Caracterização do perfil e atividade biológica de peptídeos de cavalo marinho *Hippocampus reidi*

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São Vicente
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CARACTERIZAÇÃO DO PERFIL E ATIVIDADE BIOLÓGICA DE PEPTÍDEOS DE CAVALO MARINHO *Hippocampus reidi*

CANDIDATA: CLÁUDIA NEVES CORREA
ORIENTADOR: LEANDRO MANTOVANI DE CASTRO

Tese apresentada ao Instituto de Biociências, Câmpus do Litoral Paulista, UNESP, para a obtenção do título de Doutor do Programa de Pós-Graduação em Biodiversidade de Ambientes Costeiros

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ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE CLAUDIA NEVES CORREA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE DE AMBIENTES COSTEIROS, DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DO LITORAL PAULISTA.

Aos 17 dias do mês de março do ano de 2023, às 09:00 horas, no(a) Salão Nobre do IB/CLP, realizou-se a defesa de TESE DE DOUTORADO de CLAUDIA NEVES CORREA, intitulada **Caracterização do perfil e atividade biológica de peptideos de cavalo marinho *Hippocampus reidi***. A Comissão Examinadora foi constituida pelos seguintes membros: Prof. Dr. LEANDRO MANTOVANI DE CASTRO (Orientador(a) - Participação Presencial) do(a) Departamento de Ciencias Biologicas e Ambientais / Instituto de Biociencias do Campus do Litoral Paulista da Unesp, Profa. Dra. CRISTIANE ANGÉLICA OTTONI (Participação Presencial) do(a) Instituto de Biociencias Campus do Litoral Paulista / UNESP, Prof. Dr. WAGNER VILEGAS (Participação Virtual) do(a) Departamento de Ciencias Biologicas e Ambientais / Instituto de Biociencias do Campus do Litoral Paulista da Unesp, Profa. Dra. PATRÍCIA RECKZIEGEL (Participação Virtual) do(a) Departamento de Análises Clínicas e Toxicológicas, da Faculdade de Ciências Farmacêuticas (FCF), da Universidade de São Paulo (USP), Dr. MARCÉLO JOSÉ DIAS SILVA (Participação Virtual) do(a) Universidade Federal de Alfenas. Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final:_ _ _ _ _ APROVADO _ _ . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Leandro Mantovani de Castro
Prof. Dr. LEANDRO MANTOVANI DE CASTRO

DEDICATÓRIA

Gostaria de dedicar este trabalho a pessoas que foram muito importantes em minha vida e que já não estão mais neste plano,
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Caracterização do perfil e atividade biológica de peptídeos de cavalo marinho *Hippocampus reidi*

RESUMO: A biodiversidade encontrada no ambiente marinho e a sua diversidade química é uma fonte ilimitada para a bioprospecção de substâncias com potencial para o desenvolvimento de novos fármacos. O cavalo marinho é um peixe teleósteo, reconhecido na Medicina Tradicional Chinesa por possuir propriedades medicinais. Nos últimos anos, o uso de metodologias específicas em modelos experimentais e linhagens celulares revelaram a presença de um conjunto de peptídeos provenientes do metabolismo intracelular e que podem atuar na comunicação celular. Neste contexto o objetivo deste trabalho foi caracterizar o perfil de peptídeos naturais encontrados em tecidos de cavalo marinho *Hippocampus reidi,* através de um protocolo de rápida inativação da proteólise pelo calor e espectrometria de massas, além de uma investigação inicial da atividade biológica de algumas sequências identificadas através de um ensaio para verificar um efeito antioxidante. Os resultados obtidos durante o desenvolvimento deste estudo são apresentados em três capítulos. No capítulo 1, a análise do peptidoma de brânquias permitiu a identificação de 1080 peptídeos em brânquias. Os fragmentos variaram de 5 a 38 aminoácidos e a principal localização subcelular da proteína precursora foi o citoplasma (58%) seguido pelo núcleo (22%) e mitocôndria (5%). Fragmentos N e C-terminal foram 18% e 15% respectivamente, resultados semelhantes a outros estudos de peptidoma. O mais frequente resíduo de aminoácido na posição N-terminal foi alanina, seguido por serina, na posição P1 o resíduo de aminoácido mais encontrado foi arginina, seguido por lisina. Entre as proteínas precursoras com maior número de peptídeos identificados estão proteínas ribossomais, histona e hemoglobina cadeia alfa. Fragmentos de *Moronecidin-like*, descrito como peptídeo antimicrobiano, também foram identificados em todas as amostras de brânquias analisadas. Análises de predição revelaram peptídeos com características importantes de peptídeos antimicrobianos. No capítulo 2, um ensaio de atividade antioxidante foi realizado para dois peptídeos identificados. Os peptídeos CNC1 e CNC2 não atingiram 50% de inibição de radicais com 30 minutos de reação no ensaio de DPPH, com 24 horas de reação o peptideos CNC1 atingiu 69,61% de inibição de radicais na concentração de 100 µM. No capítulo 3 é apresentado um protocolo de marcação isotópica para realização de peptidômica quantitativa. A caracterização realizada neste estudo abre novas perspectivas para investigação do potencial terapêutico de peptídeos naturais.

Palavras chave: peptidômica, espectrometria de massas, cavalo marinho, brânquias, peptídeos bioativos.

Profile of natural peptides and biological activity of seahorse *Hippocampus reidi*

ABSTRACT: The biodiversity found in the marine environment and its chemical diversity is an unlimited source for the bioprospecting of substances with potential for the development of new drugs. Among the classes of molecules, bioactive peptides with antioxidant, antimicrobial, antihypertensive or anticancer action were characterized through the preparation of protein hydrolysates from a variety of marine organisms. The seahorse is a teleost fish, recognized in Traditional Chinese Medicine for having medicinal properties. In recent years, the use of specific methodologies in experimental models and cell lines have revealed the presence of a set of peptides from intracellular metabolism that can act in cell communication. In this context, the objective of this work was the characterization in tissues of seahorse *Hippocampus reidi* the profile of natural peptides through a protocol of rapid inactivation of proteolysis by heat and mass spectrometry, in addition to an initial investigation of the biological activity of some identified sequences through an assay to verify an antioxidant effect. The results obtained during the development of this study are presented in three chapters. In chapter 1, analysis of the gill peptidome allowed the identification of 1080 peptides in gills. The fragments ranged from 5 to 38 amino acids and the main subcellular location of the precursor protein was the cytoplasm (58%) followed by the nucleus (22%) and mitochondria (5%). N- and C-terminal fragments were 18% and 15% respectively, results similar to other peptidomic studies. The most frequent amino acid residue in the N-terminal position was alanine, followed by serine, leucine and lysine, in the P1 position the most found amino acid residue was arginine, followed by lysine. Among the precursor proteins with the highest number of identified peptides are ribosomal proteins, histone and hemoglobin alpha chain. Moronecidin-like fragments, described as an antimicrobial peptide, were also identified in all gill samples analyzed. Predictive analysis also revealed peptides with important characteristics of antimicrobial peptides. In chapter 2, an antioxidant activity assay was performed for two identified peptides. The CNC1 and CNC2 peptides did not reach 50% of radical inhibition with 30 minutes of reaction in the DPPH assay, with 24 hours of reaction, the CNC1 peptide reached 69.61% of radical inhibition at the concentration of 100 μM. Chapter 3 presents an isotopic labeling protocol for performing quantitative peptidomics. The characterization performed in this study opens new perspectives for investigating the therapeutic potential of natural peptides.

Keywords: peptidomic, mass spectrometry, seahorse, gills, bioactive peptides.

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1. Introdução

1.1 Organismos marinhos como fonte de compostos bioativos

Cerca de 70% da biosfera terrestre é composta por água, com os mares e oceanos representando mais de 95% desta superfície (GLEICK; D., 1993). Estima-se que existam cerca de 2,2 milhões de espécies marinhas e que apenas 10% são conhecidas atualmente (MORA *et al.*, 2011). Os ambientes aquáticos por abrigar essa biodiversidade são locais interessantes para descobertas de novos compostos medicinais e produtos de interesse biotecnológico (BLUNT *et al.*, 2016). No entanto, informações sobre a maioria dos organismos potencialmente úteis que habitam esses ambientes permanecem inexploradas. Assim, a descoberta de moléculas naturais nesta vasta fonte pode ser utilizada no desenvolvimento de novos fármacos, produtos cosméticos e suplementos alimentares.

Devido às suas características físico-químicas, os mares e oceanos podem ser considerados ambientes extremamente exigentes e hostis. Deste modo, ao longo de milhares de anos, organismos marinhos desenvolveram aspectos morfofisiológicos e vários metabólitos secundários que garantiram sua adaptação e sobrevivência. Parte das moléculas encontradas não têm análogos em organismos terrestres, além de únicas em termos de estrutura química e atividade biológica (JASPARS *et al.*, 2016). Estes compostos bioativos incluem: polissacarídeos (ex. agar, alginato), pigmentos (ex. clorofila, carotenóides), ácidos graxos polinsaturados, polifenóis, proteínas e peptídeos, entre outros (PANGESTUTI; KIM, 2017).

1.2 Cavalos Marinhos: Características gerais do gênero, usos na medicina tradicional chinesa

Os cavalos marinhos são peixes teleósteos, pertencentes à família Syngnathidae. Possuem uma morfologia especial caracterizada pela presença de uma boca tubular, sem dentes, perda da nadadeira caudal, cauda preênsil e eixo corpóreo vertical (LIN *et al.*, 2016). Outra característica interessante é que as fêmeas de cavalo marinho depositam os ovos em uma bolsa incubadora nos machos, onde os ovos são fecundados, e os embriões são providos de nutrição, oxigênio e um ambiente controlado até o nascimento. Vivem em habitats como

ervas marinhas, manguezais, recifes de corais, estuários e macroalgas, os quais estão sujeitos a intensa ação antrópica o que acarreta danos físicos ao habitat, poluição química, eutrofização, mudanças na qualidade da água, invasão de espécies e mudanças climáticas (VINCENT; FOSTER; KOLDEWEY, 2011).

Após o nascimento, os cavalos marinhos sofrem uma série de modificações morfológicas, de comportamento e alimentação. Muitos sistemas, como o digestório, necessitam de maturação celular e funcional, fato que pode ser responsável pela alta mortalidade até o período juvenil (18-20 dias após o nascimento) em criações de *Hippocampus reidi* (NOVELLI *et al.*, 2015).

Cavalos marinhos são utilizados na Medicina Tradicional Chinesa e suas derivadas, particularmente no sudoeste da Ásia, e reconhecidos por possuírem importância medicinal. Na Medicina Tradicional Chinesa acredita-se que possuem papel no aumento e balanço do fluxo de energia vital, doenças como asma, aterosclerose, colesterol alto, doenças renais, infertilidade, disfunção erétil, incontinência e doenças cutâneas como acne e nódulos persistentes (VINCENT; FOSTER; KOLDEWEY, 2011).

Estes animais são geralmente comercializados inteiros secos ou em pó. Cerca de 95% do comércio de cavalos marinhos é destinado ao abastecimento de mercados de espécies da Medicina Tradicional Chinesa e a maior parte dos cavalos marinhos capturados vem da pesca do camarão com rede de arrasto. O gênero *Hippocampus* está relacionado no apêndice II do CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) onde estão espécies que podem se tornar ameaçadas de extinção caso o comércio não seja controlado. A superexploração é a mais significativa ameaça aos cavalos marinhos e à população marinha como um todo. Devido a características da espécie como poucos filhotes, necessidade de cuidado parenteral por longo tempo e baixa habilidade de dispersão, os cavalos marinhos são mais susceptíveis à superexploração (VINCENT; FOSTER; KOLDEWEY, 2011).

No Brasil as espécies de cavalo marinho *Hippocampus reidi* e *Hippocampus erectus* também são utilizados na medicina tradicional, sendo a espécie *H. reidi* uma das 3 espécies de peixes mais utilizadas para fins terapêuticos. Principalmente em estados da região Nordeste, é

indicado para o tratamento de doenças como a asma, gastrite, doenças cardiocirculatórias, do sistema reprodutivo e câncer (EL-DEIR *et al.*, 2012).

Fig. 1 - Exemplares de cavalo marinho da espécie *Hippocampus reidi.* (MAI & ROSA, 2009)

1.3 Cavalo marinho: fonte de bioprospecção de moléculas bioativas

Compostos bioativos descritos no gênero *Hippocampus* incluem esteróides, ácidos graxos insaturados, proteínas, peptídeos e minerais. Atualmente diversos estudos farmacológicos revelam grande número de aplicações biológicas para peptídeos bioativos identificados em cavalo marinho (MUTHURAMALINGAM *et al.*, 2019; OH *et al.*, 2018; RYU; QIAN; KIM, 2010a, 2010b).

Extratos de cavalos marinhos derivados de hidrolisados obtidos com diferentes tipos de enzimas proteolíticas *in vitro,* como alcalase, papaína, tripsina, α- chymotripisina, flavourzyme, neutrase, pepsina, protamase e Protamex® são estratégias utilizadas para a caracterização de moléculas bioativas (JE *et al.*, 2020; OH *et al.*, 2018; RYU; QIAN; KIM, 2010a, 2010b). Esta abordagem de gerar moléculas *in vitro*, depende de condições físico-químicas específicas para cada tipo de enzima (PANGESTUTI *et al.*, 2013), e produzem uma mistura de peptídeos ativos e inativos que variam em tamanho, composição e

sequência de aminoácidos, separados e purificados por técnicas cromatográficas e identificados por espectrometria de massas. Por exemplo, o hidrolisado de *Hippocampus abdominalis*, utilizando alcalase, mostrou significativa atividade antioxidante nos ensaios de DPPH e ABTS *in vitro*, assim como diminuição na geração de espécies reativas de oxigênio e diminuição na expressão de genes relacionados com a apoptose celular como p53 e caspase 3. Esses resultados evidenciam uma capacidade de diminuição do estresse oxidativo mediado pela injúria celular, importante fator na patogênese da aterosclerose (OH *et al.*, 2018).

Recentemente peptídeos isolados de *Hippocampus abdominalis* apresentaram potencial efeito na diferenciação e resistência muscular via super regulação de proteínas miogênicas (MyoD, fator de transcrição miogênica; MyoG, miogenina; MyHC, cadeia pesada de miosina). Ensaios *in vivo* em zebrafish demonstraram um aprimoramento da performance de resistência em oposição ao fluxo de água quando os animais foram tratados com o peptídeo P6 de sequência QIGFIW. Este peptídeo se mostra promissor candidato a utilização como suplemento nutricional para aprimoramento da diferenciação de células musculares e na capacidade de resistência muscular (MUTHURAMALINGAM *et al.*, 2019).

A sequência de aminoácidos AGD, isolado da fração <5 KDa e produzido por hidrólise enzimática (alcalose) do extrato de *Hippocampus abdominalis* mostrou atividade antioxidante, apresentando atividade de remoção de radicais peroxil, diminuição de espécies reativas de oxigênio, possuindo um efeito protetor ao estresse oxidativo e aumentando a viabilidade celular (KIM *et al.*, 2019).

A inibição da atividade da enzima conversora de angiotensina (ECA), que integra o sistema renina angiotensina, possui importante papel na regulação da pressão sanguínea. A inibição de ECA foi observada *in vitro* e *in vivo* em frações do extrato hidrolisado por Protamex® de *Hippocampus abdominalis*, as frações foram separadas por peso molecular. A fração de menor peso molecular (<5 KDa) aumentou, de maneira dose dependente, a produção de óxido nítrico (ON) em culturas de células assim como a expressão de eNOS responsável pela produção de ON. Desta fração foram identificados 3 peptídeos, APTL (m/z 401,240), CNVPLSP (m/z 729,397) e PWTPL (m/z 613,390). O peptídeo CNVPLSP apresentou maior inibição da ECA e menor IC50 (0,088 ± 0,002 mg/ml). Análise de tração

molecular mostrou que a ligação ECA-CNVPLSP é uma ligação estável, mostrando que o peptídeo CNVPLSP pode ser um promissor peptídeo bioativo contra a hipertensão (JE *et al.*, 2020).

Na patogenia da artrite, condrócitos e osteoblastos degradam e restringem a síntese de componentes da matriz extracelular da cartilagem (principalmente fibras colágenas tipo II) e há inflamação sinovial. Entre os fatores responsáveis pelo desencadeamento da patogenia estão metaloproteases (MMP), e citocinas inflamatórias como TPA (12-O-tetradecanoylphorbol-13-acetate), IL-1 (interleucina 1) e LPS (lipopolissacarídeos). Esses fatores aumentam a degradação da cartilagem em articulações, induzem a síntese de óxido nítrico e desencadeiam uma série de sinais intracelulares como MAPKs (proteínas kinases mitogeno ativadoras) e a via NF-κB (fator nuclear κB). O extrato hidrolisado de cavalo marinho (*Hippocampus kuda*) apresentou atividade em mediadores inflamatórios característicos do processo inflamatório da artrite reumatóide e osteoartrite. Os resultados demonstraram que o hidrolisado não afetou a viabilidade das duas linhagens celulares, MG – 63 e SW – 1353, assim como contribuiu para a diminuição de mediadores inflamatórios *in vitro*, como MMP 1 e MMP 13, ON e menor ativação da via NF-κB. O peptídeo SHP-1 de sequência LEDPFDKDDWDNWKS e peso molecular 1909 Da foi isolado (RYU; QIAN; KIM, 2010a, 2010b). Este peptídeo de cavalo marinho também apresentou correspondência de mais de 90% com a enzima α-enolase humana. Esta enzima atua como receptor e ativador de plasminogênio que participa de vários processos fisiológicos e fisiopatológicos como a degradação de matriz extracelular, remodelamento tecidual, embriogênese, cicatrização de feridas, migração e invasão celular, angiogênese e metástase celular tumoral. A superexpressão de α-enolase é considerada como marcador diagnóstico de vários tumores. Resultados *in vitro* demonstraram que este peptídeo de cavalo marinho reduziu a ligação de α-enolase e plasminogênio, diminuindo sua ativação, afetou seus níveis de expressão e inibiu a migração invasiva em células de fibrossarcoma HT1080, possuindo assim potencial para um novo agente antitumoral (KIM *et al.*, 2014).

Peptídeos antimicrobianos (PAMs) possuem amplo espectro de atividade, em geral são catiônicos e hidrofóbicos. A alta atividade hemolítica, sensibilidade à presença de sais e

susceptibilidade à proteólise muitas vezes impedem o uso clínico destes peptídeos. Através de análises genômicas em *Hippocampus comes,* um peptídeo semelhante a moronecidin, peptídeo antimicrobiano da família Piscidin foi encontrado e testado, apresentando atividade antimicrobiana de amplo espectro contra bactérias Gram positivas, Gram negativas e fungos, mesmo na presença de sais. Este peptídeo também mostrou atividade contra a formação de biofilme, importante fator de virulência bacteriana, assim como menor atividade hemolítica e citotóxica quando comparado ao peptídeo moronecidin de *morone saxatilis* (MOHAMMADI *et al.*, 2018). A busca de PAMs na espécie *Hippocampus erectus,* também realizada por análise genômica, encontrou 290 possíveis PAMs derivados de grandes proteínas intracelulares. Trombina apareceu como o principal grupo de proteínas precursoras de PAMs seguido por Histona e Lecitina (CHEN *et al.*, 2020).

Foi verificada a atividade neuroprotetora de extratos hidrolisados de *Hippocampus trimaculatus* produzidos com várias enzimas proteolíticas. Na doença de Alzheimer ocorre uma neurodegeneração progressiva, com perda da função cognitiva. Esta doença é caracterizada pelo acúmulo de substância β-amilóide (Aβ) em placas, sendo a substância Aβ42 a mais produzida. Microglias também participam da patogênese da doença de Alzheimer eliminando agregados Aβ via fagocitose, porém podem causar a morte de neurônios adjacentes devido à inflamação e liberação de substâncias neurotóxicas. O extrato produzido com pronase E apresentou alta porcentagem de proteínas (75,75 ± 0,13%) e promoveu maior viabilidade celular quando comparado a outras enzimas. Para a otimização, isolamento e identificação do peptídeo bioativo este extrato foi separado por FPLC (cromatografia líquida de proteína rápida) em frações e avaliada a atividade neuroprotetora em células PC12 contra a neurotoxicidade induzida por Aβ42. A fração V-2 apresentou maior atividade neuroprotetora. Para a identificação do peptídeo ativo foi utilizado espectrômetro de massas Q-TOF, com fonte de ionização ESI. O peptídeo foi identificado como HTP-1, com sequência GTEDLDK (PANGESTUTI *et al.*, 2013). Também foi utilizada a co-cultura de células PC12 e BV2, que simulam a patogenicidade da doença de Alzheimer quando estimuladas com Aβ42. O peptídeo HTP-1 mostrou efeito neuroprotetor contra Aβ42 pela atenuação de mediadores neurotóxicos liberados por células BV2 (células da micróglias de roedores) como PGE 2 (prostaglandina 2), TNF-α (Fator de necrose tumoral-α) e IL-1β

(interleucina 1β) e pela ativação da via de sinalização PI3K/Akt nas células PC12 o que previne a morte celular. Este peptídeo também apresenta as características estruturais identificadas como desejáveis para efeitos neuroprotetores como alto grau de hidrofobicidade, composição entre 3 a 15 aminoácidos e peso molecular < 1 KDa (PANGESTUTI; KIM, 2015).

