates and protein hydrolysates/pectin complexes when maltodextrin was used as encapsulating agent. All-SEM micrograph of spray-dried microcapsules showed well separated spherical shaped particles with smooth and dented or concave surfaces and homogenous sizes ranging from 2 to 20 μm. According to Saénz et al. (2009), dented surface can be attributed to the shrinkage of the particles during the drying process. SEM images of fractured microcapsules showing the wall and the internal structure are also presented in Fig. 7. Broken capsules show a wall thickness of approximately 3 μm and a great air bobble (void) at the centre. These central voids are formed due to the expansion of air bubbles trapped in the droplets in the last stage of the spray-drying process (Teixeira et al., 2004; Ben Amara et al., 2016).

4. Conclusion

The objectives of this study were to examine the formation of LM pectin/protein hydrolysates complexes in aqueous solution as a function of pectin concentrations and to analyse if the microencapsulation of the complexes by spray-drying affects the antioxidant capacity of the hydrolysates. Although muscle hydrolysates showed higher antioxidant activity against DPPH radical, by-product hydrolysates also showed good radical scavenging activity. These results provide evidences for the potential use of these common carp by-products as functional ingredient or nutraceutics. The complexation of LM pectin through electrostatic attraction increased the turbidity but did not affect the antioxidant capacity of protein hydrolysates. In this manner, the complex coacervation of protein hydrolysates with increasing LM pectin concentrations did not affect the antioxidant activity. Furthermore, spray-dried LM pectin/protein hydrolysates complexes with maltodextrin DE 19 as wall material resulted in the formation of independent microcapsules with smaller sizes when compared to the particle size of the complexes in solution. The results found in this study suggest that the complexation and subsequent microencapsulation by spray-drying of carp protein hydrolysates is an efficient way to protect the biological activity of protein hydrolysates. However, further studies are needed to evaluate the exact composition of the hydrolysates as well as the molecular origins of the differences observed in terms of antioxidant activity.

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Figures caption

Fig. 1. DPPH radical scavenging activity as a function of pH of common carp (*C. carpio*) muscle and by-product protein hydrolysates (5 g/L) (5 mM imidazole-acetate buffer) produced with Alcalase 2.4L® and Protamex® with degree of hydrolysis 10 and 15%.

Fig. 2. Dependence of the ζ-potential of common carp (*C. carpio*) muscle and by-product protein hydrolysates produced with Alcalase 2.4L® and Protamex® with degree of hydrolysis 10 and 15% and LM pectin on pH (5 mM imidazole-acetate buffer).

Fig. 3. Turbidity of LM pectin/common carp (*C. carpio*) protein hydrolysates complexes ratio (at 600 nm) when different LM pectin amounts (0–7 g/L) were added to a fixed protein hydrolysates concentration (1 g/L) (5 mM imidazole-acetate buffer pH 2.4).

Fig. 4. Effect of on the particle size average (D_{43}) of formed complex coacervates when different LM pectin amounts (0–7 g/L) were added to a fixed common carp (*C. carpio*) protein hydrolysates concentration (1 g/L) (5 mM imidazole-acetate buffer pH 2.4).

Fig. 5. Dependence of the ζ-potential of LM pectin/common carp (*C. carpio*) protein hydrolysates ratio when different LM pectin amounts (0–7 g/L) were added to a fixed protein hydrolysates concentration (1 g/L) (5 mM imidazole-acetate buffer pH 2.4).

Fig. 6. DPPH radical scavenging activity of formed complex coacervates when different LM pectin amounts (0–7 g/L) were added to a fixed common carp (*C. carpio*) protein hydrolysates concentration (1 g/L) (5 mM imidazole-acetate buffer pH 2.4).

Fig. 7. Scanning electron microscopy (SEM) micrographs of spray-dried non-complexed hydrolysates (MA15 and BP15) and LM pectin (5 g/L) / MA15 and LM pectin / BP15 complexes in the presence of maltodextrin DE 19 (20 wt.%) (5 mM imidazole-acetate buffer, pH 2.4).