1.4 Peptídeos intracelulares, formação, função e identificação.

Peptídeos são polímeros de aminoácidos, com menos de 10.000 Da, que possuem papel importante como moléculas bioativas atuando como neurotransmissores, hormônios, antígenos e agentes antimicrobianos. Em geral, a síntese de peptídeos, principalmente neuropeptídeos e peptídeos hormônios, se inicia com a expressão de uma proteína precursora inativa, que apresenta em sua porção N-terminal um peptídeo sinal e direciona a proteína para o lúmen do retículo endoplasmático e posteriormente complexo de Golgi, onde sofre a ação de endoproteases, processo que continua em vesículas secretórias até a liberação, por exocitose, para o meio extracelular (CASTRO, 2013).

Além da proteólise limitada em compartimentos da via secretória, proteínas intracelulares são constantemente degradadas em peptídeos e aminoácidos para serem reutilizados na síntese de novas proteínas. Este processo é altamente seletivo e precisamente regulado o que previne o acúmulo de proteínas não funcionais ou potencialmente tóxicas (GOLDBERG, 2003).

O complexo proteolítico proteassomo constitui o maior sistema de degradação proteolítica extralisossomal, com atividade de clivagem específica em aminoácidos hidrofóbicos, ácidos e básicos (ORLOWSKI, 1990). O núcleo do complexo proteassomo, chamado de 20S, é conservado entre as espécies e possui 4 anéis concêntricos empilhados, formando um tubo onde as proteínas são clivadas em peptídeos menores. Os dois anéis externos são compostos por subunidades α e dois anéis internos formados por subunidades β. As subunidades β apresentam três sítios de clivagem, subunidade β1 com atividade semelhante a caspase, clivam proteínas após resíduos ácidos, subunidade β5 de clivagem semelhante a quimotripsina, clivam proteínas após um resíduo hidrofóbico e subunidade β2, com sítio de clivagem de atividade semelhante à tripsina e clivam proteínas após resíduos

básicos (GELMAN *et al.*, 2011; HABIB *et al.*, 2022; LYAPINA; IVANOV; FESENKO, 2021). O complexo também possui uma ou duas subunidades 19S, que fazem o reconhecimento e direcionam as proteínas para o interior do complexo proteolítico (GOLDBERG, 2003). Em células eucarióticas, muitas proteínas destinadas à degradação são marcadas com uma cadeia de poliubiquitina, num processo dependente de energia, e então digeridas em peptídeos com 2 a 20 aminoácidos pelo complexo proteassomo 26S. A degradação mediada pela ubiquitina regula positivamente e negativamente uma variedade de processos celulares como o controle da divisão celular, sinal de transdução, regulação transcricional, resposta inflamatória e imune, desenvolvimento embrionário, apoptose e ciclo circadiano, garantindo a homeostase celular (HERSHKO, 2005).

A análise e identificação deste conjunto de peptídeos pode ser dificultada pela degradação enzimática *post-mortem* do tecido. A irradiação por micro-ondas, logo após a eutanásia dos animais, bloqueia a degradação de moléculas instáveis, pelo princípio da inativação térmica de proteases (CHE *et al.*, 2005). Após a inativação de proteases, a extração acidificada a frio também reduz a clivagem em ligações ácido aspártico e prolina (FRICKER, 2010).

Inicialmente, acreditava-se que apenas uma parte dos peptídeos escapavam da degradação completa a aminoácidos, porém metanálises de peptidomas em cérebro de camundongos, cérebro de zebrafish e culturas de linhagens celulares humanas identificaram muitos fragmentos de proteínas citosólicas, nucleares ou mitocondriais, com muitos fragmentos N ou C-terminal (FRICKER, 2010; GELMAN *et al.*, 2011; TEIXEIRA *et al.*, 2019). A super-representação de peptídeos N e C-terminal no peptidoma intracelular pode sugerir que ou estes peptídeos são mais estáveis que outros peptídeos ou estes peptídeos são seletivamente gerados, possivelmente pela limitada proteólise ao invés de completa degradação de proteínas em peptídeos (FRICKER, 2010). Esses achados sugerem que peptídeos intracelulares podem possuir papel na comunicação celular e não serem somente simples produtos da degradação proteica. A espectrometria de massas conferiu um avanço no estudo destas moléculas, pois possibilitou o sequenciamento de peptídeos provenientes de

misturas complexas e a identificação de moléculas com mudanças pós-translacionais (FRICKER, 2010).

2. Objetivos

Objetivo geral:

Caracterização dos peptídeos que existem naturalmente em tecidos de cavalo marinho *Hippocampus reidi,* contribuindo para o preenchimento da lacuna de dados moleculares desta espécie.

Objetivos específicos:

Avaliação da atividade antioxidante de peptídeos identificados selecionados

Descrição de método de quantificação relativa de peptídeos por marcação isotópica

3. Organização da Tese

A Tese está organizada em capítulos

3.1 Capítulo I

No primeiro capítulo apresentamos o trabalho publicado na revista Biomolecules "Revealing natural intracellular peptides in gills of seahorse *Hippocampus reidi",* onde descrevemos os peptídeos encontrados naturalmente em brânquias do cavalo marinho *Hippocampus reidi.*

3.2 Capítulo II

No segundo capítulo descrevemos a avaliação antioxidante de dois peptídeos identificados em tecidos de cavalo marinho *Hippocampus reidi.*

3.3 Capítulo III

No capítulo 3 apresentamos o trabalho publicado na revista Jove, onde descrevemos a metodologia para a marcação de peptídeos com formas leves e deuteradas de formaldeído e cianoborohidreto, para a realização de quantificação relativa de peptídeos.

Capítulo I

Revealing natural intracellular peptides in gills of seahorse *Hippocampus reidi*

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Abstract

The seahorse is a marine teleost fish member of the Syngnathidae family that displays a complex array of morphological and reproductive behavior innovations and has been recognized for their medicinal importance. In the Brazilian ichthyofauna the seahorse *Hippocampus reidi* are among the three fish species most used by the population in traditional medicine. In this study a protocol was performed based on fast heat inactivation of proteases plus liquid chromatography coupled to mass spectrometry to identify native peptides in gills of seahorse *H. reidi*. The MS/MS spectra obtained from gills allowed the identification of 1080 peptides, of which 1013 peptides were present in all samples and 67 peptide sequences were identified in an additional LC-MS/MS run from an alkylated and reduced pool of samples. The majority of peptides were fragments of the internal region of the amino acid sequence of the precursor proteins (67%), N- and C-terminal represented 18% and 15% respectively. Ribosomal proteins, Histones, and Hemoglobin were the precursor proteins which present many peptide sequences. In addition, peptide fragments from Moronecidin-like protein, described with antimicrobial activity, were found in all gill samples of *H. reidi*. The identified sequences may reveal new bioactive peptides.

Keywords: peptidomics; seahorse; mass spectrometry; gills

1. Introduction

Fish are the major component of aquatic fauna, living in a microbe-rich environment [1]. The seahorse is a marine teleost fish member of the Syngnathidae family that displays a complex variety of morphological and reproductive behavior innovations. It has been used in Traditional Chinese Medicine. It is believed that seahorse extract plays a role in diseases such as asthma, atherosclerosis, kidney diseases, infertility, erectile dysfunction, incontinence and skin diseases such as acne and persistent nodules [2,3].

Several studies have used dried seahorse powder hydrolyzed by proteases to obtain peptide extracts. Pharmacological investigation has revealed biological applications of these extracts. Antioxidant activity of enzymatic hydrolysates from the seahorse *Hippocampus abdominalis* has been reported *in vitro* and *in vivo* [4,5]. Although some protocols based on protein digestion are successful for the identification of bioactive peptides, in these preparations, the molecules are generated artificially *in vitro*.

A peptidome can be defined as the analysis of the peptides naturally generated in cells and tissues. It usually includes the following steps: sample collection, peptide extraction, fractionation, LC-MS/MS analysis, peptide identification and data mining [6]. Recently, with improvement in mass spectrometry and related proteomics analyses, it has become possible to sequence large numbers of peptides from complex mixtures. Peptides secreted from cells play roles in important physiological processes as signaling molecules (neuropeptides, hormones, growth factors), defense mechanisms (antimicrobial peptides, venoms and toxins) and modulating protein-protein interaction within cells [7]. Furthermore, fast protease inactivation techniques by heating, mainly microwave radiation, followed by molecular weight exclusion filters associated with LC-MS/MS, have revealed a constant set of peptides from cytosolic, mitochondrial and nuclear protein fragments in tissues of mice [7–9], zebrafish [10], human cell lines [11] and fungi [12] which has been termed intracellular peptides. Most of these peptides are intermediate products of protein metabolism and appear to be generated by proteasome complex [13,14], that owns caspase-like (β1), trypsin-like (β2) and

chymotrypsin-like (β5) proteolytic activities [15,6]. In addition, these intracellular peptides can play signaling roles after release from their parent proteins. Most bioactive peptides may be hidden in the sequences of functional proteins, such as hemoglobin and histone [16]. For example, Pep-H, a peptide derived from histone found in human brain tissue altered in schizophrenia patients, has shown protection from cell death [17].

The gills are organs that are constantly exposed to the surrounding water and are responsible for breathing, osmoregulation and excretion of nitrogenous waste products [18,19]. Furthermore, the gills are an immunocompetent organ due to the constant exposure to environmental challenges such as pathogens, pollutants and toxins. Several studies have demonstrated the presence of cells and molecules related to the innate immunity in gills, such as antimicrobial peptides (AMPs) and immunoglobulins, which play an important role in inhibiting pathogen invasion [20].

Antimicrobial peptides (AMPs) are related to the innate immune system of various organisms including humans, fish and plants [6,21]. AMPs usually consist of less than 100 amino acid residues, have a positive net charge and contain a hydrophobic region [22]. AMPs can kill bacteria, viruses and fungi by either disrupting their membrane integrity or inhibiting a cellular function. The contact of AMPs is initially due to an electrostatic and hydrophobic interaction with plasma membranes, usually involving pore-forming and non-pore-forming models [22]. Fish AMPs include cathelicidins, β-defensins, hepcidins, piscidins and histone-derived peptides [23].

Here we performed a protocol based on fast heat inactivation of proteases to identify native peptides in gills of the seahorse *Hippocampus reidi*. A study of the Brazilian ichthyofauna showed that the seahorse of this species is one of the three fish species most commonly used by the population for medicinal purposes [24]. Peptide fragmentation data obtained by mass spectrometry were analyzed using a genome database of the recently characterized species *Hippocampus comes* [25], which allowed the identification of peptide sequences. In addition, the protein classes with the highest number of fragments found were discussed in relation to the antimicrobial potential of the peptide sequences.

2. Materials and Methods

2.1 Animals

Three *Hippocampus reidi* adult specimens were obtained by hand in Ubatuba, São Paulo, Brazil (23° 27′ 01.41″ S - 45° 02′ 09.00″ O) while SCUBA diving and kept in the Laboratory of Marine Proteins and Peptides of the Bioscience Institute of Sao Paulo State University. Artificial seawater was prepared using commercial sea salt and mixed according to manufacturer's instructions. Animals were housed in aquaria using artificial seawater (water temperature 25°C) and fed twice a day.

This study followed the guidelines of National Council for Animal Experimentation Control (CONCEA), permission of SISBIO (Protocol Number 78669-1) and was approved by the Ethics Commission for Animal Use (CEUA) at Bioscience Institute of Sao Paulo State University (Sao Vicente, Brazil; Protocol Number 02/2021-CEUA).

2.2 Peptide extraction

Seahorse peptide extracts were prepared as previously described [10]. Adult male seahorses were anesthetized with a lethal dose of MS 222 (100 mg/L) and taken to the microwave for 10 seconds to inactivate peptidase and proteases. Next, gills were collected in centrifuge tubes. Ten one-second sonication pulses (4 Hz) were applied to homogenize each sample in 1 milliliter of deionized water, which were then maintained at 80°C for 20 minutes. After cooling in ice, HCl was added to a final concentration of 10 mM. The homogenates were centrifuged at 5000 g for 40 minutes at 4°C. A Millipore centrifugal filter unit with a molecular weight cut-off of 10.000 Da was used to filter the supernatants. C-18-like Oasis columns (Waters) were then used to purify and concentrate peptides contained in the samples, which were then dried in a vacuum centrifuge. The extract obtained was stored at -80°C for a subsequent peptidomic analysis.

In addition to identifying peptides with cysteine residues, 100 µg from a pool of gill samples were submitted to a reduction and alkylation reaction in order to identify cysteine

residues in samples. For the reduction reaction, 5 mM of final concentration of DTT (Dithiothreitol) was added to the sample and incubated for 25 minutes at 56°C. The alkylation reaction was performed with the addition of IAA (Iodoacetamide) at final concentration of 14 mM and incubated for 30 minutes at room temperature and in the dark. After incubation, a quench of free IAA was performed by adding at 5 mM final concentration, being incubated for another 15 minutes at room temperature and in the dark.

2.3 Fluorescamine

Fluorescamine was used to determine peptide concentration at pH 6.8. The reaction was performed at pH 6.8 to ensure that only the amino groups of the peptides react with the fluorescamine, avoiding that free amino acids react with it. In short, 2.5 µL of the sample was mixed with 25 µL of 0.2 M phosphate buffer (pH 6.8) and 12.5 µL of a 0.3 mg/mL acetone fluorescamine solution, then vortexed for 1 minute. Next, 110 µL of water was added, and a SpectraMax M2e plate reader (Molecular Devices, Sunnyvale, CA) was used to measure the fluorescence at an excitation wavelength of 370 nm and an emission wavelength of 480 nm. The standard reference for the determination of the peptide concentration was the peptide 5A (LTLRTKL), which has a known composition and concentration.

2.4 Liquid chromatography and mass spectrometry

The peptide mixture was suspended in 0.1% formic acid and analyzed as follows. An UltiMate 3000 Basic Automated System (Thermo Fisher®) was set up and connected online with a Fusion Lumos Orbitrap mass spectrometer (Thermo Fisher®) at the mass spectrometry facility RPT02H/Carlos Chagas Institute - Fiocruz, Paraná. The peptide mixture was chromatographically separated on a column (15 cm in length with an internal diameter of 75 µm) packed in-house with ReproSil-Pur C18-AQ 3 µm resin (Dr. Maisch GmbH HPLC) with a flow rate of 250 nL/min of 5% to 38% ACN in 0.1% formic acid on a 120 min gradient. The Fusion Lumos Orbitrap was placed in data-dependent acquisition (DDA) mode to automatically turn between full-scan MS and MS/MS acquisition with 60 s dynamic exclusion. Survey scans (300–1500 m/z) were acquired in the Orbitrap system with a

resolution of 120,000 at m/z 200. The most intense ions captured in a 2 s cycle time were chosen, excluding those which were unassigned or had a 1+ charge state. The selected ions were then isolated in sequence and fragmented using HCD (higher-energy collisional dissociation) with normalized collision energy of 30%. The fragment ions were analyzed with a resolution of 50,000 at 200 m/z. The general mass spectrometric conditions were as follows: 2.3 kV spray voltage, no sheath or auxiliary gas flow, heated capillary temperature of 175°C, predictive automatic gain control (AGC) enabled, and an S-lens RF level of 30%. Mass spectrometer scan functions and nLC solvent gradients were regulated using the Xcalibur 4.1 data system (Thermo Fisher®).

2.5 Peptide identification

The raw data files were submitted to search against the NCBI database filtered for taxonomie: *Hippocampus comes* using the software PEAKS Studio (version 8.5; Bioinformatics Solution, Waterloo, Canada). The decoy-fusion method was used to search a decoy database in order to calculate false discovery rate (FDR).The following search parameters were considered: a) precursor mass tolerance to ±30 ppm; b) fragment ion mass (tolerance of ±0.5 Da); c)variable modifications: oxidized methionine (+15.99 Da), acetylation (+42.01 Da); d)No enzyme specificity. The identified peptides were then sorted by their average of local confidence to select the best spectra for annotation, and they were filtered by FDR ≤0.1%.

3. Results

The MS/MS spectra obtained of *H. reidi* gills extracts allowed the identification of 1080 peptides from 396 proteins, of which 1013 peptides were present in all samples initially analyzed (Supplementary Table 1). Sixty-seven peptide sequences were identified in an additional LC-MS/MS run from an alkylated and reduced pool of gill samples. This run was performed to improve the detection of peptides containing cysteine residues (Supplementary Table 2).

Regarding the general characteristics of these peptides, the fragments ranged from 5 to 38 amino acid residues, with 80% having a length between 8 and 18 amino acid residues (Figure 1A). The precursor proteins of these intracellular peptides are mainly located in the cytosol (58%), nucleus (22%) and mitochondria (5%) (Figure 1B). In addition, most of the peptides found in the gills originated from ribosomal proteins (32%), histones (11%), protein related to cytoskeleton (9%) and hemoglobin fragments (5%) (Figure 1C).

In order to investigate aspects related to proteolytic processing, the amino acid sequence of the precursor proteins and the peptides generated were analyzed. Most of them were fragments of the internal region of the amino acid sequence of the precursor proteins (67%). N- and C-terminal regions represented 18% and 15% respectively (Figure 2A). About 70% of the N-terminal fragments found have an N-terminal acetyl group. The most frequent amino acid in the N-terminal region of the identified peptides was alanine, followed by serine, leucine and lysine (Figure 2B). Rare cleavage sites include tryptophan and glutamine. In addition, arginine is the most common residue in the P1 position (60,19%) followed by lysine (21,12%) (Figure 2C).

The quantification data for each peptide sequence found in all gill samples was performed and is shown in Supplementary table 1. The ratio of each peptide detected in one sample was calculated in relation to the average of the two other samples and the average ratios shown in Figure 3A. Of the 1013 quantified peptides, 72.8% had an average ratio between 1.0 and 2.0, 16.1% between 2.0 and 4.0 and the remaining (11.1%) distributed in different ratio ranges above 6 (Figure 3B).