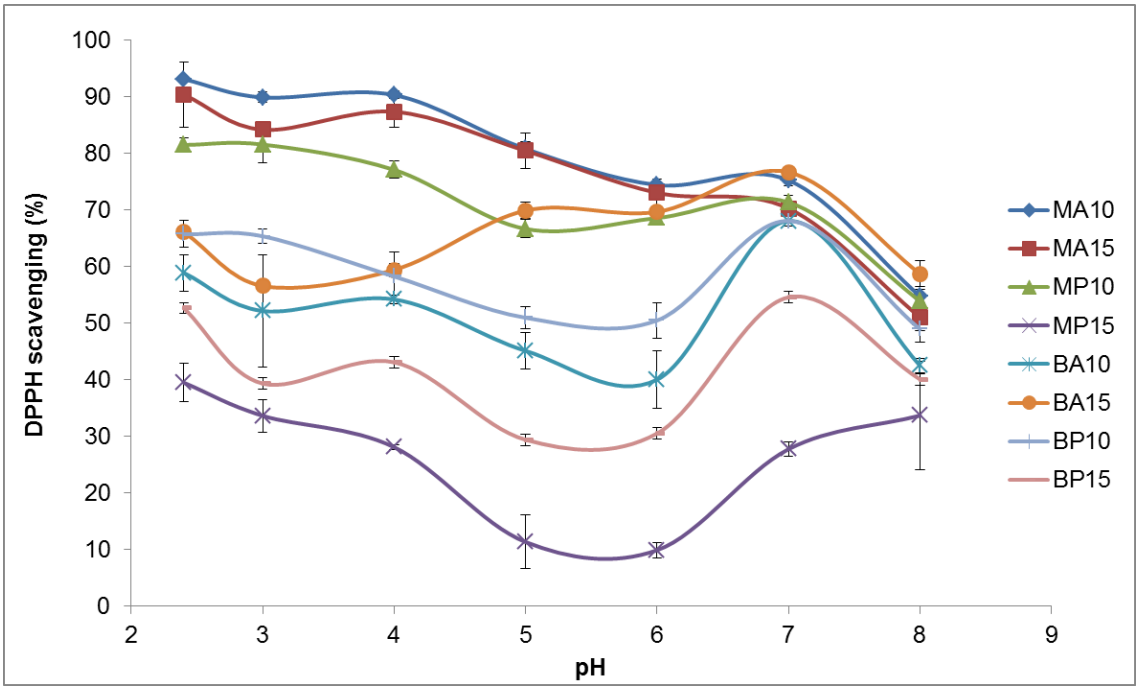
DPPH scavenging (%)
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
pH
MA10
MA15
MP10
MP15
BA10
BA15
BP10
BP15

Fig. 1

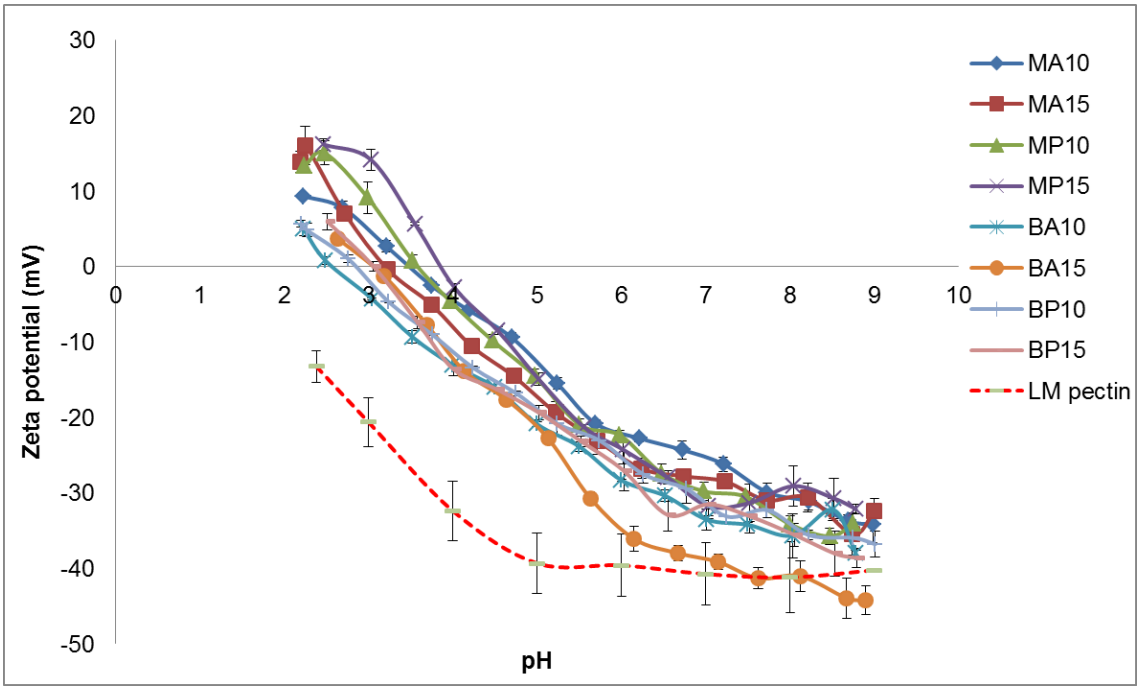
30
20
10
0
-10
-20
-30
-40
-50
0
1
2
3
4
5
6
7
8
9
10
Zeta potential (mV)
pH
MA10
MA15
MP10
MP15
BA10
BA15
BP10
BP15
LM pectin

Fig. 2

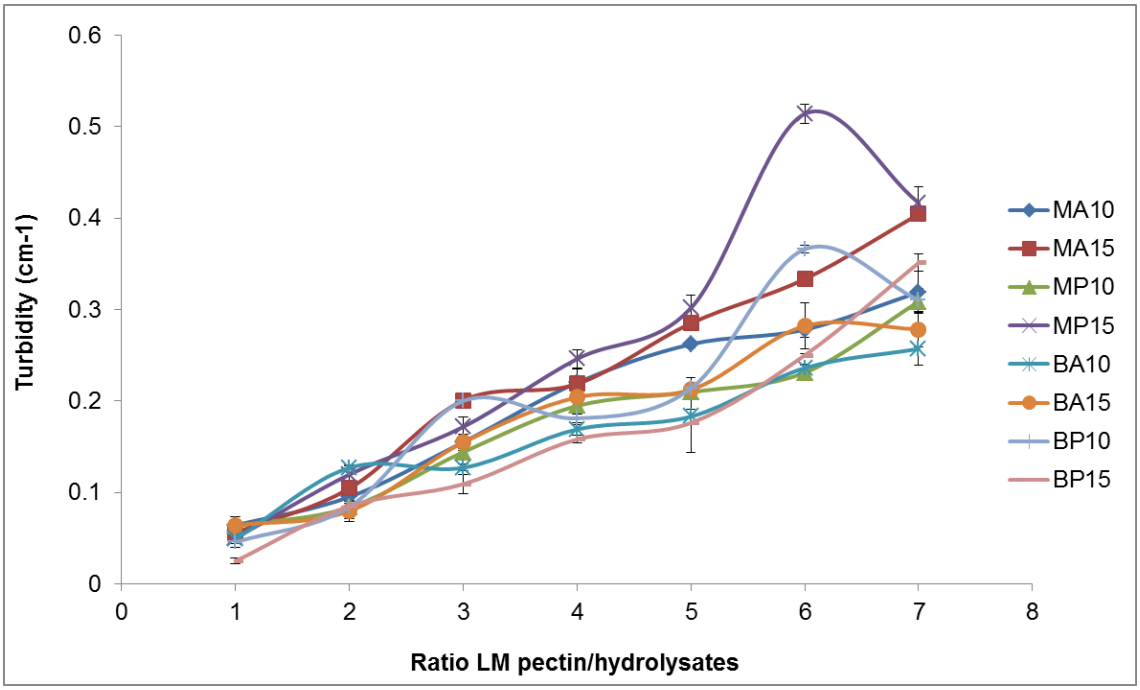
0.6
0.5
0.4
0.3
0.2
0.1
0
Turbidity (cm-1)
0
1
2
3
4
5
6
7
8
Ratio LM pectin/hydrolysates
MA10
MA15
MP10
MP15
BA10
BA15
BP10
BP15