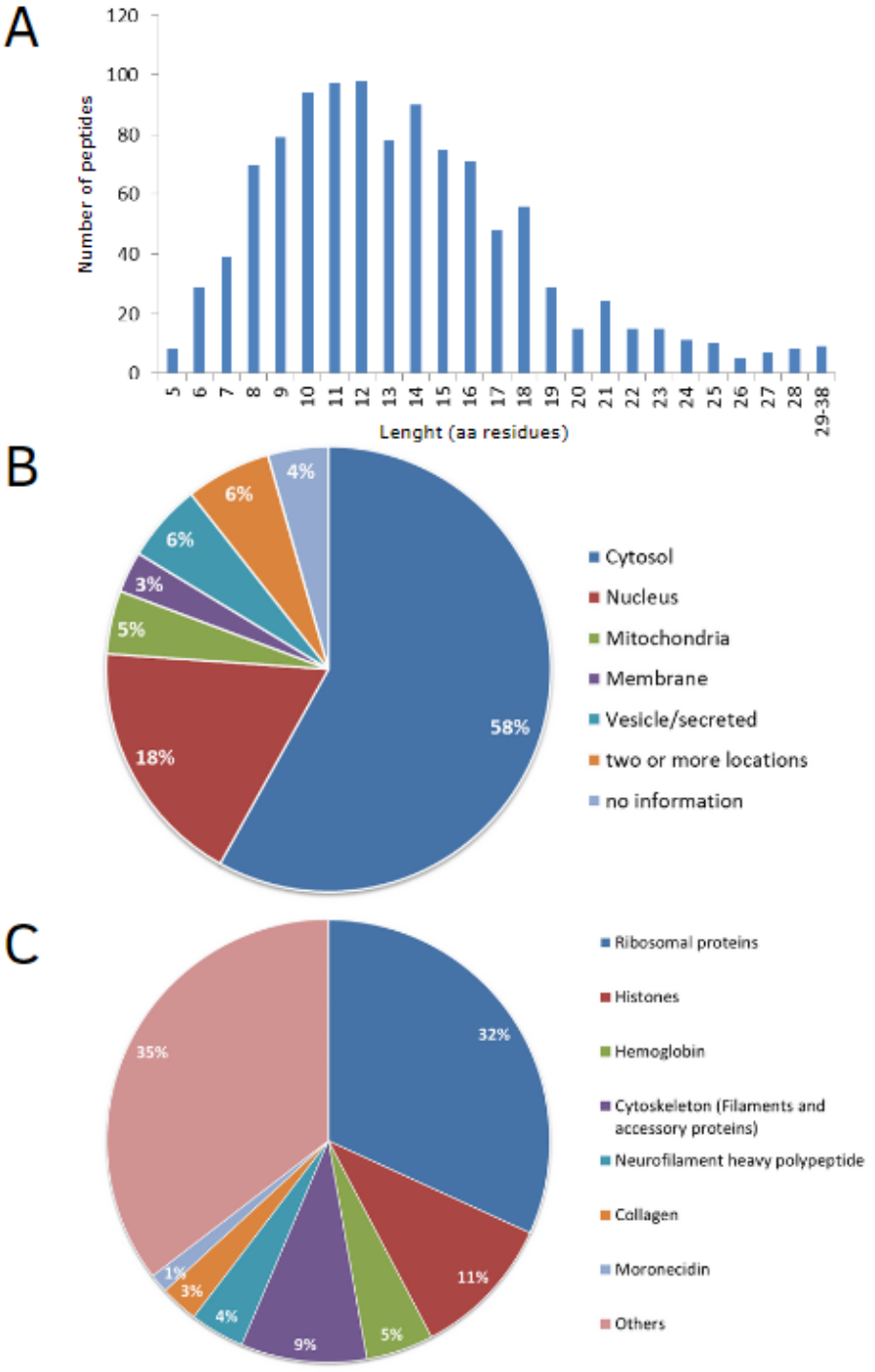

Figure 1. General features of peptide sequences found in gills of seahorse *H. reidi*. In A, peptide distribution by length (amino acid residues). In B, subcellular location of precursor proteins of peptides. In C, classes of precursor proteins.

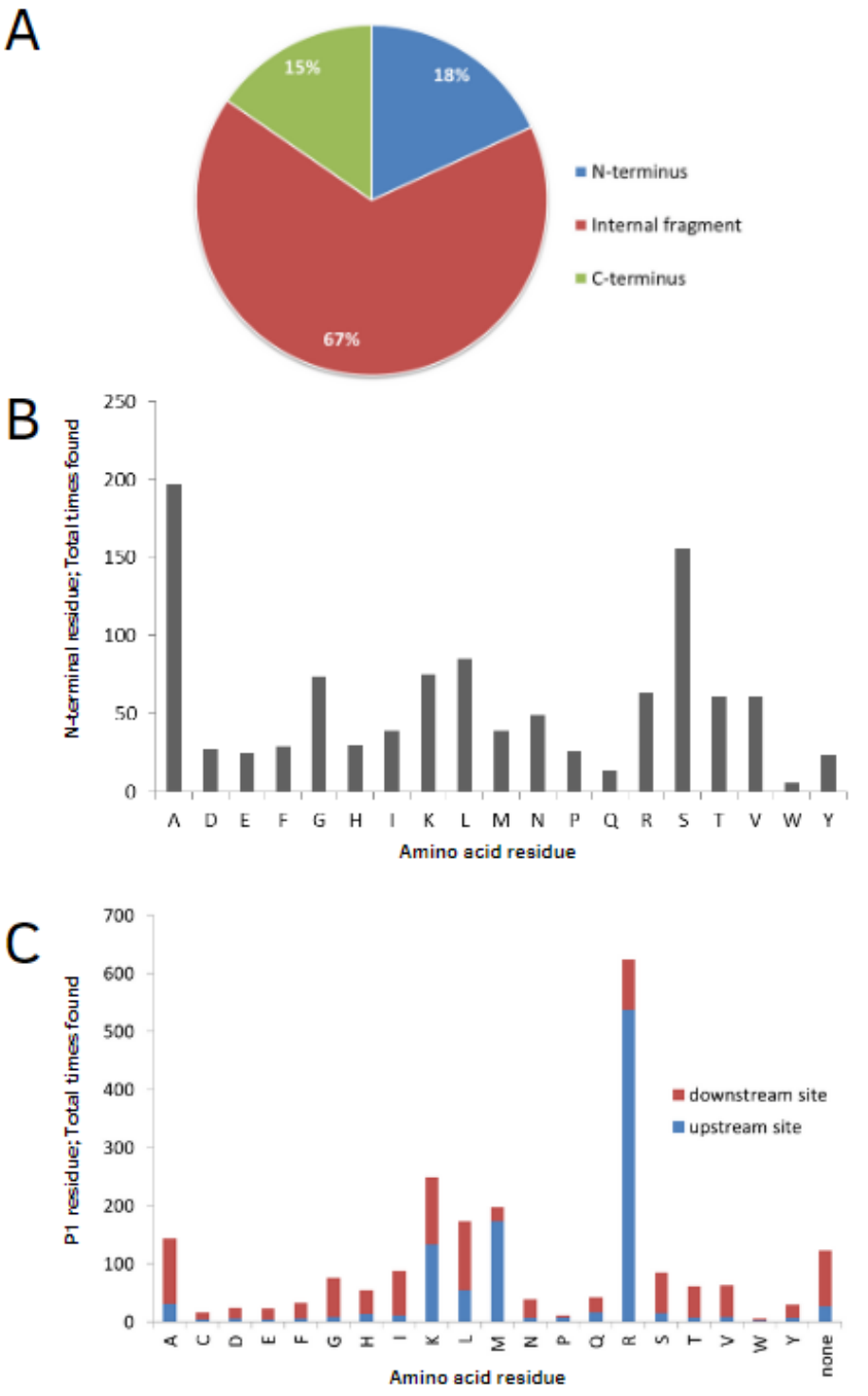

Figure 2. Proteolytic processing of peptides in gills of *H. reidi*. In A, analysis of the location of peptide within the protein precursor. In B, analysis of the N-terminal residue of the

peptides; a total number of times each amino acid was present on the N-terminus of the 1080 identified peptides. In C, analysis of the cleavage sites required to generate the observed intracellular peptides. The number of times each amino acid occurred in the P1 position of the cleavage site is indicated for both upstream and downstream sites.

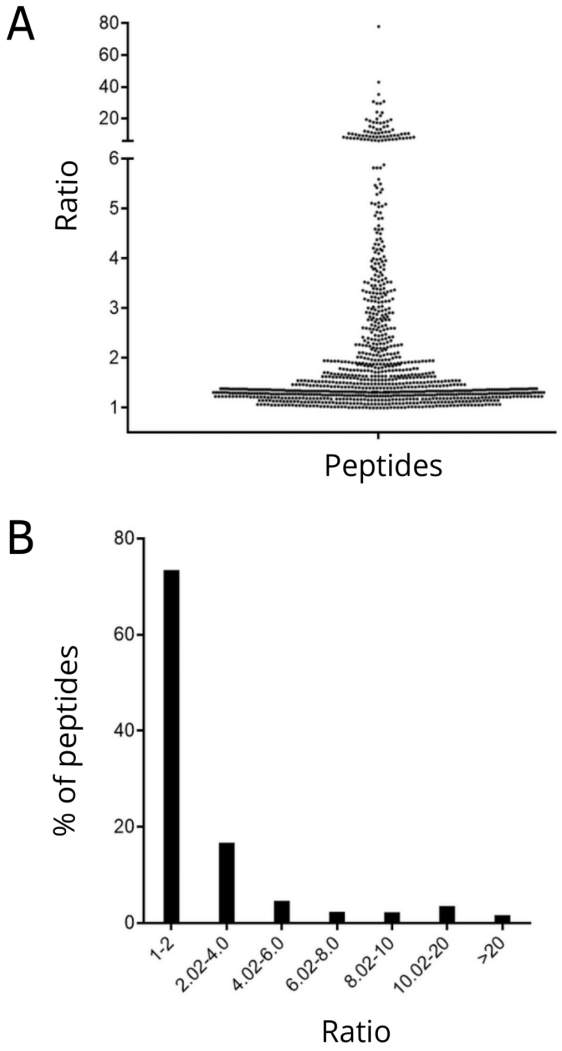

Figure 3. Average ratio of each peptide found in *H. reidi* gills (A). Percentage of peptides in different ranges of ratio (B).

4. Discussion

The main result shown here was the characterization of the peptide profile naturally present in the gills of the seahorse *H. reidi*. Most studies involving peptide extract from seahorses have used enzymes for the preparation of hydrolysates, generating sequences artificially. Specific sample preparation methods and LC-MS/MS analysis can contribute to the predominant identification of peptides with distinct physicochemical properties [6]. Peptides in seahorse gills were extracted after microwave irradiation for rapid inactivation of proteases by heat, followed by extraction in cold acidic deionized water to avoid postmortem artifacts [7]. The features of peptides identified in this study, such as the fragments size, their subcellular distribution and protein classes showed similarities with other peptidome studies that applied the same extraction protocol [10,12].

Most peptides within cells are generated during protein degradation by the large complex proteolytic proteasome that generates fragments within 5 to 22 amino acids [6,15]. This range of the sequences represented more than 90% of peptides found in gills of *H. reidi*. The three major catalytic activities associated with proteasomes are caspase-like, trypsin-like and chymotrypsin-like activities that cleave after acid, basic and hydrophobic residues respectively [15]. Despite the similarities with other peptidome studies, with respect to the general characteristics mentioned previously, differences related to proteolytic processing were observed. Our findings show strong trypsin-like activity in peptides, with predominance of basic amino acids in the P1 position of the cleavage site, in addition to the fragments being mostly from the internal region of the precursor proteins. These differences could be related to the functional aspects of the gills, as an organ that has contact with the external environment. A meta-analysis of human fluid peptidome demonstrated that, at least for serum and tears, there is a preference for and Lys, positively charged amino acids, in the P1 position [26]. The presence of basic amino acid residues in the P1 position is common in peptides generated during the classical secretory pathway, such as neuropeptides and hormones [27]. The proteasome is well known for its role in generating antigenic fragments during adaptive immunity [28,29]. However, it has been suggested that proteasome also produces antimicrobial peptides as part of the innate immune response, as shown with the

intermediate filament keratin 6a (K6a), which is constitutively processed into antimicrobial fragments in corneal epithelial cells [30]. Thus, trypsin-like protease activity seems to have a significant contribution to the shape of peptides in gills of seahorse.

Quantitatively, most peptides (72,8%) showed an average ratio between samples of 1 to 2%. In a zebrafish brain peptidome study without treatments, variations in the average ratio of peptides were also observed, with some intracellular peptides showing a greater variation than the neuropeptides [10]. Furthermore, in our analyses peptide fragments from the same protein that presented different ratios were observed, which may indicate a distinct proteolytic processing.

Mucosal barriers, such as the skin, gills and gut epithelia, provide a first line of defense against infection [31]. Antimicrobial peptides (AMPs) have been identified in gills of several species of fish [1,32–34]. Moronecidin is an AMP member of the piscidin family that has high salt tolerance and a broad spectrum of activity against microorganisms. The membrane disruption of moronecidin in microbes is due to the formation of a pore [31]. The alignment of moronecidin-like peptide, originally identified in *H. comes*, with other piscidins shows that the signal peptide is conserved, whereas the sequences of the mature peptide are variable [35]. A total of fifteen fragments from the Moronecidin-like peptide were found in gill samples of *H.reidi* (Figure 4A). Among these sequences identified here, the fragments FFRNLWKGAK, KGAKAAFR, GAKAAFR and NLWKGAKAAFR, are part of a peptide selected by in silico analysis of the *H. comes* genome and recently characterized as an antimicrobial peptide [35]. The sequence FFRNLWKGAK (Figure 4B) preserves amphipathic characteristics, such as α-helix formation, with hydrophobic residues on the same surface and also positively charged amino acid residues (Figure 4C), as described for Piscidins [23,36].

Ribosomal proteins, Histones, and Hemoglobin were the intracellular proteins that had most sequences found in the gill peptidome. Antimicrobial activity was demonstrated for peptides from these intracellular proteins in many different species [16].

Peptide sequences from ribosomal proteins represented 32% of the fragments in the gills of the seahorse *H. reidi*, with many fragments of the different ribosomal proteins that constitute the 40S small subunit and the 60S large subunit of eukaryotic ribosomes. Of the 78 described ribosomal proteins that constitute these two subunits, 61 were detected in our data,

of which 36 proteins belonged to the 60S subunit and 25 proteins to the 40S subunit. In figure 5, the number of fragments found for each ribosomal protein is shown. Ribosomal protein L13 was the one with the highest number of fragments. Hurtado-Rios and collaborators [37] have recently discussed the role of ribosomal proteins as moonlighting proteins, which are those capable of performing more than one biochemical or biophysical function within the same polypeptide chain, highlighting them as natural antimicrobials [37–39]. Recently, a C-terminal fragment of 60S ribosomal protein L27 was isolated from the skin of *S. asotus* and identified as AMP [40]. In another study, antimicrobial activity against *Bacillus megaterium*, *Escherichia coli*, and *Candida albicans* was detected in an extract from the epidermal mucus of Atlantic cod (*Gadus morhua*). Ribosomal proteins L40, L36A and L35 were identified in fractions prepared by weak cation exchange chromatography together with reversed-phase chromatography and mass spectrometry [41]. In addition, in oyster gills from *Cassostrea gigas*, a fragment of 60S ribosomal protein L29, with 54 amino acid residues, was identified as AMP. Table 1 lists some identified sequences of ribosomal proteins with similar properties of antimicrobial peptides.

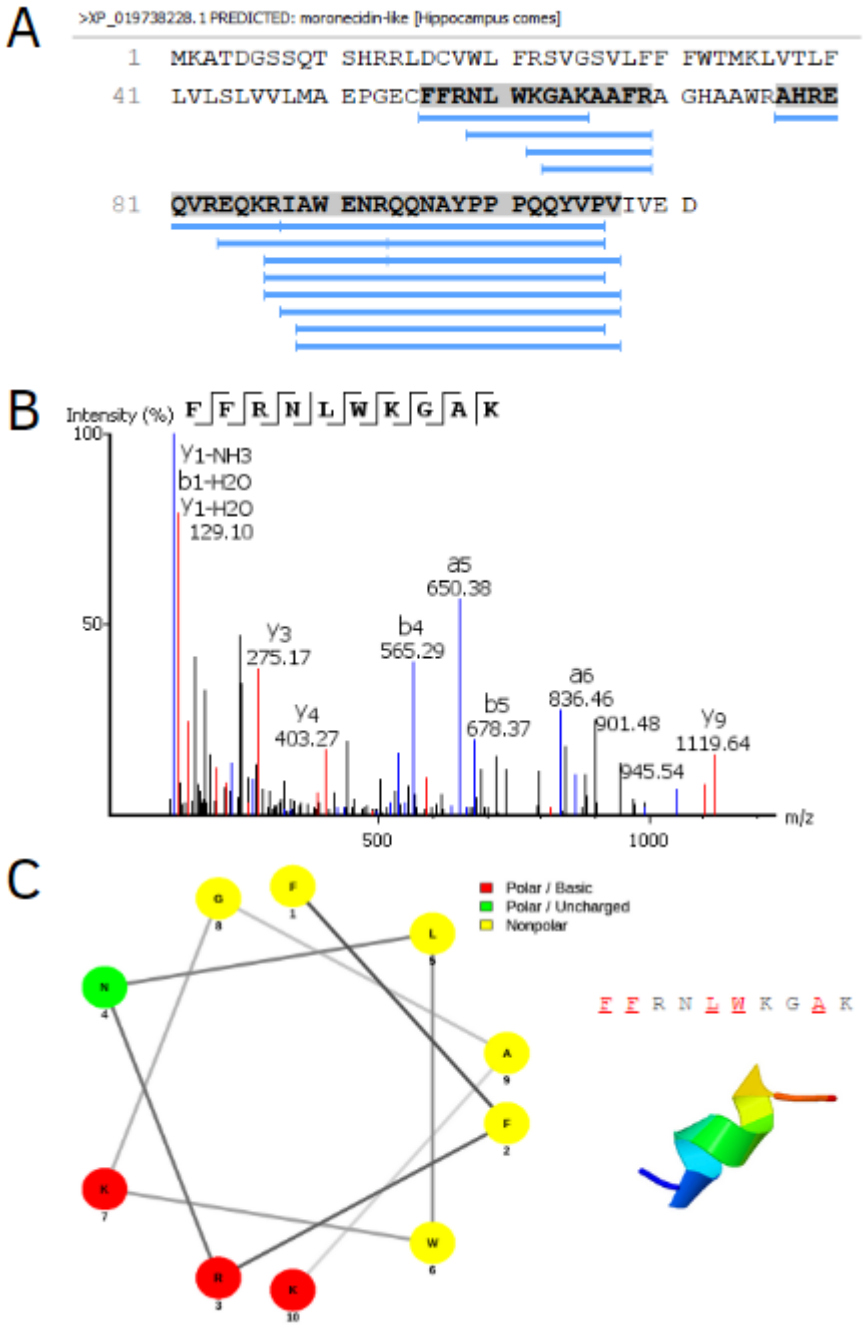

Figure 4. Moronecidin-like found in *H. reidi* gills. In A, location of peptide fragments in the precursor protein sequence. In B, MS/MS spectrum of the sequence peptide FFRNLWKGAK. In C, presence of hydrophobic (yellow) and basic (red) residues, performed using the online tool NetWheels (https://netwheels.herokuapp.com/) and alpha helix prediction analysis builtusing I-Tasser (https://zhanggroup.org/I-TASSER/).

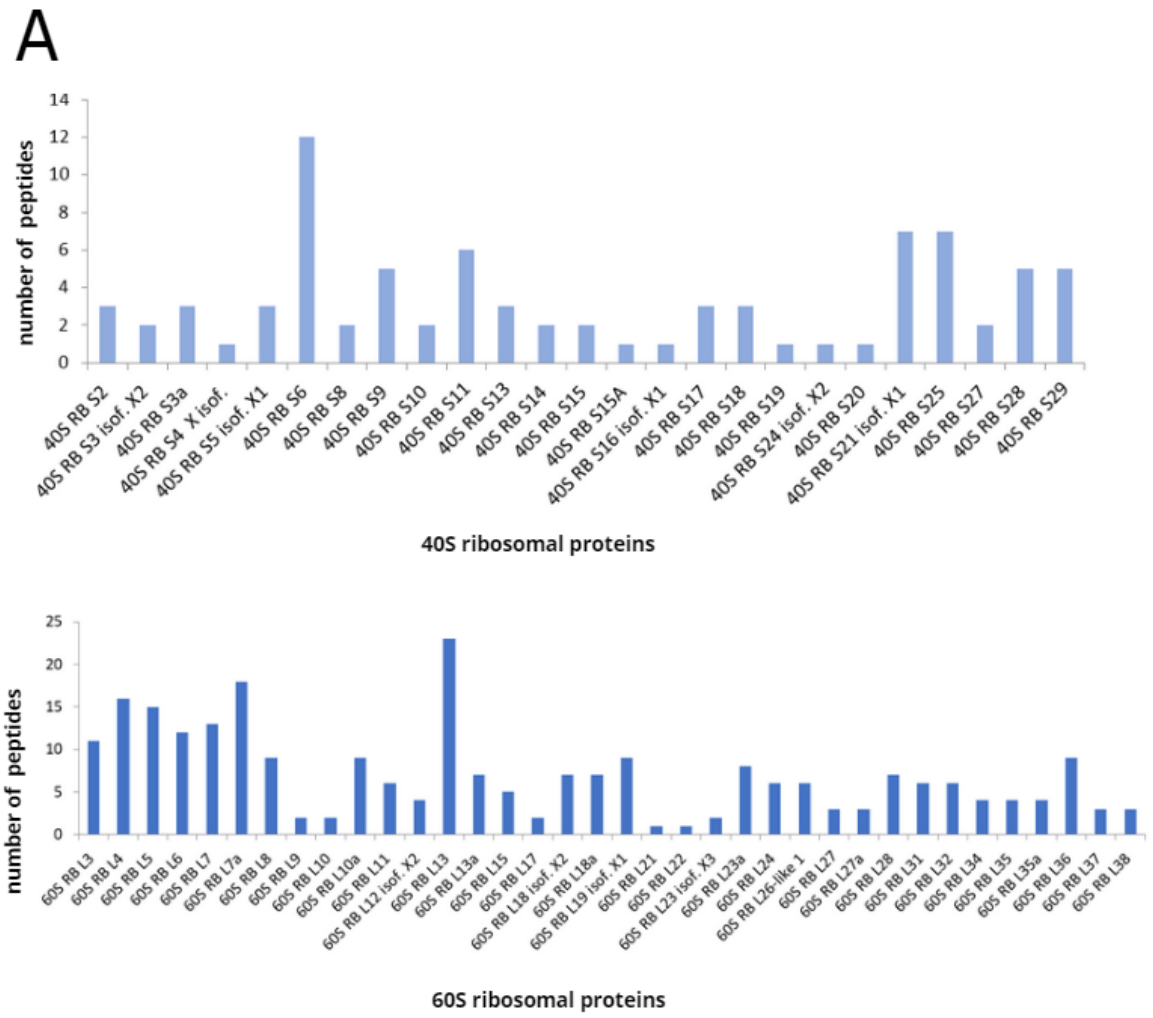

Figure 5. Number of peptides identified for each ribosomal protein, that constitute the 40S small subunit and the 60S large subunit of eukaryotic ribosomes.