Fig. 3

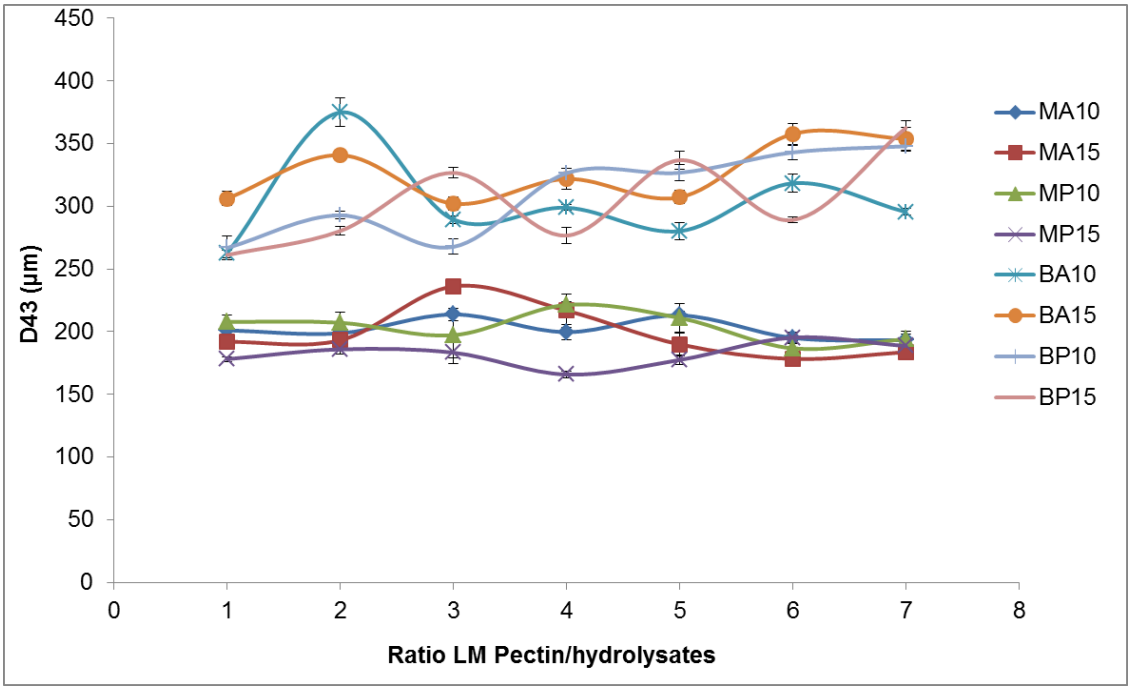
450
400
350
300
250
200
150
100
50
0
D43 (µm)
0
1
2
3
4
5
6
7
8
Ratio LM Pectin/hydrolysates
MA10
MA15
MP10
MP15
BA10
BA15
BP10
BP15

Fig. 4

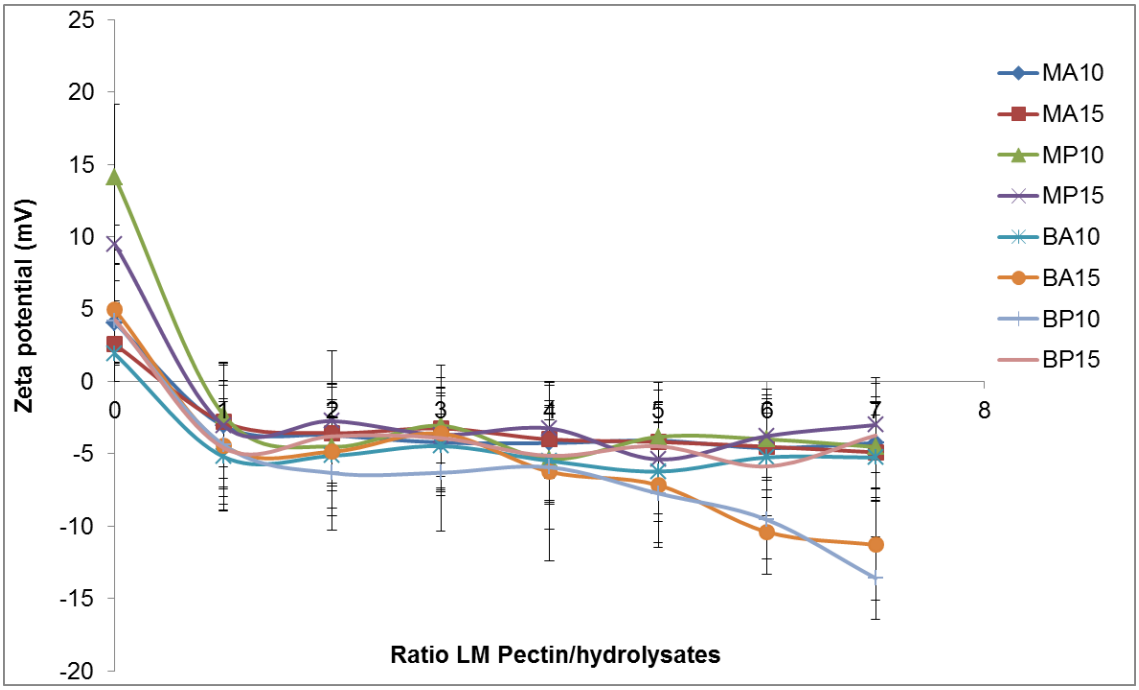
25
20
15
10
5
0
-5
-10
-15
-20
Zeta potential (mV)
0
1
2
3
4
5
6
7
8
Ratio LM Pectin/hydrolysates
MA10
MA15
MP10
MP15
BA10
BA15
BP10
BP15

Fig. 5

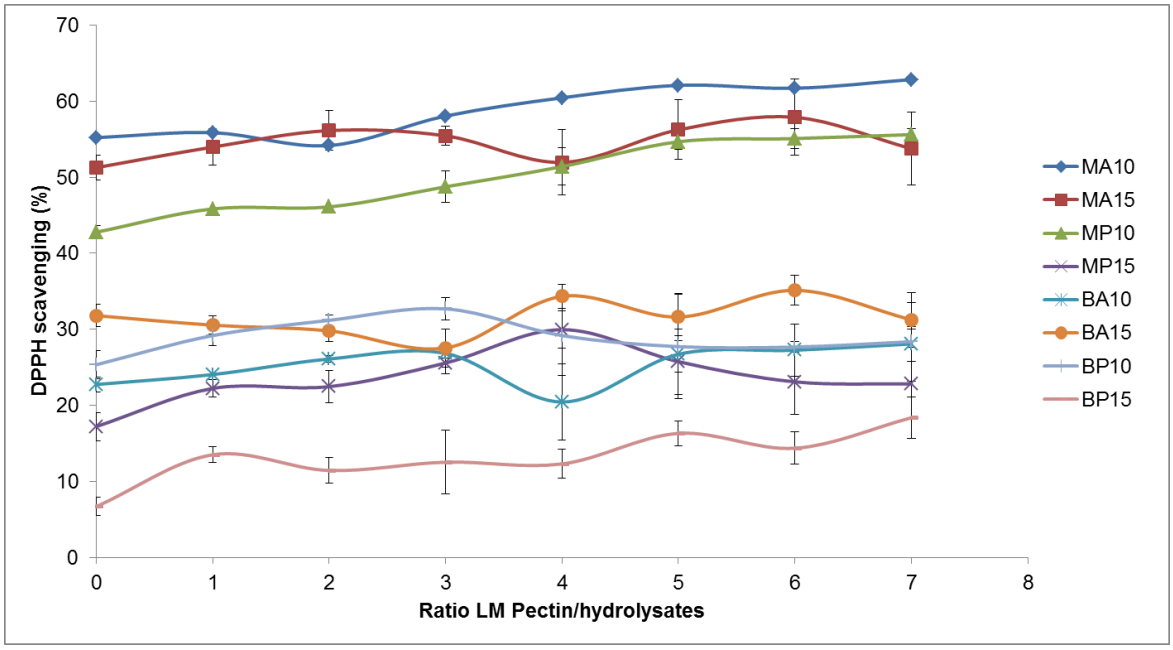
70
60
50
40
30
20
10
0
DPPH scavenging (%)
0
1
2
3
4
5
6
7
8
Ratio LM Pectin/hydrolysates
MA10
MA15
MP10
MP15
BA10
BA15
BP10
BP15