Table1. Sequences of ribosomal proteins with similar characteristics to antimicrobial peptides

Sequence	Protein	H.R.	Net Charge	GRAVY	H.R (same suface)
TVGVQPAADGKGVVVVIKKR	60S ribosomal protein L28	45%	3	0,33	0
SQGTRDLDRIAGQVAAANKKSA	40S ribosomal protein S19	36%	2	-0,72	5
AGRGFTLEELKAAGIHKKTAR	60S ribosomal protein L13	38%	3,25	-0,54	5
VRKLYDIDVSKVNTLIRPDGEKKAYVR	60S ribosomal protein L23a	33%	3	-0,64	6
NFGIGQDIQPKRDLTRFVKWPR	60S ribosomal protein L7a	32%	3	-0,99	3
PKGKKAKGKKVAPAPVVAK	60S ribosomal protein L7a	37%	7	-0,68	0
SANRAVVGVVAGGGRIDKPILK	60S ribosomal protein L8	45%	3	0,32	8

H.R.= Hydrophobic Residues

Histones are evolutionary conserved basic proteins, present in all eukaryotic cells. They are known to function in chromatin structure formation, nuclear targeting and regulation of gene expression [42]. Histone-derived AMPs have been identified in a number of fish species, with broad-spectrum activity against both human and fish pathogens, suggesting that complete sequences, N-terminal or C-terminal fragments are part of an ancient innate immune mechanism [43,44].

Histones H2A and H2B have been found in microsomes from gill epithelium of five species of primitive to advanced teleost fish, indicating that these proteins might be secreted to the extracellular environment [45]. In addition, histone H2A has been shown to be synthesized in excess, in amounts required for DNA packaging, and accumulates in cytoplasmic granules in gastric gland cells. Upon secretion into the gastric lumen, it is processed by pepsin C isozymes to yield buforin I [46,47] and proteasomal degradation of histone proteins increase after oxidative damage [48]. The peptide Buforin II (TRSSRAGLQFPVGRVHRLLRK) derived from Buforin I, demonstrates a potent antimicrobial activity, killing the bacteria without cell lise and has affinity with nucleic acids, apparently inhibiting the cellular function by binding to DNA and/or RNA [49]. A similar sequence TRSSRAGLQFPVGRVLR, identified as Histone H2A-like found here, presents hydrophobic ratio 35%, total net charge +4, four hydrophobic residues on the same surface and has 80,75% of similarity with buforin II. Here, most of the peptide sequences found for H2A and H2B represented fragments of the N- and C-terminal of these proteins (Figure 6).

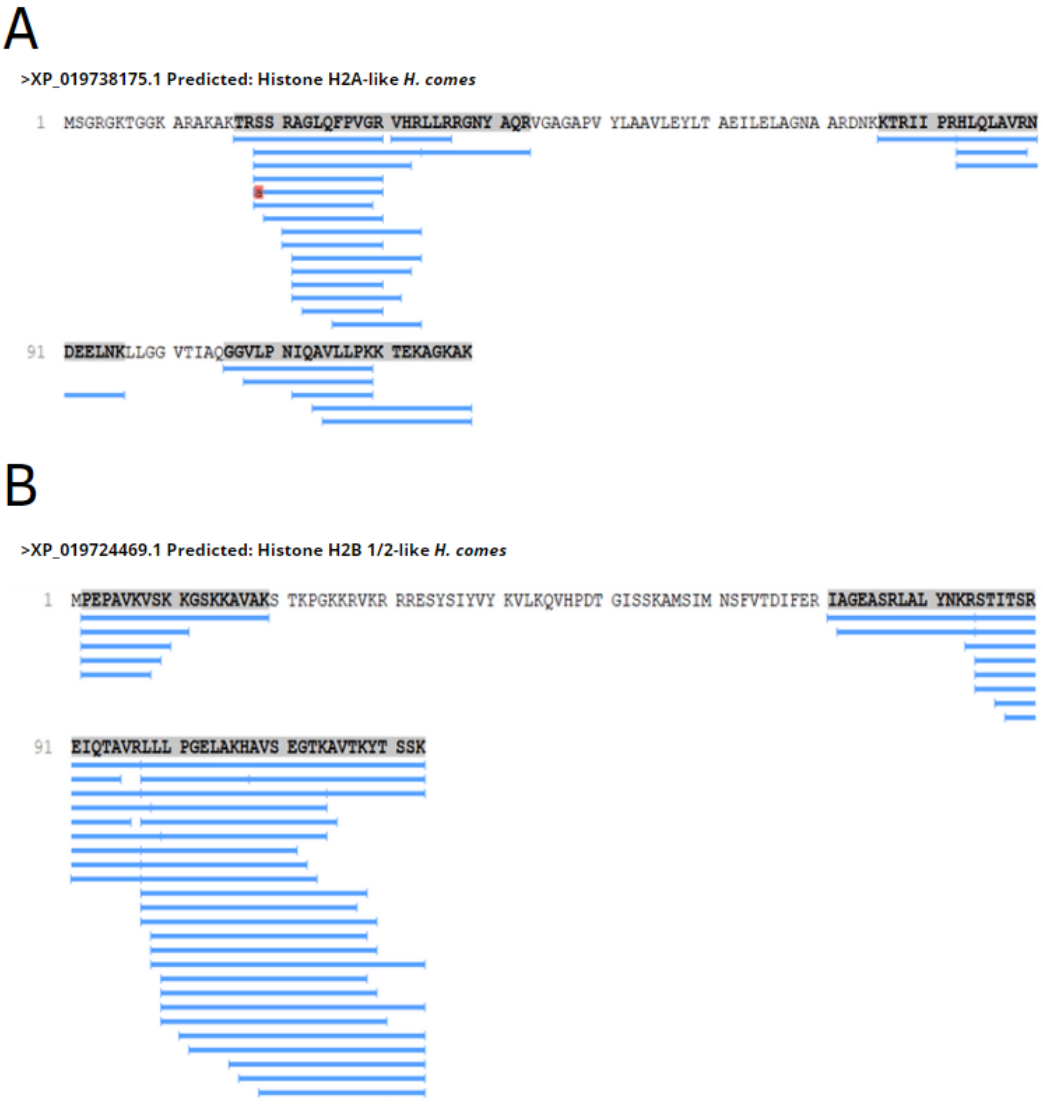

Figure 6. Histone-like derived peptides identified in gills of *H. reidi*. Location of peptide fragments (underlined in blue) in the amino acid sequence of the predicted Histone H2A-like (A) and Histone H2B-like protein (B).

Figure 7. **Hemoglobin alpha chain derived peptides identified in gills of *H. reidi***. In A, location of peptide fragments (underlined in blue) in the amino acid sequence of the precursor protein. In B, Sequence alignment of a peptide sequence found in the gills of *Hippocampus reidi* (*H.r*) and a peptide sequence described with antibacterial action in the liver of *Anguilla japonic*a (*A.j*.).

Peptide sequences derived from hemoglobin were 5% of fragments present in seahorse gills. Hemoglobin is an essential protein for maintaining cellular homeostasis due to its ability to bind and transport oxygen to the tissues. This protein has also been associated with immune response modulation, signal transduction and antimicrobial activity [50]. Hemoglobin has been known as a source of endogenous bioactive peptides that present several different functions [51–53]. Regarding innate immunity, the presence of antimicrobial peptides fragments of hemoglobin isolated from tissues such as skin, branchial epithelium and liver has been shown in fish. For example, an antiparasitic effect of hemoglobin-derived AMPs has been identified from the epithelium catfish *Ictalurus punctatus*, with changes in both HbβP-1 sequence transcribed and translated in skin and gill epithelium against infection of *Ichthyophthirius multifiliis*, where the hemoglobin concentration expressed *in vivo* appeared to be similar with the antiparasitic concentrations measured *in vitro* [54].

Furthermore, a study in sea bass detected changes in Hb-LP gene expression by real-time RT-PCR in the gills and skin in acute crowding stress, but not in other tissues [55]. Also, among the various fragments of the hemoglobin alpha chain found in *H. reidi* gill samples (Figure 7A), fifteen sequences were from a similar region corresponding to the peptide FAHWPDLGPGSPSVKKHGKVIM, derived from hemoglobin alpha in the liver of Japanese eel, *Anguilla japonica*, with strong antibacterial activities against Gram-positive or negative bacteria [56]. One of these fragments showed 56% similarity with this previously described peptide (Figure 7 B).

Our study also verified the presence of peptides containing cysteines through an additional experiment with reduction and alkylation without trypsinization, because some classes of antimicrobial peptides, such as defensins, that are small, amphiphilic and cationic peptides, are usually rich in cysteines [57]. Of the sixty-seven peptides with cysteine residues found, 10 sequences contained two residues and the others only one. However, vertebrate defensins are cationic antimicrobial peptides and have 3 pairs of disulfide bonds forming 3 intramolecular disulfide bonds [58]. Future investigations will be necessary to verify the antimicrobial activity of these sequences.

Taken together, it was possible to identify the presence of fragments similar to peptides with described antimicrobial activity and here only this aspect was explored. However, other intracellular peptide fragments identified in this study may be related to other distinct biological functions, as it has been shown in the last years [52,59,60].

5. Conclusions

The gill peptidome of seahorse *H. reidi* obtained through fast heat inactivation of proteases revealed a set of peptides consisting of many fragments from intracellular proteins residing in compartments such as the cytosol, nucleus and mitochondria. Our data bring relevant information on the levels of these molecules, showing a particular behavior for each peptide, that presents variations from animal to animal, even before being submitted to an experimental condition. Most of the sequences found showed size and properties similar to peptidomes from tissues of other organisms, where the same protocol was used. However, the

proteolytic processing analysis showed differences, such as the prevalence of internal fragments of precursor proteins and the predominance of basic residues such as arginine and lysine in the P1 position of the cleavage site. These differences may be related to some functions of gills, such as innate immune response, which may generate possible peptide sequences with antimicrobial action. Furthermore, our results confirm data from the literature that demonstrate antimicrobial activity of peptide fragments of intracellular proteins. Recognition of patterns and identifying key peptides in seahorse gills could lead to a better understanding of processes such as immune response and proteolysis dynamics. The identified sequences could yield new therapeutic peptides as an alternative to the resistance presented by many pathogenic microorganisms to antibiotics available as treatment.

Supplementary Materials:

The following supporting information can be downloaded at:

https: //www.mdpi.com/article/10.3390/biom13030433/s1

Table S1: Peptides identified in gills of sea horse *H. reidi*; Table S2: Peptides containing cysteine residues identified in gills of sea horse *H. reidi*.

The following Mass spectrometry supporting information can be downloaded at: http://massive.ucsd.edu/ProteoSAFe/status.jsp?task=274624a43f5d4e9097dc1311d5a0d140

Anexo 2- Dosagem das amostras de brânquias pelo método de Fluorescamine, página 100.

Author Contributions: CNC, LOF, GMB and LMC conceived, designed, performed the experimental procedures, analyzed the data, and wrote the manuscript.

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Capítulo II

Avaliação da atividade antioxidante de peptídeos identificados em tecidos de cavalo marinho *H.reidi*

1.Introdução

O metabolismo aeróbico produz subprodutos como radicais livres e outros oxidantes e nitrosantes altamente reativos no interior das células. Radicais livres e espécies reativas de oxigênio incluem radicais superóxido aniônico ($O2^-$), hidroxila (OH), peróxido de hidrogênio (H_2O_2) entre outros. Estas moléculas são produzidas em condições normais e removidas por mecanismos de defesa antioxidante. Há um balanço entre a geração de espécies reativas de oxigênio (ERO) e o sistema antioxidante dos organismos (BACHI; DALLE-DONNE; SCALONI, 2013).

A eficiência do sistema de defesa antioxidante é alterada em condições de patologias e a inefetiva limpeza e/ou superprodução de radicais livres leva ao estresse oxidativo e pode determinar modificações oxidativas de macromoléculas celulares, modificando sua função e eventualmente promovendo a morte celular. O estresse oxidativo está envolvido em diversas condições patológicas como câncer, doenças neurodegenerativas, diabetes mellitus e aterosclerose, assim como envelhecimento (BACHI; DALLE-DONNE; SCALONI, 2013). O acúmulo excessivo de radicais livres leva à peroxidação lipídica da membrana plasmática e destruição de estruturas naturais de proteínas intracelulares e ácidos nucleicos, diminuindo a permeabilidade da membrana interna mitocondrial e causando a fadiga oxidativa (GUO *et al.*, 2017).

Para neutralizar o estresse oxidativo, o organismo envolve respostas enzimáticas e não

enzimáticas que removem ERO diretamente ou reparam o dano oxidativo. Estas defesas incluem enzimas antioxidantes como superóxido dismutase, catalase e glutationa peroxidase e pequenas moléculas como glutationa e vitaminas C e E (QIAN *et al.*, 2008).

A reação de oxidação também é uma das maiores causas de deterioração dos alimentos. Afeta lipídeos, carboidratos e proteínas, porém a oxidação de lipídeos é a principal causa de perda da qualidade dos alimentos, levando à rancidez, diminuição da validade, comprometimento do valor nutricional, alteração do sabor e formação de derivados tóxicos (CHAI *et al.*, 2017; LI; YU, 2015). Nos alimentos, compostos antioxidantes são aptos a retardar ou prevenir o processo de oxidação.

Alguns antioxidantes artificiais como n-propil galato, BHT (butylated hydroxytoluene) e BHA (butylated hydroxyanisole) são comumente usados contra radicais livres em alimentos e sistemas biológicos, entretanto o uso destes antioxidantes artificiais é estritamente regulamentado devido ao risco à saúde de alguns compostos (WANG *et al.*, 2013). Assim, atualmente há um grande interesse na descoberta de compostos antioxidantes naturais como alternativa aos sintéticos para aplicação na conservação de alimentos, desenvolvimento de dietas funcionais, formulações cosméticas e terapias (CHAI *et al.*, 2017). A principal estratégia para reduzir o dano oxidativo é identificar e prover moléculas antioxidantes exógenas que possam induzir o sistema de defesa antioxidante nas células.

Peptídeos com atividade antioxidante foram descritos em diversas espécies marinhas (CHAI *et al.*, 2017; SENEVIRATHNE; KIM, 2012; WANG *et al.*, 2013). Grande parte dos peptídeos com atividade antioxidante possuem peso molecular entre 500 a 1800 Da, de 2 a 20 resíduos de aminoácidos e a presença de aminoácidos hidrofóbicos (A, L ou P) ou aromáticos (Y, H ou F) (CHAI *et al.*, 2017; LI; YU, 2015; WANG *et al.*, 2013). Alguns resíduos de aminoácidos possuem atividades antioxidantes. A metionina por exemplo, pode ser oxidada a metionina sulfóxido e depois desoxidada pela enzima metionina sulfóxido redutase novamente a metionina, que retoma a capacidade antioxidante inicial. A atividade antioxidante de um resíduo de aminoácido sozinho é muito menor do que a de um peptídeo contendo esse resíduo (LI; YU, 2015). A hidrólise enzimática é o método mais utilizado para

a liberação de peptídeos bioativos de várias fontes (CHAI *et al.*, 2017). Um estudo realizado em resíduos do peixe marinho *Nemipterus japonicus* mostrou que quanto maior o grau de hidrólise proteica, maior é a porcentagem de limpeza de radicais livres no ensaio de DPPH, a inibição da peroxidação lipídica, o poder de redução antioxidante do ferro (FRAP) e a atividade inibitória da enzima conversora de angiotensina (ECA)(GAJANAN; ELAVARASAN, 2016).

Peptídeos com atividade antioxidante já foram isolados de cavalos marinhos. Hidrolisados do gênero Hippocampus podem limpar radicais livres e quelar metais, suportando assim sua atividade como agente antioxidante. Guo e colaboradores (2017) observaram que peptídeo isolado de Hippocampus apresentou alta atividade antioxidante e inibiu a geração de radicais livres. Adicionalmente, este peptídeo foi hábil em limpar radicais livres produzidos durante o exercício excessivo e reduzir a produção de lactato (GUO *et al.*, 2017).

Os peptídeos HGSH, KGPSW e a combinação de ambos, isolados de cavalo marinho *Hippocampus abdominalis,* inibiram significativamente a injúria mediada por H_2O_2 em células HUVEC. Estes peptídeos aumentaram a expressão de heme oxigenase (HO1) que cataboliza heme em monóxido de carbono, ferro livre e biliverdina. Biliverdina e bilirrubina são potentes moléculas antioxidantes que provêm efeito de proteção celular pela limpeza de radicais livres. Monóxido de carbono possui ação antiinflamatória e anti-apoptótica em células endoteliais. Esses peptídeos também aumentaram a expressão no núcleo e a translocação do fator nuclear eritróide 2 (NRF2), responsável pela ativação do sistema de defesa endógeno contra o estresse oxidativo, e diminuíram a razão das proteínas Bax, BCl2 e caspase 3, diminuindo a apoptose celular. Porém não apresentaram resultados no ensaio de DPPH (OH; AHN; JE, 2021).

Um dos ensaios mais utilizados para avaliar a capacidade antioxidante é o de DPPH (2,2-difenil-1-picril-hidrazil). Este é um ensaio rápido e eficiente em predizer a atividade antioxidante de proteínas hidrolisadas, frações e peptídeos purificados. Tem sido usado para testar a capacidade de compostos de atuar como sequestradores de radicais livres ou doadores

de hidrogênio e assim avaliar a capacidade antioxidante (WANG *et al.*, 2013). Neste ensaio, ocorre uma reação de oxirredução, onde o radical DPPH, de coloração violeta, é reduzido para DPPH-H, de coloração amarela. A variação colorimétrica pode ser medida em espectrofotômetro no comprimento de 517 nm.

2. Materiais e métodos

Foram sintetizados para avaliação da atividade antioxidante o peptídeo CNC1, de sequência DKATVKAFWDKVAPKVEDIGVQALSR, identificado em todas as amostras de brânquias de *Hippocampus reidi* e o peptídeo CNC2 de sequência LRLPLALPLDEITGLTPFDDLADKVRY, identificado no rim de *H. reidi*.

A atividade antioxidante dos peptídeos CNC1, CNC2 e o padrão (quercetina) foi avaliada pelo ensaio de limpeza de radicais utilizando 2,2 diphenyl-1-picrylhydrazil (DPPH) de acordo com a metodologia descrita em (SCHERER; GODOY, 2009) com algumas modificações. Resumidamente 10 µl de quercetina nas concentrações final de 25, 50, 100 e 150 µM, preparadas utilizando etanol 80% como diluente foram colocadas em triplicata em placa de 96 poços. Da mesma forma, 10 µl do peptídeo CNC1 (2872,29 PM) e CNC2 (3055,57 PM) com 10, 25, 50 e 100 µM de concentração final, utilizando etanol 80% como diluente, foram colocados em triplicata em placa de 96 poços. 10 µl de etanol 80% foi colocado em triplicata na placa como controle negativo.