Fig. 6

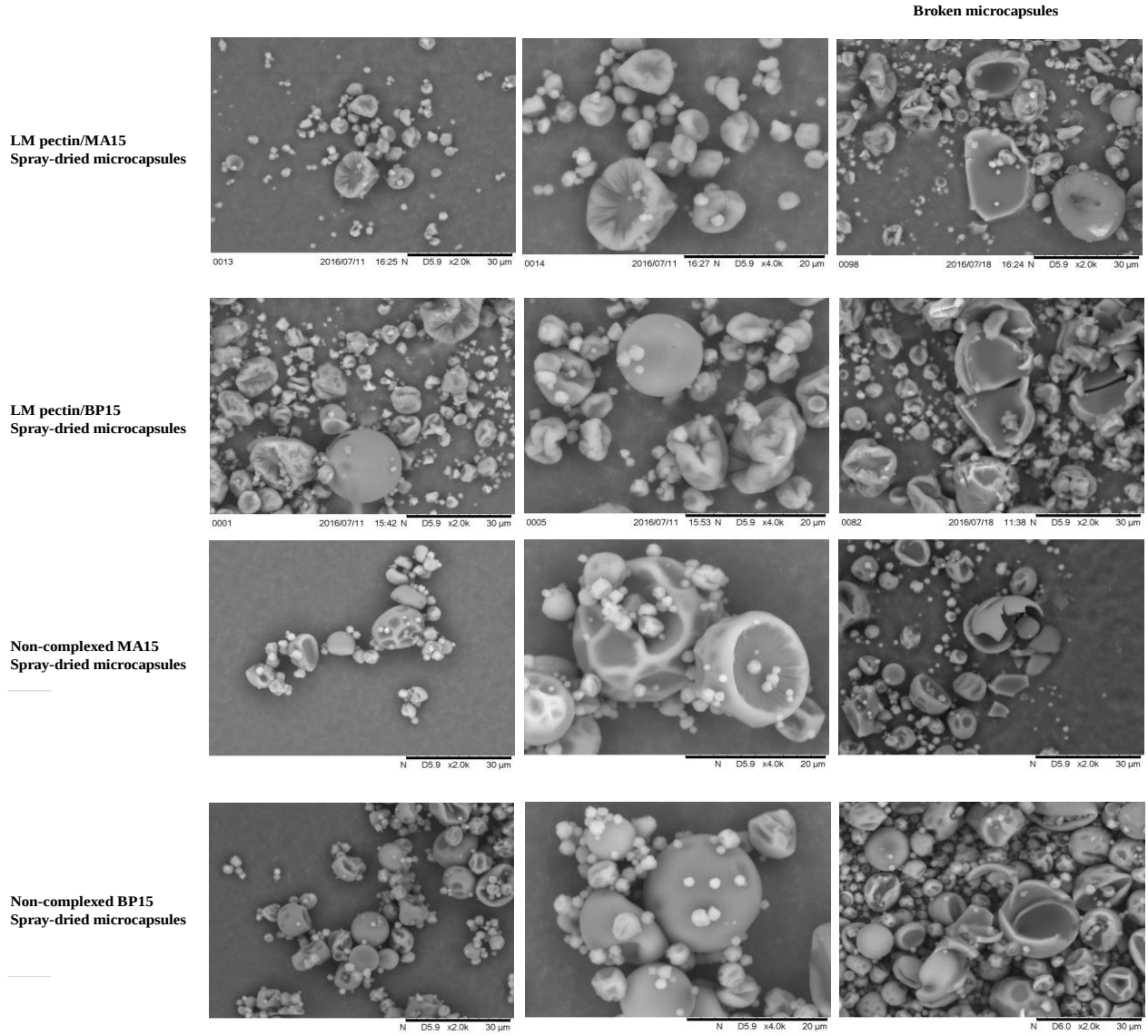
Broken microcapsules
LM pectin/MA15
Spray-dried microcapsules
LM pectin/BP15
Spray-dried microcapsules
Non-complexed MA15
Spray-dried microcapsules
Non-complexed BP15
Spray-dried microcapsules