O DPPH foi preparado com etanol 80% como diluente na concentração final de 0,08 mM e 190 µl foram aplicados em todos os poços contendo quercetina e os peptídeos CNC1 e CNC2. A leitura foi realizada em espectrofotômetro modelo VersaMax (Absorbance Microplate Reader), comprimento de onda 517 nm, após 30 minutos e 24 horas de reação, em temperatura ambiente no escuro.

A atividade antioxidante foi calculada pela fórmula: % de inibição de radicais livres =

[(Absorbância controle – absorbância da amostra)/absorbância controle] x 100 (SCHERER; GODOY, 2009).

3. Resultados e Discussão

A capacidade antioxidante é utilizada para avaliar a eficiência de antioxidantes naturais e pode ocorrer por uma série de mecanismos. Peptídeos podem diretamente eliminar os radicais livres (pela transferência de um átomo de hidrogênio ou elétron) ou na prevenção de formação de radicais livres através da quelação de íons metais. Antioxidantes também podem interromper as reações em cadeias da peroxidação lipídica retardando sua progressão (CANABADY-ROCHELLE *et al.*, 2015; CHAI *et al.*, 2017; ZOU *et al.*, 2016). O peptídeo denominado CNC1, isolado de todas as amostras de brânquias analisadas, é derivado da proteína hemoglobina alpha, possui 26 resíduos de aminoácidos, peso molecular de 2872,29 Da e apresenta características de peptídeo bioativo como carga +1, 46 % de resíduos hidrofóbicos, 7 resíduos hidrofóbicos na mesma superfície e a presença de um resíduo de triptofano em sua formação. O peptídeo denominado CNC2 possui 27 resíduos de aminoácidos, é derivado da proteína vitelogenina, possui 44% de resíduos hidrofóbicos, é rico em lisina e possui peso molecular de 3055,57 Da. Estas características são compatíveis com peptídeos antioxidantes, a presença de resíduos hidrofóbicos permite ao peptídeo antioxidante interagir com radicais livres solúveis em lipídios e retardar a peroxidação lipídica e aminoácidos aromáticos podem exercer sua atividade antioxidante pela doação de um próton a um radical livre elétron-deficiente (CHAI *et al.*, 2017).

Com 30 minutos de reação, a quercetina, utilizada como padrão, apresentou índice de inibição de radicais variando de 74,36 % na concentração de 25 µM a 77,23 % a 150 µM, o peptídeo CNC1 apresentou índice de inibição de radicais variando de 43,63 % na concentração de 100 µM a 26,74 % na concentração de 10 µM e o peptídeo CNC2 apresentou índice de inibição de radicais de 22,85% na concentração de 10 µM e 24,72% na

concentração de 100 µM (Figura 1A). Após 24 horas, a inibição de radicais da quercetina (150µM) variou para 73,81%, o peptídeo CNC1 aumentou a inibição de radicais para 69,61% na concentração de 100 µM e o peptídeo CNC2 chegou a 33,71% em 100 µM (Figura 1B). A variação da quercetina na maior concentração testada no intervalo de 30 minutos para 24 horas foi de um decréscimo de 3,42% da inibição de radicais livres. Neste mesmo período o peptídeo CNC1 apresentou uma variação de 25,98% a mais de inibição de radicais e o peptídeo CNC2 10,86%. Essa variação pode indicar que ou os peptídeos continuam exercendo uma taxa de inibição de radicais pelo período de tempo testado ou ocorre uma degradação do reagente durante este período, porém no caso de haver uma degradação, esta não ocorre igualmente entre os compostos testados.

O EC50, definido como a concentração efetiva da amostra requerida para alcançar 50% de atividade, pode variar significativamente mesmo nas moléculas antioxidantes comumente usadas como referência (CANABADY-ROCHELLE *et al.*, 2015). A maioria dos trabalhos utiliza o tempo de 30 minutos para a avaliação da capacidade antioxidante dos compostos (GUO *et al.*, 2017; MA *et al.*, 2022; OH; AHN; JE, 2021). Nos primeiros 30 minutos o índice de inibição de radicais dos peptídeos escolhidos não atingiu 50% de inibição, o peptídeo CNC1 atingiu 69,61% de inibição apenas com 24 horas de reação. Devido aos diferentes mecanismos subjacentes aos ensaios de avaliação antioxidante, uma mesma sequência de aminoácidos pode apresentar diferentes resultados antioxidantes, a depender do ensaio escolhido para a avaliação, assim, peptídeos podem apresentar resultados antioxidantes satisfatórios em um ensaio e não em outro. Por exemplo, peptídeos isolados de cavalo marinho foram eficientes na proteção contra a injúria provocada por peróxido de hidrogênio em células HUVEC pelo ensaio de MTT, porém não apresentaram resultados no ensaio de DPPH (OH; AHN; JE, 2021). Em nossas análises os peptídeos sintetizados não apresentaram resultados satisfatórios no ensaio de atividade antioxidante de limpeza de radicais DPPH em 30 minutos de reação, porém outros ensaios antioxidantes são necessários para assegurar que o peptídeo realmente não possui atividade antioxidante efetiva.

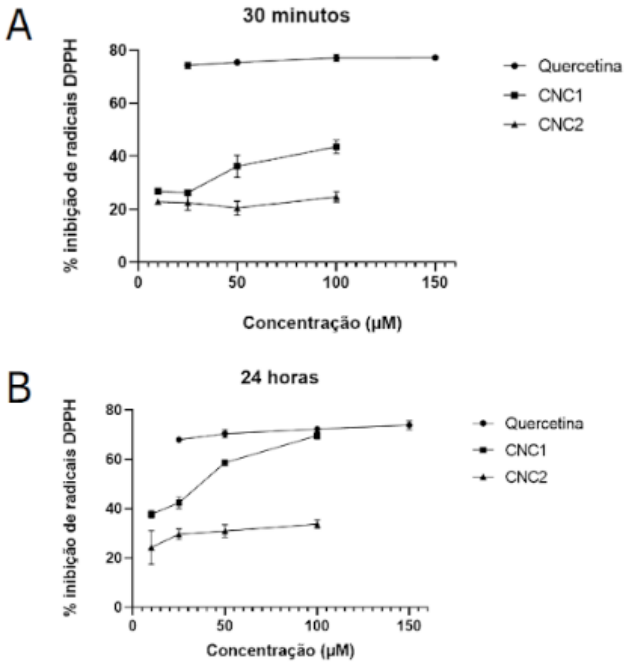

Figura 1. Porcentagem de inibição de radicais em ensaio DPPH. A- Inibição de radicais livres de quercetina, CNC1 e CNC2 em diferentes concentrações após 30 minutos. B- Inibição de radicais livres de quercetina, CNC1 e CNC2 em diferentes concentrações após 24 horas de reação.

4. Conclusão

A análise da capacidade antioxidante dos peptídeos CNC1 e CNC2 não atingiram 50% de inibição de radicais livres no ensaio de DPPH com 30 minutos de reação, porém o peptídeo CNC1 atingiu 69,61% de índice de inibição de radicais livres após 24 horas de reação na concentração de 100 µM.

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Capítulo III

Sample Preparation and Relative Quantitation using Reductive Methylation of Amines for Peptidomics Studies

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Abstract

Peptidomics can be defined as the qualitative and quantitative analysis of peptides in a biological sample. Its main applications include identifying the peptide biomarkers of disease or environmental stress, identifying neuropeptides, hormones, and bioactive intracellular peptides, discovering antimicrobial and nutraceutical peptides from protein hydrolysates, and can be used in studies to understand the proteolytic processes. The recent advance in sample preparation, separation methods, mass spectrometry techniques, and computational tools related to protein sequencing has contributed to the increase of the identified peptides number and peptidomes characterized. Peptidomic studies frequently analyze peptides that are naturally generated in cells. Here, a sample preparation protocol based on heat-inactivation is described, which eliminates protease activity, and extraction with mild conditions, so there is no peptide bonds cleavage. In addition, the relative quantitation of peptides using stable isotope labeling by reductive methylation of amines is also shown. This labeling method has some advantages as the reagents are commercially available, inexpensive compared to others, chemically stable, and allows the analysis of up to five samples in a single LC-MS run.

Introduction

Omics sciences are characterized by the deep analysis of a molecule set, such as DNA, RNA, proteins, peptides, metabolites, etc. These generated large-scale datasets (genomics, transcriptomics, proteomics, peptidomics, metabolomics, etc.) have revolutionized biology and led to an advanced understanding of biological processes[1]. The term peptidomics began to be introduced in the early 20th century, and some authors have referred to it as a branch of proteomics[2]. However, peptidomics has distinct particularities, where the main interest is to investigate the naturally generated peptides content during cellular processes, as well as the characterization of biological activity of these molecules[3,4].

Initially, bioactive peptide studies were restricted to the neuropeptides and hormone peptides through Edman degradation and radioimmunoassay. However, these techniques do not allow a global analysis, depending on the isolation of each peptide in high concentrations, time for the generation of antibodies, besides cross-reactivity possibility[5].

Peptidomics analysis was only made possible after several advances in Liquid chromatography coupled mass spectrometry (LC-MS) and genome projects that delivered comprehensive data pools for proteomics/peptidomics studies[6,7]. Moreover, a specific peptide extraction protocol for peptidomes needed to be established because the first studies that analyzed neuropeptides globally in brain samples showed that detection was affected by the massive degradation of proteins, which occur mainly in this tissue after 1 min post-mortem. The presence of these peptide fragments masked the neuropeptide signal and did not represent the peptidome *in vivo*. This problem was solved mainly with the application of fast heating inactivation of proteases using microwave irradiation, which drastically reduced the presence of these artifact fragments and allowed not only the identification of neuropeptide fragments but revealed the presence of a set of peptides from cytosolic, mitochondrial, and nuclear proteins, different of degradome[6,8,9].

These methodological procedures allowed an expansion of the peptidome beyond the well-known neuropeptides, where hundreds of intracellular peptides generated mainly by the action of proteasomes have been identified in yeast[10], zebrafish[11], rodent tissues[12], and human cells[13]. Dozens of these intracellular peptides have been extensively shown to have both biological and pharmacological activities[14,15]. Furthermore, these peptides can be used as disease biomarkers and possibly have clinical significance, as demonstrated in cerebrospinal fluid from patients with intracranial saccular aneurysms[16].

Currently, in addition to the identification of peptide sequences, it is possible through mass spectrometry to obtain data of absolute and relative quantitation. In the absolute quantitation, the peptide levels in a biological sample are compared to synthetic standards, while in the

relative quantitation, the peptide levels are compared among two or more samples[17]. Relative quantitation can be performed using the following approaches: 1) "label free"[18]; 2) *in vivo* metabolic labeling or 3) chemical labeling. The last two are based on the use of stable isotopic forms incorporated into peptides[19,20]. In label-free analysis, the peptide levels are estimated by considering the signal strength (spectral counts) during the LC-MS[18]. However, isotopic labeling can obtain more accurate relative levels of peptides.

Many peptidomic studies used trimethylammonium butyrate (TMAB) labeling reagents as chemical labeling, and more recently, Reductive Methylation of Amines (RMA) with deuterated and non-deuterated forms of formaldehyde and sodium cyanoborohydride reagents have been used[11,21,22]. However, the TMAB labels are not commercially available, and the synthesis process is very laborious. On the other hand, in the RMA, the reagents are commercially available, inexpensive compared to other labels, the procedure is simple to perform, and the labeled peptides are stable[23,24].

The use of RMA involves forming a Schiff base by allowing the peptides to react with formaldehyde, followed by a reduction reaction through the cyanoborohydride. This reaction causes dimethylation of free amino groups on N-terminals and lysine side chains and monomethylates N-terminal prolines. How proline residues are often rare on the N-terminal, practically all peptides with free amines on the N-terminus are labeled with two methyl groups[23,24,25].

Protocol

The following procedure for peptide extraction and reductive methylation was adapted from previously published procedures[24,25,26,27]. This protocol followed the guidelines of the National Council for Animal Experimentation Control (CONCEA) and was approved by the Ethics Commission for Animal Use (CEUA) at Bioscience Institute of Sao Paulo State University. The protocol steps are shown in **Figure 1**.

NOTE: Prepare all aqueous solutions in ultrapure water.

1. Peptide extraction

1. Cell culture
    1. Cultivate SHSY5Y cells in a 15 cm dish at 37 °C under 5% CO2 in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum and 1% Penicillin-Streptomycin.
    2. Use 2-3 plates for each sample. Grow the cells to 100% confluence.
    3. After treatment intended, wash the cells twice with phosphate-buffered saline. Next, add 10 mL of Phosphate-buffered saline, scrape the cells and collect into a 15 mL tube.
    4. Centrifugate at 800 x *g* for 5 min and remove the supernatant. Resuspend the pellet in 1 mL of ultra-purified deionized water at 80 °C.
    5. Transfer the contents of the tube (cellular lysate) to a 2 mL microfuge tube.
2. Animal tissues (mainly nervous tissue):
    1. Anesthetize wild-type male adult *Danio rerio* (zebrafish) with a lethal dose of MS 222 (100 mg/L) and immediately subject it to 8 s of microwave radiation to inactivate peptidase and protease.

        NOTE: A household-type microwave oven can be used. A 900 W microwave was used for 8 to 10 s at full power. The microwave used must be able to raise the brain temperature to > 80 °C within 10 s. Reproducibility in heating between

samples would also be benefited by placing the tissue in the same location in the microwave.

2. After heat-inactivation, collect the whole brain in a 2 mL microfuge tube and freeze at −80 °C until analysis.
3. Resuspend the tissue sample in 1 mL of ultra-purified deionized water at 80 °C. Sonicate the tissue with a probe using 30 pulses (4 Hz) of 1 s.

   NOTES: For liver and kidney tissues, use a mechanical homogenizer at 10,000-30,000 rpm for 20 s. For muscular tissues, grind the tissue in liquid nitrogen using a porcelain crucible and pestle. The following steps are the same for the cellular lysate or the homogenate tissue.

3. Incubate the cellular lysate or homogenate tissue at 80 °C for 20 min. Next, cool it on ice for 10-30 min.
4. Add 10 µL of 1 M HCl stock solution for each 1 mL of sample volume to obtain a final concentration of 10 mM. Mix by vortexing for 20 s and further incubate on ice for 15 min.

   NOTE: Before acidifying, ensure the sample is completely cooled to avoid breaking the peptide bonds caused by acidification at elevated temperatures.
5. Centrifuge the cellular lysate or the homogenate tissue at 12,000 x *g* at 4 °C for 15 min. Collect the supernatant in low binding protein microcentrifuge tubes and store it at -80 °C.
6. Clean the ultrafiltration devices (10 kDa cut-off filters) by adding water and centrifuge at 2300 x *g* for 3 min. Repeat this step two more times.

7. Place the supernatant in the pre-washed 10 kDa cut-off filters and centrifuge at 2,300 x g at 4 °C for 50 min in a refrigerated centrifuge. The flow-through represents the peptide extract.
8. Desalt the samples on reversed-phase cleanup columns according to the manufacturer's instructions using acetonitrile (ACN) and trifluoroacetic acid (TFA) solutions as described below:
    1. Equilibrate the column with 1 mL of 100% ACN.
    2. Wash the column with 1 mL solution of 5% ACN with 0.1% TFA.
    3. Load the complete volume of the sample in the column.
    4. Wash the column with 1 mL solution of 5% ACN with 0.1% TFA
    5. Elute the peptides from the column with a 1.8 mL solution of 100% ACN with 0.15% TFA in protein low binding microcentrifuge tubes.
9. Dry the sample completely in a vacuum centrifuge. Set the concentration method for organic solvents and temperature at 30 °C. The concentration time is monitored on display.
    1. Store the samples at −80°C until the next step.

2. Peptide quantification with fluorescamine

NOTE: The amount of peptide can be estimated using fluorescamine at pH 6.8 as previously described[11,28]. This method consists of the attachment of a fluorescamine molecule to the primary amines present in the lysine (K) residues and/or the N-terminal of peptides. The reaction is performed at pH 6.8 to guarantee that the fluorescamine reacts only with the amino groups of the peptides and not with free amino acids. The fluorescamine is measured by using

a spectrofluorometer at an excitation wavelength of 370 nm and an emission wavelength of 480 nm.

1. Prepare different concentrations of the standard peptide (0.05, 0.1, 0.15, 0.2, 0.3, 0.5 and 0.7 µg/µL) and store the aliquots at -20 °C.

   NOTE: The peptide 5A (LTLRTKL) is suggested since it has a known composition and concentration.
2. Prepare fluorescamine stock solution (0.3 mg/mL) in acetone. Aliquot quickly in microcentrifuge tubes (1 mL), seal using parafilm, and store at -20 °C in the dark.
3. Prepare 0.2 M Phosphate buffer (PB) at pH 6.8.

   NOTE: Prepare 0.2 M PB by adding 0.1 M phosphate buffer pH 6.8 (26.85 mL of Na2HPO3 1M) and 0.1 M phosphate buffer pH 6.8 (23.15 mL of NaH2PO3 1M) to 250 mL of water
4. Resuspend the peptide samples in 100-200 µL of ultra-purified water.
5. Pipette 2.5 µL of the standard peptide concentrations and samples onto the white 96-well plate for fluorescence assays in triplicate. Add 25 µL of 0.2 M Phosphate buffer.
6. Add 12.5 µL of fluorescamine with a multichannel pipette. Homogenize gently for 1 min on the orbital rotator shaker.
7. Next, add 110 µL of water with a multichannel pipette to stop the reaction.

   NOTE: Transfer the fluorescamine stock solution and ultrapure water to two reservoirs to pipette these solutions with a multichannel pipette.
8. Adjust the following reading parameters on the spectrofluorometer: Read the samples from the top, excitation wavelength at 370 nm, and emission wavelength at 480 nm.
9. Read the plate on the spectrofluorometer.

3. Reductive methylation of amines labeling

NOTE: This isotopic labeling method is based on the dimethylation of amine groups with deuterated and non-deuterated forms of formaldehyde and sodium cyanoborohydride reagents. The final product of this reaction adds 28 Da, 30 Da, 32 Da, 34 Da, or 36 Da to the final mass of each peptide at each available labeling site (lysine or N-terminal). This reaction produces an m/z difference in the peptides labeled with different forms observed in the MS spectrum (**Table 1**).

CAUTION: Proper safety equipment should be used to handle these compounds, and care should be taken to minimize exposure. Procedures with formaldehyde and sodium cyanoborohydride reagents should be performed in a fume hood because they are very toxic (including weighing the sodium cyanoborohydride). During the quenching reaction and acidification, a toxic gas (hydrogen cyanide) may be generated.

1. Prepare the following fresh solutions from the stock or reagents on the day of the procedure in ultrapure water:
    1. Dilute the stock of 37% Formaldehyde (CH2O) to 4%.
    2. Dilute the stock of 20% Formaldehyde Deuterated (CD2O) to 4%.
    3. Dilute the stock of 20 % Deuterated C13 Formaldehyde (13CD2O) to 4%.
    4. Prepare 0.6 M NaBH3CN.
    5. Prepare 0.6 M NaBD3CN.
    6. Prepare a solution of 1% Ammonium bicarbonate.
    7. Prepare a solution of 5% Formic acid.

       NOTE: Regarding the relative quantitation of peptides, as different experimental schemes can be performed depending on the number of labels used, attention is necessary during the

chemical labeling procedure. It is recommended to separate small aliquots of the labels in separate racks with the respective samples to be labeled to reduce the potential for human error in adding the wrong reagent to the sample tubes.

2. Prepare each sample containing up to 25 µg of the peptide. Samples must not contain Tris or Ammonium Bicarbonate.

   NOTE: The quantities described below are sufficient for each sample in a volume of 100 µL.

   Proceed with steps 3.3-3.8 in a fume hood.
3. Add 1/10th volume of 1 M TEAB to the samples (final concentration of the solution 100 mM of TEAB). Check the pH with a pH indicator paper; it must be between 5-8. Adjust with HCl or NaOH if needed.
4. Add 4 µL of non-deuterated, deuterated Formaldehyde or C13 deuterated Formaldehyde according to the established labeling scheme. Mix for 5 s by vortexing.
5. Add 4 µL of NaBH3CN (0.6 M) or NaBD3CN (0.6 M) according to the established labeling scheme. Mix for 5 s by vortexing.
6. Incubate in a fume hood for 2 h at room temperature, mixing every 30 min.
7. Repeat steps 3.4 and 3.5. Incubate the samples in a fume hood overnight at room temperature.
8. Add 16 µL of Ammonium bicarbonate (1%) and mix by vortexing. Place the sample on ice, add 8 µL of formic acid (5%), and mix by vortex for 5 s.
9. Combine the samples, adjust the pH to 2-4 and desalt the combined samples on reversed-phase cleanup columns as previously described in step 1.8.

10. Dry the sample completely in a vacuum centrifuge. Set the concentration method for organic solvents and temperature at 30 °C. The concentration time is monitored on display.
11. Store the samples at -20 °C.

4. Liquid chromatography and mass spectrometry

1. Perform LC-MS analysis using a nanoHPLC system coupled to a MS instrument compatible with methyl tags.

   NOTE: A variety of MS instruments are compatible with RMA labeling. A nanoHPLC system coupled to Orbitrap is typically used to perform LC-MS analysis through a nanoelectrospray ion source. First, the sample is loaded into a precolumn and peptides separated in an analytical column. The elution of peptides is performed using a linear gradient of 5%-45% acetonitrile, in 0.1% formic acid, during 90 min, with a flow of 200 nL/min. The mass spectrometer is set to function in data-dependent mode. Each full scan is acquired at an intensity of 10-30 eV, 2.3 Kv, and then the ten highest peaks are selected for collision-induced dissociation (CID) fragmentation. The injection time is set on the ion trap at 100 ms, and the Fourier Transform (FT)-MS injection is fixed with a resolution of 1000 ms 30,000 at m/z 300-1800. A minimum of 5000 counts and a dynamic exclusion of 70 s is used to perform fragmentation scanning.

5. Relative quantitation of peptides

NOTE: The MS spectra are analyzed in the mass spectrometer software. Peak groups of labeled peptides with different tags are identified in the MS spectra. The relative quantitation is calculated by the intensity of each monoisotopic peak. Each treated group is compared to the respective control group.

1. Right double-click on the raw sample file to open the spectrum analysis software. Load the Retention time (RT) and MS spectrum (EM) chromatograms in two tabs, top and bottom, respectively.
2. Right-click once sequentially on the **Display** and **Mass** options icons in the software toolbar and set the mass precision to four decimals.
3. Position the mouse cursor anywhere on the RT tab. Look for the retention time of the corresponding ion to be analyzed and click the right mouse button. The MS spectrum of the selected time will automatically be shown in the EM tab.
4. Position the mouse cursor anywhere on the EM tab. Look for the ions to be analyzed.
5. Right-click and hold in an adjacent region to the left near these ions. Then, drag the mouse to the right at the desired range to zoom the region of interest.
6. Keep the mouse positioned on the EM tab and click on the right or left keyboard arrows to define the range of ions to be analyzed.
7. Position the mouse cursor the RT tab again at the beginning of the desired time interval.
8. Right-click and drag the mouse until the chosen time value. Leave the button. The accumulated intensity of the ions will automatically be shown on the EM tab.
9. Collect the m/z, z, and ion intensity data on a spreadsheet.

   NOTE: The monoisotopic mass of each peptide without added methyl groups is calculated from the following formula:

   Mass unmodified peptide = (m/z a x z) - (C a x T) - (1.008 x z)

   m/za is the observed mass to charge value for the monoisotopic peak for each

peptide labeled with different combinations of tags (***a*** =1, 2, 3, 4 or 5, corresponding to the sample number).

z is charge state.

Ca is the monoisotopic mass of a pair of methyl groups:

For a=1, Ca = 28.0313 (the net addition of two CH3 groups to the primary amine)

For a=2, Ca = 30.0439 for two CHD2 groups

For a=3, Ca = 32.0564 for two CD2H groups

For a=4, Ca= 34.0690 for two CD3 groups

For a=5, Ca = 36.0757 for two 13 CD3 groups

T is the number of pairs of methyl groups incorporated into the peptide. This can be calculated from the following formula when five tags are used: *T=z*(m/z5 - m/z1)*/8. For peptides that contain a single primary amine and therefore are labeled with only two methyl groups present peak overlaps on the MS spectra when adjacent labels are used. The peak intensity of each labeled peptide can be corrected using the equations described by Tashima and Fricker[25].

6. Peptide Identification

1. To identify peptides, analyze the MS/MS data using a database search engine[29,30].
2. To calculate the false discovery rate (FDR) using the decoy fusion method, search a decoy database.

   NOTE: The search parameters generally used are no enzyme specificity; precursor mass tolerance set 15-50 ppm; fragment ion mass tolerance of 0.5 Da; variable modifications: reactive amines from Lys residues and N-terminus of the peptides isotopic methylated labels (L1 (+28), L2 (+30), L3 (+32), L4

(+34) and L5 (+36)), oxidized methionine (+15.99 Da) and acetylation (+42.01 Da).

3. Then, sort the peptides by their average of local confidence to select the best spectra to annotate and filter them by FDR ≤5%.

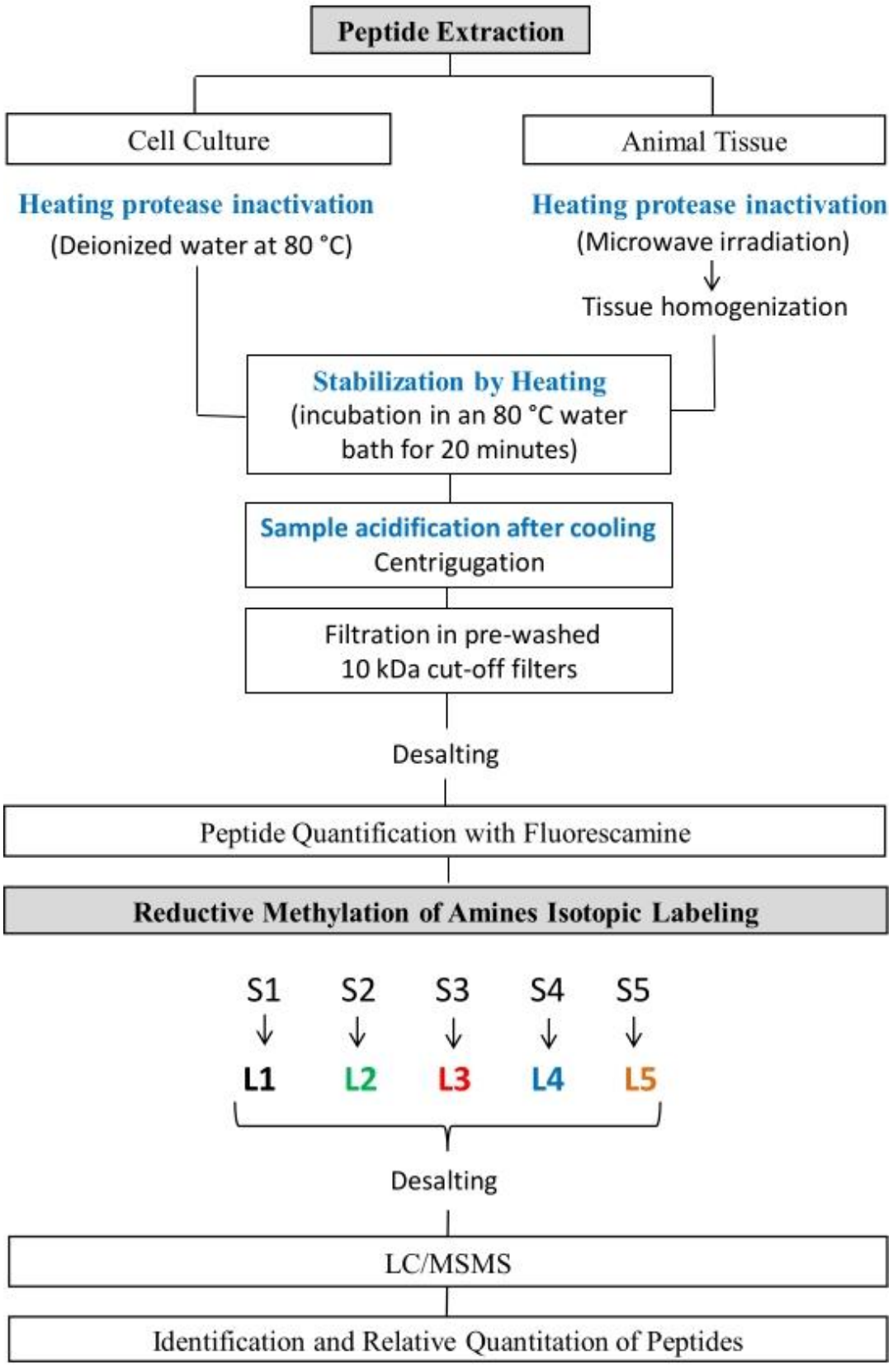

Peptide Extraction
Cell Culture
Animal Tissue
Heating protease inactivation
(Deionized water at 80 °C)
Heating protease inactivation
(Microwave irradiation)
Tissue homogenization
Stabilization by Heating
(incubation in an 80 °C water bath for 20 minutes)
Sample acidification after cooling
Centrigugation
Filtration in pre-washed 10 kDa cut-off filters
Desalting
Peptide Quantification with Fluorescamine
Reductive Methylation of Amines Isotopic Labeling
S1
S2
S3
S4
S5
L1
L2
L3
L4
L5
Desalting
LC/MSMS
Identification and Relative Quantitation of Peptides

Figure 1: Peptidomic studies workflow. Steps of peptide extraction and Reductive Methylation of amines.

Representative Results

The results obtained from the runs carried out on the mass spectrometer are stored in raw data files that can be opened in the mass spectrometer software. In the MS spectra, it is possible to observe peak groups representing labeled peptides according to the labeling scheme used, ranging from 2-5 labels. For example, in **Figure 2**, pairs of peaks detected in a chromatographic time are represented in an experiment where only two isotopic labels were used in two different samples in the same run. **Figure 3** shows other possibilities of positive results, using 3 and 4 different labels in each LC-MS run. When using 4 or 5 labels in an LC/MS run, there may be an overlap of peaks of the labeled peptides with the different tags that need to be corrected to obtain the real intensity value of each peak (**Figure 4**).

The isotopic labeling can also be used to show substrates and products *in vitro* for a given protease or peptidase, as shown in **Figure 5**. Finally, different software can be used to identify the labeled peptides, such as Peaks Studio or MASCOT. These software applications were created for proteomic analysis; therefore, the protein quantitation data should not be considered for peptidomics analysis, and each labeled peptide identified within the reliability parameters must be checked and then quantified. If the peptides have been successfully detected and labeled, these programs will provide a list of identified peptide sequences containing the labels. **Figure 6** is shown an example of the identification of a peptide sequence done by the program. In this case, only 3 different forms of the labels (L1, L3, and L5) were used to label three different samples separately, which were then mixed and analyzed by mass spectrometry in a single run.

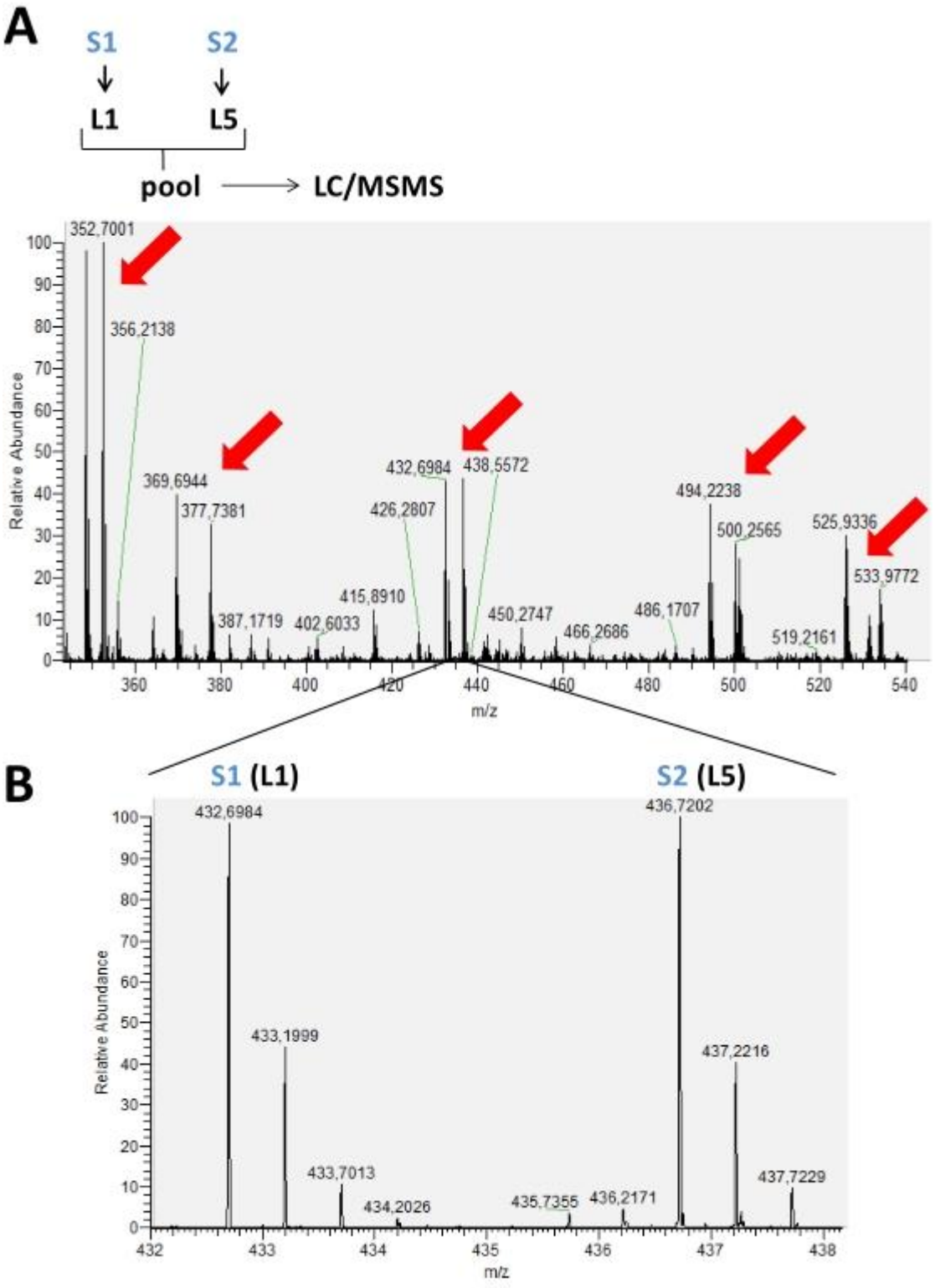

A
S1
S2
L1
L5
pool
LC/MSMS
Relative Abundance
m/z
352,7001
356,2138
369,6944
377,7381
387,1719
402,6033
415,8910
426,2807
432,6984
438,5572
450,2747
466,2686
486,1707
494,2238
500,2565
519,2161
525,9336
533,9772
B
S1 (L1)
S2 (L5)
Relative Abundance
m/z
432,6984
433,1999
433,7013
434,2026
435,7355
436,2171
436,7202
437,2216
437,7229

Figure 2: MS spectrum representative of a chromatographic time accumulated in a typical labeling experiment using reductive dimethylation of amines. In (**A**), the red arrows indicate the presence of peak pairs of different peptides labeled with 2 isotopic forms (L1 and L5) for comparison between two different samples (S1 and S2). In (**B**), an enlarged image of an MS spectrum of the same peptide showing different m/z because of the use of labels. In this case, there was no variation in the peak intensity for this peptide present in these samples.

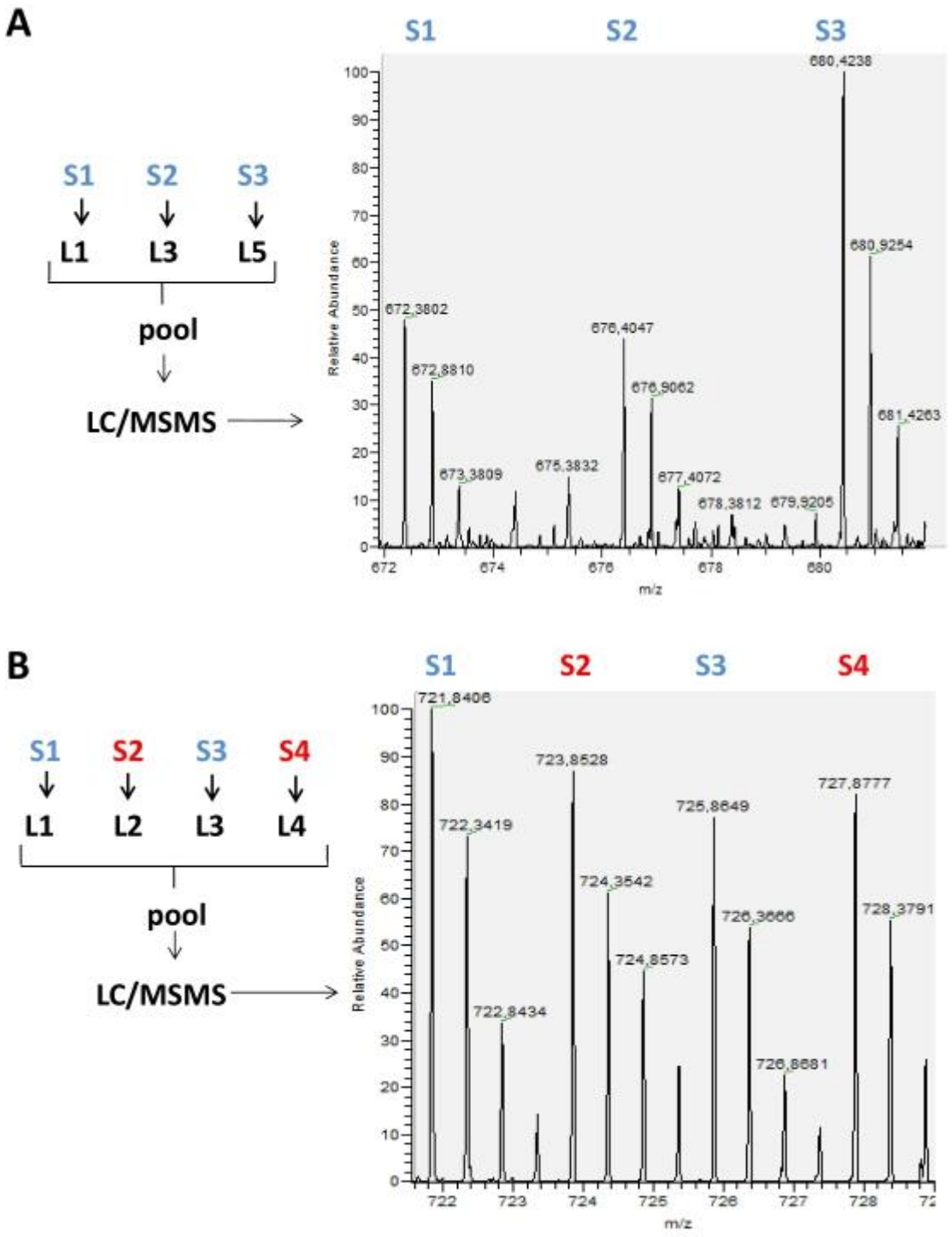
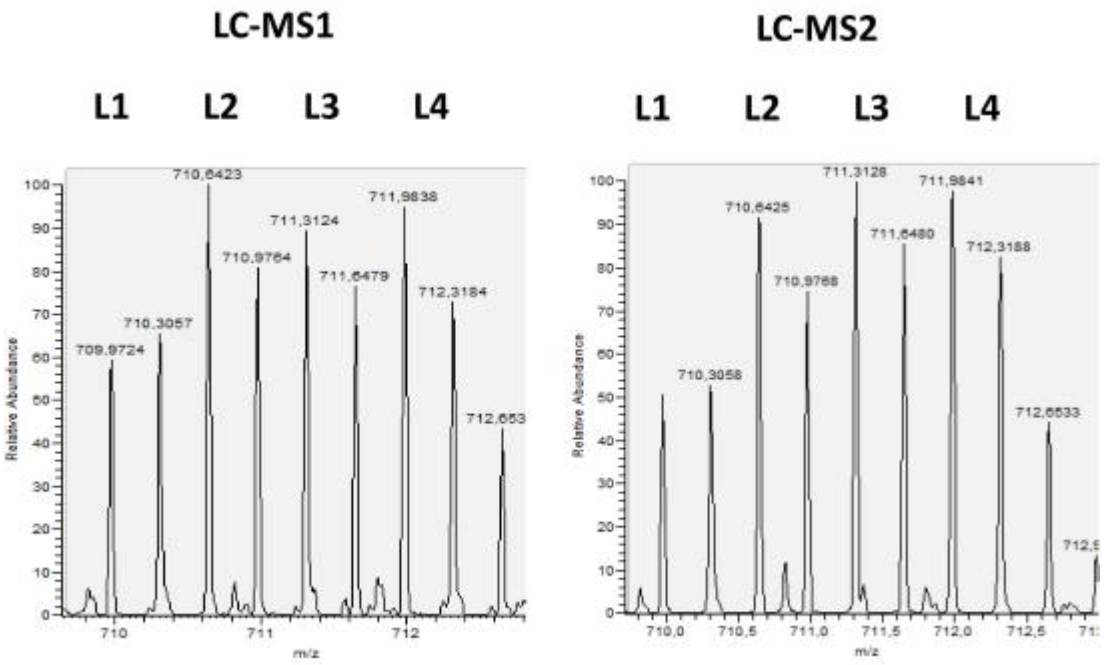


Figure 3: Representative MS spectrum of labeled peptides with reductive methylation of amines using different numbers of tags. (**A**) A triplex labeling was used. It is possible to

observe an MS spectrum of a peptide present in 3 different samples (S1, S2, and S3) labeled with L1, L3, and L5 tags, respectively. In this case, the level of the labeled peptide with L5 was twice the level observed for the same peptide labeled with L1 and L3 tags. (**B**) A quadripex labeling was performed using the L1, L2, L3, and L4 labels. In this case, control samples (S1 and S3) were labeled with L1 and L3, respectively, and compared with two experimental samples (S2 and S4) labeled with L2 and L4 labels. No significant differences were observed for this peptide between the samples.