Fig. 7

Table 1. DPPH radical scavenging activity of LM pectin/protein hydrolysates complex coacervates before (feed suspension) and after the spray-drying process (spray-dried and reconstituted suspension). Different letters in the same row indicate significant differences ($p < 0.05$).

	Feed suspension	**Spray-dried and reconstituted suspension (complexes)**	**Spray dried and reconstituted suspension (non-complexed hydrolysates)**
MA15	56.24±3.89% [a]	50.53±2.67% [a]	11.43±4.31% [b]
BP15	16.33±1.59% [a]	12.31±1.43% [a]	2.25±0.70% [b]

CONSIDERAÇÕES FINAIS

Na presente tese foi realizada uma revisão sobre a atualidade da obtenção de hidrolisados proteicos a partir de subprodutos de pescado assim como um repasso sobre os possíveis mecanismos que atuam na atividade biológica dos mesmos e os últimos estudos desenvolvidos a esse respeito.

Adicionalmente, foram obtidos hidrolisados proteicos a partir do subproduto do beneficiamento da carpa comum criada em cativeiro e foi testada a atividade biológica por métodos *in vitro* incluindo testes químicos e avaliação da atividade antioxidante em cultura celular de células de hipocampo de rato HT-22. Estes estudos revelaram que os hidrolisados proteicos obtidos a partir do subproduto da carpa possuem grande potencial como ingrediente para indústria alimentar com menos risco potencial do que os antioxidantes sintéticos para aumentar a estabilidade oxidativa dos alimentos e prevenir os processos de oxidação lipídica dos mesmos, devido a sua capacidade de sequestrar radicais, aumentando assim o tempo de vida útil do produto.

Atualmente, o teste de bioatividades do hidrolisado de proteínas de pescado é praticamente restrito apenas a experiências *in vitro*. Não obstante, a dosagem de bioatividades pelos clássicos testes *in vitro* não são suficientes, toda vez que não fornecem informação sob a potencial incorporação das moléculas no processo digestório e a incorporação celular delas. Neste sentido, estudos biológicos *in vitro*, tais como os conduzidos com culturas celulares, em conjunto com estudos mais detalhados *in vivo* precisam ser levados a cabo, a fim de compreender os possíveis benefícios para a saúde destes peptídeos, bem como o seu efeito sobre as propriedades sensoriais dos produtos alimentares, aos quais os peptídeos são adicionados. Até o momento, apenas um número

limitado de testes *in vivo* foram levados cabo em animais para responder as questões principais sobre sua absorção e biodisponibilidade no trato gastrointestinal.

Neste trabalho de tese foi demostrado que os hidrolisados proteicos de carpa lograram aumentar a atividade de desidrogenases mitocondriais de células de hipocampo HT-22 e reduzir a produção de espécies reativas de oxigênio das mesmas. Ademais, a inclusão dos hidrolisados na ração de zebrafish demostrou um efeito antioxidante no cérebro e no músculo dos peixes. Particularmente interessantes, com respeito ao potencial uso dos hidrolisados como nutracêuticos (suplementos dietéticos de uma sustância bioativa natural concentrada, presente que tem um efeito favorável sobre a saúde) ou alimentos funcionais, são os resultados antioxidantes em células de hipocampo e no cérebro de zebrafish, destacando que os hidrolisados de subproduto de carpa tem a capacidade de atravessar a barreira hematoencefálica e induzir um efeito neuroprotetor.

Respeito á diminuição da peroxidação lipídica no musculo dos zebrafish alimentados com dietas contendo hidrolisado de subproduto de carpa, é resultado de interesse para a indústria da aquicultura. O resultado abre a porta de futuras pesquisas em relação à inclusão de ingredientes naturais com atividade antioxidante que permita estender o tempo de vida útil dos files de pescado de aquicultura.