Group	Elute Time (min)	m/z CH3	m/z CH2D	m/z CHD2	m/z CD3	z	#Me	Observed Mass
LC/MS1	44.2	709.973	710.642	711.312	711.984	3	2	2098.87
LC/MS2	44.2	709.973	710.642	711.312	711.984	3	2	2098.87

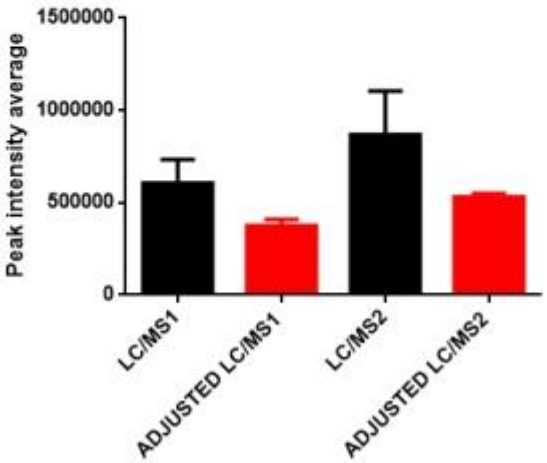


Figure 4: Representative MS spectrum of a labeled peptide presenting a peak overlap. This figure shows the MS spectrum of a peptide of charge 3, mass of 2098.87 Da with a single primary amine available for labeling. The difference between the labeled peptides is

only 2 Da, causing an overlap when 4 or 5 labels are used in the same LC/MS run. Using a model based on cubic polynomial equations, it is possible to correct these overlaps between the labels. In the graph, the red bars show the average of the intensity values adjusted for this peptide in two runs. The black bars show the average of the overlapping intensity values. After correction, this peptide showed little variation in intensity between samples (red bars).

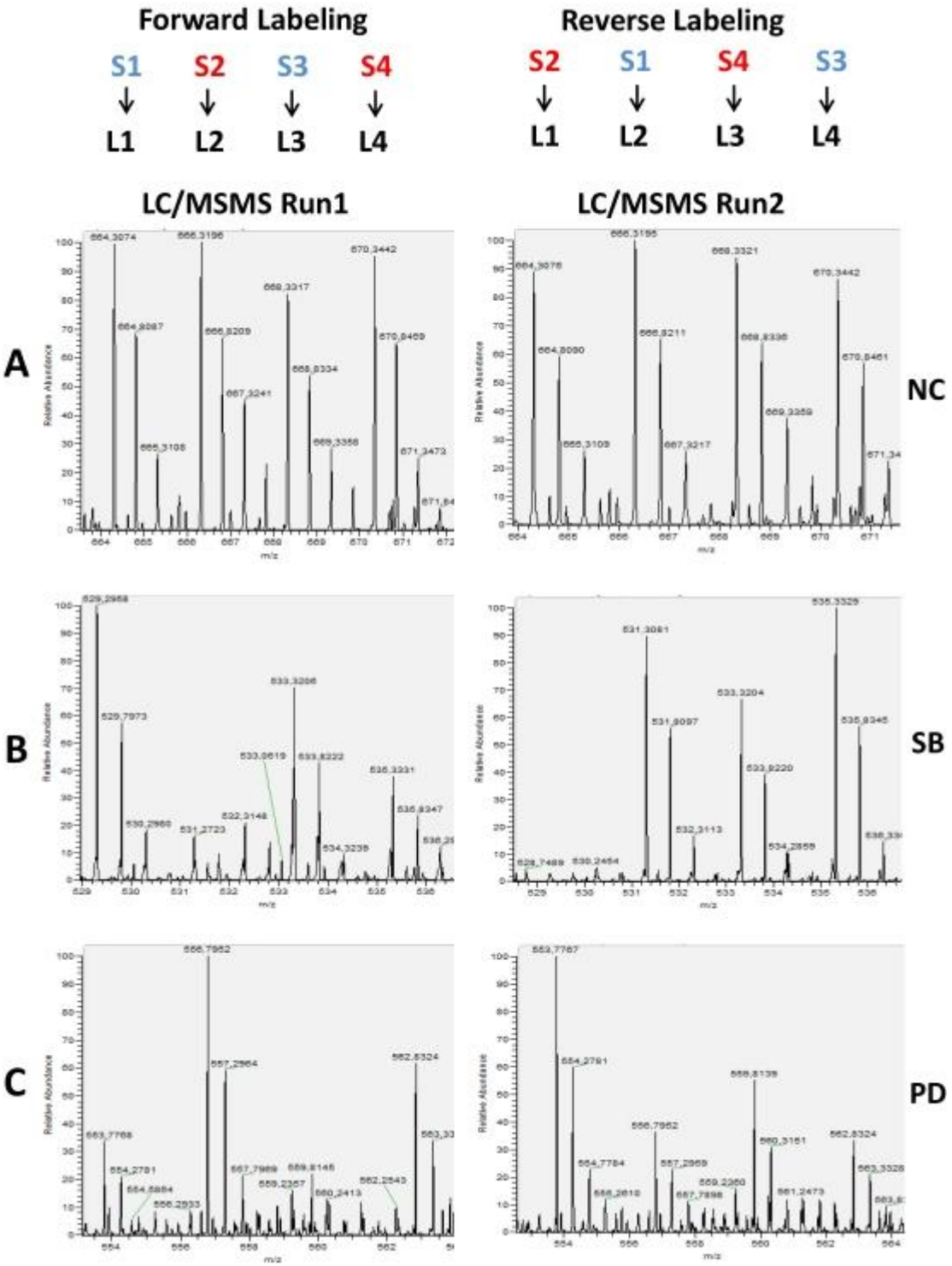


Figure 5: Representative MS spectrum of labeled peptides with reductive methylation of amines in a proteolysis study. Here, peptide extracts were incubated with 200 nM and 20

nM neurolysin to characterize their substrates and products. For confirmation of the result, two LC/MS runs with Forward and Reverse labeling strategies were performed. In the second run the position of the samples is changed in the labeling procedure in relation to the scheme used in the first run. In A, it is shown a peptide that does not change (NC) in the presence of the neurolysin enzyme. In B, a peptide that disappeared in the presence of a high concentration of the enzyme (S2) and that showed a small reduction in the low concentration of the enzyme (S4) in both forward and reverse labeling. This peptide is considered a substrate (SB) of the enzyme. In C an example of a peptide that was considered a product (PD), because its concentration was increased (S2 and S4) in the presence of the enzyme.

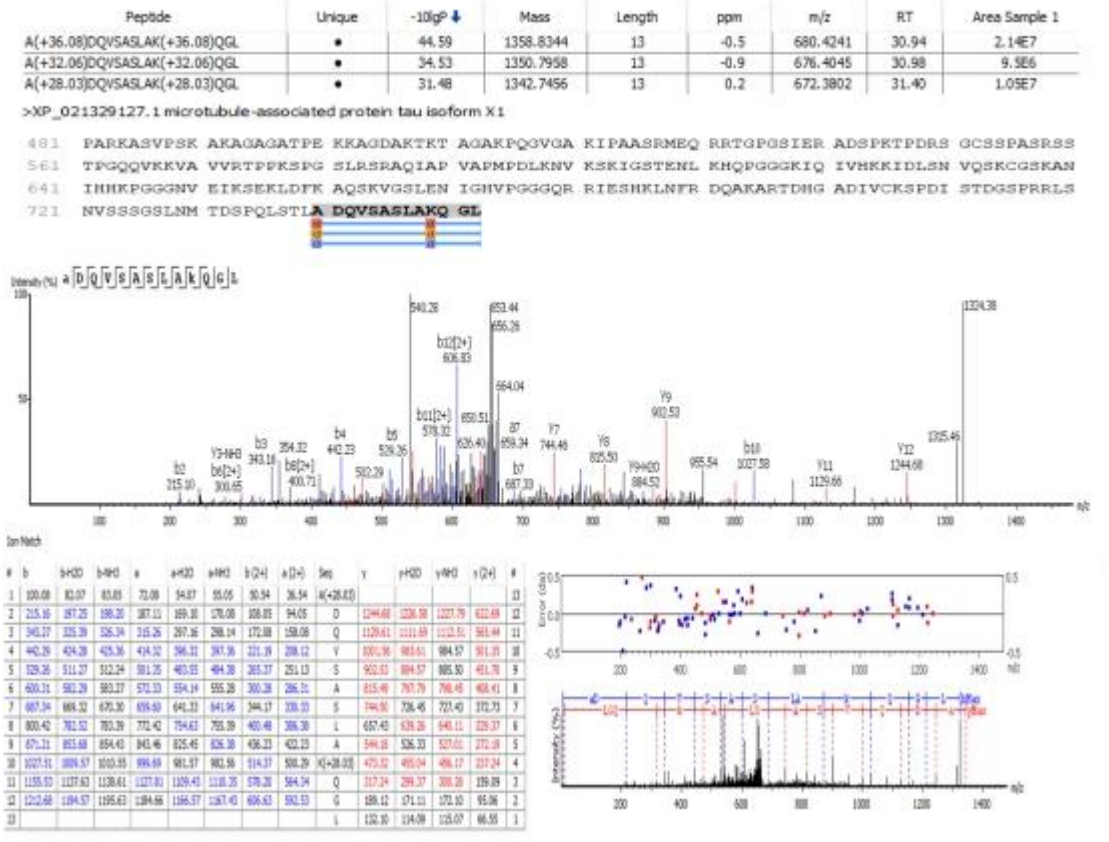


Figure 6: Representative MSMS spectrum and identification of a labeled peptide performed by a database search engine. In this example, it is possible to observe 2+ ions with m/z 672,3802, 676,4045, and 680,4241 corresponding to a peptide of the same mass

labeled with the L1, L3, and L5 methylated forms, respectively. This sequence ADQVSASLAKQGL was identified through the MS/MS spectrum as a fragment of the microtubule-associated protein tau isoform X1. This peptide has the N-terminal and a lysine available as labeling sites adding a mass difference of 8 Daltons between the labeled peptides. The MS spectrum of this peptide is shown in **Figure 3A.**

Sample	H_2CO	D_2CO	$D_{213}CO$	$NaBH_3CN$	$NaBD_3CN$	Label	Additional Mass
	2 x 4 µL	2 x 4 µL	2 x 4 µL	2 x 4 µL	2 x 4 µL	code	(Da)
1	X			X		L1	28.0313
2	X				X	L2	30.0439
3		X		X		L3	32.0564
4		X			X	L4	34.069
5			X		X	L5	36.0757

Table 1: Reagents of a typical experiment using the indicated combination of deuterated and non-deuterated forms of formaldehyde and sodium cyanoborohydride.

Discussion

In most peptidomics studies, one of the critical steps is, without doubt, the sample preparation that should be carefully performed to avoid the presence of peptide fragments generated by proteases after a few minutes post-mortem. The initial studies on brain extracts prepared from non-microwaved samples showed a large number of protein fragments to be present in the 10-kDa microfiltrates. Different approaches have been described to avoid peptide spectra from protein degradation: focused microwave irradiation animal sacrifice[6,8], cryostat dissection followed by a boiling extraction buffer[31], and post-sacrifice microwave-irradiation of tissue using a household type microwave oven[9,26]. For cell culture and some tissues, the protease inactivation can be done directly by adding water at 80 °C. However, some samples, such as nervous tissue, can be more sensitive to post-mortem change, and protease inactivation by microwave irradiation has been indicated as a choice method. Furthermore, another important point during the peptide extraction is to make sure that extracts are ice-cold before adding acid to prevent acid-labile bonds from breaking, like cleavage of Asp-Pro bonds[26].

Different strategies can be used for the relative quantification of peptides, but none of them can be considered completely ideal. To choose the method to be used, the researcher must consider factors such as availability of hours of use in the mass spectrometer, commercially available and costs of labeling reagents, and ease in analyzing the data obtained[17,25,26,32]. The label-free method has been widely used but requires many hours in the mass spectrometer. It is necessary to inject technical replicates for each sample and depends on chromatographic reproducibility among the samples. Other chemical labeling reagents, for example, ITRAQ (isobaric tags for relative and absolute quantitation) and TMT (tandem mass tag), are expensive and only provide quantitation of peptides selected for MS/MS analysis[25,33,34].

The major limitation of relative quantification through chemical labeling using RMA is the overlap that occurs for some peptides when performing a protocol using 4 or 5 labels at the

same run resulting in mass differences of 2 Da for peptides with a single primary amine and 1 Da for peptides with N-terminus proline and no internal lysine residues. However, Tashima and Fricker (2018) developed a model to correct isotopic overlapping based on cubic polynomial equations[25], which get the correct intensity of the labeled peptides in the samples. Furthermore, not all peptides can be seen by RMA. For example, some peptides lack an N-terminal free amine due to acetylation, pyroglutamylation, or another modification. If internal lysines are also absent in these peptides, they will not be labeled by the RMA reagent and will appear on the m/z spectra as unquantifiable single peaks[27].

Disclosures

No competing financial interests exist.

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CONCLUSÕES

1. O peptidoma de brânquias de cavalo marinho *H. reidi* obtido através da inativação rápida de proteases pelo calor revelou um conjunto de peptídeos constituído de muitos fragmentos de proteínas intracelulares provenientes do citosol, núcleo e mitocôndria. Muitas das sequências encontradas mostraram tamanhos e características similares ao peptidoma de tecidos de outros organismos, onde foi usado o mesmo protocolo. Entretanto, o processamento proteolítico analisado mostrou diferenças, como a prevalência de fragmentos internos da proteína precursora e a predominância de resíduos básicos, como a arginina e a lisina, na posição P1 de clivagem. Estas diferenças podem estar relacionadas com algumas funções das brânquias, como a resposta imune inata, com a geração de possíveis sequências peptídicas com ação antimicrobiana. Além do mais, nossos resultados corroboram dados da literatura que demonstram atividade antimicrobiana de fragmentos peptídicos de proteínas intracelulares. Reconhecer o padrão e identificar os principais peptídeos em brânquias de cavalo marinho pode resultar em um melhor entendimento de condições como resposta imune, assim como proteases e dinâmica de proteínas. As sequências identificadas podem revelar novos peptídeos terapêuticos.
2. A análise da capacidade antioxidante dos peptídeos CNC1 e CNC2, sintetizados a partir de sequências identificadas em tecidos de cavalo marinho *H. reidi,* não atingiram 50% de inibição de radicais livres no ensaio de DPPH com 30 minutos de reação, porém o peptídeo CNC1 atingiu 69,61% de índice de inibição de radicais livres após 24 horas de reação na concentração de 100 μM.
3. A quantificação relativa de peptídeos pelo uso de formaldeído e cianoborohidreto em formas leves e deuteradas permite identificação de até 5 amostras em uma única análise pelo sistema de cromatografia líquida acoplada com espectrometria de massas. Os reagentes são comercialmente disponíveis, acessíveis e quimicamente estáveis. A marcação de até 5 amostras simultaneamente permite a comparação de tratamentos e análises de substrato e produtos para diferentes proteases e peptidases.

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Anexo 1 - Outras colaborações

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Characterization of Intracellular Peptides from Zebrafish (*Danio rerio*) Brain

Caio Matheus Manzi Teixeira,[1] Claudia Neves Correa,[1] Leo Kei Iwai,[2] Emer Suavinho Ferro,[3] and Leandro Mantovani de Castro[1]

Abstract

Peptides represent a large class of cell signaling molecules, and they are mainly produced by the classical secretory pathway or during protein degradation. The peptide profile of *Danio rerio* (zebrafish) shows a lack of information when compared with other consolidated animal models. The aim of this work was to characterize the peptide profile of zebrafish brain by using triplex reductive methylation of amines labeling and liquid chromatography coupled to electron spray mass spectrometry. A total of 411 peptide fragments were detected and 125 peptide sequences could be solved. Further analysis suggested that most of the peptides were fragments of intracellular cytosolic and mitochondrial proteins, and that 60% of the precursor proteins were cleaved at either their N- or C-terminal. The most common residue in the P1 position was leucine whereas other common residues were lysine, alanine, arginine, and phenylalanine. Rare cleavage sites at P1 position were histidine, glutamic acid, and isoleucine. The peptide profile of zebrafish brain has similarities with results previously described in mice brain peptidome studies. Thus, this study represents an important basis for the molecular understanding of zebrafish and its use as a model for human diseases.

Keywords: intracellular peptides, peptidome, nervous system, mass spectrometry

Introduction

PEPTIDES ARE DEFINED as polymers of amino acids with <10 kDa and represent a large class of cell signaling molecules present in all living organisms from plants to mammals. Neuropeptides, peptide hormones, and growth factors are the best understood bioactive peptides because of their involvement in neuromodulation, neurotransmission, cell outgrowth, cell survival, and hormonal signaling.[1] These peptides may also participate in regulating a variety of physiologic systems, including feeding,[2] circadian rhythm,[3] pain,[4,5] anxiety,[6] and metabolism.[7,8]

These so-called "classical" peptides are initially translated in the endoplasmic reticulum as an inactive precursor protein, routed through the Golgi, and processed into peptides within secretory granules that are, subsequently, released by exocytose.[9–11] These peptides bind, by affinity to their receptors on the membrane of the target cell, initiating an intracellular signal transduction cascade.[8,12]

In addition to neuropeptides and peptides hormones produced from limited proteolysis in specific compartments of the secretory pathway, the advance of mass spectrometry technology in the past decade, together with successful techniques of peptide extraction avoiding protein degradation,[13–17] has allowed the identification of a specific peptide set derived from cytosolic, nuclear, and mitochondrial proteins in mice brain,[18] mammalian cell lines,[19] and, more recently, in yeast *Saccharomyces cerevisiae*.[20] Nearly 30%–50% of the peptides sequenced in these peptidome studies were identified as N- or C-terminal fragments of proteins and not internal fragments.[18–20] Further, cell lines treated with proteasome inhibitors such as epoxomicin or bortezomib alter the balance of intracellular peptides, suggesting that these peptides are proteasome products and/or substrates.[21,22]

Some intracellular peptides, after being chemically synthesized and reintroduced into mammalian cells, are able to regulate signal transduction,[23] glucose uptake,[24] and intracellular calcium levels[25]; potentiate interferon-γ activity[26]; and induce cell death in several tumor cells.[27]

The *Danio rerio* (zebrafish) is a fresh water specie of teleosteo fish native from Asia that has been used as a vertebrate model in the developmental biology, toxicology, and biomedical

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Redescription of the tadpole of *Thoropa taophora* (Miranda-Ribeiro) (Anura: Cycloramphidae)

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Thoropa Cope is an anuran genus of the family Cycloramphidae that comprises six species of small rock frogs peculiar for the semiterrestrial habits of their larvae, which develop on wet rock environments distributed throughout eastern and southeastern Brazil (Bokermann 1965; Frost 2019). Current knowledge on their tadpoles relies mostly on their descriptions, namely the works of Bokermann (1965) for *T. lutzi* Cochran and *T. petropolitana* (Wandolleck), Caramaschi & Sazima (1984) for *T. megatympanum* Caramaschi & Sazima, Cocroft & Heyer (1988) for *T. saxatilis* Cocroft & Heyer, and Barth (1956) and Fatorelli et al. (2018) for *T. miliaris* (Spix). *Thoropa taophora* (Miranda-Ribeiro) tadpoles were originally described by Bokermann (1965) as *T. miliaris*, but several features were overlooked. In addition, *T. taophora* was recently removed from the synonymy with *T. miliaris* (Feio et al. 2006). Herein, we present a complete redescription of the tadpoles of *T. taophora* and provide comparisons with previously published drawings and descriptions of all other species of the genus.