A microencapsulação e secagem das microcápsulas de compostos bioativos são processos que permitem melhorar a estabilidade e proteção a diversos ambientes e também permitir a liberação controlada do composto aumentando de tal modo à biodisponibilidade do nutracêutico. No presente trabalho, a microencapsulação por spray-drying dos hidrolisados reduziu significativamente a atividade biológica dos

mesmos; presumivelmente pelas altas temperaturas do processo de secado. Contudo, a coacervação complexa dos hidrolisados com pectina conseguiu proteger a atividade antioxidante dos hidrolisados quando foram submetidos à secagem por atomização. Deste modo, a coacervação complexa e posterior secagem por atomização resultou ser um método eficiente de encapsulação de hidrolisados proteicos de carpa com atividade antioxidante.

Adicionalmente, é importante destacar que a sequencia de trabalhos usada na presente tese para avaliar a bioatividade dos hidrolisados: i) ensaios químicos *in vitro*, ii) ensaios biológicos em células *in vitro* e iii) avaliação *in vivo*; demonstrou ser uma interessante e recomendável sequencia de estudos na hora de avaliar a atividade biológica de um produto assim como para compreender as suas rotas e mecanismos de ação no organismo.

Compendiando, o presente trabalho de tese demonstrou que a hidrólise enzimática dos subprodutos do beneficiamento da carpa comum é uma técnica eficaz que permite a liberação de peptídeos com atividade antioxidante e neuroprotetora com grande potencial no uso na indústria farmacêutica e de alimentos.

CONCLUSÕES

I. O subproduto do beneficiamento da carpa comum (*Cyprinus carpio*) submetido à hidrólise proteica utilizando as enzimas comerciais Alcalase e Protamex gera peptídeos com atividade antioxidante *in vitro* contra radicais peroxil e radical DPPH e tem a capacidade de reduzir a formação de espécies reativas de oxigênio em células de hipocampo HT-22 (Capítulo II).

II. A inclusão de 50 g kg^{-1} de hidrolisado de subproduto de carpa produzido com a enzima comercial Alcalase e com grau de hidrólise 15 % na dieta de zebrafish (*Danio rerio*) reduz a peroxidação lipídica do músculo dos zebrafish e do cérebro dos zebrafish, demonstrando capacidade de atravessar a barreira hematoencefálica (Capítulo III).

III. A microencapsulação por coacervação complexa com pectina e posterior secagem por atomização "*spray-drying*" demonstrou ser um método eficaz para produzir microcápsulas de hidrolisados de subprodutos de carpa sem alterar a sua atividade biológica (Capítulo IV).

ESTUDOS FUTUROS

Baseado nos resultados obtidos na presente tese propõe-se que sejam realizados estudos de purificação das sequencias peptídicas responsáveis da atividade antioxidante dos hidrolisados de subproduto de carpa a fim de esclarecer os mecanismos que desencadeiam essa bioatividade. Complementarmente, desde o ponto de vista da aquicultura, devem ser desenvolvidos experimentos de nutrição centrados na avaliação dos efeitos nos parâmetros zootécnicos e de saúde da inclusão de hidrolisados proteicos de subproduto de carpa em larvas e juvenis de espécies de interesse para a aquicultura.

Além disso, recomenda-se desenvolver estudos *in vivo* com hidrolisados de subproduto de carpa produzidos com a enzima Protamex e grau de hidrólise 15%, uma vez que apresentou aumento da viabilidade celular e atividade antioxidante em células de hipocampo Ht-22.

Igualmente, se recomenda avaliar as potenciais aplicações práticas dos hidrolisados microencapsulados com a finalidade de desenvolver rações enriquecidas para aquicultura, a produção de alimentos funcionais e/ou nutracêuticos para consumo humano, assim como as aplicações tecnológicas dos mesmos, como por exemplo, a inclusão de hidrolisados microencapsulados em filmes comestíveis para a produção de embalagens antioxidantes.