Tadpoles were collected from Ilha Porchat, Municipality of São Vicente, São Paulo State, on April 1 and September 22 of 2017. They were preserved in 5% formalin and housed in the Amphibians Collection of the Herpetology Laboratory (LHERP) of São Paulo State University (UNESP), Institute of Biosciences, São Vicente, under registration numbers HCLP-A 007 (one specimen used for description and figure drawing), HCLP-A 008 and HCLP-A 037 (seven and 16 specimens, respectively, used to describe variation between them). Development stages of tadpoles were determined according to Gosner (1960). Morphological terminology follows Altig & McDiarmid (1999). Measurements (in millimeters) of tadpoles followed Mercês & Juncá (2010): TL (total length); BL (body length); BH (body height); BW (body width); TaL (tail length); TH (tail height); TMH (tail musculature height); TMW (tail musculature width); DFH (dorsal fin height); VFH (ventral fin height); ND (nostril diameter); OD (orbit diameter); InD (internarial distance); IoD (interorbital distance); NED (nostril-eye distance); SND (snout-nostril distance); SED (snout-eye distance); ODW (oral disc width); SSD (spiracle-snout distance). TL, BL and TaL were taken using a caliper (precision 0.02 mm). All others were taken with ZEISS ZEN Digital Imaging Software and ZEISS Discovery V8 stereomicroscope (precision 0.01 mm) with attached ZEISS Axiocam ERc 5s (5 MP) camera. ND was taken by the axis of largest diameter of the structure.

Tadpole description. Measurements of tadpoles are presented in Table 1. At Stage 39, body oval in dorsal view, with slight middle constriction, and oval in profile, wider than high (Fig. 1A and 1B). Posterior margin of belly extended into flap that covers the vent tube (Fig. 1C). Snout prominent, rounded in lateral view and rounded in dorsal view. Eyes dorsal and large. Nostrils small, elliptical, both directed and located dorsolaterally, at the half distance from the tip of the snout to the eye. Spiracle is single (Fig. 1A), short, narrow, sinistral and located at the end of the first half of the body, below the lateral midline. Centripetal wall of spiracle tube absent; spiracle opening narrow, without skin projection and directed posterodorsally. Medial vent tube short, attached to the tail fin. Long tail, with thin tail musculature tapering to a pointed tip. Very low dorsal fin, continuous, arising at the final quarter of the tail. Ventral fin originates at the end of the body, flattened at the first half and higher than dorsal fin. Oral disc ventral and almost as broad as head anteriorly (Fig. 1D). Labial tooth row formula (LTRF) 2(2)/3(1). A2 and P1 rows interrupted medially by a narrow gap. Relative row length A1 = A2 > P1 = P2 > P3. Single row of marginal papillae regularly disposed on lateral and lower portions of oral disc; lateral papillae are longer and wider than posterior ones. Jaw sheath keratinized and finely serrated; very narrow, "U" shaped upper and lower jaw sheaths; lateral processes of upper jaw sheath wider than those of the lower one.

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Peptide Profile of Zebrafish Brain in a 6-OHDA-Induced Parkinson Model

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Abstract

Parkinson's disease (PD) is a chronic neurodegenerative disorder mainly attributed to the progressive loss of dopaminergic neurons in the substantia nigra, which leads to uncontrolled voluntary movements causing tremors, postural instability, joint stiffness, and speech and locomotion difficulties, among other symptoms. Previous studies have shown the participation of specific peptides in neurodegenerative diseases. In this context, the present work analyzed changes in the peptide profile in zebrafish brain induced to parkinsonian conditions with 6-hydroxydopamine, using isotopic labeling techniques plus mass spectrometry. These analyses allowed the relative quantitation and identification of 118 peptides. Of these, nine peptides showed significant changes, one peptide was increased and eight decreased. The most altered sequences were fragment of cytosolic and extracellular proteins related to lipid metabolism and dynamic cytoskeleton. These results open new perspectives of study about the function of peptides in PD.

Keywords: peptidomics, neurodegenerative disease, zebrafish, mass spectrometry

Introduction

PARKINSON'S DISEASE (PD) is the second most common age-related neurodegenerative disorder that affects ~1%–2% of the population older than 60 years, with a predicted prevalence of nine million people by 2030.[1–3] It is characterized by premature selective loss of dopaminergic neurons in the *substantia nigra* (SN), with the presence of Lewy bodies, composed of α-synuclein, which become misfolded accumulations in the nervous system and microgliosis.[4,5] As these neurons play a key role in controlling voluntary movement, the clinical symptoms observed are motor disabilities, such as rigidity, akinesia, bradykinesia, resting tremor, and postural abnormalities.[6] Furthermore, structural abnormalities in the central nervous system also occur, mainly in the upper parietal, dorsolateral frontal and anterior cingulate cortex regions, resulting in several nonmotor symptoms, as cognitive disturbances.[7,8]

Pathogenesis of PD involves several factors and the studied underlying pathways include components related to mitochondrial, proteasomal and autophagy dysfunction, protein aggregation, dopamine metabolism, and oxidative stress.[9–13] However, the cellular mechanisms involved in synaptic dysfunction responsible for the main symptoms are not fully understood.

There are many neurochemical modulators that can exert neuroprotective effects, which include peptides.[14,15] These molecules are distributed widely throughout the central and peripheral nervous systems, which function as endogenous active substances with the ability to regulate neuronal activity.[16–18] It has been shown that many neuropeptides may be associated with the pathophysiology of neurodegenerative diseases, such as Alzheimer disease[14] and retinal disease.[19]

Specific peptides have been found to be important for the regulation of cell survival in different neuronal systems.[20–25] In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP)-induced degeneration of dopaminergic neurons in mice, the octadecaneuropeptide, a diazepam-binding inhibitor-derived peptide, exerts a potent neuroprotective effect through mechanisms involving downregulation of neuroinflammatory, oxidative, and apoptotic process.[24] The neuropeptide pituitary adenylate cyclase activating polypeptide also showed neurotrophic and neuroprotective effects in 6-hydroxydopamine (6-OHDA)-induced lesion of SN.[21,25,26] On the contrary, accumulation of peptides are linked with the etiology of neurodegenerative diseases, as peptides derived from α-synuclein induced microglial activation and mitochondrial dysfunction.[27]

A recent peptide analysis performed by Van Camp *et al.* identified 105 neuropeptides in the zebrafish brain.[28] In addition, our team detected the presence of ~400 peptide fragments, of which nearly 100 sequences were characterized

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RESEARCH ARTICLE

Differential physiological responses of a biogenic silver nanoparticle and its production matrix silver nitrate in *Sorghum bicolor*

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Abstract
Silver nanoparticles (AgNP) have been extensively applied in different industrial areas, mainly due to their antibiotic properties. One of the environmental concerns with AgNP is its incorrect disposal, which might lead to severe environmental pollution. The interplay between AgNP and plants is receiving increasing attention. However, little is known regarding the phytotoxic effects of biogenic AgNP on terrestrial plants. This study aimed to compare the effects of a biogenic AgNP and $AgNO_3$ in *Sorghum bicolor* seedlings. Seeds were germinated in increasing concentrations of a biogenic AgNP and $AgNO_3$ (0, 10, 100, 500, and 1000 μM) in a growth chamber with controlled conditions. The establishment and development of the seedlings were evaluated for 15 days. Physiological and morpho-anatomical indicators of stress, enzymatic, and non-enzymatic antioxidants and photosynthetic yields were assessed. The results showed that both AgNP and $AgNO_3$ disturbed germination and the establishment of sorghum seedlings. $AgNO_3$ released more free Ag^+ spontaneously compared to AgNP, promoting increased Ag^+ toxicity. Furthermore, plants exposed to AgNP triggered more efficient protective mechanisms compared with plants exposed to $AgNO_3$. Also, the topology and connectivity of the correlation-based networks were more impacted by the exposure of $AgNO_3$ than AgNP. In conclusion, it is plausible to say that the biogenic AgNP is less toxic to sorghum than its matrix $AgNO_3$.

Keywords Antioxidant metabolism, · *Aspergillus tubingensis*, · Nanomaterials, · Photoprotection, · Reactive oxygen species

Introduction

Silver nanoparticles (AgNP) are used abundantly in industry and daily life products due to their particular anti-biological properties (Vishwakarma et al. 2017, Geranio et al. 2009; Yan and Chen 2019). AgNP are significantly different from their bulk counterpart (Ag^+), and extensive research on their effects on living systems, including plants and other organisms, has been carried out recently (Benn and Westerhoff 2008; Mueller and Nowack 2008; Rani et al. 2016). Moreover, nano-green approaches have been investigated and applied to produce AgNP with less environmental impact. According to Roy et al. (2019), plants, algae, bacteria, yeasts, and fungi have bio-active molecules that reduce salts from silver (Ag^+) to metallic silver Ag^0, and subsequently stabilizing them by coating the particle with organic materials. The coating has a multifunctional purpose preventing nanoparticle agglomeration, decreasing toxicity and improving antimicrobial activity (Roy et al. 2019).

According to the 2030 Agenda for sustainable development planned by the United Nations, objective 12 reinforces the necessity to create innovative strategies for responsible consumption and production patterns (Agbedahin, 2019). In this context, the biogenic synthesis of AgNP is an important economic and ecological alternative to produce this nanomaterial in a large scale (Zhang et al., 2020). Fungi are

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ORIGINAL RESEARCH

Relationships of mineralized dermal layer of mountain endemic miniature frogs with climate

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Keywords
altitudinal gradient; *Brachycephalus*; Eberth-Kastschenko layer; hydric stress; morphology; pluviosity; temperature; Serra do Mar.

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Abstract

The mineralized dermal layer (MDL) is found in most terrestrial anurans. Its thickness represents on average up to 8% of that of the entire skin. It has been proposed that it may reduce body water loss, act on homeostasis, support skin structure, or conversely, it may be a currently functionless trait constrained by groups' evolutionary history. We described the MDL morphology of 11 *Brachycephalus* species, terrestrial, miniaturized and microendemic anurans, and tested for its relationship with climate of higher latitude regions of the Atlantic Rainforest of Brazil. All species presented MDL, described with two distinct morphological patterns: homogeneous or heterogeneous MDL, the latter distinguishable by MDL with dorsal or lateral expansion, sometimes up to the limit of the epidermis and comprising up to 50% of the thicknesses of the entire skin. Climate differed between locations by MDL morphological group, less rainy or seasonally less rainy where species with heterogeneous MDL occur. Our results indicate that the abundance of calcium in MDL and its heterogeneous condition suggest its adaptive function in reduce water loss. Such adaptations in anurans in very humid highlands reinforce the mountains' propensity for rapid loss of humidity, demystifying them as an extremely abundant source of water. This is the third study that tested the relationship between the MDL morphology and the environment where species occur and the first that correlated this structure with the climate of anurans of the same habit and distributed in a single habitat, the Atlantic Rainforest.

Introduction

When vertebrates conquered the terrestrial environment, they had to resolve several morphological problems and reduce water loss to the environment (Toledo & Jared, 1993a). Skin adaptation to prevent water loss of these early animals could include scales (Toledo & Jared, 1993a) and harder skin provided by ossicles and bony plates (Colbert et al., 2001). Amphibians have evolved a complicated ontogeny that establishes a modern biphasic cycle of life, with aquatic larvae and terrestrial adults (Duellman & Trueb, 1994; Schoch, 2009) and are not adapted to a strictly terrestrial life (Toledo & Jared, 1993a, 1993b). In addition to numerous morphological transformations from aquatic larvae to terrestrial anurans, amphibian skin possesses several adaptations against dehydration, such as granular ventral skin, cutaneous groves, arrangement of superficial epidermal cells, concentration of cutaneous lipids, hormones and mucous, cutaneous vascularization, and secretions of granular glands (de Brito-Gitirana & Azevedo, 2005; Toledo & Jared, 1993a).

There is another particularity in anuran skin that has drawn attention, namely, the mineralized dermal layer (hereafter MDL; *sensu* Katchburian et al., 2001), also known as 'capa de Katschenko' (sic) (Porto, 1936a), 'lamina cribrosa' (Barbetta, 1958), Eberth-Kastschenko layer (Elkan, 1968), *substantia amorpha* (Elkan, 1968), ground substance (Elkan & Cooper, 1980), amorphous layer (Sampson et al., 1987), calcified layer (Toledo & Jared, 1993a), calcified dermal layer (Toledo & Jared, 1993b), cutaneous calcified layer (Toledo & Jared, 1993c), and 'lamina calcarea' (Vickaryous & Sire, 2009). MDL lies usually between the *stratum spongiosum* and *stratum compactum* (Elkan, 1968; Mangione et al., 2011; Schwinger et al., 2001; Toledo & Jared, 1993a, 1993b), two conspicuous layers that together with a very thin third layer, called *tela subcutanea*, form the anuran dermis (Elkan, 1968). It is irregularly or homogeneously distributed in dermis layers, with records of

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Anexo 2

Rendimento e dosagem de peptídeos de brânquias de *Hippocampus reidi*

Tabela 1. Amostras de brânquias de *Hippocampus reidi*, peso inicial e dosagem pelo método de fluorescamine

Amostra	Peso (mg)	Concentração (µg/µl)	10 µg (µl)
B1	48	0,63	15,8
B2	51	0,22	45,9
B3	49	0,64	15,7

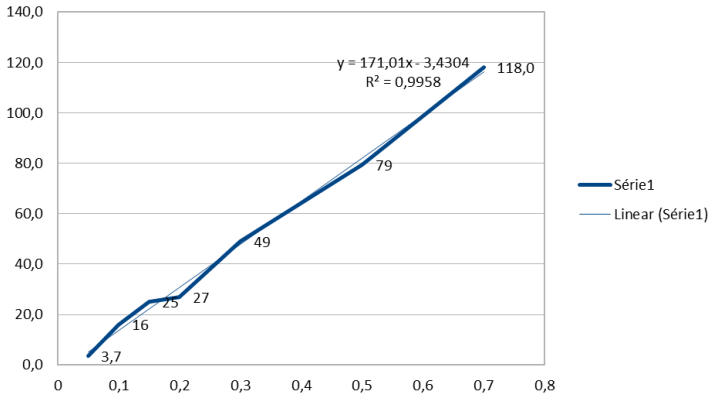

Fig.1 - Gráfico da curva de dosagem utilizando fluorescamine para peptídeos de brânquias de cavalo marinho *Hippocampus reidi*

Anexo 3 - Certificados de autorização para experimentação animal

CERTIFICADO

Certificamos que o projeto intitulado "Caracterização do perfil e atividade biológica de peptídeos de cavalo marinho *Hippocampus reidi*", Protocolo nº **02/2021-CEUA**, sobre a responsabilidade do **Prof. Dr. Leandro Mantovani de Castro** e sua orientada **Cláudia Neves Correa**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA, do Instituto de Biociências do Câmpus do Litoral Paulista, em reunião de 28/10/2022.

Vigência do Projeto:	19/03/2018 a 19/06/2022
Espécie/linhagem:	Cavalo Marinho *(Hippocampus reidi)*
Número de animais:	5
Peso/Idade:	Não definido
Sexo:	Machos
Origem:	Natureza (Ubatuba/SP)

São Vicente, 04 de novembro de 2022

Profa. Dra. Alessandra da Silva Augusto
Coordenadora – CEUA

Instituto de Biociências - Câmpus do Litoral Paulista
Praça Infante D. Henrique s/nº - CEP 11330-900 - São Vicente (SP) - Brasil
Tel. (13) 3569-7100 - Fax (13) 3569-7146 – staepe.clp@unesp.br

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Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 78669-1	Data da Emissão: 01/06/2021 19:37:52	Data da Revalidação*: 01/06/2022

De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Leandro Mantovani de Castro	CPF: 267.981.058-90
Título do Projeto: Caracterização do perfil e atividade biológica de peptideos de cavalo marinho Hippocampus reidi	
Nome da Instituição: UNIVERSIDADE ESTADUAL PAULISTA	CNPJ: 48.031.918/0005-58

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta dos Cavalos MARINHOS	07/2021	07/2021
2	Coleta dos Cavalos Marinhos	08/2021	08/2021

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Cláudia Neves Correa	aluna de doutorado	250.153.248-12	Brasileira

Observações e ressalvas

1	A autorização não eximirá o pesquisador da necessidade de obter outras anuências, como: I) do proprietário, arrendatário, posseiro ou morador quando as atividades forem realizadas em área de domínio privado ou dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso; II) da comunidade indígena envolvida, ouvido o órgão indigenista oficial, quando as atividades de pesquisa forem executadas em terra indígena; III) do Conselho de Defesa Nacional, quando as atividades de pesquisa forem executadas em área indispensável à segurança nacional; IV) da autoridade marítima, quando as atividades de pesquisa forem executadas em águas jurisdicionais brasileiras; V) do Departamento Nacional da Produção Mineral, quando a pesquisa visar a exploração de depósitos fossilíferos ou a extração de espécimes fósseis; VI) do órgão gestor da unidade de conservação estadual, distrital ou municipal, dentre outras.
2	O pesquisador somente poderá realizar atividade de campo após o término do estado de emergência devido à COVID-19, assim declarado por ato da autoridade competente.
3	Esta autorização NÃO libera o uso da substância com potencial agrotóxico e/ou inseticida e NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de atender às exigências e obter as autorizações previstas em outros instrumentos legais relativos ao registro de agrotóxicos (Lei nº 7.802, de 11 de julho de 1989, Decreto nº 4.074, de 4 de janeiro de 2002, entre outros).
4	Esta autorização NÃO libera o uso da substância com potencial agrotóxico e/ou inseticida e NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de atender às exigências e obter as autorizações previstas em outros instrumentos legais relativos ao registro de agrotóxicos (Lei nº 7.802, de 11 de julho de 1989, Decreto nº 4.074, de 4 de janeiro de 2002, entre outros)
5	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio nº 03/2014 ou na Instrução Normativa ICMBio nº 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
6	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
7	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
8	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.

Este documento foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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Dados do titular

Nome: Leandro Mantovani de Castro	CPF: 267.981.058-90
Título do Projeto: Caracterização do perfil e atividade biológica de peptídeos de cavalo marinho Hippocampus reidi	
Nome da Instituição: UNIVERSIDADE ESTADUAL PAULISTA	CNPJ: 48.031.918/0005-58

Observações e ressalvas

9	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
10	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade.
11	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.

Outras ressalvas

1		CEPSUL

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Praia do cedro, 23° 27? 01.417S; 45° 02? 09.00?O	Ubatuba-SP	Marinho	Não	Fora de UC Federal

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de espécimes da fauna silvestre in situ	Fora de UC Federal
2	Coleta/transporte de amostras biológicas in situ	Fora de UC Federal
3	Captura de animais silvestres in situ	Fora de UC Federal
4	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Atividades ex-situ (fora da natureza)

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Hippocampus reidi	-
2	Captura de animais silvestres in situ	Hippocampus reidi	-
3	Coleta/transporte de amostras biológicas in situ	Hippocampus reidi	-
4	Coleta/transporte de espécimes da fauna silvestre in situ	Hippocampus reidi	5

A quantidade prevista só é obrigatória para atividades do tipo "Coleta/transporte de espécimes da fauna silvestre in situ". Essa quantidade abrange uma porção

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territorial mínima, que pode ser uma Unidade de Conservação Federal ou um Município.

Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Peixes)	Fragmento de tecido/órgão
2	Método de captura/coleta (Peixes)	Coleta manual

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	UNIVERSIDADE ESTADUAL PAULISTA	Laboratório

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Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de Amostra	Qtde.	Data

* Identificar o espécime do nível taxonômico possível.

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